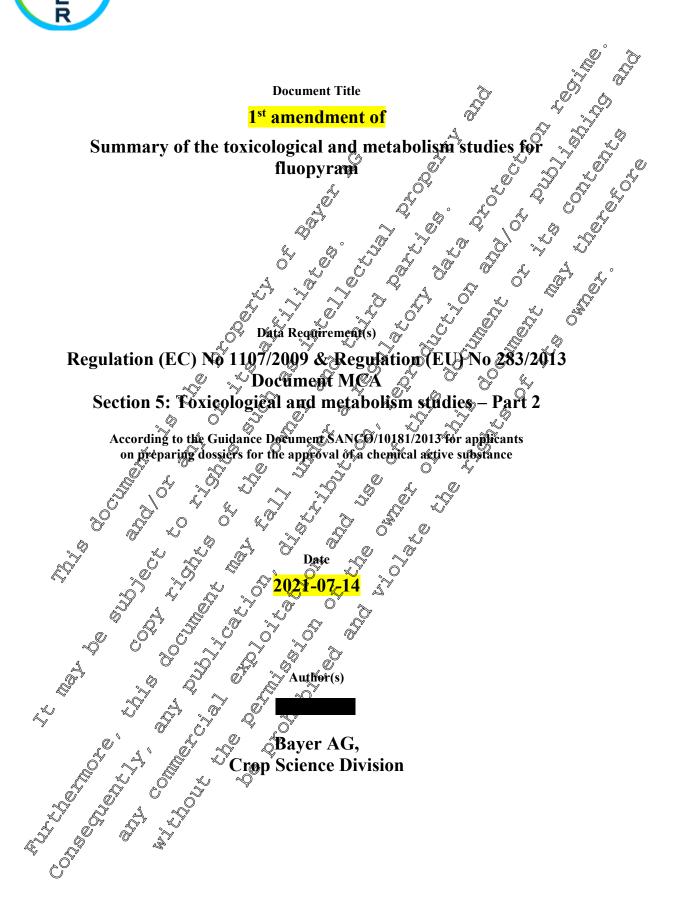


M-766370-03-2





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Version history

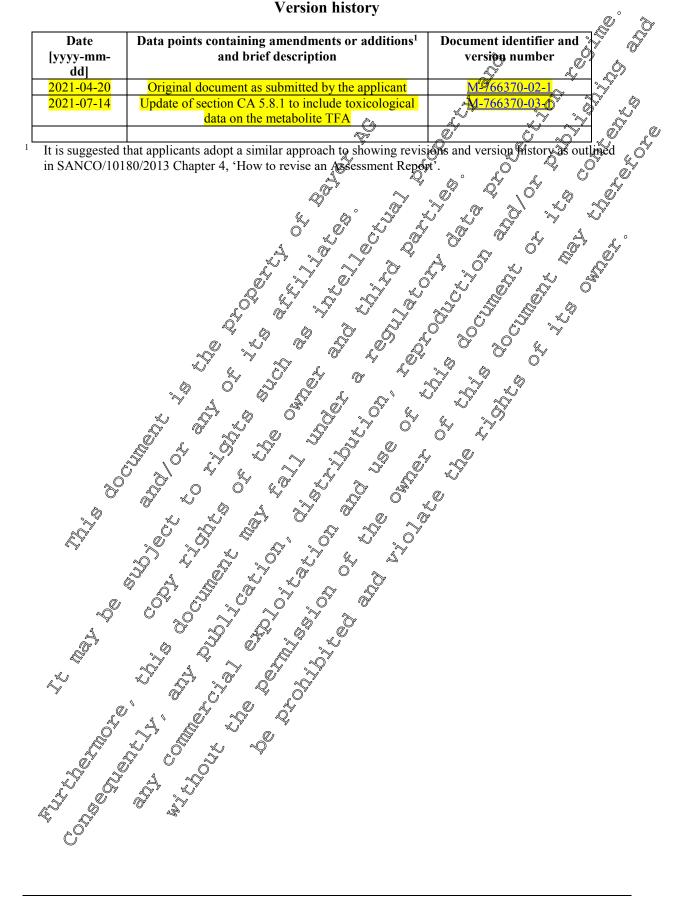




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And a stand of the CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE

This document supports the application for regulatory approval of fluory ram in Europe under Regulation (EC) No 1107/2009. a in European and a second and and a support of the second and a TRONG TO BE THE OWNER OF THE



CA 5.1 Studies on absorption, distribution, metabolism and excretion in mammals

Absorption, distribution, metabolism and excretion by other routes CA 5.1.1

 Studies are summarized in Fluopyram Active Substance, MCA Section 7, Part 1, Toxicology and metabolism studies

 CA 5.2
 Acute toxicity

 Table 5.2-1
 Summary of Fluopyram (AE C656048) acute toxicity data, with Classification of the sector of

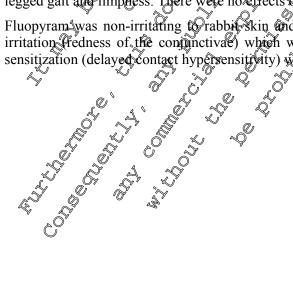
endpoints according to Regulation (EC) No 1272/2008

Study	Result & Reference	Classification according to Reg. (EC)
Acute oral rat	No mortalities observed at 200 2000 mg/kg by	5;
Acute dermal rat	Dermal LD ₅₀ 200 > 2000 mg/g bw 2 2 2 M 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	3; 1 C Loclassified
Acute inhalation rat	$> 5112 \text{ mg/m}^3$ \bigcirc \circ	006 Unclassified
Skin irritation, rabbit	Non-263302-01-	
Eye irritation, rabbit	Non-immating	
Skin sensitization (LLNA)	Noossensitizing 2006; M-2018	45 01-1

All studies were conducted in 2005-2006 and were fully compliant with Good Laboratory Practice (GLP). All tests, aside from range-finding studies, were conducted a accordance with prevailing OECD, EU, USEPA or Oapanese MAFF testing guidelines

The acute toxicity of fluopyram (AE (656948)) was fow for all routes evaluated (oral, dermal and inhalational). The oral ED_{50} was ≥ 2000 mg/kg body weight as no mortality, clinical signs or abnormalities at necropsy were reported. The rat acute definal QD_{50} was > 2000 mg/kg body weight, with no mortality or chinical signs observed. The rat acute inhabition LC_{50} (4-hour) was > 5112 mg/m³, which was the highest achievable concentration and did not cause mortality. Clinical signs (reversible) included bradypnea, labourec breathing, piloerection, ungroomed hair-coat, reduced motility, high legged gait and limpness. There were no effects on body weight.

Fluopyram was non-irritating to rabbit skin and in the rabbit eye caused only very minimal ocular irritation (redness of the conjunctivae) which was reversible within 48 hours. No evidence of skin sensitization (delayed contact hypersensitivity) was seen in a mouse local lymph node test.





CA 5.2.1 Oral

Data Point:	KCA 5.2.1/01
Report Author:	
Report Year:	
Report Title:	AE C656948 - Acute toxicity in the rat after oral administration
Report No:	AT02530
Document No:	<u>M-259398-01-1</u>
Guideline(s) followed in	OECD 423 (2001); EEC 67/548 Agnex V - Method B.1. tris; EPA 40 CER part 5 160: OPPTS 870.1100; MAFF 12 Nousan n° 8658 (Dec. 06, 2090); EPO 712 5
study:	160: OPPTS 870.1100; MAFF 12 Nousan n° 8638 (Dec. 06, 2000); EPO 712-6
Deviations from current	current guideline: Current Gardeline: OECD 423, 2001
test guideline:	Deviations: None
Previous evaluation:	Yes, evaluated and accepted in the DAR 2011
GLP/Officially	Yes, conducted under GLP/Officially becognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes y y y h A O y

Executive Summary

In an acute oral toxicity study using a stepwise proceduce, two roups of three fasted young adult female Wistar rats (HsdCpb:Wu) were given successively a single oral cose of AE Cos6948 (batch number Mix-batch:08528/0002, 94.7% purity) in 2% Cremophor EL of 2000 mg/kg by and were observed for 14 days , Ç 14 days. V C Ô \bigcirc

The dose of 2000 mg/kg bw was tolerated without nortalines, clinical signs, effects on weight gain or The dose of 2000 mg/kg bw was concurred gross pathological findings. The acute oral LD₀ was 22000 mg/kg bw (Unclassified according to Regulation (EC) No. (272/2008).

	AE C656948
1. Test material: 🖄 🔊 🤘	AE C 56948
1. Test material: Description Lot / Batch #2 Purity: CAS # 0	Beige powder
Lot / Batch the	Npx-batch:08528/0002
Purity:	ن 4.7 % مَ [*] مَ [*]
$CAS # \sqrt{2} \sqrt{2} \sqrt{2}$	658006-35-24
	Stable at @ and 200 mg/mL at room temperature for at least 2
Stadingry of test compound:	hours a s
2. Vehicle and / or positive	
control:	² % Oremonitor EL in demineralized water
3. Test animalss	
 Vehicle and / or positive control: Test animalss Species: Strain: Age: Age: Age: Age: Age: Age: Age: Age	Rat Q.
Strain:	Hsd@pb:Wu
Age: Sex Weight at dosing:	10° 12 weeks approximately
Sext 2 2	Female
Weight ab dosing	170 - 195g
Saurool 2 2	170 - 175g
Source Acclimation period:	At least 5 days
	-
Diet:	Provimi Kliba 3883.0.15 Maus/Ratte Haltung, Kaiseraugst
	Switzerland, <i>ad libitum</i>
Water:	Tap water, ad libitum



Housing:	Animals were group caged conventionally in polycarbonate cages on low dust wood granulate bedding		
Environmental conditions:			
Temperature:	$22 \pm 2^{\circ}C$		
Humidity:	55 ± 5%		
Air changes:	$55 \pm 5\%$ Approximately 10 changes per hour	4 . 4	
Photoperiod:	Alternating 12-hour light and dark cycles		

B. Study design and methods

1. In life dates: 14 July - 03 August 2005

2. Animal assignment and treatment

The substance was tested using a stepwise procedure, each step using three female cats. The animals were assigned to their groups by randomization. The random liso was based oneventy distributed chance numbers by a software application. Following an overnight tast (16to 24 hours), each group received a single dose of 2000 mg/kg of AE C656948 (94.7% perity) by gavage. The test substance was administered in demineralized water with 2% Cremophor EL at a volume of 10 mL/kg kw. Cliffical signs and mortality rates were determined several times on the day of administration and subsequently at least once daily for an observation period of at least 14 days. Bedy weights were recorded on days 1, 8 and 15. On day 15, surviving animals were sacrificed and all animals were percopsed and examined for gross pathological changes.

3. Statistics

The data did not warrant statistical analys

Results and discussion II.

A. Mortality

No mortalities occurred at 2000 mg/kg bw, the only dose tested. Details are provided in Table 5_e Ø

Doses, mortality Panimats treated Table 5.2.1-1 C

Dose (mg/kg bw)		Females	
. 20 00 (1 ⁵)	Ŏ ^Ŷ Ŵ ^Ŷ "C	0/3	
2000 (Q nd)		0/3	

B. Clinical observations

0 No clinical signs were observed with the on of an increased water intake observed in 3/6 animals (1st phase) from day 2 to 6

С. **Bod**® weight

bt or body weight gain. There was no toxicological offe

D. Necropsy

No abnormalines were observ at gross nečropsy.

Е. Deficiencies

No deficiencies are noted

onclusion

Under the conditions of this study, the acute oral LD_{50} was > 2000 mg/kg bw (Unclassified according to Regulation (EC) No. 1272/2008).



	guidance and the requirements in 283/2013. Acute toxicity via the oral route $D_{50} > 2000 \text{ mg/kg}$ bw does not trigger classification.
CA 5.2.2 Dern	
Data Point:	KCA 5.2.2/01 (3) (3) (3) (3) (3)
Report Author:	
Report Year:	
Report Title:	AE C656948 - Acute toxicity in the rat after dermal application
Report No:	AT02500
Document No:	<u>M-259275-01-1</u>
Guideline(s) followed in	OECD 402 (1987); EEC 67/548 Annex V- Method B.3; EPA OPPT\$ 870.1200,
study:	$EPA 712-C-98-192 (4998) = 0^{\circ} 0^{\circ} \sqrt{0^{\circ}} \sqrt{0^{\circ}}$
Deviations from current	current guideline QECD @02, 2097
test guideline:	Old procedure: 5' animals/sex/dose treated once rather than testing 3 animals of
	single sex in estepwise approach described @ the with the rse of up to 3 animals
	of a single sex perceiep.
	This deviation has no impact on the outcome of the study and interpretation of the
D 1 /	results Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q
Previous evaluation:	Yes, evaluated and accepted of the IZAR (2611).
GLP/Officially	Yes conducted under GLP/Officiality recognised testing facilities
recognised testing facilities:	
Acceptability/Reliability:	Yes A A A A A A A A A A A A A A A A A A A

Executive Summary

In an acute dermal toxicity study, groups of young adult Wistar rats, 5/sex were exposed by the dermal route to AE 0656948 (batch number Mix-batch 08528,0002, 94.7% purity). The test material was applied for 24 hours to 10% of each animal's body surface at a dose of 2000 mg/kg bw. Animals were observed for 14 days following exposure.

AE C656948 was of very by toxicity after dermal application. There were no clinical signs observed. Body weight and body weight gain were not affected by treatment. On the basis of this study, AE C65694 does not way ant classification for acute dermal toxicity according to Regulation (EC) No. 1272/2008.

12/20	000. v		
`,≯	34 / 11	s and metho	· × 0×
1.	Materials	s and metho	$\operatorname{ds}_{\mathcal{O}_{\mathcal{I}}}$ $\mathcal{A}_{\mathcal{I}}$
A. Ma	nterials 🕅 🔪 🛋		
		õ v	
1. Te	st material:		AE C656948
		- 0	
De	scription 🚊		Beige powder
L	t / Batch #: 🛇	, » _ <u>{</u>	Mix-batch:08528/0002
2ª		² V	
∦Pu	rity	L.S.	94.7%
[®] C A	\$ #		658066-35-4
CA	NOT		
Sta	ibility of test c	compound:	Stable at room temperature for the duration of the study.
	•	1	1



2. Vehicle and / or positive control:

3. Test animals: **Species:** Strain: Sex Age: Weight at dosing: Source: **Acclimation period:**

Diet:

Water:

Housing:

Environmental conditions: Temperature: Humidity: Air changes: **Photoperiod:**

B. Study design and methods

1. In life dates:

14 July 2005

per hour

Alternating 12-hour lig

Rat

HsdCpb:Wu Male and females

At least 5 days

9-13 weeks approximately Males: 228-259g Females: 212

2. Animal assignment and treatment

Animals were assigned by randomization to the test groups. The randomization was based on evenly distributed chance numbers generated for the study by a software application. One day prior to the treatment, the backand flanks of the rats were shorn approximately 10% of the body surface). For each animal, the required amount of pure solid test substance was weighed and transferred to a wet gauzelayer (6.0 cm \$5.0 cm) of a Cutiplast® steril" coated with air fight, Leukoflex®. The gauze strip was placed on the rat's back and secured in place using "Pena® half" corresive stretch tape (8 cm x 23 cm) and additionally covered with Longe biomedical Inc rat jacket. After 24 hours the dressing was removed and the area ensed with tepid water using soap and gently patting the area dry.

Clinical signs and mortality were observed several times on the day of administration and at least daily thereafter for an observation period of at least 14 days. Mortality and nature, duration and intensity of symptoms were recorded individually. The weight gain of the animals was checked weekly. Each animal which died or was killed in moribund condition was weighed, autopsied and examined macroscopically. The surviving animals were secrificed by carbon dioxide at the end of the study and examined macroscopically.

3. Statistics

The data did not warrant statistical analys

Results and discussion II.

Mortahity Α.

Details are provided in Table 5.2.2-1. No mortalities occurred at 2000 mg/kg bw, the only dose tested. a

Dose (mg/kg bw) Males		Females	Combined	
2000	2000 0/5		0/10	

Pure solid test material was transferred to wet gauze-layer

Provimi Kliba 3883.0.15 mate Ratte Haltung, Kaiseraugu Switzerland an libitum

Provimi Know Switzerland *fil libitum* Tap water, *ad libitum* Animals were group caged conventionally in polycarbonate new dust wood granulate bedding



B. Clinical observations

No clinical signs were observed.

C. Local dermal signs

There were no local signs.

D. Body weight

There was no toxicological effect on body weight or body, weight gain.

E. Necropsy

No abnormalities were observed at gross necropsy

F. Deficiencies

No deficiencies are noted.

Ш. Conclusions

the second secon Under the circumstances of this study, the LD50 was considered to be above 2000 mg/kg bw for both males and females. AE C656948 does not warrant classification as deing toxic or grarmful on the basis of its acute dermal toxicity according to Regulation (EC) No. 1272/2008

1 M

Assessment and conclusion by applicant:

Ò Study meets the current guidance and the requirements in 283/2013. Acute toxicity in the dermal route is low in the rats. The LD50 2000 mg/kg bw does not trigger classification

CA 5.2.3 Inha	lation & S S S
CA 5.2.3	
Data Point:	KGA 5.2001 60 2 2 2
Report Author:	
Report Year.	
Report Totle:	AE C656948 - Acute inhabition to secity in rats
Report No:	AJ03464, ~ ~ ~ ~
Document No:	<u>M-283020-01</u>
Guideline(s) followed in	OECE 403; Directive 2/69/EEC, Agnex V, Method B.2.(1992);, US-EPA
study:	ORDTS 870.1300(1998); Japan MarFF Notification no. 12 Nousan-8147 (2000)
Deviations from current	eurrent guideline; OECD/403 (2009)
test guideline:	Deviation: Transitional 2C 50 protocol. This deviation has no impact on the
	outcome of the study and interpretation of the results.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLR/Officially	Aes, conducted@nder @ZP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability.	XXX XX Q
Executive Summary	

In an acute in valation study young adult Wistar rats (5/sex) were exposed by the inhalation route to AE C656948 (batch number Mix-batch:08528/0002, 94.7% purity) in air for 4 hours (nose only) at a concentration of 5112.5 mg/m³. A concurrent control group was exposed to an atmosphere using similar exposure conditions (15 L/min; conditioned dry air). Animals were observed for 14 days following the exposure.



The inhalation LC50 for the males was $> 5112.5 \text{ mg/m}^3$

The inhalation LC50 for the females was $> 5112.5 \text{ mg/m}^3$

The inhalation LC50 for the combined sexes was $> 5112.5 \text{ mg/m}^3$

AE C656948 (solid aerosol) proved to be non-toxic via the inhalation route to rate. No mortality accurred up to the maximum technically attainable concentration. Clinical signs from exposure to AE C656948 were seen to include: bradypnea, laboured breathing patterns, reduced motility, pilo-erection, ungroomed hair-coat, limpness, giddiness, high-legged gait, flaccid paralysis of hindlegs, and mydriasis. These effects were rapidly reversible, and all animals appeared normal by the fourth post-exposure day. Several animals displayed reduced tonus and horizontal grip strength. One female had an impaired righting reflex. Rectal temperature was lowered by approximately 20% in both seves with the treatment. No treatment-related significant effects were noted on body weight evolution. At necropsy, mild discolouration of the lung was observed but this was considered not to be toxicologically relevant. On the basis of this study, AE C656948 does not warrant classification as being barmful or toxic by inhalation according to Regulation (EC) No. 9272/2008.

I. Materials and methods A. Materials 1. Test material: Beige powder Description Lot / Batch #: Mix-batch:0852 **Purity:** 947 CAS# 658066-3 April 2007 at room temperatur Stability of test compound: Stable until 5 2. Vehicle and / or positi aer@olized as dry powder test substanc control: 3. Test animals **Species:** Rat ⊖HsdCrbb: Strain: Age: Approximately 2 ponths 170-1969 (male) or 169-191g (female) Weight at dosing: Source: Acclimation period At least 5 days Provimi Kriba 3883 = NAFAG 9441 pellets maintenance diet Diet: for rats and mise, Kaiseraugst Switzerland, ad libitum Tap water, a libitum Water: Animals were individually caged in conventional Makrolon® Housing: Type IIIP cage Environmental condition Temperature: Humidity: 50@10% At least 0 air exchanges per hour Air changes: 12 hours of light, 12 hours of dark Photoperiod: B. Study design and methods 1. In life dates 08 Way -20 May 2006 2. Adimal assignment and treatment

Animals were assigned to the test groups listed in Table 5.2.3-1. The random list was based on evenly distributed chance numbers especially generated for the study by a software application. Animals were exposed to the aerosolized test substance in Plexiglas exposure tubes applying a directed-flow nose-only exposure principle. Animals were examined carefully several times on the day of exposure and at least



once daily thereafter for 2 weeks. The following reflexes were tested: visual placing response, grip strength on wire mesh, abdominal muscle tone, corneal and pupillary reflexes, pinnal reflex, righting reflex, tail-pinch response, startle reflex with respect to behavioral changes stimulated by sounds (figger snapping) and touch (back). The rectal temperatures were measured shortly after cessation of exposure Individual body weights were recorded before exposure and on days 3, 7 and 14 On day 15, all animals were sacrificed, necropsied and examined for gross pathological changes.

Table 5.2.3-1 Doses and mortality / animals treated

		·	A.	²	
N Group /sex	Target Concentration (mg/m³)	Toxicological Result	Orpset and Duration of Signs	Onset of Mortality	Rectal Temperature (CD
1/m	0	0 / 0 / 5	÷ ~	ĝ° ĝ	38.3
2/m	5000	0 / 5 / 5 🦓	0d - 1¢		36.1
1/f	0	0 / 0 / 5			38.4
2/f	5000	0/5/5	∞ 04 ^C 5d 0		of and so

N = group assignment, m = males, f = females, 0d exposure day Values given in the 'Toxicological results' columnare:

 1^{st} = number of dead animals.

 2^{nd} = number of animals with signs after ceseation of exposure

 3^{rd} = number of animals exposed.

3. Generation of the test atmosphere Champer description

Directed-flow nose-only inhelation chambers (TSE, 61348 Bad flomburg) were used. The method for dust generation employed a "Bayer Generator" system, which gave stable analyzed concentrations in the range of 1500-20000 mg/m³. The test substance concentration was determined by gravimetric analysis. Chamber samples were collected after the equilibrium/concentration/had been attained in hourly intervals. Two samples during each exposure were also taken for the analysis of the particle-size distribution using an Andersen case ade impactor.

The limit concentration of 5000 mg/m³ was attained, however, of the expense of larger particles (no cyclone used) At 5142.5 mg/m³, the Mass Median Aerodynamic Diameter was 5.6 μ m (SD = 2.02 μ m) and 19.0% of the total particulate had an aerosol mass $< 3 \mu m$. The study meets international recommendations and is acceptable.

4. Statistics

vide infr manalyze body weight gain data and rectal temperature A one-way ANC measurements

Results and discussion II.

A. Test atmosphere concentration and particle size analysis

The real-time aerosol monitoring wilter malyses) of the test atmosphere from the breathing zone indicated that the exposure conditions were temporally stable over the 4-h exposure period (for details see Appendix).

Analysis of the aerosol particle-size distribution from the breathing zone samples demonstrates that the aerosol generated was inhable. Internationally recognized recommendations such as of SOT (1992) were not faitfilled in regard to the requirement MMAD < 4 μ m. However, this MMAD was only slightly exceeded and the GSD was within guideline limits. Repeated measurements made during one exposure demonstrated temporally stable particle-size distributions.

Temperature values in the inhalation chamber were in the range suggested by the testing guidelines. Humidit Values were slightly lower due to dry air aerosolization. This deviation from the guideline had no apparent negative impact on the outcome of study.



O

Generation conditions and characterization of chamber atmosphere (Mean values) are presented in Table 5.2.3-2.

Table 5.2.3-2 Mean achieved aerosol concentration, MMAD, GSD, and inhalable fraction (% ¥µm)

for each treatmen	it group	
Parameter	Control group	Tested group
Target conc., mg/m ³	0	5000 ~~~
Gravimetric conc., mg/m ³	-	51425 9 4
Inlet air flow (L/min)	<u>i</u>	
Exhaust air flow (L/min)	¥3 _Q	
Temperature (mean, °C)	چي 22.8 °	21.80. 57 4
Relative humidity (mean, %)	6.2 Q	
MMAD, μm		2° Q, 60 60 67
GSD		
Aerosol mass $<3 \ \mu m$ (%)		19.0 [°]
Mass recovered (mg/m3)		4062.5 A
	A. O A Y A	

B. Mortality

No mortality occurred up to 5112.5 mg/m³, the maximum technically achievable concentration 12.5 mg/m^3 , the maximum technically achievable concentration 12.5 mg/m^3 , for the females was $> 5012.5 \text{ mg/m}^3$, and for the combined sexes was $> 5012.5 \text{ mg/m}^3$.

C. Clinical observations

All rats tolerated the exposure with some evidence of reversible signs. Clinical signs in both sexes exposed to AE C656948 included, brackpnear labored breathing patterns, reduced motility, piloerection, ungroomed hair oat and limphess.

A battery of reflex measurements was made on the first post-exposure day. Reflexes tested were normal in all males whereas one female showed a reduced torus and vertical grip strength together with an impaired righting response. Recta temperature was lowered in both sexes with the treatment.

All clinical signs were fully reversible with 5 days.

D. Body weight

Comparisons between the control and the exposure groups revealed no toxicologically significant changes in body weight in both sexes.

E. Necropsy

There was nothing in particular to report at the negopsy.

F. Deficiencies

No specific deficiencies were noted during the study

III, Conclusions

Under the experimental conditions of this study, AE C656948 proved to have essentially no inhalation toxicity and the LC_{50} was considered to be greater than 5112.5 mg/m³. AE C656948 does not warrant classification as being toxic or harmful on the basis of its acute inhalation toxicity according to Regulation (EC) No. 1272/2008

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013. Acute toxicity via the inhalation route is the inherat. The $LC_{50} > 5112.5 \text{ mg/m}^3$ does not trigger classification.



CA 5.2.4 Skin irritation

Data Point:	KCA 5.2.4/01
Report Author:	
Report Year:	
Report Title:	Acute skin irritation/corrosion on rabbits
Report No:	AT02737
Document No:	<u>M-263302-01-1</u>
Guideline(s) followed in	OECD 404 (2002); EEC Directive 57/548 Annex & - Method B.4 (1967); EPA
study:	40 CFR part 160; 712-C-98-196 (7998); OPPTS 870.2500; MACF 12 Yousan No
	8628 (December 06, 2000) χ
Deviations from current	current guideline: Current Gardeline: OECD 404, 2015
test guideline:	current guideline: Current Guideline: OECD 404, 2015 Deviations: None. Conductor study predates the to vitro creening recommendations.
	recommendations.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2014)
GLP/Officially	Yes, conducted under GLAOOfficially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes Q V X X X X X Y
Executive Summerv	

Executive Summary

In a primary dermal irritation study, 3 young adult New Zealand white female rabbits were exposed via the dermal route to 0.5 g of pullverized test substance (Batch-No. Mix-Batch 08528/0002, 94.7% purity) per animal. In the first step only one animal was used and three patches were applied successively to this animal. The first patch was demoved after three minutes As no serious skin reactions were observed, the second patch was demoved after three minutes As no serious skin reactions were observed, the second patch was demoved after three minutes are not been been added and removed after four hours. The test was completed using two additional animals exposed for four hours. The test substance was applied as a powder moistened with water to the skin of the animal under a gauze patch. The animals were observed for 72 hours.

No erythema, eschar or oedema was observed at any time point

Under the conditions of this study, SE C656948 was not a definal irritant and does not warrant classification as being pritating to the skin according to Regulation (EC) No. 1272/2008.

I. Materials and methods

A. Materials ~Ç 1. Test material: Description mix-Batch:08528/0002 Lot Y Batch #: Purity: 658066₁95-4 ĆAS # Juntil @ May 2007 Stability of test compound: 2. Vehicle and / or positive Aqua p.i to ensure good contact with skin control: 3. Test animals: Rabbit Species: A HsdIf:NZW - females Strain Young adult animals Age: @ Weight at dosing: 2.2 - 2.6 kg Source: **Acclimation period:** At least 5 days



Diet:	Ssniff K-Z" 4mm (manufact 59494 Soest, Germany), ad a	urer: Ssniff Spezialdiaten GmbH, <i>libitum</i>	
Water:	Tap water ad libitum	etall/Norvl by EBECO	(
Housing:	Individually in cage units M		Y
Environmental conditions:			
Temperature:	$20 \pm 3^{\circ}C$		
Humidity:	$50 \pm 25\%$		2
Air changes:	not provided in the report		y
Photoperiod:	12 hours daily 👸		Ā

B. Study design and methods

1. In life dates: 08 -11 November 2005

2. Animal assignment and treatment

One day before the application, the fur was shorn on the right and left side from the dorso-lateral area of the trunk on each animal. Fluopyram (AE C656949, Bat No. 1918-Bach:08528/0002; 94.77 purity) was applied to the skin of the animal under a gauze patch. The test substance (0.5 g) was moistened with Aqua p.i. to ensure good contact with the skin. The treated area was approximately 2.5 cm x 25 cm. The patch was held in place with non-pritating tape for the duration of exposure periods

In a first step, only one animal was used and 3 test patches were applied successively to this animal, for 3 minutes, 1 hour and 4 hours, respectively with observations of reactions offer one time-point before moving to a longer duration of exposure. Then, the test was completed with 2 additional animals.

Dermal irritation was scored approximatel at 1, 24, 48 and 72 hours after patch removal. In the case of an irritation reaction, animals were further monorored for reversibility of the effection day 7 and day 14 (maximum) after patch removal. The degree of erythema/ochar formation and ocdema formation was recorded and scored according to the Draize scheme. Any serious lestons of toxic effects other than dermal irritation were also recorded and described

3. Statistics

O Statistical analysis of the resplts was not required

Results and discussion II.

A. Findings

There was no systemic intolerance reaction to the test substance.

None of the animals showed any irritation reaction throughout the study. The individual values for skin irritation scores are shown in Table 5.2.4-1

Ø Õ Individual values for skin in three rabbits administered AE C656948 for Table 5.2 Ç four hours ~0

Animal	Observation	©24h_C	§ 48h	72h	Mean scores	Response	Reversible (days)
1	Erythema and eschar	Ŵ	0	0	0.0	-	N/A
	Ddema 🖉 🖉	0	0	0	0.0	-	N/A
2	Erythema and eschart	$\bigcirc 0$	0	0	0.0	-	N/A
<u> </u>	Edomía 🗘 🔿	0	0	0	0.0	-	N/A
3	Feythema and eschar	0	0	0	0.0	-	N/A
S.	ZEdema 🔨 💫	0	0	0	0.0	-	N/A
VA. not ap	plicable 🔊						

NA. not applicable

B. Deficiencies

There were no deficiencies in this study.



III. Conclusions

Under the circumstances of this study, it is concluded that fluopyram was not an irritant to the skin according to Regulation (EC) No. 1272/2008.

·				<u> </u>	
	and conclusion by applica			Ş	
	the current guidance and t at fluopyram was not an irr		283/2013. Base	d on those res	wlts, it was
		S S	<u> </u>		
		,	A C A		
CA 5.2.5	Eye irritation				

Data Point:	KCA 5.2.5/01 & & X & X & X
Report Author:	
Report Year:	
Report Title:	AE C656948 - Acute evolution on Pabbits A O K
Report No:	AT02738 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
Document No:	<u>M-263277-Q-1</u>
Guideline(s) followed in	<u>M-263277-64-1</u> OECD 405 (2002); EEC Directive 67/545 Annex V - Method BS. (1967); EPA
study:	40 CFR part 160, 712-5-98-195 (1998) OPP TS 870 2400; MAFF 12 Nousan No
	8628 (December 06, 2000) 5 0 0 0 0
Deviations from current	cuftent guideline: Current Guideline: OE D 405 2020
test guideline:	Deviations: None Conduct of study predates the in vitre screening
	Gecommendations.
Previous evaluation:	Yes avaluated and accepte on the BAR (2011)
X	
GLP/Officially recognised testing facilities:	Yes, conducted finder ChP/Officially recognised testing/facilities
recognised testing	
facilities:	
Acceptability Reliability.	

Executive Summary

In a primary eye irritation study, 01 g of pulverized test substance (Batch-No.: Mix-Batch 8528/0002, 94.7% purity) was placed into the conjunctival sac of one eye of a rabbit after having gently pulled the lower lid away from the eyeball. The other eye, which remained untreated, served as control. Since severe irritation was not observed one hour after treatment two further rabbits were treated as described. Eye irritation was scored and recorded at 3, 24, 48 and 32 hours after application. As no irritation indices were observed after 72 hours, the study was finished.

The degree of ocular losions was recorded as specified by Draize and any serious lesion or toxic effects other than ocular lesions were also recorded. Body weight of each animal was recorded at the beginning of the study.

A slight redness of the conjunctivae was observed after 1 hour in all animals, and at 24 hours in 2 animals (grade 1, having resolved by 2 days post-fleatment). According to the classification criteria AE C656948 was not irrelating to the spe and there were no systemic intolerance reactions.

Materials and methods 1. Test material: Description

AE C656948 Beige powder



Lot / Batch #: Mix-Batch:08528/0002 At least 5 days Standard dief "Ssniff K-Z/ 4mm (manufacturer: Ssniff Spezialdiaten Grabh, 59494 Soest, Germany) ad libitum Municipal tap water ad libitum ndividually in cage units Metall/Noryl by EBECO + 38 + 20% **Purity:** 94.7% CAS# **Stability of test compound:** 2. Vehicle and / or positive control: 3. Test animals: **Species:** Strain: Age: Weight at dosing: Source: **Acclimation period:** Standard dief "Ssniff K-20 4mm (manufactures: Ssniff Spezialdiaten GnibH, 59494 Soest, Germans) ad libitum Municipal tap water ad libitum Individually in case units Metall/Noryl by EBECO $20 \pm 3\%$ Not reported 12 hours thythm Diet: Water: Housing: **Environmental conditions: Temperature: Humidity:** Air changes: **Photoperiod:** B. Study design and methods -13 November 200 1. In life dates: 2. Animal assignment and treatment On the day of treatment, 0.1 g of Ruopycam (AE C656948, Batch No. Mix-Batch:08528/0002; 94.7% purity) was placed into the conjunctival sac of one eve of the first animal. The lids were then gently held together for about 1 second in order to preyend loss of test compound. The eye was not rinsed for at least 24 hours after instillation. The other eye remained untreated and served as control. If no severe irritation was observed 1 hour after the treatment 2 additional rabbits were used. Eye irritation was scored and ecorded approximately 1, 24, 48 and 72 hours post-application. In case of irritation, animals showing the effects were monitored usually on day 7, 14 and 21 (maximum) after application otherwise the study was finished after 72 bours. The degree of ocular lesions was recorded according to Drafze. In addition any serious lesions or toxic effects other than ocular lesions were also recorded and described.

3. Statistics

Statistica analysis of the results was not re

Results and discussion ĮK,

A. Findings

There was no systemic into brance reaction.

of the irritan effects is given in table 5.2.5-1. A summar



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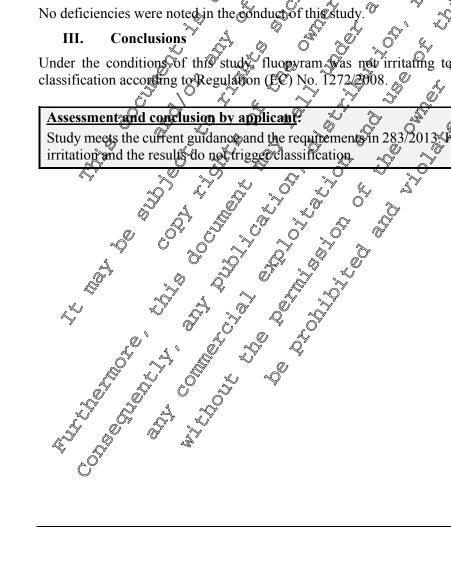
Table 5.2.5-1 Observations in treated eves of three rabbits administered AE C656948 in the conjunctival sac of one eye

	•						
					Mean		Reversible
Animal	Effects	24h	48h	72h	score	Response	(davs)
	Corneal opacity	0	0	0	0.0	<u>s</u>	¢®∕∕A ↔
1	Iritis	0	0	0	0.0	- ⁶ -	N/A S
1	Redness conjunctivae	1	0	0	0.3	.4 -	
	Chemosis conjunctivae	0	0	0	0.0 🕺	∽ - ″	NA V
	Corneal opacity	0	0	\mathcal{O}_0	0.0 0.0	- 🔊	N/A Q
2	Iritis	0	0	<u></u> 0	0.Q	- ~~	N/A N/A
2	Redness conjunctivae	0		0	0.0	, Ö	
	Chemosis conjunctivae	0	Ø	0	Q0.0		NVA ©
	Corneal opacity	0		0 📉		Q' - Q'	©N/A
3	Iritis	0	[≫] 0	。 0	0.0	~~~~``	N/AS
5	Redness conjunctivae	1		<u></u>	Q.3		
	Chemosis conjunctivae	0	<u>م</u> ع	ČÓ	0.0		A A A
Corneal op	Corneal opacity: "-" if mean score 2 3 4 5 Iritis: "-" if mean score 2 3 4 5 5						
Iritis:	"-" if mean score <1	Å.	Y a		ST .		K L
	onjunctivae: "-" if mean socie <1 conjunctivae: "-" if mean socie <1 policable	ore <2.5				Ĵ Û Â	ş O
	conjunctivae: "-" if mean	eore 🍣	S.S.		° , 5		Ď
N/A: not a	pplicable	102			1 0	õ "Š	, K
1*: conside	Chemosis conjunctivae: "-" if mean core of the second seco						4
B. Deficiencies No deficiencies were noted in the conduct of this study.							
III.	Conclusions A				s i		

B. Deficiencies

No deficiencies were noted in the conduct of this study. III. Conclusions Under the conditions of this study, fluopyram was not irritating to the eyes and does not require Ş

Study meets the cuffent guidance and the requirements in 283/2013 Fluopyram does not provoke eye irritation and the results do not trigger classification.





CA 5.2.6 Skin sensitization

Data Point:	KCA 5.2.6/01
Report Author:	
Report Year:	
Report Title:	AE C656948 - Evaluation of potential dermal sensitization in the local hymph
	node assay in the mouse
Report No:	SA 06320
Document No:	<u>M-281845-01-1</u>
Guideline(s) followed in	OECD guideline 429 (2002); 🕅
study:	Equivalent to US EPA OPPT Guideline No, 870.2600
Deviations from current	Current guideline: OECD 429, 2010
test guideline:	Deviation: None.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially	Yes, conducted under GLP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes in a construction of the construction of t

Executive Summary

In a dermal sensitisation study CAE C636948 (Batch No.: Mix-Boch:08528/0002, 940% purity) was topically administered in dissolved DMF solution at concentrations of 0%, 0.5%, 1.0%, 2.5% and 5.0% to groups of 5 female CBA/J mice Animals were treated once daily for 3 consecutive days with 25 μ L of test substance solution at the adequate concentration. On day 5, mice were injected intravenously with 3H-methyl thymidine. Appriculatelymphonodes were then reproved and processed. A stimulation index was calculated by measuring the incorporation of 3H-methyl thymidine with a beta-counter.

Compared with control animals AE C656948 did not induce any changes in stimulation index. On the basis of this study, AF C656948 did not show any sensitization potential in this assay and does not warrant classification as being a definal sensitizer according to Regulation (EC) No. 1272/2008.
 I. Materials and methods
 A. Materials

- A. Materials

1. Test material Description Lot / Baten #:	AE C656948 0 ~
Description	Beige powder S
Lot / Baten #: 2 2	Mix_Batch: 08528/0002
Purity;	94,0% 00 00
CAS	Not stated in this report
Stability of test compound:	Until 04 Max 2007
2. We hicle: $\sqrt{2}$	Dimethylformamide (DMF)
2. Vehicle: 2 A C Positive control: A C	Alpha-Hexylcinnamaldehyde (HCA)
3. Test animats: Species: Strain:	
Species:	Mice (females)
Strain Strain	CBA/J
Age: Age: Age: Age: Age: Age: Age: Age:	Approximately 8 weeks old
Strain: Strain: Source:	Not stated
Source: Source:	R Janvier, 53940 Le Genest-St-Isle, France
Acclimation periods	At least 5 days
	Certified rodent pellet diet: AO4C-10, S.A.F.E. (Scientific
Diet:	Animal Food and Engineering, Route de Saint Bris, Augy,
	France)



Water: Housing:	Municipal tap water, <i>ad libitum</i> Individually in suspended, stainl	ess steel wire-t	nesh cages
Environmental conditions:	individually in suspended, stalli		
Temperature:	20 -24°C		
Humidity:	40-70%	ð	
Air changes:	10-15 exchanges per hour		4 . 4
Photoperiod:	12 hours daily	A	8 29 . Q

B. Study design and methods

1. In life dates: 06 – 15 November 2006

2. Animal assignment and treatment

The test substance fluopyram (AE C656948, batch N° Mix-Batch:08328/0602; 9 \pm 7% purity) was topically applied to the dorsal surface of each ear of mice. Groups of 5 mice/dose were dosed once daily for 3 consecutive days with 25 µL of formulation of fluopyram at concentrations cf 0.5, 1.0, 2.5 and 5% in DMF. These dose levels were chosen on the basis of prefiminary results showing that concentrations of 10, 25, 50 and 100% caused excessive cellular toxicity. Two additional groups received the vehicle (DMF) or alpha-hexylcinnamaldehyde (HCA 25%) to serve as negative and positive controls, respectively.

Animals were checked for mortality and clinical signs at least drily during the study. In particular, the site of application was examined for signs of local irritation. Individual bod weight were also measured at study start and end (scheduled sacrifice).

On day 5, each mouse was placed in a referition box, incravenously injected via the tail vein with 250 μ L of NaCl (0.9%) containing 20 μ Ci of H methyl thymidine and then placed in a plastic cage for 5 hours. Then mice were sacrificed and the 2 auricular lomph nodes were removed. A single cell suspension (free of connective tissue) was obtained by placing the lymph nodes in an individual tube containing physiological saline and crusting them with a plastic piston.

Cell suspensions vere washed with 5 ml of physiological saline, centrifuged and then resuspended in 2 ml of 5% trick broacetic acid (TCA) and stored overnight at 4%. After a final centrifugation, the cell pellets were resuspended in 4 ml of physiological saline and placed in an ultrasonic bath for 25 mins to ensure thoroughly dispersed suspension. Cell suspensions were then added to scintillation pots containing 10 ml of scintillation fluid and assayed in a beta counter to evaluate the incorporation of 3H methyl thymidine. The results were expressed as disintegration per minute (DPM) per node. Stimulation indices (SI) were then calculated by the ratio of DPM in the treated group to DPM in the control group.

SI = DPM of treated group / DPM of Control group

A summary of animal assignment and treatment is provided in the table below.

Group	Test substance		Number of animals groups	Animal identity
1 *	Vehicle (DAF)	× ~~ 0	5	QT1F4121 to 4125
2		0.5	5	QT2F4126 to 4130
3	* AE 6536948	1	5	QT3F4131 to 4135
4		2.5	5	QT4F4136 to 4140
je ko		5	5	OT5F4141 to 4145
\$6 ¢	° H¢x	25	5	QT6F4146 to 4150
	A.			

sjoe S Ĩ . V. п. н. *с с*



Evaluation criteria

A test substance is regarded as a skin sensitizer when one concentration results in a 3-fold or more increase in ³H methyl thymidine incorporation compared to the control in the absence of skin irritation and when a dose-response is observed.

3. Statistics

No statistical analysis was required in this study.

II. **Results and discussion**

A. Findings

No mortality or clinical signs were observed during the study. In particular no cutaneous reactions were observed at the application site. Bodyweight changes were comparable between control and treated groups, of the results of proliferation assay are presented in the table below of table belo

Table 5.2.6-2	Lymph node DPM	values	and Sti	mulation	Indice	5

Test group	OP DP Mode Stimulation index Response
Control (DMF)	581.4 5 00 C 5 5 -
Fluopyram 0.5% in DMF	543 6 543 6 543 6 -
Fluopyram 0.5% in DMF	× 591.6 · · · · · · · · · · · · · · · · · · ·
Fluopyram 0.5% in DMF	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Fluopyram 0.5% in DMF 🥍 🔬	5190 <u>2</u> <u>0.9</u> <u>-</u>
HCA 25% in DMF	<u></u>

There were no confounding effects of irritation of toxicity. The results of the positive control demonstrated the validity of the assay.

ved at all dose levels Negative responses were obser

B. Deficiencies

No deficiencies were hoted

III. Conclusions

Under the conditions of the stody, fluepyrain did not show any skin sensitization potential in the local lymph node as an does for require classification according to Regulation (EC) No. 1272/2008.

Assessment and conclusion by applicant

Study meets the current guidance and the requirements in 283/2013. No sensitizing potential was noted up to the highest concentration and the sesults do not trigger classification.

CA 5.2.7 ototoxicity

According to Regulation (EU) 283/2013 an in vitro phototoxicity study is required when an active substance absorbs electromagnetic radiation in the range of 290-700 nm, and the molar extinction coefficient (c) of the UV VIS absorption maxima (calculated according to OECD TG 101) is above 1000 L 20 mol-1 x cm-1 (according to OECD 432, 2019).

UV-visible characteristics of fluopyram in aqueous solution have been determined and showed



maximum values of molar extinction coefficient on the absorbance at >290 nm in neutral medium 170.55 L x mol⁻¹ x cm⁻¹ (Fluopyram UV/VIS spectra, study submitted under point MCA 2.4, M-653943-01-1)

As the ultraviolet/visible molar extinction/absorption coefficient of fluopyram is $\frac{1}{2}$ s than 1 000 $\frac{1}{2}$ $^{-1}$ × cm $^{-1}$, phototoxicity testing is not required.

CA 5.2.8

Not applicable

CA 5.3

Potentiation/interactions of multiple active substances or products The short-term toxicity studies with fluopyram (AE C656948) were conducted between 2003 and 2008. All subchronic 90-day studies were in accordance with OFCD, EU, USEPA and Japanese M&FF testing guidelines and were fully compliant with GLP. The 28-day studies (in rats, mice and dogs), except for the 28-day study via the dermal route were not performed strictly to compliance with GLP as they were not subjected to QA inspections although the same standardized buting operating procedures applied to GLP studies were used. A summary of these results is presented in Table 5.2.1.

In rats, continuous dietary administration of AE C656948 for 28 days at dietary concentrations of 0, 50, 400, or 3200 ppm for 28 days/was/associated with reductions in body weight/gain of 12-28% in males (weeks 1 and 3) and of 1629% in females (weeks 1, 3 and 4) at the highest dose level of 3200 ppm. Overall the weight gain at 3200 ppm was comparable to controls in males but 14% lower in females. In females only, there was a slight reduction (4-10%) in food consumption throughout the study. At the hematological exampliation, makes had a 30% increase in platel count and a 32% increased prothrombin time compared with controls. At the enrical chemistry evaluation, higher mean total cholesterol and toglycevide concentrations were found in both seves, when compared to the controls, together with sightly lower aspartate aminotransferase and alkaline phosphatase activities. No effects on body weights, hematology or dinical chemistry parameters were observed at 400 ppm or below. The liver was a larget organ with increased weights in both sexes associated with centrilobular hepatocellular hypertrophy in most animals of both sexes. These effects avere mainly seen at 3200 ppm and to a lesser extent at 400 ppm, In addition at the high dose, thyroid gland weights were higher in males with hypertrophy of the follicular cells observed at the microscopic examination in both sexes. In males only, slightly higher kichey meroscopic findings characteristic of hyaline droplet nephropathy were observed at 3200 and 400 ppm, athough this finding known to be fat-specific and not to be relevant for humans.

Regarding hepatotoxicity parameters, AP C655948 caused a dose-related increase in total cytochrome P-450, BROD and PROD activities a 200 and 400 ppm. AE C656948 was therefore considered to be a moderate Phenobarbital-like cytochrome P-450 Inducer.

There were no treatment plated effects noted a 50 ppm in either sex (4.0 and 4.6 mg/kg/d in males and females, respectively) and this dose@was considered to be the No Observed Effect Level (NOEL). However, changes at 400 ppm were limited to adaptive changes in the liver and hyaline droplet nephropathy in the kidness (not relevant for humans). Therefore 400 ppm (31.0/36.1 mg/kg/d in males/females) is considered to be the No Observed Adverse Effect Level (NOAEL) for human risk assessment.

In the 90-day rat study, at the high dose of 3200 ppm, body weight parameters were reduced in both sees, and food consumption was reduced in females for the last two months of treatment. Mean prothroughin time was increased in males, whilst mean platelet and reticulocyte counts were increased in females. Slightly lower mean hemoglobin concentrations were noted in both sexes, in association with lower mean hematocrit in males and lower mean corpuscular volume and mean corpuscular



hemoglobin in females. An increase in mean total cholesterol, y-glutamyltransferase, total protein, globulin, inorganic phosphorus and calcium in both sexes, creatinine and urea in males, and triglycerides in females was observed, whereas a decrease was noted in total bilirubin and chloride in both settes, glucose in males, and alkaline phosphatase and albumin/globulin ratio in females. Urinalysis revealed an increased incidence and severity of cellular casts in males which was observed in connection with hyaline droplet nephropathy noticed at the histological examination of the kidney. An increase in mean TSH levels in both sexes at Week 3, together with an increase in mean T3 and T4 levels in females was observed. At Week 13, only an increase in mean TSH and T3 levels was noted in males. The target organs were the liver and thyroid gland in both sexes and the kidney in males. Mean there weights and thyroid gland weights were increased in both sexes, whilst mean kidner weights were increased in males only. Microscopically, in the liver, minimal to moderate centrilobular hepatocellitar hypertrophy was observed in both sexes, together with minimal to moderate perportal to midzonal hepatocellular macrovacuolation in females. In the thyroid glang minimal to slight diffuse hypertrophy of follicitar cells was seen in the majority of males and in 1/10 females. In the kidney, the incidence and sevenity of hyaline droplet nephropathy related to the accumulation of Qu-globulin in the proximal tubules and the incidence of hyaline casts were increased in males, However, hyaline droplet nephropathy is considered not to be toxicologically relevant to man as a2µ-globulin is present only in trace amounts in humans.

After 1 month of recovery in the high dose group, mean body weight was still reduced in both sexes. Affected hematology, clinical chemistry and urinalysis parameters showed some reversibility. At necropsy, mean absolute and relative kidney weights were still increased in males compared to the controls. Microscopic examination showed that hyaline droples neptropaths and hyaline casts persisted in some animals. All other affected parameters were found to be reversible after month of recovery.

At 1000 ppm, there was a decrease of 15% in body weight gain during the first week of treatment and food consumption was decreased by between 4% and 9% on most occasions, in temales, compared to controls. Lower mean hemoglobin concentrations and mean hematocrit were noted in males. Mean total cholesterol concentration was increased in both sexes, whilst creating, inorganic phosphorus and calcium were increased in males, and total bilirubin decreased in both sexes and chloride decreased in males. Urinalysis devealed an increased incidence and severity of cellular casts in males. Mean TSH levels were increased in males. Microscopically, in the liver, minimal to slight centrilobular hepatocellular hypertrophy was observed in both sexes, together with minimal periportal to midzonal hepatocellular macrovacuolation in females. In the toyroid gland, prinimal to slight diffuse hypertrophy of follierlar cells was seen in both sexes of the kidney, the incidence and severity of hyaline droplet nephropathy and the incidence of byaline casts were increased in males.

At 200 ppm, treatment related changes only consisted of adaptive changes in the liver. In addition in males, a slight increase in incidence and severity of cellular casts was observed in the urine, which was associated with the presence of hyaline droples nephropathy at the microscopic examination of the kidney, but these changes were considered not to be toxicologically relevant to man.

At 50 ppm, treatment related changes only consisted of a slight increase in incidence and severity of cellular casts in the urine in males. This finding was considered not to be toxicologically relevant to man.

The NOAEL in males was 50 ppm (equating to 3.06 mg/kg body weight/day) and the NOEL in females was 200 ppm (equating to 4.6 mg/kg body weight/day). However, since the effects observed at 200 ppm in males were limited to adaptive changes in the liver and hyaline droplet nephropathy with associated effects in the urine the relevant NOAEL for human risk assessment is considered to be 200 ppm, equivalent to 12.5/14.6 mg/kg/d in males/females).

The mouse was less sensitive than the rat to short-term exposure to AE C656948. In the 28-day study, mice were given AE C656948 in the diet at concentrations of 0, 150, 1000, or 5000 ppm. All of the males and 3/5 females at the high dose were killed for humane reasons between days 17 and 27. Clinical signs included reduced motor activity, hunched posture, piloerection, wasted appearance, coldness to



touch, abnormal respiration and distended abdomen, together with a body weight loss and reduced food consumption. In the two surviving females, one had a distended abdomen on days 8 to 10, both had elevated total cholesterol and total protein concentrations, and alanine aminotransferase activities, together with markedly increased liver weights of between 132% and 147%. Red liquid was observed in the thoracic cavity of all males at necropsy. Microscopically, treatment-related effects were seen in the adrenal glands, liver, lungs, spleen, thymus and thyroid gland. The majority of decedent animals had areas of hemorrhaging in the thoracic cavity, thyroid gland, lungs and thymus. An increased incidence and severity of extramedullary hematopoiesis in the spleen was considered, to be a reactive response to the intrathoracic hemorrhaging in these animals. Lesions observed in the adrenal glands and decreased cellularity of the thymic cortex were consistent with a stress response rather than a direct response to treatment. Findings in the liver consisted of hypertrophy of hepatocytes, hepatocyt bile duct/oval cell hyperplasia, focal necrosis, single cell hepatocellular necrosis and centulobular degeneration/necrosis. At 1000 ppm, body weight gain was slightly degreased in males during Week 2. Liver weights were increased by between 27% and 49% in both sexes, Microscopically, hypertrophy of the zona fasciculata was seen in the adrenal stands in 3/5 tomales. In the liver, shanges were seen in both sexes and included hypertrophy of centrilobular heparocytes single hepatocellular necrosits, focal necrosis, hepatocellular eosinophilia and bile daet/oval cell hyperplasia. Af 150 ppm, only adaptive changes were observed in the liver; liver weights were increased by between \$5% and 21% in both sexes and microscopically, minimal to slight hypertrophy of centrilobiliar hepatocytes was observed in the liver in all males and 2/5 females. In isolation, the liver findings were considered to be associated with adaptive changes rather than evidence of toxicity.

A dose level of 5000 ppm clearly exceeded the Maximum Tolerated Dose (MTD) due to the overt toxicity noted. The NOAEL of AE C656948 in both sexes was 450 ppm (equating to 24.7 mg/kg body weight/day in males and 31 mg/kg body weight/day in females).

In the 90-day mouse study, treatment-related indings observed at the high dos of 1000 ppm included an increase in food consumption (up to 12%) in males. Higher mean alanine aminotransferase activity, lower mean alburgen concentration in both sexes together with lower mean total cholesterol concentration, higher mean alkaline phosphatase activity and a tendency towards higher mean aspartate aminotransferate activity in males, was observed. Mean liver weights were increased by between 34% to 45% in both sexes and mean adrenal weights were increased by between 87% to 92% in males. Microscopically, minimal to moderate hyperrophy of centrilobular hepatocytes in the liver was observed in all animals together with a greater incidence of minimal focal necrosis in 3/10 males and minimative slight focal necrosis in 6/10 tomales in the adress glands, a lower incidence of ceroid pigment was noted in makes, whilst a greater incidence of minimal to slight cortical vacuolation was observed in females. At 150 ppm, mean liver weights were increased by 9% to 16% in males and 25% to 28% in females, in correlation with a minunal to hight hypertrophy of centrilobular hepatocytes noted in all males and 5/10 females at the microscopic examination. These effects were considered to be adaptive but not to be adverse. A fewer arean total cholesterol concentration was seen in males, however, as the charge was a decrease and in the absence of any other findings at this dose level apart from the adaptive changes in the liver, this finding was considered to be non-adverse. At 30 ppm, no adverse effects related to the treatment wefe observed

The NOAEL in both sexes was escablished at 150 ppm (equating to 26.6 and 32.0 mg/kg body weight/day in males and females, respectively).

The dog appeared to be less sensitive than the rat but of similar sensitivity to the mouse to short-term exposure of AFC656948. As in rats and mice, the liver was identified as the main target organ. When administered by gavage to Beagle dogs (2/sex/dose) at a dose level of 750 mg/kg/day for at least 28 days fiquid teces were noted on a few occasions in 1 male and 1 female. The 2 male dogs showed a low erythrocyte count, low hemoglobin and low hematocrit in comparison to their respective pre-test values. At clinical chemistry evaluation, 1 male and 1 female had a high alkaline phosphatase activity and a low albumin concentration (as a consequence, a low albumin/globulin ratio). In addition, the same female also had a high δ -glutamyltransferase activity and triglyceride concentration. The liver was the only



identified target organ in both sexes illustrated by an increase weight mainly associated with hepatocellular hypertrophy. No treatment-related findings were observed at 150 or 30 mg/kg/day.

The NOEL over 28-days was 150 mg/kg/day.

In the subchronic toxicity study, technical grade AE C656948 was administered in the diet to Beagle dogs (4/sex/dose) at dose levels of 0, 800, 5000, and 20000/10000 ppm for at feast 13 weeks. Due to a lack of palatability of AE C656948 in the diet, a marked reduction in food consumption was observed at the high dose in both sexes with an associated effect on body weight gains and body weights. Despite the reduction of the dietary level of the test compound from 20000 to 10000 ppm after 2 weeks of treatment, food consumption and body weight remained lower than controls throughout the study. Changes were also noted in hematology parameters (platelet count, crythrocyte counts and hemoplobic concentration) and some clinical chemistry parameters (mainly dkaline phosphatase and δ -glutamy transferase activities and bilirubin, albumin and protein concentrations).

The liver was the main target organ as shown by an ancreased weight together with the macroscopic observation "enlarged" in association with histopathological effects (hepatocellular hypertrophy, intracytoplasmic eosinophilic droplets and single will necrossis).

The thymus was found to be generally atrophic and associated with a higher severity of thymic involution compared to controls. The stroug cycle was also disturbed in females (all females were in anoestrus phase). However, these effects on the thymus and estrons cycle were attributed to the marked decrease in food consumption and ody weight at this dose level.

At 5000 ppm, although less prenounced than at the high dose, esimilar pattern of effects was seen including effects on food consumption, body weight, clinical chemistry and in the liver.

At 800 ppm, there was no beatment-related adverse effect. This level was considered to be the NOAEL for this study which was equivalent to 28.5/32.9 mg/kg/day for males/females, respectively.

In a third study in this species, technical grade AE C656948 was administered in the diet to Beagle dogs (4/sex/dose) at dose levels of 0, 100, 400 and 2000 ppm for a least 52 weeks. This study confirmed the liver as the main target organ in dogs. As in the 90-day study, the boor paratability of the compound in the diet was also confirmed. At 2000 ppm there was an initial body weight loss during the first week of treatment in both series concomitant to lower food consumption. Thereafter, food consumption and body weight were comparable to controls in males whereas food consumption remained slightly lower than controls in females which resulted in an overall 10% reduction in food consumption in this sex. The liver was the target organ. Although no change in liver, weights was observed in either sex, histopathology revealed centrilobular hepatocellular hopertrophy in 3/4 males. In addition, an increase in circulating alkaline phosphagese (A&P) was also seen in both sexes at this dose level.

At 400 and 100 ppm no treatment related adverse effects were observed. Therefore the NOAEL for this study was 400 ppm (equivalent to 13.2/14.4 mg/kg/day for males/females).

Dermal route

Technical grade AE C656948 was administered by topical (dermal) application to male and female Wistar rats five days/week for at least four weeks by applying the test substance to a commercially available adhesive bandage that had been moistened with deionized water. The dose groups for this study were 0 control proup 100, 300, and 1000 mg/kg/day. The dose was based on each animal's body weight on days 0, 2, 14, 25, and 28.

Compound-related findings were only observed at 1000 mg/kg/day and consisted in an increased cholesterol concentration in females, an increased prothrombin time in males and effects on the liver (increased liver weights for males and females associated with hepatic hypertrophy). The increased liver weights and hypertrophy in the high-dose group were attributed to hepatic enzyme induction and thus were considered to be an adaptive response to AE C656948.

No compound-related effects were observed at 100 or 300 mg/kg/day.



In conclusion, the NOAEL for dermal application over 28 days was 300 mg/kg/day.

Study	NOEL/	NOEL/	LOAEL	LOAEL	Main findings 🔊	Reference
Doses tested:	NOAEL	NOAEL	ppm	mg/kg	Č,	Ű S
opm or	ppm	mg/kg		bw/d	1 and	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
mg/kg bw/d		bw/d			-	
GLP					X	<u>, , , , , , , , , , , , , , , , , , , </u>
28-day rat	400*	31.0/36.1*	3200	254/26	↓ body weight gain	2004 0 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
study,	(M/F)	(M/F)	(M/F)	(M/F))	(M/F) , \uparrow platelet count	
0, 50, 400,				A.	and protoombin time	<u>M-089510-0421</u>
3200 ppm				A.	(M), [†] Cholesterol and ^O triglyceridesconc. [•]	
0 1 0 21 0					(M)F), ↓aspartate	
0, 4.0, 31.0, 254 M			,	× °		
, 4.6, 36.1,			×		alkaline phosphatase	
263 F			0		↑ liver weight with	
203 F			A		hepatocelkular	
			\mathcal{L}		hypertrophy (M/F)	
			O in	×, ×	thypoid weight with	ã Õ
		Ć		, \$	follignalar cel	
		A A	Ø		hypertropoy (M)	Š. 4. Š
		~~	Q Q	Č,	follioalar cel hypertrophy (M) Sidney nyaline	, 😽
				- A	Urople hephropathy	<u> </u>
28-day mouse	150	24.7/31.1	1000	162/197	Mortality at \$000 ppm	
study,	(M/F)	(M/F)	(\$\$#/F)	∑ (M/fØ)	(M/F), ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	2004
0, 150, 1000,	4	γ	\$~_\$~	Å.	J body weight gain	<u>M-088486-01-1</u>
5000 ppm	×1	A C			$(M) \downarrow ALT (M),$	
0 24 7 162	S	or L	an .		↑ liver weights, 27	
0, 24./, 102, 747 M		L . 5	~~~ ^		49% (M/P) associated	
/4/ IVI 0 31 1 107		pʻʻʻxʻ	\sim		hyperwophy, single	
0, 51.1, 177, 051 F	0°				celland focal necrosis,	
////			s .		hepatocellular	
Ó.	*0 . /	<u>_</u>	AN		eosinophilia and bile	
	Ĉ		Q'		duct/oyal cell	
je starter and the second seco	. Ű				hyperplasia (M/F).,	
·	~^~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		, 0× ~	× &	Adrenal gland:	
		Ũ	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	O' A	hypertrophy of zona	
				S - S	fasciculata (F)	
28-day dog	$p n.aO^{*}$	6 ¹⁵⁰	n@`	0. 750.0	\downarrow erythrocyte count,	2004
study,		QM/F		(Mgg)	hemoglobin, (M)	<u>M-242097-01-1</u>
0, 30, 150 <u>, 1</u>	Ro	J.		×,	hematocrit (M), ↑ alkaline phosphatase	
ng/kg/ðav					(M/F) , \downarrow albumin conc.	
sing/Kg/duy ≪∥	, S	AN	Ő X	¥	(M/F) , $\uparrow \delta$ -	
No.	\sim		Q 29	č	glutamyltransferase	
					activity and	
0	× 1	l d' ŝŜ	- Qi		triglyceride	
, C			Q		concentration (F).	
L'		p* "×	~Q		↑ liver weights	
	Û,	\sim			associated with	
J A	Ž A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~			hypertrophy (M) Adney, hyaline diropho hephropathy Mortality, at \$000 ppm (M/T), ↓ body, weight sam (M) ↑ ALT (M), ↑ liver weights, 27 49% (M/T) associated with : centrilobalar hypertrophy, single cell and focal necrosis, h¢atocel alar cosinoparlia and bile duct oval cell hypertrophy of zona fasciculata (F) ↓ erythrocyte count, hematocrit (M), ↑ alkaline phosphatase (M/F), ↓ albumin conc. (M/F), ↑ δ- glutamyltransferase activity and triglyceride concentration (F). ↑ liver weights associated with hepatocellular hypertrophy (M/F).	
× 0,					hypertrophy (M/F).	



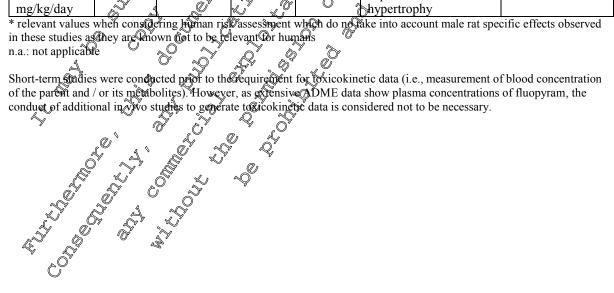
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Study.	NOFL /	NOFL/	LOAEL	LOAFI	Main findings	Deference
Study Doses tested:	NOEL/ NOAEL	NOEL/ NOAEL	DOAEL ppm	LOAEL mg/kg	Main findings	Reference
ppm or	ррт	mg/kg	ppm	bw/d		° >
mg/kg bw/d		bw/d				
GLP					<u>~</u>	
90-day rat	200*	12.5/14.6*	1000	60.5/70.1	↓ body weight & food	
study, 0, 50, 200,	(M/F)	(M/F)	(M/F)	(M/F)	consumption, <i>of</i> hematology parameters	
1000, 3200					affected cholesterol	<u>IVI-2-0940-04-1</u>
ppm				Č V	conc & δ-	2005 <u>M-259946-6071</u> ¹ ¹ ¹ ¹ ¹ ¹ ¹ ¹ ¹ ¹
				- Va		
0, 3.06, 12.5,				a s	activity TSH, T3 & T4.	
60.5, 204 M				A	Target organs were	
0, 3.63, 14.6, 70.1, 230 F			4		inver anominitrono giano	
70.1, 2501			K,	, Ø		
			0″		Miver, (hyroid grand &	L A co
			A.	σ _v ũ	kidnov weights.	
					Liver: centrilobular hypertrophy &	
			°, in		periportal to undzonal	
		Ć		S 5	hepatocellutar	
		Q,Y	0' 20 20		hepatocellutar macrovactolation Dhyroid gland	
		<i>Q</i> 1	LÍ (VÍ	S.	follice Qar cell	<i>4</i> .
		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		10° - K	hypertroph Kidney:	Ó [×]
				\$ O	hyaline droplet	b
	~		6 S	<u> </u>	nephropathy. 🔨 🔬	8
90-day mouse	150	26.6432.0	1000		ALT & J albumin	2011
study,	(M/H)	ANT/F) L	(M/F)	(M/F)	cono (M/F) ↓ · · · · · · · · · · · · · · · · · ·	2011 M-251136-01-1
1000 ppm	, S	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	s s		alkaline phospleatase	<u>IVI-231130-01-1</u>
PP					(M), Hiver, weights,	
0, 5.4, 26.6, 🛛			K.	N D	34,45% (M/F), ↑	
188 M	Ø	N D	.4 .		adrenal gland weights,	
0, 6.8, 32.0,			<u>o</u> r or	.~~ ~~	%7-92% (M) Liver centrilobular	
210 F	. C			Ô V	hypertrophy and	
, v			, Ô ^y , K		focal necrosis (M/F)	
		Ŭ ĮŬ	j z z		hypertrophy and focal necrosis (M/F) Adrenal gland: ↓ ceroid pigment (M), ↑ cortical vacuolation (F)	
	a. R			S S	ceroid pigment (M), ↑	
				× _×	cortical vacuolation (F)	
A		°° _° °	A S			
Q [*]	, Ô	Q, (	U ja			
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~			~Q`		
N N N N N N N N N N N N N N N N N N N	*©*		Q N	2		
	a,`		v A			
	§ A	je S	, _N			
Į,	, "N".	Å. 4.,	~			
Ő	ŝ č	ĭ Ď	У			
~~~ /	P A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~				
E S	чU' А	\$″				
Î Î						
90-day mouse study, 0, 30, 150, 1000 ppm 0, 5.4, 26.6, 188 M 0, 6.8, 32, 0 216 F						



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Study	NOEL/	NOEL/	LOAEL	LOAEL	Main findings	Reference
Doses tested:	NOAEL	NOAEL	ppm	mg/kg	8	
ppm or	ppm	mg/kg	11	bw/d		, CU°
mg/kg bw/d	r r	bw/d				
GLP						~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
90-day dog	800	28.5/32.9	5000	171/184	↓ body weight & food	
study,	(M/F)	(M/F)	(M/F)	(M/F)	consumption,	2006
0, 800, 5000,	(141/1)	(101/1)	(101/1)	(11/1)	hematology parameters	M-236047-6021
20000/10000					affected at high dose	
ppm				Ĉŝ	only. ↑ alkaline	
ppin				- T	phosphatase δ-	
0, 28.5, 171,				L	glutamy@ansferase ⊀	2006 <u>M-27-0047-6071</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u>
332 M				a U V	activity, ALT & AST,	
0, 32.9, 184,				A A	$\downarrow$ albumin conc and	Å
0, 32.9, 184, 337 /F			4	Ŕ	bilirubin.	
JJ / / F				́ до°	bluidubins Diver weights associated with hen the cellulo	
			Ň	Ű, ž	associated with	e 4
			4	K C	hepgocellular	
				$\sim$	hypertrophy S	
			$\mathcal{L}$		intracytoriasmic	
			V in		heparocellular hypertrophy, nutracytoplasmie eosinophilic aroplets and single cell	
		ć	V V	, Q , Y	and single coll	
		S.	°	ing' w	necrosis. Both serves	
		Q.		Č,	necrosis. Both serves	
1 year dog	400	13,294.4 %	2000	67,6/66.1	bod weight & food	2007
study,	(M/F)		[♥] (MAE)	(M/F)	consumption $(M/F)$ , $\uparrow$	M-294279-01-1
0, 100, 400,	()			~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	alkaline phosphatase	
2000 ppm	0	Ø O	6 C	<i>.</i>	(M/F), Liver: 🛇 🐇	
		YA.	A B	NO É	centrilobular 🗸 🖓	
0, 3.0, 13.2,	$\sim$			jo in	hypertrophy, >>	
67.6 M				S L		
<i>0, 3.8, 14.4,</i>						
66.1 F		Υ _λ γ,		N A		
<b>UU.1</b>	ŏ ò			XX ~.		
Doroutor	nfa.			1060 (M/F)	AQualactori (E) A	
Percutaneous 28-day rodent	nvær.	· ≈ 300	A ^{n.a.}		$\uparrow$ cholesterol (F), $\uparrow$	A : 2007
		(MI/¥Q) ≈	Ø ^r O	(M/F)	Oprothrombin time (M)	A.; 2007
(rat) study,	. @		× ~. ^	Ô ^y V	↑ liver weights	<u>M-293833-01-1</u>
0, 100, <b>3</b> 00,	, Š	ŠÝ D			associated with	
1000	, P	× 5	N M		hepatocellular	
mg/kg/day			V V	Ĺ	hypertrophy	





# CA 5.3.1 Oral 28-day study

Data Point:	KCA 5.3.1/01
Report Author:	
Report Year:	
Report Title:	AE C656948 - Exploratory 28-day toxicity study in the ray by dietary of administration
Report No:	SA 03332
Document No:	<u>M-085510-01-1</u>
Guideline(s) followed in	Not applicable
study:	
Deviations from current	Current guideline: not applicable
test guideline:	
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially	No, not conducted under GID/Officially recognised esting facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes y y y y y y y

# **Executive Summary**

AE C656948 (batch No: FLH, 999; 98.6% purity) was administered to Wostar rats at dietary concentrations of 0, 50, 400, of 3200 ppm for 28 days, corresponding to 0, 4.0, 3P.0, 254 mg/kg bw/day in males and 0, 4.6, 36.1, 263 mg/kg bw/day in females

Males and females given the highest dose experienced reductions in body weight gain of 12-28% in males (weeks 1 and 3) and 16.29% internales (weeks 1, 3 and 4). Overall the weight gain at 3200 ppm was comparable to controls in males but 14% lower in females. In temales only, there was a slight reduction (4-10%) in food consumption broughout the study. At the hematological examination, males had a 30% increase in platelet count and a 32% increased pothronion time compared with controls. In clinical chemistry, higher mean total chelesterol and triglycerise concentrations were found in both sexes, when compared to the controls together with slightly lower aspartate aminotransferase and alkaline physphatase activities. The liver was a target organ with increased weights in both sexes associated with centril bular depatocellular hypertrophy in most animals of both sexes. These effects were mainly seen at 3200 ppm and to a lesser extent at 400 ppm. In addition at the high dose, thyroid glands weights were higher in males with hypertrophy of the follicular cells observed at the microscopic examination. In males only, slightly higher kidney microscopic findings characteristic of hyaline droplet nephropathy were observed at 3200 and 400 ppm although this finding is known to be rat-specific and not to be relevant for humans.

Regarding Repatotoxicity parameters *R*E C656948 caused a dose-related increase in total cytochrome P-450, BROD and PROD activities at 400 and 3200 ppm. It was therefore considered as a moderate Phenebarbital-like cytochrome P-450 inducer.

There were no treatment related effects (NOEL) noted at 50 ppm in either sex (4.0 and 4.6 mg/kg/day in males and females respectively) Flowever, changes at 400 ppm were limited to adaptive changes in the liver and ryaline dropler nephropath in the kidneys (not relevant for humans), therefore 400 ppm (31.0/36.1 prg/kg bw/d in males females) is considered to be NOAEL for human risk assessment.

Materials and methods

1. Test material: Description Lot / Batch #:

AE C656948 Pale yellow powder FLH 999



98.6% **Purity:** CAS# 658066-35-4 Stable in rodent diet for a period covering the study duration **Stability of test compound:** 2. Vehicle and / or positive none control: 3. Test animals: **Species:** Rat Wistar Rj: WI (IOPS HAN) Strain: 7 weeks approximately at start of dosing Age: 255 -283 g (males); 171-193g (females) Weight at dosing: Source: **Acclimation period:** 7-8 days Certified rodent powdered and irradiated Bet AQ4C-10B1 from Jern, **Diet:** S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), and libitum n suspended stainless-steel Water: Tap water, ad Hbitum, Rats were housed indivi **Housing:** mesh cages **Environmental conditions: Temperature:** 40-70% **Humidity:** 10J15 air@hanges per bour Air changes: 12 hours light, 12 hours dark **Photoperiod:** B. Study design 10 December 2003 January 2003 1. In life dates: 2. Animal assignment and treatment ¢ a n

There were 5 animals of each sex per dose group. Animals were assigned to dose groups randomly by body weight. AB C656948 was administered in the piet for 28 days to Wistar rats at the following doses – 0, 50, 400 and 3200 ppm (equating to 40, 310 and 294 mg/kg/dag in males and 4.6, 36.1 and 263 mg/kg/dag in females). A negative control group received plain dict. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health NIH publication N 86-23, revised 1985) and "Le Guide du Journal Officiel des Communautes Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986"

# 3. Diet prepagation and analysis

AE C656948 was incorporated into the diet by fry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. The stability was demonstrated during the course of the study at concentrations of 20 and 10000 ppm for a time which covered the period of usage and storage for the study. AE C656948 concentrations were verified to be within 95-105% of nominal. Homogeneity at the lowest and highest dietary concentrations were within the range 99-115%.

	2 Concentration	<b>Dose per animal</b>	(study averages)	Animals	assigned
	in diet (ppm)	Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
		0	0	5	5
	50	4.0	4.6	5	5
$\int$	400	31.0	36.1	5	5
4	3200	254	263	5	5

Table 5.3.1-1 Study design and group sizes



# 4. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous, a Kruskal-Wallis ANOVA was performed followed by the Dunn test if the Kruskal-Wallis was significant. For some parameters, when data were not homogenous, they were transformed (log transformation or square root transformation), then reanalyzed as above. When one or more group variance(s) equaled 0, means were compared fusing non-parametric procedures. Group means were compared at the 5% and 1% levels of significance Statistical analyses were carried out using Path/Tox System V4.2.2. (Medule Enhanced Statistics), *O* 

# C. Methods

# 1. Observations

The animals were observed twice daily for morjbundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature onset severity, reversibility, and duration of any clinical signs were recorded

# 2. Body weight

Body weights were recorded three times during the acclimatization phase. In the first day of test substance administration, then at weekly intervals throughout the treatment periods Diet-fasted animals were weighed before necropsy.

# 3. Food consumption and compound in take

Food consumption was recorded weekly the weekly mean achieved dosage intake for each week and for weeks 1 to 4 was calculated for each sex

# 4. Ophthalmoscopic examination

Ophthalmoscopic amination was not conducted.

# 5. Clinical chemistry

On study day 29, prfor to necropsy, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital vendes plexus. Animals were diet-fasted overnight prior to blood sampling and anesthetized by inhalation of Isoflurane. Blood was collected on EDTA for hematology, on lithium heparin for plasma and clot activator for serum for elinical chemistry and on sodium citrate for coagulation parameters.

Ø

The following hematology parameters were assauld using a Advia 120 (Bayer Diagnostics, Puteaux, France): red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular bemoglobin, concentration, reticulocyte count, white blood cell count and differential count evaluation and plateter count. A blood smear of Wright's stain was examined when results of Advia 120 determinations were abnormal. Prothrombin time was assayed on an ACL 3000 (Instrumentation Laboratory, Paris France).

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glueose, urea, creatinine, total cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase activities, total protein and albumin concentrations were assayed on serum ample susing an Hitachi 911 (Roche Diagnostics, Meylan, France).

6. Urinalysis Urmalysis was not conducted.



# 7. Sacrifice and pathology

On study day 29 a complete necropsy was performed on all surviving animals. Animals were deeply anesthetized by Isoflurane inhalation, then exsanguinated before necropsy. All animals were diet-tested prior to scheduled sacrifice. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopicalby. Adrenal gland, brain, kidney, liver, ovary, spleen, testis, thyroid gland (with@parathyroid_gland). weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

Tissues samples were fixed by immersion in neutral buffered 10% formalin with the ception testes which were fixed in Davidson's fixative.

The following tissues were sampled: adrenal gland, brain, liver, kidney, lung, overy, piturtary, testis, and thyroid (including parathyroid).

Histopathological examinations were performed on all tissues from all the animals in the control and high dose groups. The liver, kidney, lung, pituitary gland and thyroid gland, were also examined in all animals in the intermediate doses in the study.

# 8. Hepatotoxicity testing

At final necropsy, the remaining portions of the liver from all surviving animals were homogenized for microsomal preparations in order to determine total cytochrome 19450 content and specific octochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) to check the hepatotoxic potential of the test substance. Besults were compared to those generated with well known reference compounds.

### II. Results and discussion

# A. Clinical signs and mortality

# 1. Clinical signs of foxicity"

sions evident in any group. There were no climical

# 2. Mortality

There was no mortality in any group.

# Bock weight and body weight gain

Body weight gain was reduced at \$200 ppm in both serves, by \$2-28% in males (week 1 and 3) and by 16-29% in females (weeks 1, 3 and 4), (However, overall body weight gain was comparable to controls in males, whereas it was 14% power in females. Body weight was unaffected at 400 or 50 ppm in either sex.

Ø

 $\bigcirc$ 

# C. Food consumption and compound intake

At 3200 ppm, food intake was reduced by 440 10% in females throughout the study compared to control values, there was no impact on food consumption in either sex at dietary concentrations of 400 or 50 ppm.

The achieved doses in the study were 0, 4.6236.1, and 263 mg/kg/day for females, and 0, 4.0, 31.0, and 254 mg/kg/d@ in males.

# D. Ophthalmoscopic examination

Ophthalmoscopic examination was not conducted in this study.

# E. Hematology, clinical chemistry, and urinalysis

# 1. Hematology



Hematological examination showed an increase of 30% in platelets and of 34% in prothrombin time in males at 3200 ppm.

### Changes in hematology parameters in the 28-day rat study with AE C656948 Table 5.3.1-2 SD)

52)			Q [°]	e s
Dosage level (ppm)	0	50	400 🔊	3200 . 5
		Males	.1	
Platelet count $(10^9 / 1)$	$1183\pm193$	$1331 \pm 105$	134 150	\$1,539 ± 126** ≪
Prothrombin time (s)	$13.4 \pm 1.5$	14.0 ± 007	$150 \pm 0.6$	₩18.0₩2.9*
*: p≤0.05; **: p≤0.01		"Y"	Q.	
		al a	0×	

# 2. Clinical Chemistry

Clinical chemistry findings included higher total cholesterol and trigly ceride conceptrations together with slightly lower aspartate aminotransferase and alkaline phophatase activities in both series

No relevant changes were observed at 400 or SO ppp in either sex

### Table 5.3.1-3 Changes in clinical chemistry parameters in the 28-day rate udy with AE C $(mean \pm SD)$ Ø

(				A O
Dosage level (ppm)	<u>رې ۵ کې ا</u>	<u>\$</u>	r <u></u> 400 [°]	ال ا
	<u> </u>	Males		Š, V
Total cholesterol (mmol/L)	$1.45 \pm 0.07$	2 1.50 ± 0.280	1.52×9.28	$2.64 \pm 0.52 **$
Triglycerides (mmol/L)	0.81 \$ 0.24	$0.083 \pm 0.025$	$0.76 \pm 0.23$	2.01 ± 0.38**
Aspartate aminotransferase (IU/L)	\$6 ± 23 °	\$ 80 <del>4</del> 14	×67 ± 50	$63 \pm 7*$
Alkaline phosphatase (IU/b)	√ 127 ± <b>%</b> 6	× 143±13	124 11	$84 \pm 10$
×.		Females . O		
Total cholesterol (mmol/L)	$0^{\circ}$ 1.90 ± 0.19,	\$2.09 <b>≭</b> Ø.38 [©]	$2321 \pm 0.32$	$3.50 \pm 0.47 **$
Triglycerides (mpol/L) 🛴	$0040 \pm 0.03$	∞ 0.47¥ 0.10⊘	$0.40 \pm 0.05$	$1.01 \pm 0.41$ **
Aspartate [®] O [®] aminotransferase (IU <del>B</del> )	√ 76±7 √	$70\pm4$	Q 74 ± 16	63 ± 5
Alkaline phosphatase (U/L)	90°±25 ≶	\$ 86 <del>5</del> 21 \$	@95 ± 24	$54 \pm 8*$
*: p≤0.05; **: p≤0.01				

# 3. Urinativsis

Urinalysis was not conducted in

### F. Sacrifice and pathology

# 1. Terminal body weights and organ weight

There were no changes in terminal body weights at any dose in either sex. Liver weights were increased in the 400 and 3200 prom doso groups in both sexes compared with controls, the effect being slightly more pronounced in females. At 3200 ppm the increase was more than 50% compared to controls. This increased weight was associated with managed and dark livers at macroscopic examination and with minimal to moderate centrilobular hepatocellular hypertrophy in most animals in both sexes. Similar effects were observed at 400 ppm, however with lower magnitude, incidence and severity.

In addition to the liver effects, the thyroid gland weights (absolute and relative) were higher in males at 3200 ppm, in association, hyperrophy of the follicular cells was observed in 3/5 males. Thyroid gland weight was not affected in tomales although 2/5 animals showed a minimal follicular cell hypertrophy.

In makes, increased adney weights were also seen at 3200 and 400 ppm in association with microscopic changes of hyaline droplet nephropathy (basophilic tubules, hyaline droplets in the proximal tubule and granular@ast in the medulla). This typical nephropathy is a well know lesion occurring in male rat kidney and which is linked to accumulation of  $\alpha 2\mu$ -globulin in the proximal tubule. This protein is naturally reabsorbed and accumulated in the renal tubular epithelium of young rats. The accumulation is



dependent upon the interaction between a chemical and the  $\alpha 2\mu$ -globulin specifically in the rat. As humans secrete only trace amounts of this globulin, this mechanism is generally accepted as being nonrelevant for humans. Therefore, although this effect was considered treatment-related in the rat f is considered not to be relevant to man.

Dosage level (ppm)	0	50	400	3200
Dosage level (ppill)	U		4002	<u></u>
		Males		
Liver weight	$11.13 \pm 0.93$	$11.18 \pm 0.93$	$12.4 \pm 1.15$	Ŭ7.1 ₩ 1.76* \$54%}\$
Absolute (g)	11110 0190	(+0.4%)		
Bodyweight-relative	$2.864 \pm 0.210$	2.811 0.137	@.055 ± 0.176	$4,401 \pm 0$ $239$ **
body weight-relative	2.004 ± 0.210	Q=2%)	(*****) Q	(+54%) ^{(*}
During and the	5(7042 + 57254	565.078 ± 48.426	$607.382 \pm 500971$	878.09¥±86.733
Brain-relative	$567.042 \pm 57.354$	× (0%) ~	∠ (+7%)	(+55%)
Kidney weight		2.49 ± 0.18	3.16±9.24**	3.13 ±€20** €°
Absolute (g)	$2.63 \pm 0.09$	°~~ (-5%)~~~~	(420%)	(+0%)
	4	°~0.627 ≠0.053 ~	0077±0.052	0.801 ± 0.094
Bodyweight-relative	$0.676 \pm 0.046$	(\$%) ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	(+18%)	(+18%)
	_0× ×	125.616 ± 7.214	¥54.282 ± 9.52 ₽**	£§1.284 <b>₽</b> 9.292**
Brain-relative	133.726 ± 6.952	(-6%)	Q+15%	č ( <del>*</del> 21%)
		$0.0497 \pm 0$		(\ <u>\</u> 2176) \(0.0252 ±
Thyroid weight	~0.0176¥	0.0023	\$ \$222 ± 0.0026	$0.0232 \pm 0.0033 **$
Absolute (g)	^س 0.0040 ک	(+12%)	~ ( <del>*2</del> 6%) Q	© (+43%)
%			×01005 (19) ×	
Dedunisht valation	$0.00456 \pm$	$0.00499 \pm 0.00499 \pm 0.00499 \pm 0.00499 \pm 0.004999 \pm 0.00499 \pm 0.00499 \pm 0.004999 \pm 0.004999 \pm 0.00499 \pm 0.004999 \pm 0.0049999 \pm 0.0049999 \pm 0.004999 \pm 0.004999 \pm 0.004999 \pm 0.0049999 \pm 0.0049999 \pm 0.0049999 \pm 0.0049999999999999999999999999999999999$	₩.00548£ %_0.00086 . Ô	$0.00653 \pm 0.00109**$
Bodyweight-relative	50.001 8	0 030083 0 .0+9%k		(+41%)
				``´´´
	0.89067 ±	$0.99568 \pm 0.99568$	$1.08461 \pm$	$1.29779 \pm$
Brain-relative	<b>\$0.18060</b>		(+22%)	0.20244**
			as a second s	(+46%)
	× ×	Females		
Liver weight 🔬	$554 \pm 0.36$	© 6.24 ± 0.32 @	6.76±0.46	$9.64 \pm 1.00 **$
Absolute (g)		\$\$7%) \$\$	(+16%)	(+65%)
Bodyweight-relative	≰ 2.665¥ 0.200	2.773 ± 0.064	3.072 ± 0.151	$4.599 \pm 0.349 **$
		(+46)	s (+15%)	(+73%)
		330.241, ± 27,788	$384.924 \pm 27.400$	550.595 ± 63.589**
Brain-relative	315044 + 91.373	Q+4%)	(+21%)	(+74%)

	Organ weight changes in the 28-day rat study with AE C656948 (mean $\pm$ SD)
Table 5.3.1-4	Organ weight changes in the 28 day ratistudy with AF $C(55604)$ (mean $\pm$ SD)
1 abic 3.3.1-4	Organ weight changes in the 20-day rat study with AE C030240 (mean ± SDS)

*: p≤0.05; **: p20.01; Figures in Parentheses are odifferences from control No other treatment-related effects were observed in other organs.

-Q

# 2. Liver enzyme induction

°~ 1 Changes that were observed are described in Table 5.3.1-5. A dose-related slight increase in total cytochrome P-450, BROD and PROPactiveres was observed in both sexes at 3200 and 400 ppm. No significant effects were observed at 50 ppm. Given the hepatic enzymatic profile exhibited by AE C656948, it was considered to be a moderate Phenobarbital-like cytochrome P-450 inducer.



Table 5.3.1-5: Results of the hepatotoxicity testing in the 28-day rat study with AE C656948 (mean ± SD )
Tuble 5.5.1 5. Results of the nepatotoxicity testing in the 26 day fat study with TEL (650) to (incar = 55)

Dosage level (ppm)	0	50	400	3200 @"°
		Males		
Total P-450	$1.19 \pm 0.19$	$1.24 \pm 0.25$	$1.43 \pm 0.11$	$1.63 \pm 0.25\%$
(nmol/mg prot.)	$1.19 \pm 0.19$	(-)	(+20%)	× (+37%)
BROD	$8.90 \pm 1.00$	$9.86 \pm 2.89$	71 ± 20.66	171.82 <b>£</b> ∕29.Q2√
(pmol/min/mg prot.)	0.90 ± 1.00	(-)	(+698%)	(+1831%)
EROD	$59.24 \pm 6.94$	$46.80 \pm 4.14$	$66.78 \pm 2.26$	7,9,93 ± 8,92 4
(pmol/min prot.)	J9.24 ± 0.94	(-) 🖏	(-)	L' (2) 2 2
PROD	$6.56 \pm 1.30$	$4.68 \pm 1.00\%$	29.54 11.80	68.31 6.05
(pmol/min/mg prot.)	$0.50 \pm 1.50$	(-) 🔍	$(+ \mathfrak{W}))$	<u>~ (+941%)</u> ~~ 0
		Females		
Total P-450	$0.85 \pm 0.07$	0.89 ± 0.01	$0.98 \pm 923$ Q ²	$0.13$ $1.20 \pm 0.13$
(nmol/mg prot.)	$0.03 \pm 0.07$	~~~	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	(+A) %)
BROD	$2.79 \pm 0.54$	\$4,32 ± 0663		86.35×± 37.92
(pmol/min/mg prot)	2.79 ± 0.34	° (P č	¥842%¥	× (+3002%) .
EROD	52.16 ± 3.31	54.40± 3.92∅	& .57 ± 8.61	87.90 ± 3.33 (=
(pmol/min prot.)	52.10 ± 5.51		$\sim$ $(-)$ $(-)^{v}$	
PROD	2.81 ± 0.5	°3/.49±@64 .	$1202 \pm 4.87$	45 68 ± 24 ≠ 3
(pmol/min/mg prot.)	j v s		<u> (</u> +360%)	<u>7</u> -152 <u>6</u> %)
No statistical analysis performed	d. 🛴 🦷	$a^{y} \sim a^{y} \sim a^{y}$		

(%) as compared to control

(*o) as compared to control
(-) no relevant change
G. Deficiencies
No specific deficiencies were noted in the study.
III. Conclusions
III. Conclusions
III. Study was 50 ppm (4.0 and 4.6 mg/kg/day for males and females, respectively). However, changes at 400 ppm were kimited to adaptive changes in the liver and hvaline respectively). However, changes at 400 ppm were kimited to adaptive changes in the liver and hyaline droplet nephropathy in the kidneys (not relevant for humans), therefore 400 ppm (31.0/36.1 mg/kg bw/d in males/females) is considered to be NQAEL for human risk assessment.

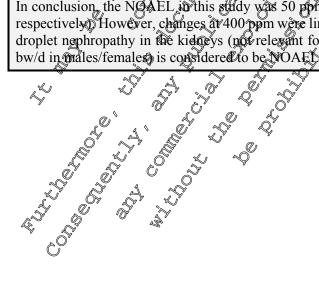
# Assessment and conclusion by applicant:

The study is acceptable as it provides preliminary information on the effects and target organs of AE Car. C656948 in the rat. O K)

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In conclusion, the NGAEL in this study was 50 pm (4 frand 4.6 mg/kg/day for males and females, respectively However, changes at 400 ppm were limited to adaptive changes in the liver and hyaline droplet nephropathy in the kidneys (nor relevant for numans), therefore 400 ppm (31.0/36.1 mg/kg bw/d in males/females) is considered to be NOAEL for human risk assessment





Data Point:	KCA 5.3.1/02
Report Author:	
Report Year:	
Report Title:	AE C656948 - Preliminary 28-day toxicity study in the mouse by dietary
	administration
Report No:	SA 04013
Document No:	<u>M-088486-01-1</u>
Guideline(s) followed in	Not applicable
study:	
Deviations from current	Current guideline: not applicable 2
test guideline:	
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially	No, not conducted under LP/Officially recognized testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes y y y y y y

### Executive Summary

AE C656948, (batch number FLH 5046, 994% purity) was administered via the dier to separate groups of C57BL/6J mice (5/sex/group) at dose avels & 0, 150, 1000 and 5000 ppm (equivalent to 0, 24.7, 162 and 747 mg/kg body weight/day in males and 0, 39.1, 197 and 954 mg/kg body weight in females, respectively), for at least 28 days.

At 5000 ppm, all males and 3/5 females were scorificed for humane to asons between study Days 17 and 27. Clinical signs in these decedent animals included reduced motor activity, hunched posture, piloerection, wasted appearance, coldness to tooch, abnormaDrespiration and distended abdomen, together with a loss in body weight and reduced food consumption. Red liquid was observed in the thoracic cavity of al males. Microscopically, hypertrophy, vacuolation and degeneration/necrosis of the zona fasciculate were seen in the actenal glands in all animals rogether with perivascular and intraalveolar hemorhage and degeneration/inframmation of pulmonary geins in the lungs and erythroid extramedulary hematopoiesis in the spleen. Focal honorrhage was seen in the thyroid gland in 3/5 males and decreased cellularity of the cortex and focal tomorrhage were seen in the thymus in all animals where examination was possible. In the liver, hyperbophy of hepatocytes, hepatocellular eosinophilia, bile diot/oval cell hyperplasa, focal necrosis and single cell hepatocellular necrosis were seen in all animals, and centrilogular degeneration/necrosis in 1/5 males. In the two surviving females, distended abdomen warnoted between study Days 8 and 19 in one animal. Higher total cholesterol and total protein concentrations as well as higher alanine aminotransferase activities were observed for both females. Mean absolute and repative fiver weights were increased by between 132% and 147%. Microscopically, changes were seen in the advenal grand and liver in both animals which were consistent with those observed in decedent animals.

At 1000 ppm, there were no mortalities of clinical signs. There was a slight decrease in the body weight gain during study Week 2 in mates. Mean absolute and relative liver weights were increased by between 27% and 49% to both sexes. Microscopically, hypertrophy of the zona fasciculata was seen in the adrenal glands in 3/5 females. In the liver, hypertrophy of centrilobular hepatocytes was observed in all animals in both sexes, single hepatocellular necrosis was seen in all males, focal necrosis was noted in 3/5 males and 2/5 females, and hepatocellular eosinophilia and bile duct/oval cell hyperplasia were seen in 1/5 females. Microscopically, hypertrophy of centrilobular hepatocytes was observed in all 21% in both sexes. Microscopically, notice and relative liver weights were increased by between 15% and 21% in both sexes. Microscopically, notice and relative liver findings were considered to be associated with adaptive changes rather than evidence of toxicity.



At 150 ppm, there were no adverse findings. Mean absolute and relative liver weights were increased by between 15% and 21% in both sexes. Microscopically, minimal to slight hypertrophy of centrilobular hepatocytes was decreased in the liver in all males and 2/5 females. In isolation, the liver findings were considered to be associated with adaptive changes rather than evidence of toxicity.

A dose level of 5000 ppm clearly exceeded the Maximum Tolerated Dose (ATD) due to the overt toxicity noted, whilst a dose level of 150 ppm represented the NOAEL of AB C656948 in both sexes (equating to 24.7 mg/kg body weight/day in males and 31.1 mg/kg body weight/day in females)

I. Materials and methods A. Materials 1. Test material: AE C656948 Beige powde Description Lot / Batch #: FLH 1046 **Purity:** 99.4% © osoure -35-40 Stable in rodent diet at 20 and 10000 ppin after 95 days frozen storage and 10 days at room temperature. CAS# **Stability of test compound:** 2. Vehicle and / or positive control: 3. Test animals: **Species:** Strain: C57BA 6J Age: Approximately 6 weeks at start of treatment 18.7 -21 6 g (mates), 151-18.3 g (feroales) Weight at dosing: Source: A, Acclimation period O ĭ13 d&ys Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E Scientific Animal God and Engineering, Epinay-sur-**Diet:** Orger Prancey ad libitum Municipat tap water, ad libitum Water: Appimals Were caged individually in suspended stainless steel Housing: wire mesh cages. Environmental conditions Temperature 20**°-2**4°C Humidity: 5Quir changes per hour Air changes: -hour light and dark cycles (7 am- 7 pm) **Photoperiod:** B. Study design 35 February -97 In Jife dates: 1. 2. Animal assignment and meatment

There were 5 animals per poup. Animal were randomly assigned to treatment groups by body weight, and were even AL C656948 in the diet at concentrations of 0, 150, 1000, or 5000 ppm for 28 days. Animal housing and husband were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals Public Health Service, National Institute of Health, NIH publication N°86-23, Devised 1985) and "De Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".



A

### 3. Diet preparation and analysis

AE C656948 was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. The formulated diet was stored at approximately  $-18^{\circ}$ C when not in use. AE C556948 concentrations were shown in a previous study (M-085510-01-1) to be stable at 20 and 1000 ppm for 95 days frozen followed by 10 days at room temperature.

le 5.3.1-6 Study	design and achieved doses	de la companya de la comp	
Test group	Achieved dose (mg/kg/day)	Q Animals	assigned x
	Males		Q O A
Control	0		5 4 . 4
150	24.7 🖉		
1000	162		5 7 1
5000	747*		
	Éemales ?	× A Ô ×	
Control			5
150			5. ⁰ . ¹
1000			
5000	Q × 9578 S Q		5 🔬
culated for Weeks 1 to 3 of	only the to mortality or early sacrifice in this s	group.	$\bigcirc^{\nu}$

### 4. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dumett's test of parameters showing a significant effect by ANOVA. When the data were not homogeneous, a Kruskal-Wallis ANOVA was performed follower by the Dum'r's test if the Kruskal-Wallis was significant. For some parameters, when data were not homogeneous, they were transformed using a log transformation, then reanalyzed as above. When one or more group variance(s) equated 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

### C. Methods

### 1. Observations

The animals were observed twice daily for noribundity and mortality (once daily on weekends or public holidays). Observed elinical signs were recorded at least once daily for all animals and detailed physical examinations were performed an east weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were vecorded. Cages and cage trays were inspected daily for signs of adverse effects, such a blood or loos feces.

### 2. Body weight

Body weights were recorded three times during the acclimatization phase, on the first day of test substance administration, then at weekly intervals throughout the treatment period. Diet-fasted animals were weighed before necessary.

### 3. Food consumption and compound intake

Food consumption was recorded weekly; any spillage was noted, and the weekly mean achieved dosage intake for each week and for weeks 1 to 4 was calculated.

### 4. Ophthalmoscopic examination

Ophthalmoscopic examination was not conducted in this study.



### 5. Clinical pathology: Hematology and clinical chemistry

On study Day 29, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane. Blood (0.6 ml) was collected on lithium heparin for plasma chemistry determinations.

Clinical chemistry: Plasma samples were analysed for total bilirubin, urea, total protein, abumin total Meylan, cholesterol, AST, ALT, and alkaline phosphatase on a Hitachi 911 (Roche Diagnostics France). 

### 6. Urinalysis

Urinalysis was not conducted.

### 7. Sacrifice and pathology

On study Day 29 a complete necropsy was performed on all surviving anothals. Animals were deeply anesthetized by Isoflurane inhalation. All animals were fasted prior to scorduled sacrifice. Macroscopic abnormalities were recorded, sampled and examined incroscopically. Adrenal gland, bron, kidney, liver, spleen, testis and ovary were weighed fresh af scheduled sacrifice only Paired organs were weighed together. Ľ

The following tissues were sampled Adrenal gland, liver, kidney, lung ovary, spleen restistand thyroid gland (including parathyroid). Tissue samples were rixed by impersion in neutral buffered 10% formalin with the exception of the testis which was fixed in Day alson's fixative. These tissues were embedded in paraffin wax, and histological examinations were performed for all tissues in the control and high dose groups. Histologica examinations were performed on the adrenal gland and spleen, on all animals in all dose groups. 

or V

### II. Results and discussion

### A. Clinical signs and mortality

### 1. Clinical signs of toxicity

Clinical signs in the decedent animals comprised reduced moto activity, hunched posture, piloerection, wasted appearance and/or coldness to touch in both sexes together with labored respiration in 3/5 males and distended abdomer in 2/2 remales. These clinical signs were noted mainly on the day of sacrifice or for a few days prior to sacrifice A loss of body weight accompanied these signs, as did reduced food intake. In the surviving females at 5000 ppm, distended abdomen was noted between study Days 8 and 10 in one animal

There were no clinical signs in either the 1000 paper or 150 ppm dose groups.

### 2. Mortality

de la companya de la comp All males and 3/5 females at 3000 ppm were humanely sacrificed between study Days 17 and 27. No mortalities occurred in the other dose groups.

### B. Body weight and body weight gain

At 5000 ppm in the surviving females, mean body weight and mean body weight change parameters were comparable to the control values

There was a slight decrease in the daily mean body weight gain during study Week 2 in males at 1000 ppm (% Ø3 g/day vs. 0 g/day in the control group, not statistically significant).

Norreatment-related effect on mean body weight and mean body weight change parameters was noted at 1000 ppm in females or 150 ppm in either sex.



### C. Food consumption and compound intake

No effect on mean food consumption was noted in animals surviving to terminal sacrifice.

Achieved doses are shown in Table.5.3.1-6.

### D. Hematology, clinical chemistry, and urinalysis

### 1. Hematology

No hematology parameters were measured

### 2. Clinical Chemistry

The two surviving females at 5000 ppm, had elevated for al cholester (+118%) and total protein  $O^{1}16\%$  (+384%).

At 1000 ppm, mean alanine aminotransferase activity was higher in males (+259%, not statistically significant) and mean albumin concentration was lower in temales (-12%  $p \le 0.05$ ). Lower mean total bilirubin and albumin concentrations were noted in frales (56%)  $p \le 0.05$  and >10%  $p \le 0.01$ , respectively, compared to controls). These changes were considered not to be toxicologically relevant as the individual values were within historical control ranges.

At 150 ppm, lower mean total bilirubar and albumin concentrations were noted in males (-50 and -5%, respectively,  $p \le 0.05$  compared to controls). These changes were considered not to be toxicologically relevant as the individual values were within historical controls range.

### E. Sacrifice and pathology

### 1. Terminal body weight and organ weights 🖉

Mean absolute and relative liver weights were higher at 5000 ppm in females and at 1000 and 150 ppm in both sexes. These changes were found to be dose-related. The magnitude of the changes relative to the controls and statistical significance between treated and control values were as follows:

Due to the low number of values obtained (only two surviving animals), the statistical analysisperformed on female Group 4 was considered not to be relevant.

			•	
Sex 2		N O W M	alles	
Dietary level (ppm)		× 150	× 1000	5000
Terminal body wt	19 8±0.9 ×	. ⊘ 19.2⊖0.9 🔬	19.2±0.8	а
Liver wt, g	\$0±0,40	0.94*±0.05	$1.13^{+}\pm0.08$	а
Liver to body 🐲, %	€¥.052±9.495 O	_4®96±0.10°1	5.905 ⁺ ±0.223	а
Liver to brain $\mathbb{W}$ t, % $\mathbb{O}$	Q75.232±18.260	24 F.332 - 12.975	261.966 ⁺ ±15.650	а
Sex A		م 🖉 Fen	nales	
Dietary evel (ppm)	Q 0 g	× 150	1000	5000
Terminal body wt, g	15.1¥1.5	\$\$15.0±0.3	16.4±0.8	15.9±0.5
Liver wt, g	0.68 ± 0.05	~©*0.79 ± 0.05	$0.94 \pm 0.12$ **	$1.66 \pm 0.12 \#$
Liver to body wt, %	$4 502 \pm 0.163$	0.294*	$5.712 \pm 0.594 **$	$10.435 \pm 0.43 \#$
Liver to brain \$4, %	101.379 11.017	185.388 ± 14.792	214.903 ± 35.597**	398.576 ± 8.592#
*: p<0.05: ** p<0.04				

Table 5.3.1-🖗	veight and absolute and	

 $a = no surgiving apprials \hat{C}$ 

#: the statistical significance was not calculated as there were only two surviving animals.

### 2. Gross and microscopic pathology

All males and 3/5 females dosed at 5000 ppm were killed for humane reasons prior to the scheduled termination date. A pale pancreas was observed in all males and in 2/3 females. Rounded borders were observed in the liver in 3/5 males and 1/3 females. Dark livers were observed in 4/5 males and in all



females, and enlarged livers were observed in 1/5 males and 2/3 females. The size of the thymus was clearly reduced in 4/5 males and 1/3 females, and distended abdomen was noted in 3/5 males. Red liquid was observed in the thoracic cavity in all males.

Enlarged livers were observed in both surviving females at 5000 ppm and in all males and 4/5 females at 1000 ppm. Dark livers were observed in 1/2 surviving females at 5000 ppm and in 3/5 males and 2/5 females at 1000 ppm.

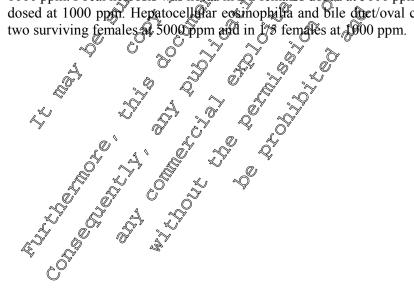
In the decedent animals at 5000 ppm, treatment-related effects were seen in the adrena glands liver, lungs, spleen, thymus and thyroid gland. Hypertrophy, vacuolation and degeneration necrosis of the zona fasciculata were seen in the adrenal glands in all animals, together with perivascular and intraalveolar hemorrhage and degeneration/inflammation of pulmonary veins in the tungs and ersthroid extramedullary hematopoiesis in the spleen. Focal themorrhage was seen in the thyroid gland in 3% males and decreased cellularity of the cortex and focal hemorrhage were seen in the thynus in all animals where examination was possible. In the liver, hypertrophy of hepatocytes (mannly centrilobular), hepatocellular eosinophilia, bile duct/oval cell hyperplasia, focal necrosis and single hepatocellular necrosis were seen in all animals, and centrilobular degeneration/necrosis in 1/5 males

It was considered that premature sacrifice in all mates and in 1/3 female dosed at 5000 ppm was associated with intrathoracic hemorrhage. The majority of decedent mimals had areas of hemorrhage in the thoracic cavity, thyroid gland, lungs and thymus. Hemorrhaging into the thoracic cavity (and other tissues) was considered to have contributed to the clinical condition of moribuid animals. Hence, the increase in the incidence and severary of extramedullar hematopoies in the spleen mostlikely represents a reactive response to intrathoracic hemorrhaging in moribund animals. The lesions in the adrenal glands and decreased cellularity of the thymic cortex are consistent with stress as a non-specific reaction rather than a direct effect of treatment.

In animals surviving to terminal sacrifice, effects of reatment with AE C656948 were seen in the liver in both sexes and in the adrenal glands in females only.

Hypertrophy of the zona fasciculata was seen in the adrenal grands in the two surviving females at 5000 ppm and in 3/5 females at 1000 ppm. This effect was considered to be treatment-related, but most likely resulting from an adaptive change.

Hypertrophy of centrilobular hepatocytes was seen in the liver of both females at 5000 ppm, in all animals at 1000 ppm and in all males and 2/5 females at 500 ppm, with evidence of a dose-response. Single cell hepatocell flar necrosis was seen in 1/2 females dosed at 5000 ppm and in all males dosed at 1000 ppm. Focal necrosis was noted in 2/2 females dosed at 5000 ppm and in 3/5 males and 2/5 females dosed at 1000 ppm. Hepatocell flar ecsinophila and bile doet/oval cell hyperplasia were noted in the two surviving females at 5000 ppm and in 1/3 females at 1000 ppm.





Sex		Ma	ales			Fen	nales	a,°	
Dietary level (ppm)	0	150	1000	5000	0	150	1000	5000	, P
Liver, N examined	5	5	5	0*	5	5	5	2* 4	de la compañía de la comp
Hypertrophy, hepatocellular, centrilobular - Minimal	0	3	0	-		2	4Ç		
Hypertrophy, hepatocellular, centrilobular - Slight	0	2	0	- 🖌	1 0	0	\$ ^{\$4} .		Ņ
Hypertrophy, hepatocellular, centrilobular - Moderate	0		5		0	Ø			Ļ
Total	0	£, 5	5	.0 <u>¥</u>	0	K) 2	ð ⁵ 5	S 2 @	°.
Necrosis, hepatocellular, single cell - Minimal	0,1	0	5 🖉	۶ - ،	0	0	S 0 ≷	1.0	¥
Necrosis, focal - Minimal	0000	0	3	Ę,	Q v	6	2	Ø.≯	
Necrosis, focal - Slight	~@	_0	₽Ø	م م			×.0	Ĩ	
Total	0		^ک 3	× - ×	<b>ي 0 ک</b>	0	≫ 2	[∞] 2	
Eosinophilia, hepatocellular - Minimal		<u>0</u> 0	0	-~~	00	0 🏑	14	, 2 _e •	
Hyperplasia, bile ducts / oval cells - Minimal,	<u>`</u>	~®	6Rø	4-	, Or	Ø	, de la companya de l	a y	
* 0/2 survivals in males/females, respectively	$\sim$	$\sim$	ð	\$ ·	, O'	2		S.	

Table 5.3.1-8 Incidence of microscopic changes in the liver.

Hypertrophy of centrilobular hepatocyces is associated with the proliferation of sub-cellular organelles, although their identity cannot be confirmed in hematoxylin and cosin sections. The change is predominantly centrilobular but appears to be more diffuse in a few animals doset at 5000 ppm. This finding in isolation is associated with adaptive changes rather than demonstrating evidence of toxicity and is consistent with an increase in liver weights at hecropsy.

Necrosis and eosinophilia of hepatocytes and tocal necrosis represent some degree of toxicity and hyperplasia of bile ducts/oval cells was considered to have occurred as a reactive change adjacent to areas of necrosis.

### F. Deficiencies

No specific deficiencies were ported in this study.

III. Conclusions "

A dose level of 5000 ppm clearly exceeded the MTD due to the over toxicity noted, whilst a dose level of 150 ppm represented the WOAEL of AE C656948 in both exes (equating to 24.7 mg/kg body weight day in females).

### Assessment and conclusion by applicant;

The study is seceptable as it provides prefining information on the effects and target organs of AE C656948 in the mouse.

A dose havel of 5000 ppm clearly exceeded the MPD due to the overt toxicity noted, whilst a dose level of 150 ppm represented the NOARC of AC C656948 in both sexes (equating to 24.7 mg/kg body weight/day in males and 3101 mg/kg body weight/day in females).

The stand 3 to mg kg box



Data Point:	KCA 5.3.1/03	
Report Author:		
Report Year:	2004	ð
Report Title:	AE C656948 - Preliminary 28-day toxicity study in the dog by gavage	<u>S</u>
Report No:	SA 04049	0"
Document No:	<u>M-242097-01-1</u>	
Guideline(s) followed in	Not applicable	
study:	A OY XY I	Ŷ
Deviations from current	Current guideline: not applicable	,
test guideline:		Ø
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011) yes	Ő,
		Ý
GLP/Officially	No, not conducted under GEP/Officially recognised testing/facilities	
recognised testing	No, not conducted under GEP/Officially recognised testing/facilities	
facilities:		
Acceptability/Reliability:	Yes O Q Y Y A R A	

### **Executive Summary**

Technical grade AE C656948 was administered by gavage to Beagle dogs (2/sex dose) or dose levels of 0, 30, 150, and 750 mg/kg/day for at least 28 days. Gage side observations were conducted daily, detailed clinical observations were conducted weekly, food consumption was measured daily, and body weights were taken weekly. Ophthalmic examinations were performed once pre-exposure and just prior to necropsy. Clinical chemistry, hematology, and urinallysis measurements were taken once pre-exposure and at the end of treatment. A gross necropsy was performed, selected organ weights were taken, and a range of tissues were collected and processed for histopathological examination.

### At 750 mg/kg/day:

Liquid feces were noted on a few occasions in T male and 1/2 female. The 2 male dogs showed a low erythrocyte count low hemoglobin and low hematocrit in comparison to their respective pre-test values. At clinical chemistry valuation, 1 male and 1 female showed a high alkaline phosphatase activity and a low albumin concentration (as a consequence a low albumin/globalin ratio). In addition, the same female also showed a high  $\delta$ -glutamyltrapsferate activity and trigly or ide concentration.

The liver was the only dentified target organ in with sexes illustrated by an increased weight mainly associated with hepatocellular hypertrophy.

At 150 or 30 mg/kg/day:

- No treatment-related findings were observed
- The NOEL for this study was 150 mg/kg day in both sexes
  - I. & Materials and methods
- A. Materials

1. Test mater fal:	XE C606948
Description	^J Beige powder
Lot / Batch #	P₩00304
Purity:	99.0%
CASH R A S	658066-35-4
Stability of test compound:	Stable at $25 \pm 5^{\circ}$ C (room temperature)
2. Vehicle and / or positive control	none
3. Test animals:	
Species:	Dog



Strain:	Beagle
Age:	Approximately 8-10 months old
Weight at dosing:	6.9 -8.5 kg (males), 6.4 – 8.1 kg (females)
Source:	
Acclimation period:	At least 21 days Certified canine meal 153C3 from S.A.F.E. (Scientific Animal
Diet:	Food and Engineering, Augy, France, 7
Diet.	Food and Engineering, Augy, France, Each animal received approximately 300 g of dict daily approximately 2.5 hours after gazage administration for 1.5
	Food and Engineering, Augy, France, Each animal received approximately 300 g of dict daily approximately 2.5 hours after gazage administration for 1.5 hours Municipal tap water, <i>ad libition</i>
Water:	Municipal tap water, ad libition
	Animals were housed individually in stainless steel kennels with
	a floor surface area of 1 29m ² .
	Supervised exercise in inside tuns was permitted daily for dogs
Housing:	of the same sex and seatment group throughout the
iiousing.	acclimatization and treatment periods except on weekends and
	public holidays. At the end of Working day, dogs were pairs
	Roused overnight by opening the partition between 2 animals of
Environmental conditions: <b>A</b>	the same sex and dose group. 18-21°C 2 40-70% 10-15 air changes for hour Alternative 12-hour light and dark cytops (7.5m-7 nm)
Temperature:	18-21°C° 5
Humidity:	40-70% ⁰ ⁴ ⁰ ⁶ ⁶
Air changes:	10-1 Sair changes per hour so so so
Air changes: Photoperiod:	Alternative 12-hour light and tark cycles (7 am - 7 pm)
B. Study design	
1 In life datase	
1. In me dates: 5 502 June	
Photoperiod: B. Study design 1. In life dates: 2 2. Animal assignment and create	- 30 June 2004
Shortly after arrival, all dogs were	examined by a vetermarian for signs of ill-health and were subjected
to a detailed clinical examination	to assess their physical and behavioral status. Body weight was
recorded and food intake determine	red. All animals were subjected to an ophthalmologic examination,
hamatalagy and alightal alphinistre	InvestOrations and wingly and during the acclimatization phase
Animals were assigned to dosage	groups using a computerized randomization procedure in order to
ensure a similar body weight distr	ibution among groups of each sex. Test groups were as described in
the table 5.3 09.	
Table 5.3/09 Testgroups and	

Kest Group (Group number)	Sex : Number of animals	Dose levels (mg/kgbw/day)
Control (1)	Afale: 2 Female:2	0
Dow (2)	Male: 2 Female:2	30
(Mid S) O S	Male: 2 Female:2	150
$\operatorname{High}(4)_{4}$	Male: 2 Female:2	750

# 3. Diet preparation and analysis

AE C656948 was administered orally by gavage at a dosage volume of 5 mL/kg bw for at least 28 days. The dosing formulations were prepared by suspending the test substance in aqueous solution of 0.4%



methylcellulose 400. Four preparations of 3 concentrations (6, 30 and 150 g/L) were prepared during the course of the study. Stability in solution, homogeneity and concentration were checked.

Results:

- Stability Analysis: Stability of AE C656948 in aqueous methylcellulose suspensions was demonstrat at 0.0868 and 250 g/L over 33 days under the storage conditions used in this study.

- Homogeneity Analysis: 99-103% of nominal concentration (checked at 6 and 150 g/L)
- Concentration Analysis: 93-101% of nominal concentration (checked at 30 g/L)

Results were within the in-house target range of 90 to 110% of nominal concentration and were considered to be acceptable for use on the current study.

### 4. Statistics

Not applicable due to the low number of animals per group. The results of body weight parameters, food consumption and clinical pathology parameters were compared individually with the respective pre-test values, each animal serving as its own control.

### C. Methods

- 1. Observations
- a. Cage side observations

All study animals were observed at least twice daily for ill health moribundity mortality and clinical signs of toxicity (except once daily on weekends and holidays), Baily examination of the kennels was also carried out for vomitus diarrhea or bood.

### b. Clinical examinations

Detailed clinical observations for clinical signs of toxicity were performed of all animals at treatment initiation (study day 0) and on a weekly basis thereafter. The physical examination included but was not necessarily restricted to the following examinations: fur and slan, eyes, ears, teeth, gum, mucous membranes, rectal temperature, gait, stance general behavior, chest including heart and respiratory rate, abdomen including palpitation, external genitalia and mammary glands.

### 2. Body weight

Each animal was weighed at least weekly before the gavage administration during the acclimatization and treatment periods and prior to necropsy.

### 3. Food consumption and compound intake

Food intake was measured for a minimum of 5 consecutive days immediately before start of treatment and daily throughout the study.

### 4. Ophthalmoscopic examination

During the acclimation period and at the end of reatment, ophthalmic examinations were conducted on all animals by means of ar indirect ophthalmoscope after instillation of an atropinic agent  $(mydriaticum)_{c}$ 

### 5. Clinical pathology: Hematology and clinical chemistry

Clinical differentials, were performed on all animals once ptor to administration of the test substance (day -9) and on day 27. Animals were diet fasted overnight prov to blood collection, which was drawn via jugular vein puncture. The following parameters were evaluated:

Hematology:



Hematocrit, Hemoglobin concentration, White blood cell count, Red blood cell count, Platelet count, Activated partial thromboplastin time, Leukocyte differential count, Mean corpuscular hemoglobin, Mean corpuscular volume, Reticulocyte count and % Reticulocytes, Mean corpuscular hemoglobin concentration, Prothrombin time.

Clinical chemistry:

Calcium (Ca), Chloride (Cl), Magnesium (Mg), Phosphorous (Phos), Potassium (K), Sodium (Na), Alkaline phosphatase, Alanine aminotransferase, Aspartate aminotransferase, Gamma glutamyltransferase, Albumin, Creatinine, Urea, Total Cholesterol, Glacose, Totak bilirubin, Total protein, Triglycerides.

### 6. Urinalysis

Urinalysis was performed on all animals twice prior to administration of the test substance (Day -14 and -8) and on all animals on study Day 28. Urine volume was collegeed overnight. The following parameters were measured:

Appearance, Volume, Specific gravity/ osmolality/ Refractive Index, pH, Sediment (microscopic), Protein, Glucose, Ketones, Bilirubin, Blood/red Plood cells, Tobilinogen, O

### 7. Sacrifice and pathology

On study Days 29 to 30, all animals from all groups were sacrificed. All animals were tranquilized by intramuscular injection of acepromazine (50 µL/kg body weight) and then deeply anesthetized by intravenous injection of pentobarbital (i.v. injection of 60 mg/kg body weight). Animals were then exsanguinated and necropsice

The necropsy included the examination of all major organs, stissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically. The following organs or tissues were sampled and/or weighed at necropsy:

were	sampled and/or weighed at	neecopsy, , U	Ň	af a
	Digestive system	Cardiovasc. / Demat	0	Neurologic
Х	Tongue N N	XX WAorta S	XX	Brain (with cerebellum)
Х	Submaxillary (salivary)		📡 X 🎝	
	gianu 🖉 🖉 🔘	X Bone marrow, steenum	X	Spinal cord (cervical,
Х	Esophagus 🞯 🔍	QX ALymph node, mesenteric		thoracic, lumbar)
Х	Stoppach 🔬 🔬		ŴΧ	Eyes
Х	Aprodenum O	v K Lymph node, V	X	Optic nerves
Х	*Yejunum	X Lymph note, repropharyngeal	XX	Pituitary gland*
Х	Ileum 🐒 😽 🖉	XX Spleen		
Х	Cecum of A	XX Thymus		Glandular
Х	Colon S		XX	Adrenal gland
Х	Rectum	X Lymph node, mesenteric X Lymph node, reprophasyngeal XX Spleen XXX Thynnis	Х	Parathyroid gland
XX			Λ	
Х	Pancreas	X Urinary bladder	XX	Thyroid gland (weighed
	* .		7171	with parathyroid gland)
X	Gallbladder 🖓 🔬	XX Destis		
A.	🖉 Respiratory	XX @Epidicymis		Other
Х	Trachea	XX Prostate gland	Х	Bone (sternum)
Х	Lung 🖉 🔥 🖉	XX Overy	Х	Skeletal muscle
Х	Phary@x 🔿 🖉	XX Uterus (with cervix)	Х	Skin
Х	Pharwax Lagonx	↓ X ^A Mammary gland	Х	All gross lesions and masses
		X Vagina	Х	Articular surface
	V & A S	_	11	(femorotibial joint)
(X) T	ssues were collected for histol	logical examination.		

(XX) Organ@were weighed@resh at scheduled sacrifice. Paired organs were weighed together.

For sacrificed animals, a bone marrow smear was prepared from one rib and stained with May-Grünwald Giemsa, but not examined.



Samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eve, optic nerve, epididymis and testis that were fixed in Davidson's fixative.

Histopathological examinations were performed on all tissues from all the animals in all dose group

#### II. **Results and discussion**

### A. Clinical signs and mortality

### 1. Clinical signs of toxicity

The only clinical sign observed at the high dose was liquid teces that were beserved on a few occasions in 2 animals at the high dose. On 1 or 2 occasions only, figuid feces was also observed in 24 animals at 150 mg/kg/day and in 1/4 animals at 30 mg/kg/day. However as liquid feees is occasionally observed in control animals and there was no evidence of a treatment-related increase in this finding over a 90-day period at a dose level of up to c. 330 mg/kg/day (M-2%6047%)1-1), this finding at 150 and 30 mg/kg/day was considered to be incidental.

aninations throughout the study. No abnormal findings were noted at the detailed

Rectal temperature was within the normal range

### 2. Mortality

There were no mortalities throughout the stud

### B. Body weight and body weight gain

vergent galas at any dose level in either There was no treatment-related effect on body weights bØď sex

### C. Food consumption and compound mitake

Mean food consumption was not affected throughout the

### D. Hematology, clinical chemistry, and urinary

### 1. Hematology

The 2 males a 750 mg/kg/day showed a Tower erythrocyte wunt , lower hemoglobin and a lower hematocrit values compared to their respective pre-test values.

thereroun No change was observed

### 2. Clinical Chemistr

setivity and a flow albumin concentration (and consequently a low A high alkaline phosphatase albumin/globulin ratio were observed in 1 male and 1 female at the high dose. This female also showed a high  $\delta$ -glutamyltransferase activity and triglyceride concentration.

g/kg/day. No relevant changes were observed at 130

### 3. Urinalysis

There were no treatment-related findings in either sex at any dose level.

### Sacrifice and pathology Ε.

### 1. Terminal body weight and organ weights

Liver weights (absolute and relative) were clearly higher in both sexes at 750 mg/kg/day. This weight increase was associated with histopathological findings (see below).

Liver weights wergalso stightly higher in both sexes at 150 mg/kg/day and in males at 30 mg/kg/day but were not associated with any histopathological findings. They were therefore considered as non adverse.

There were no other organ weight changes that were considered to be treatment-related.



### 2. Gross and microscopic pathology

At microscopic examination, minimal to slight centrilobular to panlobular hepatocellular hypertrophy and eosinophilic inclusion bodies were observed in most animals at 750 mg/kg/day. 

No other treatment-related changes were observed.

T-11- 52110	11 $4$ $4$ $1$ $1$ $1$ $1$ $1$ $1$ $1$ $1$ $1$ $1$
Table 5.3.1-10	Histopathological changes in the 28-day dog study with AE C656948
14010 2.2.1 10	Thistoputhological changes in the 20 day dog stady with the copp to

Sex		M	ales	, si a construction de la construcción de la constr	ŧ "	Fen	ales 🏷		, and the second s
AE C656948, dietary concentration in mg/kg	0	30	150	750	0	Â	<b>1</b> 50	250	Ľ
bw/day		s a		Ô¥			N.	Q a	0″
Liver, N examined	2	ŧ [≫] 2	2	<b>√</b> 2	2 (	2 '	ي 2 🌾	D´2 🎽	1
Centrilobular to panlobular hepatocellular hypertrophy: diffuse - Minimal		0	$\sim 0^{2}$		Ŵ	୍କ	8	<u></u>	
Centrilobular to panlobular hepatocellular hypertrophy: diffuse - Slight		¢°0 ×				0	×0 4	$\mathcal{O}_2$	
Total	0~	<b>%</b>	6	20	0	0	0	2 🕵 °	
Eosinophilic inclusion bodies: focal/	NO C	$\tilde{\gamma}^{0}$			× ×	$\overset{\circ}{\gg}^0$	N S	AN AN	
Eosinophilic inclusion bodies: focal/		, de se la companya de la companya d				00		1	
Total	۵ (	0	Ň	Ã	Õ	Ň	. <b>10</b>	2	
F. Deficiencies							1		
No specific deficiencies were noted in this stud	ly 🎣		A		, Ô	i de la companya de l			
No specific deficiencies were noted in this stud III. Conclusions	bôth se					L Y			
The NOEL was found to be 150 tog/kg/day in	bốth sẹ	gêš.		<u>د</u>	Ŵ				

### F. Deficiencies

The NOEL was found to be 150 not kg/day in both seves. Assessment and conclusion by applicant: Non-guideline study considered reliable. Following 28 dosing the NOEL was found to be 150 mg/kg/day in both seves.

the contraction of the contracti



### CA 5.3.2 Oral 90-day study

Data Point:	KCA 5.3.2/01
Report Author:	
Report Year:	
Report Title:	AE C656948 - 90-day toxicity study in the rat by dietary administration
Report No:	SA 04048
Document No:	<u>M-250946-01-1</u>
Guideline(s) followed in	OECD 408; Directive 2001/59/EC Annex V, Method B.26; US-EPA, OPPTS
study:	870.3100; JMAFF notification 12 Nousan No. 847
Deviations from current	Current guideline: OECD 408(2018
test guideline:	Deviations: no measurement of HDL, LDL but the study acceptability was not
	affected because total choresterol were measured $\mathcal{Q}$ $\mathcal{Q}^{*}$
	These deviations have not impact the outcome of the study and interpretation of
	the results. $\langle \chi \rangle = \langle \chi \rangle =$
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
	Vac. conducted in Agr (DP/Officially exceeding the stimulation of the state of the
GLP/Officially	Tes, conducted ander OI21/Opticially recognised testing factures
recognised testing	$\begin{array}{cccccccccccccccccccccccccccccccccccc$
facilities:	
Acceptability/Reliability:	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$

### **Executive Summary**

AE C656948 (batch number PFI 0304, 29.0% parity) was administered continuously via the diet to separate groups of Wistar rats (10/sex/group) at close levels of 0, 50, 200, 1000 and 3200 ppm equivalent to 0, 3.06, 12.5, 60.5 and 204 ng/kg/day in males and 0, 3.66, 14.6, 70.1 and 230 ng/kg/day in females, respectively, for at least 90 days. An additional 10 males and 10 females fed either 0 or 3200 ppm of test diet for at least 90 days were maintained on control diet for a further 28 days to examine the reversibility of any effects seen.

At 3200 ppm mean body weight was decreased by between 4% and 6% in males and 4% and 8% in females throughout the course of the study, with an overall mean body weight gain reduced by 9% in males and 17% in femates, compared to controls. Mean food consumption was slightly decreased by between \$% and 12% from Days 29% o 90 in females, compared to controls. Mean prothrombin time was increased in males, whilst mean placelet and retioulocyte (absolute and percentage) counts were increased in females. In addition, slightly lower mean hemoglobin concentrations were noted in both sexes, in association with lower mean hematocrit in males and lower mean corpuscular volume and mean corpusc far hepoglobit in females. On increase in mean total cholesterol, γ-glutamyltransferase, total protein, globulin, inorganic phosphorus and calcum in both sexes, creatinine and urea in males, and triglycerides in females was observed, whereas a decrease was noted in total bilirubin and chloride in both sexes, glucose in males, and alkaline prosphatase and albumin/globulin ratio in females. Urinalysis revealed an increased incidence and severity of cellular casts in males, compared to controls which was observed in connection with hydrine droplet nephropathy noticed at the histological examination of the kidney. An increase in mean TSH levels in both sexes at Week 3, together with an increase in mean T3 and T4 vevels in females was observed, whereas at Week 13, only an increase in mean TSH and T3 levels was noted in males. At necropsy, mean liver weights and thyroid gland weights were increased in both sexes, whilst mean kidney weights were increased in males only. At the microscopic examination treatment-related changes were observed in the liver and thyroid gland in both sexes and in the kinney in males. In the liver, minimal to moderate centrilobular hepatocellular hypertrophy was observed in both sexes, together with minimal to moderate periportal to midzonal hepatoce failar macrovacuolation in females. In the thyroid gland, minimal to slight diffuse hypertrophy of follicalar cells was seen in the majority of males and in 1/10 females. In the kidney, the incidence and severity of hyaline droplet nephropathy related to the accumulation of  $\alpha 2\mu$ -globulin in the proximal



tubules and the incidence of hyaline casts were increased in males. However, hyaline droplet nephropathy is considered not to be toxicologically relevant to man as  $\alpha 2\mu$ -globulin is present only in trace amounts in humans.

After 1 month of recovery in the high dose group, mean body weight was still reduced in both sexes. Affected hematology, clinical chemistry and urinalysis parameters were partially reversible. At necropsy, mean absolute and relative kidney weights were still increased inomales, compared to the controls. Microscopic examination showed that hyaline droplet nephropathy and hyaline exists persisted in some animals. All other affected parameters were found to be reversible after 1 month of recovery.

At 1000 ppm, a decrease of 15% in mean body weight gain per day was noted in females during the first week of treatment, when compared to the control group. Mean food consumption was decreased by between 4% and 9% on most occasions in females, compared to controls. Lower mean hemoglobility concentrations and lower mean hematocrit were poted in males. An increase in mean total cholesterol in both sexes, an increase in creatinine, inorganic phosphorus and calcium in males, and a decrease in total bilirubin in both sexes and chloride in males were observed In connection with hyaline droplet nephropathy seen at the histolopathological examination, utimalysis revealed an increased incidence and severity of cellular casts in males, compared to controls. Hormonal assessment revealed an increase in mean TSH level in males on Week 13 only. At necropsy, mean liver weights were increased in both sexes. In addition in males, mean kidney weights were increased Microscopic examination revealed treatment-related changes in the liver and thyroid sland in both sexes and in the kidney in males. In the liver, minimal to slight centrilobular hepatocellular hepertrophy was observed in both sexes, together with minimal periportal to midzonal hepatocedular macrover dolation in females In the thyroid gland, minimal to slight diffuse hypertrophy of follicular cells was seen in both sexes. In the kidney, the incidence and severity of hyaline droplet hephropathy, considered not to be toxicologically relevant to man, and the incidence of fryaline casts overe increased in males.

At 200 ppm, treatment-related changes were noted at the microscopic examination in males only and consisted of minimal centrilobular hepatocellular hypertrophy in the Over of 2/10 animals. In view to the very low incidence and severity; these changes were considered to be adaptive but not adverse changes. In addition in males, a slight increase in incidence and severity of cellular casts was observed in urine in connection with the presence of Fyaline dropler nephropathy at the microscopic examination of the kidney, but these changes are considered not to be toxicologically relevant to man.

At 50 pprov treatment-related changes consisted only of a flight prcrease in incidence and severity of cellular easts in the uffine in males, compared to controls. These changes were considered not to be toxicologically relevant to man.

In the Wistar rat, when administered at C656948 in the met over a 90-day period, a dose level of 50 ppm represented the NOAEC in males (equating to 3.06 fig/kg body weight/day), whereas a dose level of 200 ppm represented the NOAEC in females (equating to 14.6 mg/kg body weight/day). However, since the only relevant effect observed in males at 200 ppm was related to hyaline droplet nephropathy (known not to be relevant for humans), the appropriate NOAEL for human risk assessment purpose is considered to be 200 ppm (equivalent to 12.5 mg/kg bw/day).

	Materials and method	
	I. Materials and methods	s V O
A.	Materials A &	
1.	Test materiate	AE C656948
	Description	Beige powder
	Lot / Batch #:	PFI 0304
	Burity 0 S	99.0%
~	CAS#	658066-35-4
	Stability of test compound:	Stable at 20 and 10000 ppm at room temperature for 105 days.



2. Vehicle and / or positive control:	none
3. Test animals:	
Species:	Rat Wistar Rj:WI (IOPS HAN)
Strain:	Wistar Rj:WI (IOPS HAN) 6 weeks approximately 182-221 g (males), 159-190 g (females) 7 days
Age:	6 weeks approximately
Weight at dosing:	182-221 g (males), 159-190 g (females)
Source:	7 days Certified rodent powdered and irradiated diet A04C-10P1 from
Acclimation period:	7 days
	Certified rodent powdered and irradiated diet A04C-10P1 from
Diet:	S.A.F.E. (Scientific Animal Food and Engineering, Qugy, S
	Certified rodent powdered and irradiated diet AP4C-toP1 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), <i>ad libitum</i> Tap water, <i>ad libitum</i>
Water:	Tap water, addibitum
Housing:	Individually, suspended stainless steel and way mesh eages
<b>Environmental conditions:</b>	Tap water, addibitum Individually, suspended stainless steel and wro mesh cages 20-24°C 40-70% 15 per hour 12 hours dark, 12 hours light (7 am 7 pm)
Temperature:	20-24 $C$ $W$ $Q$ $Q$ $O$ $O$ $O$ $O$ $O$ $O$ $O$
Humidity:	$40-70\%$ $\gamma$
Air changes:	40-70% 15 per hour 12 hours dark 12 hours light (7 ame) pm)
Photoperiod:	Q hours dark 12 hours light (7 am 7 pm)
B. Study design	
1. In life dates:05 May 2	$0.044 \neq 0.8$ September 2004 $2004$ $2004$ $2004$ $2004$
2. Animal assignment and treat	pent of a a a a a

2. Animal assignment and treatment There were 10 animals of each sex per dose group. Animals were assigned to dose groups randomly by weight. AE C656948 was administered in the diet for at least 90 days to Wistor rats at the following doses – 0, 50, 200, 4000, and 3200 ppm (equating approximately to 0, 3.06, 12.5, 60.5 and 204 mg/kg/day in males and 0, 3.63, 14, 6, 700, and 230 mg/kg/day in females). An additional 10 males and 10 females fed either 0 of 3200 ppm of test diet for at least 90 days were maintained for a minimum of 28 days to examine the reversibility of any effects seen. Aniftial housing and husbandry were in accordance with the regulations of the Guide for the Care and USe of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication 10 86-23, revised 1985) and "Le Guide du Journal Officiel des Communantes Exceptences L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Ď

	Arest C	Diet O	No. Of animals	Animal	identity	
Group			Per group	Dosing phase	Recovery phase	
L. L	¢ "		Males			
1	Control		°0 +10*	OT1M1773 to 1782	OT1M1783 to 1792	
20	$\mathbb{Z}$		~ 10	OT2M1813 to 1822	-	
3″	AE C65ô948	Ø (Q00 )	0° 10	OT3M1833 to 1842	-	
4	AE C 00948	1000 A	) 10	OT4M1853 to 1862	-	
5	ja zi	\$ 3209	10 + 10*	OT5M1873 to 1882	OT5M1883 to 1892	
		.0° & ~9	Females			
1	Connol	C ~ 0	10+10*	OTIF1793to 1802	OTIF1803to 1812	
2	1 2 1	<u>50</u>	10	OT2F1823to 1832	-	
3	ALC C656948	200	10	OT3F1843to 1852	-	
LA.	ASE C030948	× 1000	10	OT4F1863to 1872	-	
5	Ĵ ^v	3200	10 + 10*	OT5F1893to 1902	OT5F1903to 1912	

Table 5.3.2-1	Details of	group sizes and	treatment
---------------	------------	-----------------	-----------

*These animals were sacrificed following a 28-day recovery period after cessation of treatment.



### 3. Dose selection

The dose levels were set after taking into account the results from a preliminary range-finding rat toxicity study where AE C656948 (batch No: FLH 999; 98.6% purity) was administered to Wistar rats at detary concentrations of 0, 50, 400, or 3200 ppm for 28 days (M-085510-01-1). There were no treatment related effects (NOEL) noted at 50 ppm in either sex (4.0 and 4.6 mg/kg/day in males and remales, respectively). However, changes at 400 ppm were limited to adaptive changes in the lover and hyaline droplet nephropathy in the kidneys (not relevant for humans), therefore 400 ppm (31.0/36.1 mg/kg bw/d in males/females) is considered to be NOAEL for human risk assessment.

### 4. Diet preparation and analysis

AE C656948 was ground to a fine powder before being incorporated into the det by dry mixing to provide the required concentrations. There were two preparations of each concentration for the whole study.

Results:

Homogeneity Analysis: Homogeneity of the diet was verified on the first preparation at 50 and 3200 ppm and was within a range of 93-97% of the popninal concentration.

Stability Analysis: The stability was demonstrated before the start of the study. Diet samples of 20 and 10000 ppm were found to be stable over a 105-day period at ambient temperature or over a 95-day freezing period followed by 10 days at ambient temperature

Concentration Analysis: Concentration was checked for each concentration for both dietary preparations. Measured concentrations for the four dose groups and were within the range of 92-98% of the nominal concentration. Hence all values were within the in-house target range of 85-115% of the nominal concentration.

### 5. Statistics

For the 90-day phase, continuous data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett test (2-sided) on parameters showing a significant effect by ANOVA. When the data were not homogeneous, a Kruskal-Wallis ANOVA was performed followed by the Dunn test (2-sided) to identify statistical significance between groups. For some parameters, when data were not homogeneous, they were transformed using a log transformation or sphare pot transformation, then reanalyzed as above.

For urine analysis (pH), group means were compared using the non-parametric Kruskal-Wallis test which was followed by the Dunnitest (2-sided p if the Kruskal-Wallis test indicated significance.

For the recovery phase continuous data were analyzed by the F test for homogeneity of variances. When the data were homogeneous, a t-test (2-sided) was performed. When the data were not homogeneous, a modified t-test was performed. For some parameters, when data were not homogenous, they were transformed using a log transformation or square root transformation, then reanalyzed using the F test.

For usine analysis (pH), group means were compared using the non-parametric Mann-Whitney test.

When one or more group variance (s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Pate Tox System \$4.2.2. (Module Enhanced Statistics).

### C. Methods

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays) Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed once during the acclimatization phase and at least weekly during the treatment period. On study weeks 11 to 12, a neurotoxicity assessment was performed for all animals



(except for animals of the recovery groups) by observers who were blind with respect to the dose level. Animals were tested individually for motor activity using an automated photocell recording apparatus designed to measure quantitatively spontaneous exploratory motor activity in a novel environment. Motor activity was recorded for 90 minutes with data being collected at 15-minute intervals throughout the session. For sensor reactivity, the following reflexes and responses were recorded: pupillary reflex, surface righting reflex, corneal reflex, flexor reflex, auditory startle response and tail pinch response. The fore- and hindlimb grip strength of all animals were measured quantitatively using a grip strength apparatus equipped with one pull and one push strain gauge. The mean of three successive measurements was determined for both fore- and hindlimb grip strength.

### 2. Body weight

Body weights were recorded twice during the acclimatization phase, on the first day of test substance administration, then at weekly intervals throughout the treatment and recovery poliods and before necropsy.

### 3. Food consumption and compound intake

3. Food consumption and compound intake The weight of food supplied and of that remaining at the end of the food the weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment and recovery periods. From these records the mean daily consumption was calculated. Any food spillage was also noted.

The weekly mean achieved dosage intake in ing/kg day for each week and for Weeks 1 to 13 was calculated for each sex using the formula:  $\bigcirc$ 

Dose level (ppm) x Group mean food consumption (g/day) Test substance intake (mg/kg/day) € Group mean body weight (g) at the end of the food consumption period

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### 4. Ophthalmoscopic examination

O During the acclimatization period all animals were subjected to an ophthalmic examination. After instillation of an aropine agent (Mydriaticum, Merck Sharp and Dohmey each eye was examined by means of an indirect withtalmoscope. During Week 13 of the gratment period, all surviving animals from control and high dose group were re examined

### 5. Neuropogical examination

During study Weeks of to 12 a neurotoxicity assessment was performed for all surviving animals (except for animals of the reversibility group by observers who were blind with respect to the dose level. The assessment consisted of measurements of motor activity, sensory reactivity and grip strength.

### 6. Hematology and clinical chemistry

On study days 91, 92, 93, or 94, and on recovery phase days 29 and 30, prior to necropsy, blood samples were taken from all surviving animals of all groups by puncture of the retro-orbital venous plexus. Animals were diet fasted overnight prior to blocking and anesthetized by inhalation of Isoflurane. Blood was collected on EDTA for hematology, or dithium heparin for plasma and clot activator for serum for clinical chemotry and on softum citrate for coagulation parameters.

Hematology parameters investigated:

Red blood cell count, henoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemediobin@mean@orpuscular hemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation and platelet count. A blood smear was prepared and stained with Wright main. It was examined when results of Advia 120 determinations were abnormal. Prothrombin time was assayed on an ACL 3000 (Instrumentation Laboratory, Paris, France).



Clinical chemistry parameters investigated:

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, catelum and inorganic phosphorus concentrations and aspartate aminotransferase, alanine aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed on plasma samples, total protein and albumin concentrations were assayed on serum samples using an Hitachi 911 (Roche Diagnostics, Meylan, France).

Thyroid Hormone Assessment:

During weeks 3 and 13 of the treatment phase, and week 5 of the regivery phase, blood samples were collected from the retro-orbital venous plexus of all sarviving animals diet fasted overnight. Blood was collected in heparin, and plasma separated and frozen until used for hormone analyses

Thyroid hormones, including T3, T4, and TSH, were assayed by radiommunoassay kit (Amersham for TSH, and Backman-Coulter for T3/T4).

### 7. Urinalysis

In the morning, overnight urine samples were collected on study Days 85, 86, or 87 from the exposure phase animals and on recovery Day 25 from the surviving extra animals in the control and high dose groups allocated to the recovery phase. Food and water were not accessible during urbe collection.

Any significant change in the general appearance of the urine was recorded. The urine volume was measured. pH was assayed using a Chnitek 200+ and Ames Mutistix dipstices (Bayer Diagnostics, Puteaux, France). Urinary retractive index was measured using a RFM320 refractometer (Bioblock Scientific, Illkirch, France).

The following semi-quantitative parameters were assayed sing a Clinitek 200 and Ames Multistix dipsticks: glucose, bilirubin ketone bodies, occult blood, potein and urobilinogen. Microscopic examination of the grinary sediment was performed after centrifugation of the urine. The presence of red blood cells, while blood cells, epitherial cells, bacteria, casts and crystals was graded.

### 8. Sacrifice and pathology

On study days 91, 92, 93, and 94 and on recovery days 29 or 30, a complete necropsy was performed on all surviving animals. Animals were deeply anesthetized by inhalation of Isoflurane, and then exsangunated before tecropsy All animals were fasted prior to scheduled sacrifice. All animals, either found dead or killed for humane reasons were necropsied. The necropsy included the examination of all major organs, itsues and budy cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically. Adrenal gland, brain, epididynides, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testas, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

The following organs of tissues were sampled: advenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, esophagus, exorbital (lachrymal) gland, eye and optic nerve, Harderian gland, heart intestine (duodenum, jejunum, ileum, cecum, colon, rectum), kidney, larynx/pharynx, liver, tung, bymph todes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary pancreas, pitetary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymus, thyroid grand (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagina.

A bone parrow smear was prepared from femur, stained with May-Grünwald Giemsa, but not examined. Tissues samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye, optic nerve, Hardenan gland, epididymis and testis that were fixed in Davidson's fixative. Histopathological examinations were performed on all tissues from all the animals in the control and high dose groups and all decedents in all groups. The liver, kidney, lung, thyroid gland and significant gross findings were examined in all animals in the study. For the reversibility phase, liver, kidney, lung,



thyroid gland and macroscopic observations were processed and liver, lung, kidney and thyroid gland were examined.

In addition, immunohistochemical staining for  $\alpha 2\mu$ -globulin was performed on male kidney sections in all dose groups. 

#### II. **Results and discussion**

### A. Observations and mortality

### 1. Clinical signs of toxicity

There were no treatment-related clinical signs in either sex throughout the study.

### 2. Mortality

Two males were sacrificed prematurely for human reasons on Days 2 and 57. One male (2000 pm) was sacrificed on Day 37 having had a distended abdomen between Days 22 and 32 and 32 and 36 and generak pallor between Days 27 and 37. This animal was noted to have a pale appearance and an enlarged, irregular and red mottled liver at the macroscopic examination. One male (50 ppm) was sacroficed on Day \$7 having been noted to have labored and noisy respiration, a wasted appearance, pilgerection and ocular discharge on both eyes on the day of sacrifice, together with a body weight loss of 8.2 g/day and a food consumption reduced to 18 g/day during the week prior to sacrifice. The condition of this animal was attributable to an accidental trauma as malitoscopic examination revealed sorted fur around both eyes and a fracture of the nasal cavity. These two promature sacrifices were considered not to be treatmentrelated.

There were no mortalities during the recovery phase of the stud

### 3. Neurological examinations

Ő Motor activity - Motor activity was indistinguishable between the treated and control groups.

Sensory reactivity Sensory reactivity was unaffected by treatment. The few changes in the tail pinch response noted in the male high dose groups were considered to reflect inter-individual variations rather & () than any treatmont-related effect.  $\hat{\bigcirc}$ 

Grip strength - At 3200 ppm, fore- and hindligh grip strength were essentially comparable to control values in males and slightly decreased by 16% and 23% respectively, in females (not statistically significant), compared to controls. This slight effect in fernales was considered most likely to be due to a decrease in body weight tather than to be a specific freatment-related neurotoxic effect. At 1000, 200 and 50 ppm in both sexes, for and hindling grip strength were essentially comparable to control values. The few changes noted in temales at 1000 ppm were considered to reflect inter-individual variations rather than any treatment related effect.

### Body weight and body weight gain B.

At 3200 ppm, mean body weight was decreased by between 4% and 6% in males ( $p\leq0.01$  or  $p\leq0.05$ ) and 4% and 8% in temales  $p \le 0.00^{\circ}$  or  $p \le 0.05$  or most occasions) throughout the course of the study, compared to controls. The effect on body weight was primarily due to an initial decrease in mean body weight gain per day during the first week of freatment in males and females (-26 and -29%, respectively, p≤0.01), compared to controls. Thereafter, mean body weight gain per day was essentially comparable to the controls in both sexes, but mean overall body weight gain was reduced by 9% in males ( $p \le 0.05$ ) and 17% on females (p\$0.01) The effect on mean body weight was still observed after 4 weeks of recovery in both sexes, as the magnitude of the decrease was similar to that observed at the end of the treatment phase.

At 1000 prim, mean body weight parameters were unaffected by the treatment in either sex, with the exception of a slight decrease of 15% (not statistically significant) in mean body weight gain per day in females during the first week of treatment, compared to controls. Other minor differences (reaching



statistical significance) from controls were noted but they were considered to reflect inter-individual variation rather than a treatment-related effect.

At 200 and 50 ppm, no effect on mean body weight and mean body weight change was noted in other sex.

### C. Food consumption and compound intake

At 3200 ppm, mean food consumption in males was essentially comparable to the control values and in females was slightly decreased by between 5 and 12% from Days 29 to 90 ( $p\leq0.01$  or  $p\leq0.05$  on most occasions), compared to the controls. No treatment-related effect on mean food consumption was nated in either sex during the recovery phase. The slight decrease of 8% in mean food consumption noted in males during the last two weeks of the recovery phase was considered to be incidental.

At 1000 ppm, mean food consumption was similar to the control values in males and Sightly decreased by between 4 and 9% on most occasions in females (not statistically significant), compared to controls.

At 200 and 50 ppm, no effect on food consumption, was noted in either sec.

The mean achieved dosage intake per group was as follows:

### Table 5.3.2-2 Mean achieved dose fevels of AE @656948 in mg/kg bw/day

	$\cap$	V X,	£C.Y					
Sex	Ś	<b>M</b> ale:	s 🤊		/ () ²	Fema		)
Dietary level (ppm)	50 🗳	200	3 <b>5000</b> 🖒	[,] 3200	59	<b>∑</b> 200 0	1000/	3200
Weeks 1-13 (mg/kg/day)		\$2.5 0	⁷ 60.5	204	<b>3</b> .63	° 14.6°	70,1	230
			6.//	(A) (A)	av		<u></u>	

### D. Ophthalmoscopic examination

No treatment-related ocular abnormalities were observed at the ophthalmoscopic examination.

### E. Hematology, clinical chemistry, and urinalyse

### 1. Hematology

### a) Dosing Phase

Higher mean prothrophin time was noted at 3200 ppm in males only ( $\pm$  74%, p $\leq$ 0.01), when compared to the control values. Slightly lower mean hemoglobin concentrations were noted at 3200 ppm in both sexes and at 1000 ppm in males only. This variation was associated with lower mean hematocrit in males and lower mean corpuscular volume and mean corpuscular hemoglobin in females at 3200 ppm. The magnitude and statistical significance relative to the control groups were as follows:

# Table 5.3.2-3 Hematological finefings (Vlain study (dosing) phase) (mean ± SD)

	Main s	study (dosing) j	ohase		
Dese levels (ppm)		× .50	200	1000	3200
		Males			
Hemoglobin (g/dL)	€ 15.5⊕0.3 €	15.4 ± 0.3	$15.2 \pm 0.6$	$14.9 \pm 0.5$ (-4%)	14.6±0.7** (-6%)
Hematocrit (9)	47.6±00	47.6 ± 0.9	$47.0 \pm 1.9$	45.7 ± 1.2*	45.4 ± 1.9**
		Females			
Hemoglobin (gedL)	) 15.0 ± 0.5	$15.2 \pm 0.5$	$15.4 \pm 0.3$	$15.1 \pm 0.6$ (-4%)	14.4±0.6* (-4%)
Mean corpuscular volume (fl)	53 ± 1	54 ± 1	54 ± 2	53 ± 1	49 ± 1** (-8%)



Mean corpuscular hemoglobin (pg)	$17.8 \pm 0.5$	$17.8 \pm 0.4$	$17.9 \pm 0.7$	$17.5 \pm 0.5$	$16.3 \pm 0.4 **$ (-8%)	~
$* = p \le 0.05$ . $** = p \le 0.01$						Ş

Additionally, higher mean platelet (+24%, p $\leq$ 0.01) and reticulocyte (+50% for absolute count and +4 for percentage, p $\leq$ 0.01) counts were noted at 3200 ppm in females.

There was no evidence of treatment-related changes at 1000 ppm in females or at 200 and 50 ppm in either sex.

### b) Recovery Phase

After 1 month of recovery in the high dose group, there was a tendency towards reversibility attroughts mean hemoglobin concentration was still lower after the recovery period in males and females (-4%,  $p \le 0.01$  and -3%, not statistically significant, respectively), compared to controls. However, these variations were lower than at the end of the dosing phase.

Male prothrombin time and female platelet and reticulocyte variations observed at the end of the dosing phase were considered to be reversible, as after the 1-month recovery period, to significant differences were noted between the high dose and the control groups.

### 2. Clinical Chemistry

a) Dosing phase:

At 3200 and/or 1000 ppm, several treatment-related variations were observed in males and/or females. The magnitude and statistical significance relative to the control groups were as follows:

Main Study (Dosing) phase K & K										
Sex	S.	- O ^Y		les S	í "N	0	- O -	Fema	les	
Dietary level	ି ୧୦	50	200	1090	×3200 5	0	50 50	^{′′} 200	1000	3200
Total bilirubin µmol/L		Q7±	₽.8 ± 0.5 ∢	(-3)	) [*] 1.3+ 0.5** (\$5%)		2,1± 0.3**	1.9± 0.5**	1.6 ± 0.3** (38%)	1.7 ± 0.04** (-35%)
Triglycefrides (mmol/L)	₽ °		N	NC S	NC NC	0.44 0.12	± 0.12	$\begin{array}{c} 0.45 \\ \pm \\ 0.04 \end{array}$	$\begin{array}{c} 0.50 \pm \\ 0.08 \end{array}$	0.85 ± 0.33** (+102%)
Total cholesterol		1.730 0.25	2.23) 2.23) 2.0,57	2,729± *0,84* \$445%	2.95 ± 0.47* (+58%)	1.88 ± 0.17	2.13 ± 0.39	$2.11 \\ \pm \\ 0.43$	$2.79 \pm 0.65^{**}$ (-48%)	3.78 ± 0.78** (-101%)
γ- glutamylt <del>ra</del> usferase (IU/L)			0 + 0		₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩ ₩	$0 \pm 0$	$0\pm 0$	1 ± 1	$1 \pm 1$	9 ± 4** (+4300%)
Alkaline Phosphatase (IU/L)	NC C	NC	v Q	NÇY	NC	47 ± 10	45 ± 9	44 ± 10	42 ± 9	31 ± 6** (-34%)
Total protein (g@)	72±3	73-£	72+ ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	₹¥±4	78 ± 4** (+8%)	72 ± 3	$70 \pm 4$	72 ± 5	$75\pm4$	78 ± 7* (+8%)
Globulin (gds)	$28\pm 2$	* 1 ĸ	29± 29	29 ± 2	31 ± 2	24 ± 2	25 ± 2	27 ± 2	$27 \pm 2$	30 ± 3** (+25%)
Albumin Globulin ratio	ANC		NC	NC	NC	1.96 ± 0.26	1.82 ± 0.15	1.74 ± 0.06	$\begin{array}{c} 1.78 \pm \\ 0.10 \end{array}$	1.64 ± 0.10** (-16%)
Chatinine mmol/L	35 🔊	35 ± 4	$33 \pm 2$	$39 \pm 5*$ (+11%)	40 ± 3** (+14%)	NC	NC	NC	NC	NC

## Table 5.3.2-4 Selected clinical chemistry parameters (mean ± SB)



Main study (dosing) phase										
Sex			Ma	les				Fema	les	0
Urea (mmol/L)	5.14 ± 0.64	4.96 ± 0.39	4.86 ± 0.44	5.45 ± 0.79	$\begin{array}{c} 6.01 \pm \\ 0.57^{**} \\ (+17\%) \end{array}$	NC	NC	NC	NC	
Glucose (mmol/L)	6.64 ± 1.04	7.05 ± 1.38	6.87 ± 1.15	$\begin{array}{c} 5.85 \pm \\ 0.49 \end{array}$	$\begin{array}{c} 4.88 \pm \\ 0.32^{**} \\ (-27\%) \end{array}$	NC	NC	Â	NC	NG
Inorganic	1.79	1.86	1.86	1.96 ±	2.11 ±	1.46	1.45	1.48	£ 58 ± /	°∼J.68 ±~
phosphorus (mmol/L)	± 0.09	± 0.15	± 0.18	0.14* (+9%)	0.15	± 0.13	Q.13	± 0.11	0.18	¥ 0.23€
	2.71	2.71	2.73	2.81 ±	<b>2</b> 86±	2.67	2.65	2.76	2.74¥	(+15%) $200 \pm$ 0.12**
Calcium mmol/L	± 0.07	$\overset{\pm}{0.05}$	$\overset{\pm}{0.06}$	0.07* (+4%)		±Q 10,07	. @06	0.95	607	♥.12**© (+5%)
Chloride (mmol/L)	103 ± 2	103 ± 1	$\begin{array}{c} 102 \pm \\ 1 \end{array}$	$100 \pm$ 1 (-3%)	$\mathbb{Q}^{1}$	± 104	@06 Л03 ★ ↓	0103 + 20	103 ± 1	101×± 1**
Significant at * $p \le 0.05$ ;	** $p \leq 0$	0.01;	S	NC =	ot relevant o	change	A	6 ⁵ «	-O'	

b) Recovery phase

After 1 month of recovery in the logh dose group, the general tendancy was towards reversibility. Nevertheless, statistically significant differences were still noted in mean total cholesterol, globulin concentrations and albumin/globulin ratio in temales (+19%) +14% and 94% respectively, p≤0.05), compared to the control values. However, the variations were lower than at the end of the dosing phase.

The other changes observed at the end of the dosing phase were considered to be reversible, as after the 1-month recovery period, to significant differences were noted between the high dose and the control groups. The slightly higher mean alkaline phosphatese activity seen in females was considered not to be biologically or toxicologically relevant.

### Thyroid hormone

### a) Dosing phase

At 3200 ppm, an increase in mean TSH level was noted at Week 3 in both sexes (+63%, p $\leq$ 0.05 and +71%, p $\leq$ 0.01, respectively), together with an increase in mean T9 and T4 levels in females (+24%, p $\leq$ 0.05 and +54%, p $\leq$ 0.01, respectively), whereas at Week 13, only an increase in mean TSH and T3 levels was noted in males (+88 and +40%) respectively, p $\leq$ 0.01).

At 1000 ppm, mean TSH level was increased by 54% (not statistically significant) in males on Week 13 only.

There was no evidence of Piologically or toxicologically relevant changes at 200 or 50 ppm in either sex.

b) Recovery phase

All changes observed at the end of the cosing phase were considered to be reversible, as after the 1month recovery period, no statistically significant differences were noted between the high dose and the control groups. The slight changes in thyroid hormone levels observed after 1 month of recovery were considered not to be biologically or toxic logically relevant.

### 3. Urinalysis

### a) Dosing phase

The incidence and severity of cellular casts in urine were increased in all male treated groups in a doserelated manner. The presence of casts in the urine is to be seen in connection to the hyaline droplet nephropathy observed at histopathology examinations.



Table 5.3.2-5 Incidence and severity of cellular casts in urine at the end of the Main study (dosing) phase

F	Main st	udy (dosing) ph	ase					
Sex	Males 😞							
Dietary level (ppm)	0	50	200	<b>\$1000</b>	~3200 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~			
Number of animals examined	10	9	10	4 9	Q 10 X			
Slight	1	4	2 🖋	0 %				
Moderate	0	2	1	3				
Severe	0	0,	204	5	ð 7 <u>5</u> 0			
Total	1	18						

Other changes were considered to be incidental and not treatment-related.

outer changes were considered to be incidental and not treatment related. b) Recovery phase After 1 month of recovery, cellular casts in grine were stillebbsered in male high dosceroup filowester, the incidence and severity were lower than at the end of the dosing phase. Supporting a progression towards reversibility.



Table 5.3.2-6	Incidence and severity of cellular casts in urine at the end of the recovery phase
---------------	------------------------------------------------------------------------------------

Recovery phase							
Sex		Μ					
Dietary level (ppm)		0	3200 0				
Number of animals examined		10					
Slight		0	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~				
Moderate	, S	0	C 20 Y QY				
Severe	Å.	0 0					
Total	A.	6°, s°					
	QQ ⁰	~~. Ű					

### F. Sacrifice and pathology

### 1. Terminal body weight and organ weight

### a) Dosing phase

Mean terminal body weight at 3200 ppin was lower than in controls in males and females (-5%, not statistically significant and -9%,  $p \le 0.93$ , respectively).

Mean absolute and relative liver worghts were statistically significantly higher at 3260 and 1000 ppm in both sexes, when compared to controls, A tendency towards higher liver worghts was also noted at 200 ppm in both sexes (not statistically significant).

Mean absolute and relative kidney weights were statistically significantly higher at \$200 and 1000 ppm in males, when compared to controls. Altended by towards higher kidney weights was also observed at 200 ppm in males (not statistically significantly higher than controls, but this change was attributable to lower terminal body weight and was thus considered not to be toxic@togically relevant.

There was a tendency towards higher thyroid weights at 3200 ppm in both sexes.

### Table 5.3.2-7

6.2-7 Mean terminal body weight and absolute and relative weights of liver, kidney and thyroid (mean + SD)

Main study dosing) phase							
Dietary level (ppm)		50 0	200	1000	3200		
		National Action	es 🖧				
Terminal body wt, g	₩19.2±Q).9	¥ 476 1±35 7	500.7±38.8	498.2±19.6	457.5±42.7		
Liver weg	10.94 ± 0.80		2 12.02 ± 1.19 (+10%)	$13.63 \pm 1.13^{**}$ (+25%)	16.77 ± 1.70** (+53%)		
Liver wt, % body wt	2.282 20.096	2.297 ± 6001	$2.400 \pm 0.151 \\ (+5\%)$	2.736 ± 0.188** (+20%)	3.669 ± 0.223** (+61%)		
Liver wt, %	\$18.50 \$1.102	516 <b>67</b> ± 41.111 (0%)	569.50 ± 75.647 (+10%)	657.050 ± 57.55** (+27%)	$839.82 \pm 83.091 ** (+62\%)$		
Kidney wt, g		2.89 ± 0.20 (+3%)	$3.10 \pm 0.30$ (+10%)	$3.65 \pm 0.32 ** (+30\%)$	3.60 ± 0.38** (+28%)		
Kidnex Wt, % body wt	$0.587 \pm 0.045$	0.608 ± 0.029 (+4%)	$0.620 \pm 0.049$ (+6%)	$\begin{array}{c} 0.732 \pm \\ 0.043^{**} \\ (+25\%) \end{array}$	0.788 ± 0.069** (+34%)		



Main study (dosing) phase							
Dietary level (ppm)	0	50	200	1000	3200 °		
		Male	es				
$\mathbf{V}$	$133.04 \pm 12.503$	$137.98 \pm 10.830$	$146.70 \pm 17.950$	175.96±	180.5⊅⊭		
Kidney wt, %		(+4%)	(+10%)	15.175**	21,128**		
brain wt			~ /	$(+2 \hat{Q}^{*})$	(+62%)		
TT1 '1 1 1	$0.0272 \pm 0.0052$	$0.0224 \pm 0.0058$	$0.0269 \pm 0.0048$	0 <u>1</u> 0282 ±	0.6299±0.9951		
Thyroid gland,		(-18%)	(-1%)	×0.0060	× (+10%)		
g			<u> </u>	(+4%)			
	$0.00568 \pm$	$0.00471 \pm$	0.00496 ±	0.00565 ±. @	0.000121 (+06%) %		
Thyroid wt, %	0.00107	0.00123 (-17%)	0.00081 (-13%)	0.00106	0.00121 (+36%)		
body wt		Â		° (-1%)			
T1 1 4 44	$1.287 \pm 0.25164$	$1.068 \pm 0.2720^{\circ}$	1.178 ± 0.23204 @	∫ 1.352/± ∖	01.500@0.2640		
Thyroid wt, %		(-17%)	. (-8%)	0.23465 న	、 <b>√</b> 417%€≶		
brain wt		È S (		345%)~	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		
		, Ferma	les O O	Se v			
Terminal body	070 1:02 0			2630±23.3			
wt, g	270.1±23.0	269-3±16.8	274.522.5	263©±23.3	245.3*±19.6		
, 0		\$.94 ± 9.32		×7.09 ±	9.09 ± 1.55**		
Liver wt, g	$5.74 \pm 0.58$	\$3.94 <b>±</b> °0.32	$0.40 \pm 0.49$	0.58	$9.09 \pm 1.55^{**}$		
, 0				(+24%)	\$ ( <del> </del> \$8%)		
T · · 0/	~	2Q35 ± 00+05		~2697±	2 (00 + 0 502**		
Liver wt, %	$2.123 \pm 0.096$	$22/35 \pm 0005$	2.366 ± 0.07	0.172	$3.699 \pm 0.503 **$		
body wt	Ĵ,		~(+11%) ()	Q (+27%)	O [≫] (+74%)		
Linen and 0/	ia (	20(4) 10 00	222 45 + 15 250	367,12±0	≥ 501.61 ±		
Liver wt, %	292.99 3 24.236	$30643 \pm 18640$	323.45 ± 15.25	24.632**	70.554**		
brain wt			5910%	(+25%)	(+71%)		
Viduou and a	$1.65 \pm 0.10^{4}$	≪ 1.74 ± 0.13 ↔	$1.73 \pm 0.15$	¥1.73 ∉ 0.18	$1.70 \pm 0.17$		
Kidney wt, g		\$ ~(\$5%) ~	\$ (+ <b>5</b> %)	(+5%)	(+3%)		
Vidnov ut 0/		0.655±0.941	0.64@± 0.030	≈.659 ±	$0.693 \pm 0.069 **$		
Kidney wt, %	0 🔊 🔦	(+ <b>7</b> %) .~~	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	د ^ک 0.062	(+13%)		
body wt			Ö Å	(+7%)			
Kidney wt, 🕉	84.540 ± 0.618	89.904 ± 7.905	87.468 ± 5.139	89.817 ±	$94.027 \pm 10.455$		
brain wt	\$2 \$2 [*]	(+6%) (+6%)		9.611	(+11%)		
				(+6%)			
Thyroid gland,	$0.0185 \pm 0.0021$ x	(_0.0203 € 0.0040	$0.0164 \pm 0.0042$	0.0184 ±	$0.0206 \pm 0.0026$		
i nyroiu gland,		~+10%	(-11%)	0.0038	(+11%)		
g				(-1%)			
	0~000689 ക്	0.00767 ± 0	Ø.00610±	0.00696 ±	$0.00841 \pm$		
Thyroid wt, %	0.000	× 0.90163 ×	0.00156	0.00116	0.00097*		
body wt	Or _N O,	<u>11%</u>	Ø (-11%)	(0%)	(+22%)		
Thursday	0.94& 0.12	12050 ± 0/2168	0.829 ± 0.1925	0.954 ±	$1.140 \pm 0.1423$		
Thyroid vt, %		$(+1)^{3}$	(-13%)	0.2046	(+20%)		
brain wt				(0%)			
ignificant at * p ≤0	0.05; ** p 🔊 0.01	O. N					

4 3-

b) Recovery phase After 1 month of recovery in the high dese groups, mean terminal body weight was still lower than in controls in males and females (9%,  $p \le 0.01$  and -7%, not statistically significant, respectively). Mean fiver to body weight ratio in the high dose male and females groups was still statistically significantly higher than in controls, but this change was related to the lower body weight and was considered to be incidental.

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Mean absolute and relative kidney weights in the high dose male group were still statistically significantly higher than in controls. However, the magnitude of variation compared to control was clearly lower than at the end of the dosing phase.

Mean absolute thyroid weight and mean thyroid to body weight ratio in the female high dose group were statistically significantly higher than in controls, but this change was considered not to be treatments related as it was not associated with any microscopic finding.

### Table 5.3.2-7continued

	(3)		
	<b>Recovery phase</b>		
Dietary level (ppm)	0 🗸	0×	<b>3200</b> Star
	Make	Â.	
Terminal body wt, g	522 7 31.5		Q178.2+32.4**
Kidney wt, g	2.89 ± 0.16		$3.08 \pm 0.22* (47\%)$
Kidney wt, % body wt	$0(554 \pm 0)(24)$	, a a	645 ± 0.044** (+16%)
Kidney wt, % brain wt	193.05 466	P47	7.31 @ 12.280 ** (+14%)
* = p<0.05. **= p<0.01	$A$ $\phi$ $\phi$	Q ,	

2. Gross pathology

### a) Dosing phase

<u>Terminal sacrifice</u>: Enlarged and thirk liver and/or prominent bobulation of the liver were observed at 3200 and 1000 ppm. These findings corroborate the centrilobular hypertrophy noted at the microscopic examination.

Enlarged and/or pale kidneys were found in some males at 3200 ppm and 1000 ppm

Table 5.3.2-8Incidence macroscopic changes in the liver and kidneys terminal sacrifice

Liver: Obvious P large         0/10         0/9         24/9         46/9         90/10         9/10         0/10         1/10         7/10         10/           Liver: Dark         0/10         0/10         0/10         2/9         9/10         0/10         0/10         0/10         0/10         9/10         0/10         0/10         0/10         0/10         0/10         0/10         9/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0	Ĩ		Main study	(dosing) phas	se	O [×] ×	8		
Dose level (ppm)         0         50         200         1000         3200         50         200         1000         32           Liver: Obvious Varge         0/10         0/90         2410         50         60/10         0/10         0/10         1/10         7/10         10/           Liver: Dark         0/10         0/10         0/90         2410         50/9         9/10         0/10         0/10         1/10         7/10         10/           Liver: Dark         0/10         0/10         0/10         2/9         9/10         0/10         0/10         0/10         9/10         9/10         0/10         0/10         1/10         9/10         0/10         0/10         0/10         9/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/10         0/1	Sex 🔊			ŝ, O	Å.		Female	s	
Liver: Obvious large $0/10$ $0/9$ $24/9$ $6/9$ $0/10$ $0/10$ $1/10$ $7/10$ $10/10$ Liver: Dark $0'10$ $0/40'$ $0/9$ $0/10$ $2/9$ $9/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ <t< th=""><th></th><th><b>∑0</b> √50 ∢</th><th>200</th><th>1000 3200</th><th><b>A</b></th><th>50</th><th>200</th><th>1000</th><th>3200</th></t<>		<b>∑0</b> √50 ∢	200	1000 3200	<b>A</b>	50	200	1000	3200
Liver: Dark $0'$ $0/40'$ $0/9'$ $0/10'$ $2/9'$ $9/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ $0/10'$ <t< td=""><td>Liver: Obvious large</td><td>0/10 0/90</td><td></td><td></td><td></td><td>0/10</td><td>1/10</td><td>7/10</td><td>10/10</td></t<>	Liver: Obvious large	0/10 0/90				0/10	1/10	7/10	10/10
Liver: Prominent $0/10$ $0/9$ $0/10$ $4/9$ $6/10$ $0/10$ $0/10$ $1/10$ $0/1$	Liver: Dark 🔗		0/10	2/9 \$9/10	₽0/10	0/10	0/10	0/10	9/10
	lobulation	0/10 0/9	Ø [°] 0/10		0/10	0/10	0/10	1/10	0/10
Kidneys: Obviously 0/10 1/9 3010 4/9 6/10 0/10 0/10 1/10 0/10 0/1					0/10	0/10	1/10	0/10	0/10
Kidney: Pale $4/10$ $0/9$ $1/10$ $7/9$ $9/00$ $0/10$ $1/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0/10$ $0$	Kidney: Pale	<u>م /10</u>		7/9 9/00	0/10	1/10	0/10	0/10	0/10

b) Recovery please

Terminal sacrifice: After month of recovery in the high dose group, enlarged kidneys were noted in 2/10 males:

### 3. Microscopic pathology

a) Dosing phase

<u>Terminal sacrifice</u>: Effects of treatment with AE C656948 were seen in the liver, kidney, thyroid gland and lung.

In the liver, minimal to moderate centrilobular hepatocellular hypertrophy was observed with a doserelated increase in incidence and severity at 3200 and 1000 ppm in both sexes and minimal centrilobular hepatocellular hyperrophy, was observed at 200 ppm in males. In addition, minimal to moderate perportate midzonal hepatocellular macrovacuolation was observed in females at 3200 and 1000 ppm.

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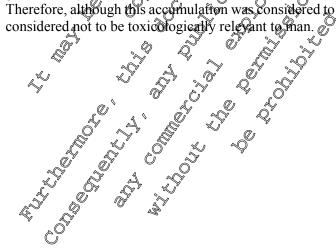
## BAYER E R

 Table 5.3.2-9
 Incidence of treatment-related microscopic changes in the liver after 90 days of treatment

										a,°	
Main study (dosing) phase     Sex       Males     Sex											
Sex			Male	8				» Fema	les		0
Dietary level (ppm)	0	50	200	1000	3200	0	50	200	1000	3200	
Number of animals examined	10	9	10	9	10	10	10	10 。	Ôľo	\$ ⁷ 10 "	Ċ,
Centrilobular hepatocellular hy	Centrilobular hepatocellular hypertrophy: diffuse										
Minimal	0	0	2	پ ج	0 (	$\mathbb{P}_0$	0	× B			Ô
Slight	0	0	01	4	2Q	Q °	0 4	0	2	ې ۲ 5 و	
Moderate	0	0		0	<u>~</u> 8 ~	Ċ		$\hat{\rho}_{O}$		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	
Total	0	0	2	<u>ُ</u> 9	10	0	<u>ر</u> و ر	$\bigcirc^{0}$	°≫7	10	
Periportal to midzonal hepatoce	llular ma	crova	cuolatio	n : <b>fo</b> cal	/m@tifo	cal	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		Y D		
Minimal	0 #		$\sqrt[n]{0}$		<u>ь о Ф</u>	<b>)</b> 0 %	$\bigcirc_0^{\circ}$	£3	6	\$3	
Slight		Q.	0,5	<u>j</u>	¢.	¢~	0	0	§ 0	° 1	
Moderate	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	Ŵ	Ĩ	- V	$\sim 0$	Ň	Ì	<b>A</b>	ູຢິ	1	1
Total	7, 0,	0 0		0	0 5	0 🗞			6	5	1

In the kidney, hyaline droplet nephropathy (characterized by basophilic tubules, hyaline droplets in proximal tubules and granular casts in the medulta) and hyaline casts were higher at 3200 and 1000 ppm in males, in comparison with controls. Hyaline droplet nephropathy was also slightly higher at 200 ppm in males. Hyaline droplet nephropathy is a recognized lesion of the male rat kidney that is related to the accumulation of  $\alpha_2\mu_3$  globulin in the proximal babules of affected animals. Accumulation of  $\alpha_2\mu_3$  globulin in the proximal babules of affected animals. Accumulation of  $\alpha_2\mu_3$  globulin in the proximal babules of affected animals. Accumulation of  $\alpha_2\mu_3$  globulin in the proximal babules of affected animals. Accumulation, which showed a higher storage of  $\alpha_2\mu_3$  globulin in the proximal convoluted tubules at 1000 ppm and above, and in the proximal straight tubules at 200 ppm and above, when compared to controls.

 $\alpha 2\mu$ -globulin is naturally reabsorbed and accumulated in the renal tubular epithelium of the young male rat. The sequence of renal events leading to its additional accumulation is dependent upon the interaction between a chemical and the  $\alpha 2\mu$ -globulin and is male rat-specific (G.C. HARD et al., Susceptibility of the kidney to toxic substances, in MOHRU. et al., Pathobiology of the aging rat, vol. 1, p. 252, ILSI Press, 1992, KIIA 3.3.2 /03; Hard, G. C., Alder, C.L., 1992, M-344988-01-1). As humans secrete  $\alpha 2\mu$ globulin only in trace for outs' this dechamping is generally accepted as being not relevant to humans. Therefore, although this accumulation was considered to be treatment-related in the current study, it was considered not to be toxicologically relevant to man.



## BAYER E R

 Table 5.3.2-10
 Incidence of treatment-related macroscopic changes in the kidney after 90 days of treatment

treatment										<i>a</i> .°	
	Main study (dosing) phase										
Sex			Males	5				≽ Fema		$\hat{\mathcal{D}}$	<i>.</i>
Dietary level (ppm)	0	50	200	1000	3200	0	50	200	1000	3200	
Number of animals examined	10	9	10	9	10	10	» 10	10 、	ÔĨO	\$ ⁹ 10 🔬	ĝ,
Hyaline droplets : proximal tubul	es			Č		<u>Í</u>		, K			
Minimal	1	3	7	ç Ö	0	$\mathbb{P}_0$	0	Ň	Ĩ	ãõ .	Ô
Slight	0	0	3.1	0	₩	Q °	0 🗸	0	, 0 、	^م ر و ک	
Moderate	0	0	Q	9	$\sim 1$	Ø		, Og			
Marked	0	0			9,5	0 🐇	0	$\bigcirc 0$	~~	$\mathbb{V}_0$	
Total	1	3	H			ð	<u>e</u>	00		<u> </u>	, 
<b>Basophilic tubules : focal/multifoc</b>	al "	$\langle \rangle$					Ŏ ^Ÿ	$\overset{\checkmark}{\sim}$	4.1	<u>S</u>	
Minimal	20	4%	3	3	1 KU	¢	0	0	§ 0	0° 0	
Slight	×0	Ŷ	Ŷ	¥*	$\gg$	õ	Ì	<u>o</u>	, P	0	
Moderate	0 K	1			1	0 🔊		$\begin{array}{c} 0\\ 0\\ 0\end{array}$		0	
Total	2	~ <b>G</b> 7	3	9 ⁵ /	<b>AN</b>	, Ô		0 0	× 0	0	
Granular casts: medulla 🚬 🖗	Ň,	S ^o	Ű,	Ø		, y , y , , y		Ŝ,			
Minimal	Ø	6	Ĩ LÔ	<u></u> 20 ⁵	, (6, °	_0 [%]	00	0	0	0	
Slight		<b>@</b> /0	Ĩ	×6	$\mathbb{O}_2^{\cdot}$	$\widetilde{00}$	- A	0	0	0	
Moderate	$p = 0 \sqrt{2}$	0	0	0.0	1	0	0	0	0	0	
Total	<b>K</b>	U O	J.	~8	J.S	ď v	0	0	0	0	
Hyaline casts: focal/multifocal	<u> </u>	× .		Ş.	0° _<	<u> </u>					
Minimar &	L.	0	00		<u> </u>	0	1	1	0	2	
Slight 5		Ő.	$\sim 0$		°≫0	0	0	0	0	0	
Total		0	° 0 €	4~	6	0	1	1	0	2	

In the thyroid gland, togher incidences of minimation slight diffuse hypertrophy of follicular cells was seen at 3200 and 1000 ppn in both sexes compared to controls and internal historical control data.

Table 5.3.2-11	Incidence of treatment-related macroscopic changes in the thyroid gland after 90 days
4 .	offreatment a frage

Main study (dosing) phase										
Sex & A &	<u></u>	Q,	Males	5				Fema	les	
Sex S S S S S S S S S S S S S S S S S S	0~0	D 50	200	1000	3200	0	50	200	1000	3200
Number Fanimer examined	10	9	10	9	10	10	10	10	10	10
Folligular celorypertrophy diffuse	;									
AMinimal &	0	0	1	3	5	0	0	0	2	1
Sligh	0	0	0	1	3	0	0	0	0	0
Total	0	0	1	4	8	0	0	0	1	1



### b) Recovery phase

After 1 month of recovery in the high dose groups, basophilic tubules, hyaline droplets in proximal  $\sqrt{2}$ tubules, granular casts in the medulla and hyaline casts were persistent in the kidney of some males.

	Main study (de	osing) phase	1	S S
Sex	Ma	les 🔊	Fema	ales à t
Dietary level (ppm)	0	∕∛3200		
Number of animals examined	10	<u>10</u>		Q 100 x
J	Hyaline droplets	proximal tubules		
Minimal	0	es 20 ×	× .00 ×	
Total	0 0 *			
I	Basophilic tubules	: focal/multifocal	A ô ^r	
Minimal				
Slight		× 277		
Total				°≫ 0
	Sranular cas	🚱: medulla 🔗		×
Minimal		~ 5 ×		0
Slight 📎 🔬				0
Moderate 🖇			& 07	0
Total L	\$\$0\$ [*]			0
	Hyaline casts: f	cal/multifocal		
Minimal 2		96	Ø 0	1
Total 🔬 🖉		Ø Ø	0	1

Incidence of treatment-related changes in the kidney after 1 month of recover Table 5.3.2-12

After the recovery period, liver and thyroid gland were comparable between the high dose and control groups, indicating that the changes noted after 90 days of treatment were reversible in these organs

### G. Deficiencies

G. Deficiencies

### III. Conclusions

In the Wistar rat, when administered AFC656978 in the diet over a 90-day period, a dose level of 50 ppm represented the NOMEL in males (equation to 3.06 mg/kg body weight/day), whereas a dose level of 200 ppm represented the NGEL in females (equating to 14.6 mg/kg body weight/day). However since the only relevant effects observed in males at 200 ppm were related to hyaline droplet nephropathy (known not to be relevant for humans) and minimal adaptive liver changes, the appropriate NOAEL for human rise assessment purpose is considered to be 200 ppm (equivalent to 12.5 mg/kg bw/day).

St Contraction of the second s



### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013.

In the Wistar rat, when administered AE C656948 in the diet over a 90-day period, a dose level of 50 ppm represented the NOAEL in males (equating to 3.06 mg/kg body weight/day), whereas addose level of 200 ppm represented the NOEL in females (equating to 14.6 mg/kg body weight/day) However since the only relevant effects observed in males at 200 ppm were folated to hyaline droplet nephropathy (known not to be relevant for humans) and minimal adaptive liver dranges the appropriate NOAEL for human risk assessment purpose is considered to be 200 ppm requivalent to 12.5 mg/kg bw/day).

12.0 mg/ng 0 (() awj):	
Data Point:	KCA 5.3.2/02
Report Author:	
Report Year:	
Report Title:	AE C656948 - 90-day toxicity study in the mouse by dietary administration
Report No:	SA 04052
Document No:	M-251136-01-4 ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~
Guideline(s) followed in	OECD 408; Streetive 2001/59/EC, Method B.26; US-EPA Series 870, No
study:	870.3100; @MAFF\$2 Nousan no \$47, @
Deviations from current	Current grideline. OECD 408, 2018 2 2 2 2 2
test guideline:	Deviations: normeasurement Othyroig hormone, no preasurements of HDL,
	LDL. These deviations have no impact on the outcome of the study and
	interpretation of the results.
Previous evaluation:	Yes, evaluated and submitted in the DAR (2014)
<b>`</b>	
GLP/Officially	Yes conducted under GLR Officially recognised testing fabilities
recognised testing	
facilities:	
Acceptability/Reliability:	$\mathcal{D}^{\mathbf{Y}}$ es $\mathcal{N}^{\mathbf{Y}}$ $\mathcal{N}^{\mathbf{Y}}$ $\mathcal{N}^{\mathbf{Y}}$ $\mathcal{N}^{\mathbf{Y}}$ $\mathcal{N}^{\mathbf{Y}}$

## Executive Summar

AE C656248 (batch number PFI 03040 99.0 % purity), was administered continuously via the diet to groups of C57BL/6J, wice (10) sex/group) at concentrations of Q. 30, 150 and 1000 ppm for at least 90 days (equating approximately to 0, 5.4, 26.6 and 188 mg/kg/day in males and 0, 6.8, 32.0 and 216 de L ,° Q mg/kg/day in females). Ľ

At 1000 ppm_mean bod consumption vos increased by up to 12% on a few occasions in males, compared to controls. Clipeal chemistry assessment revealed a higher mean alanine aminotransferase activity and a slightly lower mean albumin concentration in both sexes, together with a lower mean total cholesterol concentration, a higher mean alkaline phosphatase activity and a tendency towards higher mean aspartate aminotransferase activity in males. At necropsy, mean liver weights were increased by between 34% to 45% in both sexes and mean areanal weights were increased by between 87% to 92% in males. Microscopic examination revealed treatment-related changes in the liver and adrenal glands in both sexes. In the liver, minimal to moderate hypertrophy of centrilobular hepatocytes was observed in all animal logether with a greater incidence of minimal focal necrosis in 3/10 males and minimal to slight focative resist in \$90 females. In the adrenal glands, a lower incidence of ceroid pigment was noted in males, whilst a greater incidence of minimal to slight cortical vacuolation was observed in females, compared to controls.

At 30 ppp, at necropsysmean liver weights were increased by 9% to 16% in males and 25% to 28% in female, in correlation with a minimal to slight hypertrophy of centrilobular hepatocytes noted in all males and 5/10 females at the microscopic examination. These changes were considered to be adaptive but not adverse. Clinical chemistry assessment revealed a lower mean total cholesterol concentration in



males, compared to the control group. As this change was a decrease and in the absence of any other changes at this dose level apart from adaptive changes in the liver, this finding was considered to be non-adverse.

At 30 ppm, no adverse treatment-related change was observed.

A dose level of 150 ppm AE C656948 administered by dietary administration to the C57BL/6J mouse over 90 days, represented a NOAEL in males and females (equating to 26.6 and 32.0 mg/kg body weight/day, respectively).

	I. Materials and methods	AE C656948 Beige powder PFI 0304 99.0% 658066-35-4 Stable at 20 to 10000 ppm at room temperature for ±05 days None C57Bb/6J Approximately 6 weeks old Mates: 18 22.3(g; Females: 145 – 179 g
A.	Materials	
1.	Test material:	AE C656948
	Description	Beige powder
	Lot / Batch #:	PFI 0304
	Purity:	99.0% ~ ~ ~ ~ ~ ~ ~ ~ ~
	CAS #	658060-35-4
	Stability of test compound:	Stable at 20 to 10000 ppm at room temperature for 105 days
2.	Vehicle and / or positive	
	ontrol:	Over y is a si si si
3.	Test animals:	
	Species:	Mouse of St of St St k
	Species:	Č57BL6J
	Age:	Approximatory 6 works of a second sec
	Age: Weight at dosing: $\sqrt[n]{2}$	Mates: $1822.3$ g; Females: $1475 - 169$ g
	Source: Acclimation period:	
	Acclimation neried · 🔊 🕺	J days a w o o o o
	& & . )	7 days Certified rodent powdered and irradiated diet A04C-10 P1 from
	Diet:	S.A.F.E. Scientific Animal Food and Engineering, Epinay-sur-
	Water: O of to	Tap water, tal libitum of Q
	Housing:	Individually in suspended, standerss steel and wire mesh cages
	Housing: Environmental conditions: Temperature: Humidity:	Individually in suspended, staintess steel and wire mesh cages
	Air changes	40-70% 2 % A 10415 perhour %
	Humidity: Air changes Photoperiod:	10-15 per hour $\sim$
		$\int 2 \ln \theta d\theta d$
B.	Temperature: Humidity: Air changess Photoperiod: Study design	
1.	Humidity: Air changess Photoperiod: Study design In life dates: Animal assignment and treatment	02 - 07 October 2004
2	Animal assignment and treatm	ant and a definition of the second seco

There were 10 animals of each sex per dos Ogroup. Animals were assigned to dose groups using a randomization procedure by weight AE C656948 was administered in the diet for at least 90 days to C57BL/6J mice at the following doses -0, 30, 150 and 1000 ppm (equating approximately to 0, 5.4, 26.6 and 188 mg/kg/day in males and 0, 6.8, 32.0 and 216 mg/kg/day in females). A negative control group received plain diet. Animal housing and husbandry were in accordance with the regulations of the Guide for the Gare and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N 86-22, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Decembre 1986, N°86/609/CEE du 24 Novembre 1986".

Ô



Group	Test substance	Diet concentration (ppm)	No. Of animals per group	Animal 0 2 identity
		Males	<b>^</b>	
1	Control	0	10	OTIM279262801
2		30	10 🔗	OT2M2812to282
3	AE C656948	150	10 🛋	OT3M3832to2841
4		1000	10	OT4112852tg2861
		Females 🛷		
1	Control	0	168	UTIF2\$92to28
2		30	~\$0	O OT2F2822to2831
3	AE 1170437	150	~%10 ĝ° ĵ	✓ OT\$F2842to2851√
4		1000	× 10 ⁰	QT4F2862to287
<b>D</b> ! /		· «. »		

### 3. Diet preparation and analysis

AE C656948 was ground to a fine powdet before being incorporated into the diet by dry mixing to provide the required concentrations. There were two preparations of each concentration during the dudy. The stability had been demonstrated in a previous study where diet camples of 20 and 10000 ppm were found to be stable over a 105-day period at ambient temperature or over a 35-day freezing period followed by 10 days at ambient temperature. The homogeneity of the diet was verified on the first preparation at 30 and 1000 ppm and was within a range of 86-100% of the nominal concentrations. Concentration. Hence all values were within the target range of 85-115% of the nominal concentration.

### 4. Statistics

Data were analyzed by the Barbett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett test (2-sided) on parameters showing a significant effect by ANOVA. When the data were not homogeneous, a Kruskal-Wallis ANOVA was performed followed by the Dunn test (2-sided) to identify statistical significance between groups. For some parameters, when data were not homogenous, they were transformed using a log transformation, then reanalyzed as above. When one of more group variance(s) equaled 0, means were compared using non-parameteric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Jex System V42.2. (Module Enhanced Statistics).

### C. Methods

### 1. Observations

The animals were observed twice daily for morifundity and mortality (once daily on weekends or public holidays) observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed once during the acclimatization phase and at least weekly during the treatment period.

### 2. Body weight

Body weights were recorded twice during the acclimatization phase, on the first day of test substance administration, then at weekly intervals throughout the treatment period and before necropsy.

### 3. Food consumption and compound intake

Food consumption was recorded weekly; the weekly mean achieved dosage intake for each week and for weeks to 13 was calculated for each sex.

The group mean achieved dosage for each sex, expressed as mg/kg body weight/day, was calculated for each week and the overall mean subsequently derived for Weeks 1 to 13 using the following formula:



Mean weekly food consumption  $(g/day) \times Dose level (ppm)$ Mean achieved dosage (mg/kg body weight/day) =

Mean body weight at the end of the week (g)

### 4. Ophthalmology

Ophthalmological examinations were not conducted during the study.

### 5. Clinical chemistry

On study days 91, 92 or 93, in the morning, prior to necropsy, blood samples were taken from all mimats in all groups by puncture of the retro-orbital venous plexue. Animals were diet fasted vernight prior to bleeding and anesthetized by inhalation of Isoflurane. Blood was collected on lithiun reparation plasma chemistry determinations. Any significant change in the appearance of the plasma was recorded and the following clinical chemistry parameters were measured on a Hitacki 911 (Roche Diagnostics, Meylan, France) for: total bilirubin, total protein, albumin, total cholesterol and use a concentrations, and aspatiate aminotransferase, alanine aminotransferase and alkaline phosphatase activities.

### 6. Urinalysis

Urinalysis was not conducted in this study

### 7. Sacrifice and pathology

7. Sacrifice and pathology On study days 91, 92 or 93, a complete neoropsy was performed on all animals. Animals were deeply anesthetized by Isoflurane inhalation, then exsanguinated before necropsy. An animals we're fasted prior Ì to scheduled sacrifice.

The following organs or tissues were sampled:

The following organs or tissues were sampled:	
Adrenal gland	Eye , , , , , , , , , , , , , , , , , , ,
Aorta	Exorbiol (lachrymal) gland
Articular surface (fergoro-tioral)	Gallblädder
Bone (sternum)	Harderiareland
Bone marrow (sternum)	Heart N Q S
Brain of a start of a	Intestine Aileum, jejunum)
Cecum o a ar	Kinghey 🖉
Colon Colon	Aarynx pharynx
Duodenam	Liver
Epididymis	Lung
Oesophagus Q A S S S	Lymph nodes (submaxillary)
Lymph nodes@mesenteric)	Spina cord (cervical, thoracic, lumbar)
Mammary gland	Spleen
Nasal capities	Stomach
Optic nerve	Submaxillary (salivary) gland
Ovary & Strand	Testis
Pancreas	Thymus
Pituitary gland A A	Thyroid gland (with parathyroid gland)
Prostate & A &	Tongue
Rectum of the contract of the	Trachea
Sciatic herver A S	Urinary bladder
Adrenal gland Adrenal gland Aorta Articular surface (femoro-tibiral) Bone (sternum) Bone marrow (sternum) Brain Cecum Colon Duodenum Epididymis Oesophagus Lymph nodes (mesenteric) Mammary gland Nasal capitles Optic nerve Ovaty Pancreas Pituitary gland Prostate Rectum	Uterus (including cervix)
Skeletal muscle	Vagina
Skin 🖉	



A bone marrow smear was prepared from femur, stained with May-Grünwald Giemsa, but not examined. Tissues samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eve, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative. Histopathological examinations were performed on all tissues from all the animals in the confront and high dose groups and all decedents in all groups. The liver, kidney, lung, thyroid gland and were examined in all animals in the study. In addition, the adrenal gland was also examined in the intermediate dose groups as necessary to identify the no-effect-level, as it was identified as a possible target organ Significant macroscopic findings were also examined in all dose groups.

#### II. **Results and discussion**

### A. Clinical signs and mortality

### 1. Clinical signs of toxicity

There were no treatment-related clinical signs of toxicity observed in animals at any dose revel 2 Mortality

### 2. Mortality

No treatment-related mortality occurred in the test animals at any dose level

One male at 30 ppm was found dead on Day 30 after & body weigh loss of 6.9 g between Day 15 and 29 and a reduced food consumption between Days and 29. Clinical signs recorded prior to death for this animal were reduced motor activity on Days 22 and 23 together with waster appearance and hunched posture from Days 22 to 29 Spontaneous hydrocephalus observed at the macroscopic examination and confirmed microscopically was considered to be the cause of death for this animal. Hence, the death of this animal was attribused to a spontaneous lesion and was not treatment-related. A second male, from the control group, was killed for humane reasons on Day 69 after an accidental trauma.

### B. Body weight and body weight gain

The body weight evolution of animals was unaffected by treatment in any dose group.

### C. Food consumption and compound intake

Food consumption was unaffected by treatment at 1000 ppm in females or at 150 or 30 ppm in either sex.

The mean achieved dosage intake of AE C6569480er group is presented in the following table: K.

Sex 🖉	Å,		C Mates	S P		Females	
Dietary level (ppm)		≫ <b>30</b> ~~	Q.50	1900	30	150	1000
Weeks 1.03		5.4	Ŵ 26.	188	6.8	32.0	216

### Mean achieved dose levels of AE C696948 in mg/kg bw/day, Table 5.3.2-14

### D. Blood analyses

### 1. Clinical Chemistry

At 1000 ppm mean alanine aminotransferase activity was higher in males and females (+205 and +109% after removal of a poutlier in the female control values, respectively,  $p \le 0.01$ ), compared to the control groups. Jy addition in males, mean alkaline phosphatase activity was higher (+21%, p≤0.01), mean albumin and mean total cholesterol concentrations were lower (-12 and -40%, respectively,  $p \le 0.04$ ) and a lender by towards higher values was noted in aspartate aminotransferase activity (+46%, p<0.05). In females, a tendency towards lower mean albumin concentration was also noted (-9%, p≤0.01).



At 150 and 30 ppm, the only treatment-related change consisted in a lower mean total cholesterol concentration in males (-41 and -30%, respectively,  $p \le 0.01$ ), compared to the control group. As it was a decrease and was observed in the absence of any other changes apart from adaptive changes in the liver, lower mean cholesterol concentrations were considered not to be adverse.

Table 5.3.2-15	Mean values for alanine aminotransferase, albumin,	aspartate amino	transferase and
	cholesterol (mean ± SD)	- "0"	

Sec		Ma	ales		🔊 Females 💊 🔬			
Dietary level (ppm)	0	30	150	1000	0	<b>Ø</b> 30	150	J 1000
Alamine aminotransferase IU/L	40±13	88±79	55±16	122±35*	83±Q8	56±20 4	64±24	96±32*0
Albumin, g/L	41±1	39±2	38±2* ~~	36±2*	_@ <b>4</b> 3±3_°^	₹ 43#?	2±2, *	¥ 39≇ <b>2*</b>
Aspartate aminotransferase , IU/L	108±4 0	139±50	92±21	\$58±51*		\$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$		466±71 °
Cholesterol mmol/l	1.35±0.17	0.94±0.21*	g79±0-14	0.81 0.16*	Q.22±033		0.93±0.15*	1.3300.1
Significant at * p < 0.0	01; ** p <0.	05;		\$ \$		5 \$	i di	<u> </u>

### E. Sacrifice and pathology

### 1. Organ weight

There was no relevant change in terminal fordy weight in either sex. Mean absolute and/or relative liver weights were statistically significantly ligher at 1000 and 150 ppm in both sexes with a dose-related effect. At 30 ppm, mean absolute liver weight and relative liver to body weight ratio were statistically significantly higher in females only, but this change was considered not to be toxicologically relevant since it was not observed in all parameters and not associated with any histological change.

Table 5.3.2-16	ÛLiv	erweight	changes	at term	inaksa	crifice	% chai	nge wh	* compared to controls)
8	) (m	an ± <b>SD</b> )	Õ	K)	<i>K</i> [°]	ð,			<b>I</b> ,

	y Ku		0,0	
Sex 🔊		<u>, S^Y O' M</u>	ales 🖉	
Dietary level (ppm)		30%	<b>&gt;</b> 150	1000
Terminal body wt, g -	© 2,990±1.2 [™]	23.9±0.8 ×	≥ 22.5±0.9	22.6±0.6
Liver wt, g	$^{\circ}$	O ^v 0.94±0.15	≤″ 1.04±0.07	1.24±0.14*
Liver wt, % body wt	3.973±@/312 √	40064±0.640	4.590±0.147*	5.497±0.688*
Liver wt, % brain wt	_Q10.400±21.300	×212.555 36.690	230.188±14.668	282.470±33.447*
	° ∕ ° ∕ ′	<b>Fen</b>	nales	
A A A A A A A A A A A A A A A A A A A			150	1000
Terminal body wt, g	18.30.7	∞ 18.5≠0.5	$18.8\pm0.6$	19.1±0.5*
ronning ouy we, 5			10.0±0.0	17.1±0.5
Liver weg	0.79+0.09	\$ 0.84±0.06**	0.96±0.08*	1.09±0.07*
Liver we'g Liver wt, % body wt	$0.75 \pm 0.09$ 4 $4.02 \pm 0.452$	€ 0.84±0.06** 4.566±0.294**		
Liver we g	0.75±0.09 4.102±0.452 1.65.237±22.807	€ 0.84±0.06** 4.566±0.294**	0.96±0.08*	1.09±0.07*

Statistically significant at p < 0.01; p < 0.05;

Mean absolute and relative drenat gland weights were increased by between 87% and 92% at 1000 ppm in males compared to controls, the offference being statistically significant for mean adrenal gland to body weight ratio only  $(p \le 0.05)$ .

### 2. Gross and histopathology

Terminal sacrifice: at 1000 ppm, enlarged livers were observed in 8/10 males and 9/10 females, and dark lives were observed in 5/10 males and 10/10 females. Other gross pathology changes were considered as incidental and not treatment-related.



<u>Microscopic pathology</u>: effects of treatment with AE C656948 were seen in the liver and adrenal gland in both sexes.  $^{\circ}$ 

In the liver, there was a minimal to moderate hypertrophy of centrilobular hepatocytes in both seves at 1000 and 150 ppm. This finding was considered to be the explanation for the enlarged livers observed at necropsy in animals given 1000 ppm and the statistically significant increase in liver weight seen in animals given 1000 or 150 ppm. In isolation, the finding observed at 150 ppm was considered to be associated with adaptive changes rather than evidence of toxicity. In addition in the liver, there was a greater incidence of minimal or slight focal necrosis in both sexes at 1000 ppm, when compared to controls. Minimal or slight focal necrosis was present in 10 males and 6/10 females given 1000 ppm, compared to one female in the control group.

Liver, N examined     9     29     010       Centrilobular hepatocellular hypertrophy:     0     0     0       Minimal     0     0     0     0       Slight     0     0     0     0       Moderate     0     0     0     0       Focal necrosis:     0     0     0     0       Minimal     0     0     0     0       Slight     0     0     0     0       Total     0     0     0     0       Slight     0     0     0     0	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$		<b>1</b> 50	<b>1000</b> 10 <u>°</u>
Liver, N examined     9     9     9     0     0       Centrilobular hepatocellular hypertrophy:     10     10     10     10       Minimal     0     0     20     7     10       Slight     0     0     0     0     10       Moderate     0     0     0     0     10       Focal necrosis:     10     10     10     10       Minimal     10     10     10     10       Slight     10     10     10     10	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$		10 4 5	1000
Centrilobular hepatocellular hypertrophy:     Image: Centrilobular hepatocellular hypertrophy:       Minimal     Image: Centrilobular hepatocellular Minimal       Slight     Image: Centrilobular hepatocellular Moderate       Total     Image: Centrilobular hepatocellular Moderate       Focal necrosis:     Image: Centrilobular hepatocellular Minimal       Slight     Image: Centrilobular hepatocellular Minimal       Slight     Image: Centrilobular hepatocellular Minimal       Image: Centrilobular hepatocellular Minimal     Image: Centrilobular hepatocellular Minimal			5	
hypertrophy:     Image: Constraint of the second seco			5	
Minimal     O     O     O       Slight     V     0     0     7       Moderate     O     0     0     0       Total     O     O     O     O       Focal necrosis:     V     V     0     0       Minimal     V     0     0     0       Slight     V     V     0     0			5	× 1
Minimal     O     O     O       Slight     V     0     0     7       Moderate     O     0     0     0       Total     O     O     O     O       Focal necrosis:     V     V     0     0       Minimal     V     0     0     0       Slight     V     V     0     0				^{\$} 1
Moderate     0     0     0       Total     0     0     0       Focal necrosis:     0     0     0       Minimal     0     0     0       Slight     0     0     0	$\begin{array}{c} 0 \\ 10 \end{array} \begin{array}{c} 0 \\ 0 \end{array} \begin{array}{c} 0 \\ 0 \end{array}$		0	1
Total     0     0     0       Focal necrosis:     7     7     7       Minimal     7     7     0     0       Slight     7     0     0     0	100 06		0	8
Focal necrosis:     Image: Control of the control of th			0 0	1
Minimal     Image: Minimal     Image: Minimal     Image: Minimal       Slight     Image: Minimal     Image: Minimal     Image: Minimal	19 ~9	Se ^	3	10
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$				
Slight         V         0         0         0           Total         V         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q         Q	3 1	10	0	3
Total 2 C C C		Ø	0	3
	Y A	<u>1</u>	0	6
Adrenal, N examined 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	10 10 10	D 10	10	10
Cortical ceroid nigneent: $\mathcal{D}$				
		0	0	0
Slight $\sqrt{2}$ $\sqrt{2}$ $\sqrt{2}$ $\sqrt{2}$ $\sqrt{2}$ $\sqrt{2}$ $\sqrt{2}$		0	0	0
Total $\mathcal{O}$ $\mathcal$	0 🖤 0	0	0	0
Cortical vacuolation , C				
Minimad Q A Q Q	Ø	1	2	9
	<b>V</b> 3	1	~	1
$\frac{Takat}{Takat} \underbrace{\mathcal{O}}_{A} \underbrace{\mathcal{O}} \underbrace{\mathcal{O}}_{A} \underbrace{\mathcal{O}} \underbrace{\mathcalO} $	//	0	0	1

Table 5.3.2-17	Incidence of microscopic changes in the liver and adremal gland	
1 abic 3.3.2-17	Incluence of finctoscopic changes in the river and auteural grand	

In the adrenal glands at 1000 ppm, there was a lower incidence of cortical ceroid pigment in males and a greater incidence of minimat to slight cortical vacuolation in females, compared to controls. The change noted in males at 1000 ppm was considered to be light compared to the magnitude of increase in adrenal gland weights scen in this sex. No treatment-related changes were observed in the adrenal glands at 150 or 30 ppm in either sex.

In the fore-stomach of males given 1000 ppm, there was a slightly greater incidence of focal epithelial hyperplasia than in controls However, as these changes in males were only focal and isolated, they were considered not to be tradiment related. In females, the incidence and severity of this finding were comparable between controls and treated animals.

F. Deficiencies

Nødeficieræcies were nøted

IIK Conclustons

Under the conditions of this study, a dose level of 150 ppm AE C656948 by dietary administration to the C57BE/6J mouse for 90 days, represented a NOAEL in males and females (equating to 26.6 and 32.0 mg/kg body weight/day, respectively).



Under the conditions of	guidance and the requirements in 283/2013. This study, a dose level of 150 ppm AE C656948 by dietary administration for 90 days, represented a NOAEL in males and females (equating to 26.6
Data Point:	KCA 5.3.2/03
Report Author:	
Report Year:	
Report Title:	AE C656948 - 90-day toxicity study in the dog by dietary administration
Report No:	SA05046 $\mathcal{O}$ $\mathcal{O}$ $\mathcal{O}$ $\mathcal{O}$ $\mathcal{O}$
Document No:	<u>M-276047-01-1</u>
Guideline(s) followed in	O.E.C.D. guideline 409 (1998); E.E.O. Directive 2001/59/EC, Method B.27 (August 21, 2001); U.S. E.P.A., OPPTS Sories 870, Health Effects Testing
study:	(August 21, 2001); U.S. EP.A., OPPTS Series 870, Health Effects Testing
	Guidelines, No \$70.3150 (August, 1998); M.A.F.F. in Japan, notification 12
	Nousan n°8147 November 24, 2000 V Sy
Deviations from current	Current guidefine: @ECD 409, 1998
test guideline:	Deviation Wine & S S & S & G
Previous evaluation:	Yes, evaluated and submitted in the DAR (2010)
GLP/Officially	Yes conducted under GLP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes Q Y Y Q Y Y

#### Executive summary

Executive summary Technical grade AF C656948 was administered in the diet to Beagle dogs (4/sex/dose) at dose levels of 0, 800, 5000 and 20000/10000 ppm for at least 13 weeks (equivalent to 0, 28.5, 171, 332 mg/kg bw/day in males and 0, 32,99 84, 397 mgArg bw/day in females).

Cage side Observations were conducted daily detailed clineral observations were conducted weekly, food consumption was preasured dails, and body weights were taken weekly. Ophthalmic examinations were performed once pre-exposure and just prior to necrops Clinical chemistry, hematology, and urinalysis measurements were taken once pre-exposure and approximately during study weeks 8 and 13. A gross necropsy was performed selected organ weights were taken, and a range of tissues were collected and process of for histopathological examination

#### At 20000/10000 ppm:

Due to a tack of palatability of AE C656946 in the diet, a marked reduction in food consumption was observed in both seves with an associated effect on body weight gains and body weights. Despite the reduction of the dietary level of the test components from 20000 to 10000 ppm after 2 weeks of treatment, food consumption and body weight remained lower than controls throughout the study. Changes were also noted in hematology parameters platefet count, erythrocyte counts, hemoglobin concentration and hematology) and some clinical chemistry parameters (mainly alkaline phosphatase and  $\delta$ -glutamyl transferase activities and pilirubir, albumin and protein concentrations).

The liver was the main target organ as shown by an increased weight together with macroscopic observation & enlarged"), in association with histopathological effects (hepatocellular hypertrophy, intracytoplasmic eosinophilic droplets and single cell necrosis).

The them was found to be generally atrophic and associated with a higher severity in them the severity in the severity in the severity in the severity in the severity is the severity in the severity is the severity in the severity in the severity in the severity is the severity in the severity in the severity in the severity is the severity in the severity is the severity in the severity is the severity in the severity in the severity is the severity is the severity in the severity is the severity in the severity is the severity in the severity is the severity is the severity in the severity is the involution compared to controls. The estrous cycle was also disturbed in females (all females were in



anestrus phase). However these effects on the thymus and estrous cycle were attributed to the marked decrease food consumption and bodyweight at this dose level.

#### At 5000 ppm:

Although less pronounced than at the high dose, similar pattern of effects were seen at this dose level including effects on food consumption, body weight, clinical chemistry and the liver. At 800ppm: There was no treatment-related adverse effect. toom temperature toom tempera

The NOAEL for this study was 800 ppm (28.5/32.9 mg/kg/day for mates/females).

AE C656948 🛛

Mix-Batch:08528/

Beige powder

Mm. 94.6%

**©**58066-35-4

Stable at 25

Beagle 5

Males: 6.3 9.0 ko & females: 4.9 - 7.1

None

Dog

#### I. Materials and methods

- A. Materials
- 1. Test material: Description Lot / Batch #: **Purity:** CAS# Stability of test compound: Q

2. Vehicle and / or positive

- control:
- 3. Test animals: **Species:** Strain: Age: Weight at doside

Source: Acclimation period

20 days Centified canine meal 125C3-Pt from S.A.F.E. (Scientific Amimal Food and Engineering, Augy, France). Three hundred grams of die moistened with 450 ml of water at  $\swarrow$  the tone of distribution was given daily to each animal for approximately 1.9 hours each morning, except on study Day 337 When animals were for in the afternoon due to a change in >>> planning.

In addition due to a low food consumption observed on several consecutive days and/or to a marked body weight loss, a thin layer of commercialized dog food (Beef pâté from Pedigree®) ² was [©] wer the normal dietary ration of 2 animals (PT4M1291 and PT4F1295) to stimulate their appetite as

		layer of commerciali	zed dog food (Beef	pâté from Pedigree®
Diet:		layer of commerciali was opread over the r (PT4M1291 and PT4	normal dietary ratio	on of 2 animals
$\sim$	No. No.	(PT4M1291 and PT4	F1295) to stimulat	e their appetite as
		described below:		
Ó	Y A & K	<u>Animal</u>	Study days	Beef pâté added to
		~0		the diet
ő,	S 0 5	⁹ PT4M1291	16	200 g
~~~			17 to 19	100 g
	n A V	PT4F1295	16	200g
			17 to 21	100g
			66 to 69	100g
¢Q'			90	400g



	Water:	Filtered and softened tap water from the municipal water supply,
	vi ater.	ad libitum
		Animals were housed individually in stainless steel kennels with
		a floor surface area of 1.2 m^2 .
		When possible, they were pair housed overhight by temporary
		opening of the partitions between 2 dogs from the same sex and
		dose group.
	Housing:	Supervised exercise in inside runs was permitted daily for dogs 🖉
		of the same sex and treatment group throughout the acclimatization and treatment periods except on weekends and public holidays. Additional supervised exercise was permitted in a
		acclimatization and reatment periods except on weekends and
		public holidays. Additional supervised exercise was permitted in &
		inside runs for an extended tige of approximately 1 hour at leas \mathbb{Q}^{2}
		once per weel for dogs of the same sex and treatment group.
	Environmental condit	
	Temperature:	18-21°C 2 2 4 2 2 2
	Humidity:	
	Air changes:	Target of 15 per hour during the dosing period
	Photoperiod:	12 hours dark, 12 hours light (Fam - Typm) a w
ъ	•	
в.	Study design	12 bours dark, 12 hours light (Fam – 7 pm)
1.	In life dates: 06	5 Aprik 005 گر 2005 کې

2. Animal assignment and treatment

Shortly after arrival, all dogs were examined by a veterinarian for signs of ill-health and were subjected to a detailed clinical examination to assess their physical and behavioral status. Body weight was recorded and food intake determined All animals were subjected to an ophthal pological examination, hematology and clinical chernistry investigations and urinalysis Once during the acclimatization phase. Animals were allocated to dosage groups using a randomization procedure in order to ensure a similar body weight distribution among groups of each sex. Test groups were as described in the table 5.3.3-1. Control animals received untreated dist. All other groups received the appropriate dietary concentrations at a constant (ppm) level. Control animals receiver unitered at a constant (ppm) byel.

Ê.

Test Group (Group number) Sex : Number of Animals	Achieved dose to animal (Weeks 1-13) (mg/kg/day)
Control Contro	0
Low (2) Male 4 5 800 Female: 4 5 800	Male: 28.5 Female:32.9
Mid (3) Male: 4 5000	Male: 171 Female: 184
High (4) Mate: 4 20000 (Day 1 to 14) Feesale: 4 10000 (Day 15 to sacrifice) Diet preparation and analysis	Male: 332 Female : 337

3. Diet preparation and analysis

The appropriate amount (weight/weight concentration) of test substance was incorporated into the ground diet to provide the required dietary concentrations of 800, 5000 or 10000 or 20000 ppm. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. One preparation at 20000 ppm and three preparations at 800, 5000 and 10000 ppm were performed to provide the treated diet required for the study.



Homogeneity of the test substance in the diet was checked at 800, 10000 and 20000 ppm on the 1st formulation. Concentrations were checked for each dose levels and all preparations. Stability of the preparations under storage conditions was determined at 800 and 20000 ppm. In addition, stability in the moistened diet distributed to the dogs was determined for a period covering the time for food preparation and distribution.

The homogeneity analysis demonstrated that the concentration was av 87-115% of nominal concentration confirming that the formulations were homogeneously distributed.

AE C656948 was found to be stable in the diet at 800 ard 20000 ppm wer a period that covered the usage and storage conditions used in this study and for at least 4 additional hours, which covered the time of food preparation and distribution.

The mean concentrations for the study were 89-10% of the nominal levels; Results were within the fahouse target range of 85 to 115% of nominal concentration and were therefore considered to be acceptable for use on the current study.

4. Statistics

Statistical analyses were carried out using Path/Tox system version 4.2.2 (Module Enhanced Statistics). Continuous data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett test (2-sided) of parameters showing a significant effect by ANOVA. When the data were not homogeneous a Kruskal-Wallis ANOVA was performed followed by the Dunn test (2-sided) to identify statistical significance between groups. For some parameters, when data were not homogeneous, they were transformed (log transformation or square root transformation), then reanalyzed as above.

For urine analysis (pH), group means over compared using the non-parametric Kruskal-Wallis test which was followed by the Durn test (2-side), if the Kruskal-Wallis test indicated significance.

When one or more group variance (spequaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

C. Methods

1. Observations

All study animals were observed at least twice daily for in-health, moribundity, mortality and clinical signs of toxicity (except once daily on weekends and bolidays). This included but was not limited to changes in general behavior and appearance, skin and fur, teeth and gum, eyes, ears, mucous membranes. Any deviations from normal were recorded in respect to the nature and severity. Daily examination of the kennels was also carried out for vomitus, diarrhea or blood.

Detailed clinical observations to clinical signs of to city were performed on all animals at treatment initiation (study day 0) and on a weekly basis thereafter. The physical examination included but was not necessarily restricted to the following examinations: fur and skin, eyes, ears, teeth, gum, mucous memoranes, rectal temperature, gait, statue, general behavior, chest including heart and respiratory rate, abdomen including palpitation, external genitalia and mammary glands

2. Body weight

Body weights were recorded weekly throughout the treatment. Body weights were also taken immediately prior to necropsyco allow for calculation of organ-to-body weight ratios.

3. Food consumption and compound intake

Food intake was measured for a minimum of 4 consecutive days immediately before the start of treatment. The weight of food supplied to each animal and that remaining were recorded daily throughout the treatment period. From these records, the mean weekly consumption was calculated for each dog. Food spillage was also recorded.



The group mean achieved dosage for each sex, expressed as mg/kg body weight/day, was calculated for each week and the overall mean subsequently derived for Weeks 1 to 13.

4. Ophthalmology

Following the acclimation period and prior to initiation of dosing, ophthalmic examinations were conducted on all animals after instillation of an atropinic agent (mydriaticum). Sphthalmic examinations were also conducted on all animals just prior to termination of the study.

5. Hematology and clinical chemistry, and bioanalytical examination

Clinical chemistry and complete blood count, including differentials, were performed on all animals once prior to administration of the test substance and on days 55-57 and 86-87. Animals were dier fasted overnight prior to blood collection, which was drawn via jugular with puncture.

The following parameters were evaluated:

Hematology:

Red blood cell count, hemoglobin, hematocrie, meta corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocore count, white blood cell count and differential count evaluation and plateter count were assaded using ap Advia 120 Bayer Diagnostics, Puteaux, France). A blood sincar was prepared and stailed with Wright stain. It was examined when the results of Advia 20 determinations were abnormat Protherminiprime and activated partial thromboplastin time were assayed on an ACL 2000 (Instrumentation Laboratory, Paris, France).

Clinical chemistry:

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus concentrations, and aspartate minofransferase, alarine-aminotransferase, alkaline phosphatase and gamma-glutamytransferase activities were assayed on plasma samples. Total protein and albumpr concentrations were assayed on serum samples using an Hitachi 911 (Roche Diagnostics, Merjan, France). Globulin and albumpr globulin ratie values were calculated.

6. Urinalysis

Once before dosing and on study Days 49 (or 50) and 84 in the moving, overnight urine samples were collected from all animals in all groups. Access to stater was not estricted during urine collection.

Any significant change in the general appearance of the urine was recorded.

Urine samples ware weighed to determine, winary volume pH was assayed using Clinitek 200+ and Ames Multistic dipsticks (Bayer Diagnostics, Putcaux, France). Urinary refractive index was measured using a RFM 20 refractoreter (Bjoblock Scientific, Hkirch, France).

Glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen were assayed using Clinitek 200+ and Ames Multistix dipsticks (Bayer Diagnostics, Puteaux, France).

Microscopic examination of the urinary rediment was performed after centrifugation of the urine. The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was graded.

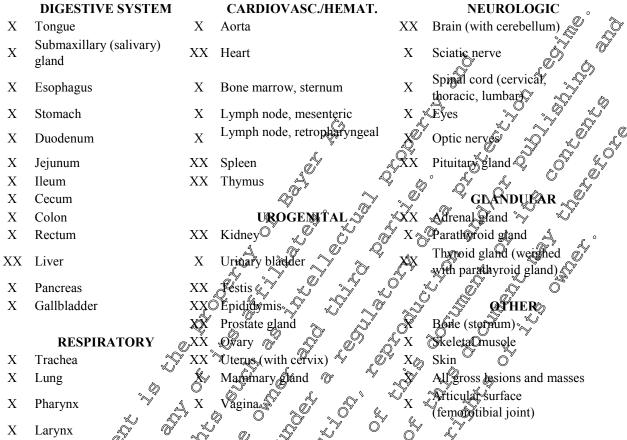
7. Sacrifice and pathology

On study days 91 to 94, all animals from all groups were sacrificed. All animals were tranquilized by intramuscular injection of acopromazine (50 μ /kg body weight) and then deeply anesthetized by intravenous injection of pentobarbital (0.5 mL/kg body weight). Animals were then exsanguinated and necropsied

The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically.



The following organs or tissues were sampled and/or weighed at necropsy:



(X) Tissues were collected.

(XX) Organs were weighed fresh at scheduled sacrifice. Paired organs were weighed together.

For sacrificed animals a bone marrow smear was prepared from one rib and stained with May-Grünwald Giemsa, but not examined.

Samples were fixed by immersion in neutral buffered 10% formation with the exception of the eye, optic nerve, epididymis and estist that were fixed in Davidson's fixative.

Histopathological examinations were performed on an tissues from all the animals in all dose groups. After the examination by the study pathologist, an independent review by a second pathologist was conducted. The result presented in the report are a consensus view of the 2 pathologists.

II. Results and discussion

A. Clinical signs and mortality

1. Chnical signs of toxicity and physical examination

The only clinical sign observed at the bigh dose was a wasted appearance noted for 1 male and 2 females. This sign was observed in conclusion with a reduced food consumption attributed to a lack of palatability of AE C656948 at this dietary level.

Animals & 5000 and 800 ppm and no treatment-related clinical signs.

There was no reatment-related change in rectal temperature.

2. Mortality

There were no mortalities throughout the study.



B. Body weight and body weight gain

At 20000 ppm, there was a mean body weight loss noted in both sexes during the first 2 weeks. This was observed together with a reduced food consumption in animals of these groups which was attributed to a lack of palatability of the test substance in the diet. Thereafter, the dietary level was reduced from 20000 ppm to 10000 ppm, however, mean body weight gain remained reduced in comparison to controls on most occasions. Overall, a body weight loss of 0.8 kg and 1.1 kg was noted in males and females, respectively compared to a body weight gain of 1.0 kg in controls which resulted in an 5% and 29% reduction in final body weight for males and females, respectively compared to controls.

At 5000 ppm, mean absolute weight gains were slightly reduced in both sexes compared to controls. Overall, body weight gains of 0.6 kg and 0.2 kg were noted for males and temales respectively compared to 1.0 kg in both male and female control groups. This corresponded to 7% and 11% reduction in final body weight in males and females respectively, compared to controls.

At 800 ppm, body weight parameters were comparable to controls in males while there was a slight reduction in body weight gain in females. All the end of the study mean female body weight was 8% lower than controls.

In all cases, effects on body weight parameters were concomitant with a lower food consumption of comparable magnitude due to a lack oppalatability of the test substance in the diff.

3			~~. (()) [*]	
Dosage level (ppm)		2° 8 00 °	5000 Q	20000/10000
Sex			illes 🗸 🔊	ý.
Initial BW (%C)	7.4 20.8	7.5 ± 0.4 400%)	₹ .2 ± 0,7 (97%)	7.7 ± 0.3 (104%)
BWG Week 1		0.2 ≠ 0.1 ×	O 0.1, ¥0.1 √	-0.3 ± 0.1 **
BWG Weeks 1-4	0.6 ± 0	0.0 ± 0.20	0.4 ± 0.3	-0.7 ± 0.4 **
BWG Weeks 1-85	, 00.9 ± 0½ ∽	0.8 ± 0.4	Ø.4 ± 0.6	$-0.8 \pm 0.4 **$
BWG Weeks 1	>> 1.0 ± 0.3 ≪	1.0,40.7	£ 0.6 ±0 .8	$-0.8 \pm 0.5 **$
Final BW ()	3.0 ± 0.8 °	8.4±07(1000)	₹7.8±∂,1 (93%)	6.9 ± 1.0 (82%)
Sex	r °, ô 1	Fem 🖓 🖓 🖓		
Initial BW (%C)	5.5 ± 9.6 0	5.6 ± 1.1 (102%)	5.€ ± 0.6 (102%)	5.7 ± 0.7 (104%)
BW& Week 1	0.30,0.1	0.1⊕0.2 √	$^{\circ}$ 0' 0.1 ± 0.1	-0.4 ± 0.2 **
BWG Weeks 1-4	$\beta = 0.7 \pm 0.1$	0.3 ± 0.2	0.1 ± 0.4	$-0.8 \pm 0.3 **$
BWG Weeks 1-8	0.8 ± 62	@.4 ± 0@* 🔊	0.0 ± 0.5	$-1.1 \pm 0.6 **$
BWG Weeks 1-13	j>1.0 € 0.3 j	0.4 ≠0.5	0.2 ± 0.5	$-1.1 \pm 0.5 **$
Final BW (%)	0 65±0.6	06.0 ≠ 02 (92%)	5.8 ± 0.8 (89%)	4.6 ± 0.7 (71%)

Table 5.3.2-19 Body weight and weight gains (kg) in the 90 day dop study with AE C656948 (mean ± SD)

*: p≤0.05; **: p≤0.01; BWG Gody weight gain; (%G)=% forsus control

C. Food sonsumption and compound intake

At 20000 ppm, there was a marked reduction of food consumption in both sexes (- 25 to - 53% and - 53 to - 58% in males and females, respectively) during the first 2 weeks of treatment compared to controls. Thereafter, although the dietary level of AE 0556948 was reduced to 10000 ppm, the food consumption was still reduced by 238% in males and 2853% in females.

To a lesser extent the food consumption was also reduced at 5000 ppm in both sexes (overall, 7% and 22% reduction in males and females, respectively compared to controls). In addition there was still a slightly reduced food consumption in females at the low dose (-9% overall in comparison to controls).

The decrease in food consumption was attributed to a lack of palatability of the test substance when incorporated into the diet. Hence, lower dietary concentrations were associated with better food intake and the use of commercialized dog food moistened with the diet to improve the palatability of the diet and to stimulate appetite of the dogs resulted an improved food intake.



0	800	5000	20000/10 000	0	800	5000	20000/10° 000
	Ma	les			Fem	ales	
641	671	548	478*	638	524	> 490	£99** A
	(105%)	(85%)	(75%)		(82%)	[°] (77%)	47%)
701	722	653	528	676	61,2	527 🔍	368
	(103%)	(93%)	(75%)		(9,1%)	(78%) [©]	(54%)
	-	Ma 641 671 (105%) 701	Males 641 671 548 (105%) (85%) 701 722 653	0 800 5000 000 Males 641 671 548 478* (105%) (85%) (75%) 701 722 653 528	0 800 5000 000 0 Males 641 671 548 478* 638 (105%) (85%) (75%) (75%) 701 722 653 528 676	0 800 5000 000 0 800 Males Fem 641 671 548 478* 638 524 632 (105%) (85%) (75%) (82%) 632 612 701 722 653 528 676 612	0 800 5000 000 0 800 5000 Males Females 641 671 548 478* 638 524 490 (105%) (85%) (75%) (82%) (77%) 701 722 653 528 676 612 527

T 11 5 2 2 20	
Table 5.3.2-20	Food consumption (g/day) in the 90-day dog study with AE C656948

*: $p \le 0.05$; **: $p \le 0.01$; (%) = % versus control

No treatment-related ocular abnormalities were observed at opfithalmic examination: (Week 13) than at week 8. In addition, lower erythrocyte counts Remoglobin concentrations and , Loo »Ó hematocrit values were observed in females. \sim

	~ Y	* ~~~~		U
Table 5.3.2-21	Homotology rocal	te in the Ol_day /	dag study with AFSC 8560/A	$(moon \pm SD)$
1 abic 5.5.2-21	inclinatology result	is in the ju-uay t	dog study with AE Co56948	(mean + SD)

là-		.~~		0 .	~~~	No C) í	
Dosage level (ppm)	A A	800	5000	20000/ 0000		800	5000	20000/ 10000
Sex 🖉	Ø S				ales O	\$* *		
Week			ek 8 🔊			🖉 Wee	ek 13	
	361 ± 4		396⁄±	548±	≫ 379 ¥	$406 \pm$	$390 \pm$	$491 \pm$
Platelet count (10%L)	Q 77 ^Q	46/	©71 _	° 151°	.78	32	84	103
	, Q	£+6%)	~(+10%) ³	(+52%)		(+7%)	(+3%)	(+30%)
Sex O			<u>s</u>	S Fem	ales			
Week		ŚWe	ek 8 🔬			Wee	ek 13	
	370 ±	435 ±	∲¥15 ₽°	577 ±	411 ±	$470 \pm$	492 ±	$570 \pm$
Platelet count (10%/Lo	\$70	Ø 47×~~	164	233	82	57	100	183
	0 - 😽	(+18%)	»(1 2%)	(+56%)	-	(+14%)	(+20%)	(+39%)
Della	7.16±	39 6 ± 6	\$6.93 €	6.45 ±	7.27 ±	$6.89 \pm$	$6.70 \pm$	$6.49 \pm$
Red blood cell count $(10^{12}/L)$	0.57	@ 0.35 ×	0,59	0.65	0.73	0.50	0.47	0.82
(10 ¹² /L)	- 🗸	(-1%)	(3 %)	(-10%)	-	(-5%)	(-8%)	(-11%)
	16,2⊈	10.6 ± 8	£15.8±	$14.8 \pm$	$16.5 \pm$	15.8 ±	15.0±	14.7 ±
Hemoglobin concentration	¢!9	1.0 °C	0.9	1.3	1.4	1.2	0.8	2.0
	0 - ×	(-2%)	(-7%)	(-12%)	-	(-4%)	(-9%.)	(-11%)
Heinatocrit (TL)	0.480±	0 47 6±	$0.456 \pm$	$0.429 \pm$	$0.479 \pm$	$0.457 \pm$	$0.436 \pm$	0.431 ±
Hematocrit (1/L)	0,028	[*] 0.031	0.020	0.038	0.035	0.041	0.023	0.061
	<u></u> ~-	(-1%)	(-5%)	(-11%)	-	(-5%)	(-9%)	(-10%)
%) = % variation versus contr	N.	•						

variation versus control

2. Chinical Chemistry

At 2000 ppm, higher alkaline phosphatase and δ -glutamyl transferase activities were noted in both sexes, whereas aspartate and alanine aminotransferase activities were higher in males only when compared to controls. In addition, lower total bilirubin, mean albumin (and as a consequence



albumin/globulin ratio) and mean total protein were observed in both sexes. To a lesser extent, the same parameters were also affected at 5000 ppm. No adverse effects were noted at 800 ppm in either sex. Table 5.3.2-22 Changes in clinical chemistry in the 90-day dog study with AE C656948 (mean 3.5D)

parameters were also affected at 5000 ppm. No adverse effects were noted at 800 ppm in either sex.								
Table 5.3.2-22 Cha Dosage level (ppm)	nges in cl	inical chei 800	mistry in the 5000	e 90-day dog 20000/ 10000	g study v 0	vith AE C 800	656948 (m 5000	ean 5 SD) 2000000 10900
Sex				Mal	es	A	. 08	
Week		W	eek 8	Ĉs		5 W	Veek 43	
Alkaline phosphatase (IU/l)	97 ± 16	129 ± 15	325 ± 150*	461 ± 309**	93 £	139 ± 15	Ø72 ± 3 172≉Q	\$\$\$\$± \$20**\$
γ-glutamyl transferase	- 1 ± 1	(+33%) 2 ± 0	(+235%) 3 ±	(+375%) $10 \pm 7**$	~Q`- 7 2 ±_1©	(+49%) 3 ± 1	(+300%)	\$13 ± 8 *
(IU/I) Aspartate aminotransferase (IU/I)	- 28 ± 5 -	(+100%) 25 ± 8 (-11%)*	(+200%) $(-11%)$	<u>(+900%)</u> 497≠23 √75%		$(\pm 50\%)$ 31 ± 7 (+ $(\pm 5\%)$)	Q(+50%) 26⊕ ⁴ 5 (- √ ^{7%})	(+550%)
Alanine aminotransferase (IU/l)	32 ± 12	27 4 (16%)	\$34 ± 12 © (+6%)	115 98 (+259%)		28 ± 6 (-105)	38 £15 (523%) *	215 ± 189 2(+594%)
Total bilirubin (μmol/l)	4.4 ±	y 2.7 ≰y 0.4** (√39%).	200 ± 0.3** (-55%)	\$ 1.9 0.5** @57%)		0.6 (-31%)	2,2 ± (©)* (\$51%)	2.8 ± 1.4 (-38%)
Albumin (g/l)	36 ± 2,2	34 ± 1 (-	30 ² 3* (2 17%)	29 + *** (***) (36±	33 ± 1 5 8%	28 ± 3** (-22%)	27 ± 4** (- 25%)
Total protein (31)		\$55 ± 1+(- \$%)	54≠3 (≤ 5%),5%	$52 \pm 9(-$	59± 04	5€±2(- √7%)	53 ± 4 (-10%)	52 ± 1* (- 12%)
Albumin globulin ratio	1.72 ± 0.160	91.61 + 0.05 (-6%)	120± 0.16**©	0.25 ** (-28%)	k6 4 ⊘± 0.16	1.45 ± 0.04 (-12%)	1.09 ± 0.12** (-34%)	1.11 ± 0.25** (-32%)
Sex 🔊	A é	<u>v</u> s		^{O'} Fema	les			
Week	Q"	С ^р W	/eek 8 5	<u> </u>		W	veek 13	
Alkaline phosphatase		$\begin{array}{c} 190 \pm \\ 43 \pm \\ (+74\%) \end{array}$	∑ 2992¥ 4661 €¥169%)	375 ± 73** (+238%)	111 ± 28 -	190 ± 72 (+71%)	355 ± 208 (+220%)	383 ± 85* (+245%)
γ-glutamyl transferase (IU/I)			4 ± 5 (+ 500%)	$10 \pm 5^{*}$ (+400%)	2 ± 2 -	3 ± 1 (+50%)	5 ± 3 (+150%)	14 ± 12**
Total bilumbin (µmcQI)	3.4 ±	2.8# 2.8# 2.7 (-18%)	√1.9± 0.3* (-44%)	2.2 ± 0.5* (-35%)	3.3 ± 0.9 -	3.2 ± 1.1 (-3%)	2.1 ± 0.6 (-36%)	2.0 ± 0.9 (-39%)
Albumin (2/1)		36 ± 1 (0%)	30 ± 1** (-17%)	30 ± 2** (-17%)	36 ±1	37 ± 1 (+3%)	29 ± 1** (-19%)	28 ± 2** (- 22%)
Totapprotein (g/l)	57 ± 2 -	58 ± 3 (+2%)	53 ± 3 (-7%)	51 ± 4* (-11%)	58 ± 1 -	59 ± 2 (+2%)	53 ± 3** (-9%)	50 ± 1** (-14%)



Albumin/globulin ratio	1.67 ± 0.28	1.71 ± 0.21	1.32 ± 0.10	1.41 ± 0.14	1.61 ± 0.17	1.63 ± 0.27	1.24 ± 0.04 *	1.28 ± 0.20 °	
ritounini gioounii tutto	-	(+2%)	(-21%)	(-16%)	-	(+1%)	(-23%)	(-20%)) j
*: p<0.05: **: p<0.01: (%		()		(-16%)	-	(+1%)	(-23%)		2085) ~

*: $p \le 0.05$; **: $p \le 0.01$; (%) = % versus co

F. Urinalysis

There were no treatment-related findings in either sex at any dose level.

G. Sacrifice and pathology

1. Organ weight

Absolute and relative liver weights were higher in both sexes at 20000/10000 ppm and 5000 ppm, compared to controls. In addition in females, absolute and relative thomas weights were lower in comparison to control. These changes were associated with gross and histopathological changes see below).

Table 5.3.2-23	Mean terminal body	weight	andarb	solute	and r	ative v	weights o	f liver	and thymus	ó,
	(moon + SD)	s s	\sim	×¥		A	Oľ	~// ~	N° (7

((mean ± SD)			
Dosage level (ppm)	0 0	800	5000	20000/10000
Sex	Q [°]	h h h h h h h h h h h h h h h h h h h		
Terminal body wt, kg	8.49 0.83	$\frac{1}{8.47 \oplus 0.81}$	\$ 8.00 ± 1.20 °	6.95 ± 1.01
Liver Absolute wt, g	280.9±366	(+25%)	(+590) V	$410.3 \pm 60.9 **$ (+46%)
Liver wt, % body wt	3.32 0.38	4.18,±0.50*	5.61 ± 0.37** (+69%)	5.91 ± 0.11** (+78%)
Liver wt, % brain wt	378.2±40,9	$\begin{array}{c} & 473.4 \pm 47.0 \\ \hline & (+2.5\%) \\ \hline & & & &$	$620.7 \pm 86.6**$ \swarrow (+6426)	$580.6 \pm 49.0 ** \\ (+54\%)$
Sex Ö		Fêmal	eşç 🖓	
Terminal body wt, kg	6:47 ± 0.63			$4.68 \pm 0.46*$
Liver Absolute wt, g	0228.7915.8	295.8+26.7 (Q9%)	308.2 ±41.2* (+35%)	307.8 ±55.1* (+35%)
Liver wt, % body wt	3.55 ± 0.04	3.95 ± 0.69 $(+39\%)$	[♥] 5.30 ± 1.11* (+49%)	$6.57 \pm 0.90 **$ (+85%)
Liver wt, % brand wt	€315,5€ 29.6°	448.7 ± 40.52	478.6 ± 68.7* (+52%)	487.1 ± 115.7* (+54%)
Thymus Absolute wt, g	©32±240	$ \begin{array}{c} $	6.97 ± 2.69 (-16%)	$2.38 \pm 0.59 **$ (-71%)
Thymus wt, % body wt	°~ 0.128 ¥ 0.034	0.102 = 0.018 20%)	$0.115 \pm 0.034 \\ (-10\%)$	0.050 ± 0.008** (-61%)
Thymus wt, % * brain wt	\$.55 ± 3.77	$\sqrt{2}39 \pm 2.48$	10.75 ± 3.96 (-7%)	3.71 ± 0.86** (-68%)
*: p≤0.05; **: p≨Ø⁄01		2 (-19%)		

2. Gross and histopathology

At the matroscopic examination, enlarged liver was noted in both sexes in 2/4 and 1/4 animals at the high and find dose, respectively and generalized atrophy of thymus was noted in all females at the high dose.

At the moroscopic examination, minimal to slight hepatocellular hypertrophy and intracytoplasmic eosinophilic droplets were observed in all animals of both sexes at both 20000/10000 and 5000 ppm. In addition at these dose levels, hepatocellular single cell necrosis was observed in males.



In the thymus, a slightly higher severity of thymic involution was observed in both sexes compared to controls. In addition in females at the high dose, disturbance of the estrous cycle was observed as only anestrus phase was seen. However, these 2 latter effects (thymus and estrous cycle) were attributed to a secondary effect linked to the decrease in food consumption and bodyweight.

Table 5.3.2-24	Histopathological changes in the 90-day dog study with AE C656948
	mistoputiological changes in the yo day dog study with the webby to

Dose level (ppm)	0	800	5000	20000/ 10000	0,7	800	50000	20000 10000
Sex		N	lales		a y		Female	es N O
Liver, N examined	4	4	¥4	4	4	4	@4	
Hepatocellular hypertrophy: diffuse:			<u> </u>	()	·	Š	<u> </u>	<u>č</u>
Minimal	0	01	3	-Q″	00		30	
Slight	0	<i>and a</i>	1	- ·	Q O	~Q;	\mathbb{Q}^{n}	
Total	0	0	۰ 4 ،	@`4 <i>`</i> ^	10_0	70	⊳ ^4 .,	
Intracytoplasmic eosinophilic droplets	: multifocal	× ¢	<u>2</u>				×	× ×
Minimal	0	14) 200	Į0		ð,	10	13	
Slight	×~0	~0	~ 2			- 90	2	
Moderate			VO L		2/	0	V 1 🖌	
Total			3~	<u>4</u>	Ø	10	4.	Q
Hepatocellular single cell necrosis: fo		1.5	S.		<u>s</u>	S.		, Òj
Minimal	× °00	0	2	S* 3 6		<u>0</u> ر	Ň.	× 0
Moderate					080	0_0		× 0
Total	<u>`~~</u>	00	2,00	_ð∛	ູ0ັ	00	15	0
Thymus, N examined	č¥	4	4	~4 v	4	i A	4	3
Decreased size of cortex: insolution		<u>v</u>		<u> </u>		1 4	Ş	r
Minimal 🍾	× 4		1	<u> </u>	40	<u></u>	2	0
Slight 🔬 🖓	Q Q	ð	×30	\$¥	0	$\sim 0^{\circ}$	2	1
Moderate S O		50	×0	<u>1</u>	-	€ Ø	0	2
Marked					0	0	0	0
Total		4~	45	L Č	L AÇ	3	4	3
Estrus phases @ the u@rus:		<u> </u>	<u>~</u>		~			
Proestrus Or By C			Ş.		2 1	1	4	0
Early metostrus					1	2	0	0
Midmetestrus O A	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		, S	Ň	1	0	0	0
Anestrus and a		<u> </u>		\sim	1	1	0	4

None

III. Conclusion

The NOARL for this stady was 809 ppm for both sexes (equivalent to 28.5 and 32.9 considered to mg/kg bw/day for males and females, respecti Ø

Assessment and conclusion by applicants

Study meets the current gundance and the requirements in 283/2013.

The NOAKE for this study was considered to be 800 ppm for both sexes (equivalent to 28.5 and 32.9 mg/kg by/day for males and tomales, respectively).



Data Point:	MCA 5.3.2/04
Report Author:	
Report Year:	
Report Title:	AE C656948 - Chronic toxicity study in the dog by dietary administration
Report No:	SA 05047
Document No:	<u>M-294279-01-1</u>
Guideline(s) followed in	OECD test guideline 452; EEC Directive 88/302, Method B.30 (1992); OPPTS
study:	870.; MAFF in Japan, 12 Nousan No 8147 🍂 🔗 🖉
Deviations from current	Current guideline: OECD 452, 2018
test guideline:	Deviation: None
Previous evaluation:	Yes, evaluated and accepted in the DAR (201)
GLP/Officially	Yes, conducted under GLP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes a v v v v v

Executive summary

Technical grade AE C656948 was administered in the diet to Beagle dogs (4/sex/dose) at dose levels of 0, 100, 400, and 2000 ppm for at least 52 weeks (equivatent to 0, 3.0, 03.2 and 67.6 mg/kg bw/day in males and 0, 3.8, 14.4 and 66.1 mg/kg bw/day in females).

. Cage side observations were conducted daily, detailed clinical observations were conducted weekly, food consumption was measured daily, and body weights were taken weekly. Ophthalmic examinations were performed once pre-exposure and cust prior to necropsy. Clinical chemistry and hematology measurements were taken once pre-exposure, during months 3 and 6, and at the end of the study. Urinalysis measurements were taken once pre-exposure, during months 4 and 6 and at the end of the study. A gross necropsy was performed, organ weights were taken, and ussues were examined microscopically.

At 2000 ppm:

There was an paitial body weight loss during the 1st week of treatment in both sexes concomitant to lower food consumption most likely due to a lack of pattability of the test compound. Thereafter, food consumption and body weight were comparable to controls in males whereas food consumption remained slightly lower than controls in females, which tesulted in an overall 10% reduction in food consumption.

The liver was the target organ. No change in liver weights was observed in either sex however histopathology revealed centribulat hepatocellular hypertrophy in 3/4 males. In addition, an increase in circulating alkaline phosphatase (ALP) was also seen in both sexes at this dose level.

At 400 and 100 ppm:

No treatment-related adverse effects were observed. The NOAEL for this study was 400 ppm (13.2) 4.4 mg/kg/day for males/females).

I. Materials and methods

- A. Materials
- 1. Test material: Description Lot / Batch #: Purity: CAS # Stability of test compound:

AE C656948 Beige powder Mix-Batch 08528/0002 Min 94.6% 658066-35-4 Stable at $25 \pm 5^{\circ}$ C / room temperature



2. Vehicle and / or positive none control: 3. Test animals: **Species:** Dog Strain: Beagle Approximately 8 months old Age: 7.0 - 9.4 kg, males; 5.2 - 7.7 kg, females Weight at dosing: Source: 33 days **Acclimation period:** Certified canine meal 125C3-P1 from S.A.F.E Scientific Diet: Animal Food and Engineering Augy, Frances. Three hundred grams of diet poistened with 450 ml of water at the time of distribution was given daily to each mimal for approximately 1.5 hours each morning exception study Day 337 when animals were fed in the afternoop due to a change in planning. Ő Supplementary food ration: due to a degrease in body weight of between Q8 to 18 kg during the study for anomals PF1M607 Control group, PT3, 46632 (mid dose group), PT, F6620 and PT1F6621 (control group), despite the fact these animals ate their entire daily food ration, a supplementary untreated pelleted diffet ration (153 53 from S.A. F.E.) was given to these animals starting on Day 149, 130, 199 and 209, respectively, until study v termination Approximately 100 g per day of untreated pelleted diebwas distributed to each dog after, the measurement of its empty feeder of the detary mixture, providing that the dog had eaten approximately its entire ration. The weight of the supplementary food supplied and that remaining was recorded on the day of distribution or the day after (supplemental food pation was left overnight where necessary on weekends and holidays). Data were kepton the study file. Fiftered and softened tan water from the municipal water Water 🎗 supply, ad libitum. Animals were housed individually in stainless steel kennels with Housing: a floor surface area of 1.2 m^2 . When possible, they were pair housed overnight by temporary opening of the partitions between 2 dogs from the same sex and dose group Supervised exercise in inside runs was permitted daily for dogs of the same sex and treatment group throughout the acclineatization and treatment periods except on weekends and public holidays. Additional supervised exercise was permitted in inside runs for an extended time of approximately 1 hour at least Environmental conditions: Sonce per week for dogs of the same sex and treatment group. 18921°C Temperature: 40-70% Humidity: Target of 15 per hour Air/changes: 12 hours dark, 12 hours light (7 am - 7 pm)iotoperiod: Study design B. 31 January 2006 to 01 February 2007 1. In life dates:



2. Animal assignment and treatment

Shortly after arrival, all dogs were examined by a veterinarian for signs of ill-health and were subjected to a detailed clinical examination to assess their physical and behavioral status. During the acclimatization phase, animals were checked twice daily for moribundity and mortality. Clinical signs were recorded daily and a detailed physical examination was performed approximately weekly. All animals were weighed at least weekly before food distribution and food intake was measured for a minimum of 5 consecutive days before start of treatment. All animals were subjected to an ophthalmological examination, hematology and clinical chemistry investigations and prinalysis once during the acclimatization phase. Animals were selected and randomized for use in the study on the basis of acceptable findings from physical and ophthalmological examinations. Fody weight food of consumption, clinical chemistry, hematology analyses and urinalysis. Animals were allocated to dosage groups in order to ensure a similar body weight distribution among groups of each set whilst ensuring full siblings were not placed in the same treatment group.

Animals were randomly assigned to the test groups noted in the following table. Control animals received untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Dose Group	No Animats/	Males & Stemales
(ppm)	Dose/Sæx	(mg/kg/day) (mg/kg/day)
0 (control)		
100	4 2 L	3.8
400	0 0 4 × Q	
2000	Y 1 4 S	Ø 67,6 € 66.1

Table 5.3.2-25: Test groups, dose levels and achieved dosages

3. Diet preparation and analysis

The test substance formulations were prepared to cover the dietary requirements over approximately 6week periods. The appropriate amount (weight weight concentration) of test substance was incorporated into the ground diet to provide the required dietary concentrations of 100, 400 or 2000 ppm. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. Nine preparations at each dose level were performed to provide the treated diet required for the study.

Homogeneity analysis. The pomogeneity of the test substance in the diet was verified before the start of the study for all concentrations on the first preparation from the first formulation to demonstrate adequate formulation procedures. In addition, the homogeneity of the test substance in diet was verified at the lowest and highest dietary levels on the first preparation from the fifth formulation. The mean values obtained from the homogeneity check were taken as measured concentration. The dietary level of the test substance in the diet was verified for all concentrations on all preparations from the first, third and fifth formulations and on the first preparation from the seventh formulation. Homogeneity Analysis: 93-108% of nominal concentration

Stability analysis. The stability of the test substance in the diet supplemented with 2% of diet enhancer (E.A. 125 from S.A.F.E.) was determined in a previous study at concentrations of 800 and 20000 ppm for a period which covers the period of storage and usage for the current study. The stability of the test substance at the lowest and highest dosage levels was determined during the current study in the moistened diet distributed to the dogs after a 55 day period of storage of dry diet at room temperature. AE C656948 was found to be stable in the diet at 100 and 2000 ppm over a 55-day day period of storage at room temperature and then moistened for 4 hours, which covered the time of food preparation and distribution.

<u>Conceptation analysis:</u> 93-107% of nominal concentration. Results were within the in-house target range of 85 to 115% of nominal concentration and were therefore considered to be acceptable for use on the current study.



4. Statistics

Statistical analyses were carried out using Path/Tox system version 4.2.2 (Module Enhanced Statistics). Continuous data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by the Dunnett test (2-sided) on parameters showing a significant effect by ANOVA. When the data were not homogeneous, a Kruskal-Wabis ANOVA was performed followed by the Dunn test (2-sided) to identify statistical significance between groups. For some parameters, when data were not homogenous, they were transformed (log transformation or square root transformation), then reanalyzed as above.

For urine analysis (pH), group means were compared using the nonparametric Kuskat Wallis test which was followed by the Dunn test (2-sided), if the Kruskal-Wallistest indicated significance

When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% fevels of significance. Statistical analyses were carried Jtat. out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

C. Methods

1. Observations

a. Cage side observations

All study animals were observed at least twice daily for clinical signs of toxicity (except once daily on weekends and holidays). This included but was not limited to change in general behavior and appearance, skin and fur, teeth and guin, eyes, ears mucous memoranes. Any deviations from normal were recorded in respect to the nature and severity. Daily examination of the kennels was also carried out for vomitus, diarrhea or blood

b. Clinical examinations

Detailed clinical observations for chinical signs of toxicity were performed of all animals at treatment initiation (study day 0) and on a weekly basis thereafter. The physical examination included but was not necessarily restricted to the following examinations: fur and skin, eyes, ears, teeth, gum, mucous membranes, rectal temperature, gait stance general behavior, chest including heart and respiratory rate, abdomen including palpitation, external genitalia and mammary glands.

2. Body weight

Body weights were feconded weekly throughout the treatment. Body weights were also taken immediately prior to necropsy to allow for calculation of organ-to-body weight ratios.

3. Food consumption and compound intake

Food intake was measured for a munimum of 5 consecutive days immediately before start of treatment. The weight of food supplied to each animal and that remaining were recorded daily throughout the treatment period. From these records, the mean weekly consumption was calculated for each dog. Food spillage was also recorded.

The group mean achieved dosage for each sex expressed as mg/kg body weight/day, was calculated for each week and the overall mean subsequently derived for weeks 1 to 52.

4. Ophthalmology

Following the acclimation period and prior to intitiation of dosing, ophthalmic examinations were conducted on all animals. Ophthalmic examinations were also conducted on all animals just prior to termination of the study.

5. Hematology and clinical chemistry, and bioanalytical examination

L.

Clinical chemistry and a complete blood count, including differentials, were performed on all animals (pre-exposure and during study weeks 13, 26, 39, and 52). Animals were fasted overnight prior to the



collection of blood, which was drawn via jugular venipuncture. The parameters evaluated are marked (x) in the lists below.

a. Hematology

- Х Hematocrit (HCT)*
- Х Hemoglobin (HGB)*
- Х Leukocyte count (WBC)*
- Х Erythrocyte count (RBC)*
- Х Platelet count*
 - Blood clotting measurements:

- Х Leukocyte differential count*
- Mean corpuscular henroglobin (MCH) Х
- Х
- al Cholesterol (Chole ilobuths (Giob) A/Cratio (AG) Gheose (gluc)* total phrubin (T-Bili) Totalsprotein (TP)* Triglycerifies (Trig) Х X Reticulocyte ount and % refiellocyces

Ľ Х Activated partial thromboplastin time

*Recommended for chronic studies based on OPPTS Guideline 870.4100

b. Clinical Chemistry

- **ELECTROLYTES**
- XX Calcium (calc)*
- XX Chloride (Cl)* Magnesium (Mg)*
- XX Phosphorous (Phos)*
- XX Potassium (K)*
- XX Sodium (Na)* 0 ENZYMES (more than the pathy enzymes)* ©XX
- XX Alkaline phosphatase (ALK)
- Alanine aminotransforase (SLT/SGDT)* XX
- Aspartate aminotransferase (AST/SGOT) XX
- Gamma Glutany transferase (GGT)* O XX
- *Recommended for chronic studies based on OPPTS (Sindeline 870.4 100
- (X) Parameters examined on serum samples

(XX) Parameters examined on plasma samples.

Globulins and A/G ratio by calculation of TP

6. Urinalysis

and and a second Urinalysis (including parameters mentioned in the table below) was performed on all animals once prior to administration of the test Substance and on al Panimal's during study weeks 14, 24 and 52. Urine A volume was collected overhight.

XX

- Х Appearance
- Sucose (Glu)* Х Volume (UVol) Ketones (Ket)
- osmolalitv Х Speciffe gravity Bilirubin (Bil)
- Х pH (pH)*
- Х Sediment (microscopic
- Х Protein (Pro)*

Urobilinogen (Uro)

Blood (Bld)*

*Recommended for chronic studies based on OPPTS Guideline 870.4100

7. Sacrifice and pathology

On study Da@ 365 0 368 all animals from all groups were sacrificed. All animals were tranquilized by intramyscular injection of acepromizine (50 µl/kg body weight) and then deeply anesthetized by intravences injection of pentobarbital (60 mg/kg body weight). Animals were then exsanguinated and necropsied.

The vectors included the examination of all major organs, tissues and body cavities. Macroscopic abnormativies were recorded, sampled and examined microscopically.



 \bigcirc

The following organs or tissues were sampled and/or weighed at necropsy:

	DIGESTIVE SYSTEM		CARDIOVASC. / HEMAT.		NEUROLOGIC
Х	Tongue	Х	Aorta*	XX	Brain with cerebellun
Х	Submandibular (salivary)	XX	Heart*	ΛΛ	
Λ	gland*	Х	Bone marrow, sternum*	Х	Scienc nerve*
Х	Esophagus*	Х	Lymph node, mesenteric*	Х	Spanal cord (cervical,
Х	Stomach*	Λ	Lymph node, mesenterie	Λ	Schute nerve* Spinal cord (cervical, spinal cord (cervical, spinal cord) thoracic, lumbar * Eyes* Optic nerves* Pituitary@land* GLANDULAR
Х	Duodenum*	Х	Lymph node,	X 🕺	Éyes*
Х	Jejunum*	Λ	submaxillary* 💭	X	Optic nerves \sim
Х	Ileum*	XX	Spleen*	XX	Pituitary@land*
Х	Cecum*	XX	Thymus 🕺		
Х	Colon*		<u>A.</u> Q	γ ,	GLANDULAR O
Х	Rectum*		UROGENTAL 👡	XX	Advinal gland*
XX	Liver*	XX	UROGENTTAL Kidney* Urinaty bladdet*	XX XX XX XX XX	Optic nerves* Pituitary@land* GLANDULAR Advenal gland*
Х	Pancreas*	Х	Urinary bladder*		Thyroid gland* (weighed
		XX	Testis* 🖉 🖉	XX	with parathyroid grand)+
		XX	Epididymas*	4	
	RESPIRATORY	XX	Prostate gland*		Cacrymal exorbital glar
Х	Trachea*	X	Ovidagi* , O , S , (⊃X 🔬	Hardervan gland
Х	Lung*	XX	Ogary* ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	, O	OTHER O
Х	Pharynx*	XX	Ugerus (ŵith cervix)* 🍼	X	Bone (stephum)
Х	Larynx*	×	Mammary gland*	, X	Skeletatimuscley
Х	Nasal cavities*	X	Vaginan S 🖉	∀X Č	Sking 🖉 🔬
	, S	Х 🕅	Seminal vesicle* 🔧 🖉	vÒ	All gross looions and
	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	×,	Epididymar Prostate gland* Ovidinet* Ovary* Uterus (with cervix)* Mammary gland* Vagina Seminal vestcle*		with parathyroid gland)+ Cacrymal exorbital gland* Hardevan gland OTHER Bone (stephum) Skeletat muscle Skint All gross legions and masses
	. Q	0		$\tilde{\mathbb{A}}^{v} \approx$	Articular surface
	× .4	4		$\sim \Lambda \ll$	(femorotibial joint)

recommended for chronic stoffes based on USEPA guideline \$70.4100

- X tissues were collected for histological exampations
- XX organs were workhed tresh at cheduled sacrifice only with parted organs weighed together, and collected for histological examination

For sacrificed animals a bone marrow smear was prepared from the rib and stained with May-Grünwald Giemsa, but not examined.

Samples vere fixed by immersion in routral buffered 10% formation with the exception of the eye, optic nerve, Harderian gland, epidietymis and textis that were fixed in Davidson's fixative.

Histopathological examinations were performed on albussues from all the animals in all dose groups.

- II. Results and discussion
- A. Clinical Signs and moctality
- 1. Clinical signs of toxicity and physical examination

No treatment-related finical signs were noted in other sex.

2. Mortality

There were no mortalities throughout the study

### B. Body weight and body weight gain

At 2000 spin during the 1st week of treatment, there was a mean body weight loss of 0.2 and 0.1 kg in males and females, respectively compared to controls. There after body weight gains were comparable to controls in both sexes. The initial decrease in body weight gain corroborated with a lower food in both sexes consumption over this period.

At 400 and 100 ppm, body weight and weight gain were not affected by the treatment in either sex.



#### Body weight and weight gains (kg) in the 1-year dog study with AE C656948 (mean $\pm$ Table 5.3.2-26 SD)

Dosage level (ppm)	0	100	400	2000
Sex		Ma	les	
Initial BW (%C)	$8.3\pm0.6$	8.1 ± 0.6 (98%)	$8.2 \pm 0.9$ (90%)	8.2 ± 0 3 (99%)
BWG Week 1	$0.1 \pm 0.2$	$0.1 \pm 0.1$	$0.0\pm 0.0$	$-0.27 \pm 0.20$
BWG Weeks 1-52	$0.4 \pm 1.0$	$2.3 \pm 1.3$	1.3 ₄ ± 1.8	0.2 ± 1 1
Final BW (%C)	8.7 ± 0.8	$10.4 \pm 1.7$ (120%)	9.6 ± 1.3 √(110%)	8:5 ± 0:8 (98%)
Sex		🕅 Fen	ales	
Initial BW (%C)	$6.9 \pm 0.6$	6.6 4 0.9 (96%)	<b>@.8</b> ± 0.8 (99%)	6,7)¥ 0.4 (\$7%) ( ^O
BWG Week 1	$0.0 \pm 0.1$	0.2 ± 0.1	$0.2 \pm 0.1$	-0.1 0.2
BWG Weeks 1-52	$1.1 \pm 0.6$	1.4 ± 0.9	$9.2 \pm 00^{\circ}$	$1.0 \pm 0.2$
Final BW (%C)	8.0 ± 1.0	8.0 ± 1.3 (100%)	× 8.1 ± 1.4 (101%)	7.6 ¥ 0.3 (25%)
BWG: body weight gain; (%C) =	= % versus contro		y de la	& A co

#### C. Food consumption and compound intake

During the 1st week of treatment, there was a 30% and 24% reduction in food consumption at 2000 ppm in males and females, respectively. This initial offect was most likely due to lack of palatability of the test compound as already observed in the previous 28 and 90 day studies in dogs (M-226047-01-1, <u>M-276047-01-1</u>). Overall, food consumption was comparable to control in males whereas this parameter remained slightly lower in pemale throughout the study resulting in an overall 10% reduction.  $\cap$ 

#### Food consumption (g/day) in the 1-year dog study with AE C656948 Table 5.3.2-27

0		400		$\bigcirc^{\mathbb{N}}$		× 400	2000
	Ma		S .~~		⊃´ 19∕em	ales	
	r 648)	\$ 656	498	to CAE LA	626	625	493
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	(90%)	(93%)	(70%)		<i>(</i> 99%)	(97%)	(76%)
		4.0	× 720 ×		702	602	620
7,20	<i>l</i> n	<i>°</i>		Ø88 .«			
					(102%)	(101%)	(90%)
	5709 5709 790	7,009 0 730 0 7,009 0 730 0 7,000 0 730 0 7,000 0 730 0	Males Males <th< th=""><th>0, 100 400 500, 5000, 5000, 5000, 5000, 5000, 5000, 500,</th><th>$\begin{array}{c ccccccccccccccccccccccccccccccccccc$</th><th>0 100 400 5000 € 100 0 0 100 5000 € 100 5000 € 100 5000 500 0 0 100 5000 € 100 500 500 500 500 500 500 500 500 500</th><th>0, t00 400 2000, t0, 100 400 3709 648 656 498 666 625 (90%) (93%) (70%) 645 666 625 (90%) (93%) (70%) 645 720 688 703 693 (101%) (97%) (101%)</th></th<>	0, 100 400 500, 5000, 5000, 5000, 5000, 5000, 5000, 500,	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	0 100 400 5000 € 100 0 0 100 5000 € 100 5000 € 100 5000 500 0 0 100 5000 € 100 500 500 500 500 500 500 500 500 500	0, t00 400 2000, t0, 100 400 3709 648 656 498 666 625 (90%) (93%) (70%) 645 666 625 (90%) (93%) (70%) 645 720 688 703 693 (101%) (97%) (101%)

‰~versus control

Ophthalmic examination

No ocular abnormalities or females any dietary level.

Е. Blood analyses

1. Hematology

changes in wither sex at any dose level. There were no treatment Į,

2. Clinical Chemistry

The only consistent change throughout the study concerned the alkaline phosphatase activity which was at least doubled at 2000 ppm compared to controls in both sexes. Values were also higher than the pre- \sim test values.

The slightly elevated ACP activity seen in males at 400 ppm was judged not to be related to treatment since this elevation was mainly due to one animal only and since there was no other finding at this dose.

ct was seen at 100 ppm.



Table 5.3.2-28Alkaline phosphatase activity (IU/L) in the 1-year dog study with AE C656948 (mean± SD)

Dosage level (ppm)	0	100	400	2000 🖉 .
Sex		Ma	ales 🔬	
Pre-test	111 ± 19	142 ± 35	119±405	119±16
Month 3	120 ± 51 (+8%)	107 ± 17 (-25%)	$162 \pm 41 (+36\%)$	256 ± 108* O(+115%)
Month 6	154 ± 121 (+39%)	108 = (-24%)	\$15 ± 122 (+81%)	344 + 135 987%
Month 12	117 ± 46 (+5%)	80 ± 30 (-43%)	0 176 ± 67 (+48%)	299±66 (+150%)
Sex	Q	°° ∕¥jen	nalee 🕺 🖓 🔪	
Pre-test	121 ± 18 🔬	$34 \pm 40^{\circ}$	∠ ⁷ 13 <i>5</i> , ⁹ 34 ℃	°~)45 ± €)
Month 3	$119 \pm 27^{\circ}$	2123 5 30 G	(-4%)	£ 229 ±62** ° € (±98%) €
Month 6	147, 49 (21%), ×		134 ² 24 461%)	334 ± 123 (+130%)
Month 12	040 ± 43 (+16%)	₹ 135¥72 (+1%) ₹	169°± 61, 519%) \$	© 285 ± 126

*: p≤0.05; ** p≤0.01

3. Urinalysis

There were no treatment related findings in either sex at any dose level.

F. Sacrifice and patholog

1. Organ weight

The only change in organ weight was a higher mean absolute and brain relative thyroid gland weight in females at 2000 ppm. However this change was considered not to be adverse since there was no associated bistopathological effect.

2. Gross and histopathology

No treatment-related changes were observed at the matroscopic examination.

At the microscopic examination, the liver was characterized by a minimal diffuse centrilobular hepatocellular hypertrophy in 3/4 mates at 2000 ppm. This was the only treatment-related finding.

A minimal diffuse hypertophy of the folicular cells was observed in the thyroid gland of 2/4 and 1/4 males at 2000 and 100 ppm, respectively. In the absence of a dose relationship this minor change was judged to be incidental.

Table 5.3.2-29 Histopathological changes in the 1-year dog study with AE C656948

Dose wever (ppm)	0.0	100	400	2000	0	100	400	2000
Sex Sex		Ma	les			Fem	ales	
Cempflobular hepatocellular	~© 0/4	0/4	0/4	3/4	0/4	0/4	0/4	0/4
Follicular epithelium hypertrophy (minimal, diffuse)	0/4	1/4	0/4	2/4	0/4	0/4	0/4	0/4

G. Deficiencies

There are no deficiencies noted in this study.



III. Conclusions

The NOAEL for this study was 400 ppm for both sexes (equivalent to 13.2 and 14.4 mg/kg bw/day for males and females, respectively) based on liver effects (increased alkaline phosphatase activities and liver cell hypertrophy).

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013. The NOAEL for this study was 400 ppm for both sexes (equivalent to 16.2 and 14.4 mg/kg.bw/day for males and females, respectively) based on liver effects (increased affaline phosphatase activities and liver cell hypertrophy).

CA 5.3.3 Other routes

Data Point: KCA 5.3.3/01 Report Author: 2007 Report Year: 2007 Report Title: A subacute derma toxicity study of rats with technical grade AIE 656948 Report No: 201617 Document No: M-293833-014 Guideline(s) followed in study: US CPA OPPTS 870.3200 (1998) Deviations from current test guideline: Deviation: none Previous evaluation: Yes conducted and acceptor in the DAR (2011) GLP/Officially Yes Acceptability/Reliability Yes		
CA 5.3.3 Other routes Data Point: KCA 5.3.3/01 Report Author: Image: Comparison of the point of		
Data Point: KCA 5.3.3/01 Report Author: 2007 Report Year: 2007 Report Title: A subacute dermal toxicity study in rats with technical grade AFE 656948 Report No: 201617 Document No: M-293833-01 Guideline(s) followed in study: USASPA ORPTS 870.3200 (1998) Deviations from current test guideline: Current guideline: OE (0 410, 1981) Previous evaluation: Yes evaluated and acceptor in the DAR (2011) GLP/Officially Yes, conducted under GCP/Officially recognised testing facilities: Acceptability/Rehabilitive Yes		
Data Point: KCA 5.3.3/01 Report Author: 2007 Report Year: 2007 Report Title: A subacute dermal toxicity study in rats with technical grade AFE 656948 Report No: 201617 Document No: M-293833-01 Guideline(s) followed in study: USASPA ORPTS 870.3200 (1998) Deviations from current test guideline: Current guideline: OE (0 410, 1981) Previous evaluation: Yes evaluated and acceptor in the DAR (2011) GLP/Officially Yes, conducted under GCP/Officially recognised testing facilities: Acceptability/Rehabilitive Yes	CA 5.3.3 Other	r routes
Report Author: Report Year: 2007 Report Year: 2007 Report Title: A subacute dermal toxicity study in rats with technical grade AE 656948 Report No: 201617 Document No: M-293833-01-9 Guideline(s) followed in study: US CPA OPPTS 870.3200 (1998) Deviations from current test guideline: Deviation: none Previous evaluation: Yes evaluated and acceptor in the DAR (2011) GLP/Officially Yes, conducted under GCP/Officially recognised testing facilities Acceptability/Reliabilityr Yes		
Report Author: 2007 Report Year: 2007 Report Title: A subacute derma toxicity study if rats with technical grade ARC 656948 Report No: 201617 Document No: M-293833-014 Guideline(s) followed in study: USAPA OPPTS 870.3200 (1998) Deviations from current test guideline: Current guideline: OE (D 410, 1981) Previous evaluation: Yes evaluated and acceptor in the DAR (2011) GLP/Officially Yes, conducted under GLP/Officially recognised testing facilities: Acceptability/Refiability Yes	Data Point:	KCA 5.3.3/01 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
Report Title: A subacuto dermal roxicity study in rats with technical grade AEC 656948 Report No: 201617 Document No: M-293833-01-2 Guideline(s) followed in study: USAPA OPPTS 870.3200 (1998) Deviations from current test guideline: Current guideline: OE (0 410, 1981) Previous evaluation: Yes evaluated and acceptor in the DAR (2011) GLP/Officially recognised testing facilities: Yes Acceptability/Reliability Yes	Report Author:	
Report No: 20161/0 Document No: M-293833-01-4 Guideline(s) followed in study: US DPA OPPTS 870.3200 (1998) Deviations from current test guideline: Current guideline: OE (0 410, 1981) Previous evaluation: Yes evaluated and acceptor in the DAR (2011) GLP/Officially recognised testing facilities: Yes Acceptability/Reliability Yes	Report Year:	
Report No: 20161/0 Document No: M-293833-01-4 Guideline(s) followed in study: US DPA OPPTS 870.3200 (1998) Deviations from current test guideline: Current guideline: OE (0 410, 1981) Previous evaluation: Yes evaluated and acceptor in the DAR (2011) GLP/Officially recognised testing facilities: Yes Acceptability/Reliability Yes	Report Title:	A subacutedermal toxicity study in rats with technical grade AEC 656948
Guideline(s) followed in study: USACRA OPPTS 870.3200 (1998) Deviations from current test guideline: Gurrent Quideline: OE(D 410, 1981) Previous evaluation: Yes evaluated and acceptor in the DAR (2011) GLP/Officially recognised testing facilities: Yes Acceptability/Reliability Yes	Report No:	
Study. Study. Deviations from current test guideline: Surrent guideline: OE (D 410, 1981) Previous evaluation: Yes, evaluated and acceptor in the DAR (2011) GLP/Officially recognised testing facilities: Yes, conducted under GCP/Officially recognised testing facilities Acceptability/Rehability Yes	Document No:	M-293833-01-9
Study. Study. Deviations from current test guideline: Surrent guideline: OE (D 410, 1981) Previous evaluation: Yes, evaluated and acceptor in the DAR (2011) GLP/Officially recognised testing facilities: Yes, conducted under GCP/Officially recognised testing facilities Acceptability/Rehability Yes	Guideline(s) followed in	US OF A OPPTS 870.3200 (0998) ()
Previous evaluation: Yes evaluated and acceptor in the DAR (2011) (GLP/Officially Yes, conducted under GLP/Officially recognised testing facilities recognised testing Acceptability/Rehability Yes Acceptability/Rehabilit	study:	
Previous evaluation: Yes evaluated and acceptor in the DAR (2011) (GLP/Officially Yes, conducted under GLP/Officially recognised testing facilities recognised testing Acceptability/Rehability Yes Acceptability/Rehabilit	<u></u>	GurrentQuideling: OEQD 410, 1981
Previous evaluation: GLP/Officially recognised testing facilities: Acceptability/Rehability Yes, conducted order GLP/Officially recognised testing facilities Acceptability/Rehability Yes, conducted order GLP/Officially recognised testing facilities		Deviation: none Si A Si Si Si
GLP/Officially Yes, conducted ander GLP/Officially recognised testing facilities facilities:	Previous evaluation:	Vescevaluated and Ocception the PAR (3011)
	GLP/Officially	Yes, conducted under GCP/Officially recognised testing facilities
	recognised testing	
	facilities:	
	neceptuolinty/nendoling.	
	O A	

Executive Summary

Technical grade AE C6569 was administered by topical (dermal) application to male and female Wistar rats five dags/week/for at least four weeks by applying the test substance to a commercially available adhesive bandage that had been morstened with deionized water. The dose groups for this study were 0 (control group), 100, 300, and 1000 mg/kg/day. The dose was based on each animal's body weight on day 0, 7,04, 21, and 28. During the study the animals were evaluated for the effect of the test compound on body weight food consumption, inical signs, the eyes, clinical chemistry, and hematology. Gross nectopsy evaluations were performed on all animals. Histopathologic evaluation of selected tissues was conducted on the control and high-dose groups, and for tissues from the low- and mid dose groups for tissues for which findings were observed in the high-dose group.

Compound-related findings were only observed at 1000 mg/kg/day and consisted of an increased cholesterol concentration in gemales, an increased prothrombin time in males and effect in the liver (increased liver weights formales and fernales associated with hepatic hypertrophy). The increased liver weights and hypertrophy in the high-dose group are attributed to hepatic enzyme induction, based on findings in another study (see 9.3.1) and thus are considered to be an adaptive response to AE C656948.

No compound-related effects were observed at 300 or at 100 mg/kg/day.

In conclusion, the NOAEL for dermal application over 28 days was 300 mg/kg/day.



- I. Materials and methods
- A. Materials
- AE C656948 -- N-[2-[3-Chloro-5-(trifluoromethyl)-2-1. Test material: pyridinyl]ethyl]-2-(trifluoromethyl)benzanide Beige powder Description Mix-batch:08528/0002 Lot / Batch #: 94.7% **Purity:** CAS# 658066-35-4 Stable at room température **Stability of test compound:** For treated animals gauze pads were moistered with deion 2ed water and the deit substance was the formula the deiter and the d 2. Vehicle and / or positive water and the test substance was then applied to the gauze. The control animals were treated with gauze pads moistened with control: deionized water. 3. Test animals: **Species:** Rat Strain: (nulliparous and nonpregnant) M weeks Age: ð g (females) 281.3-351.0 g (males), 203,1-23 Weight at dosing: Charles River Laboratorios, Incorporated, Raleigh, North Source: Carolina, U.S.A L **Acclimation period:** 7 days prior to release for the study PMJCertified Rodent Diet 50025m "meal" form provided Diet: continuously for ad librar consumption, except when fasted prior to bleeding. ° Tap water provided continuously for ad Wibitum consumption. The water was sampled monthly by the KCMO Water Department and was periodically sampled and analyzed by a Water: Continental Analytical Services, Inc., Salina, KS for a variety of potential impurities (e.g., aflatoxins, chlorinated hydrocarbons, heavy metals, etc.). mary metals, etc.). Midividually boused in stain as steel cages. Hoasing: Environmental conditions: S Temperature 1**8-2⁄6°**C Humidity: veraged at Rast 12? per hour Air changes: Approximately 12 hours of light alternating with 12 hours of Photoperiod: darkness, except when lights were turned off for eye exams. B. Study design 25 September 2006 i 30-31 October 2006 1. In life dates: 2. Animal assignment and preatment The rats were randomly assigned to dose groups, based on weight, using INSTEM DATATOX[®]. Weight variation of animals used were targeted not to exceed $\pm 20\%$ of the mean weight for each sex.



Dose Group (mg/kg/day)	No. Animals/ Dose/Sex	a °
0 (control)	10	Ţ,
100	10 🔊	
300	105	4 . 8
1000	410 Å	

Table 5.3.3-1 Study Design

3. Preparation and treatment of animal skin

On study day -3, the hair was removed from the dorsal and lateral areas of the trunk of each rat using electric clippers. During the dosing period, the animals were shaved as necessary due to the growth.

Individual doses of the test substance were weighed out for each animal and applied to a commerciallyavailable adhesive bandage (2 in. x 4 in.; dose area 2 in. x 2 in.) that was moistened before close application with 1 ml of deionized water. The bandage was placed on the shaved skin of the rat and the torso of the animal was then wrapped with porous tape to assure that the bandage remained in contact with the skin during the dosing interval. The same procedure was performed for control animals, except that only a bandage moistened with 1 mL of detonized water was applied to the dose site.

The test substance/bandage was held to contact with the skin for a minimum of six hours/day for five consecutive days/week for four weeks. During the fifth week, the animals were dosed daily until the day the rat was sacrificed (rats were not dosed on the day of sacrifice). Each day the bandage and tape were removed and the dose site was weped with deionized water-dampered and dry gauge to remove as much test substance residue as feasible without damaging the skin.

4. Statistics

Statistical significance, was determined at p0.05 for all tests with the exception of Bartlett's test, in which a probability value of p ≤ 0.001 was used. All tests were two-tailed, except for gross and histopathologic lesion evaluation that were one-tailed.

Continuous data were analyzed by Bartlett's test for homogeneity. If the data was homogeneous, an ANOVA was performed followed by Durnett's test on parameters showing a significant

effect by ANOVA. If the data were non-homogeneous, a Kruskal-Wallis ANOVA was performed followed by the Mann-Whitney U-text to identify statistical significance between groups.

Frequency data that were examined statistically were evaluated using the Chi-Square and/or Fisher's Exact tests.

C. Methods

1. Observations

All animate were observed at least twice daily (AM and PM) for clinical signs of toxicity, except once daily on weekends. Findings were recorded when the beginning of each week thereafter.

2. Body weight

Individual body weights were measured weekly throughout the study. Body weights were also taken immediately prior to necropsy to allow Or calculation of organ-to-body weight ratios.

3. Food consumption

Food sonsumption was measured weekly during the study.



Leukocyte differential coun

Reticulocyte count C Blood celomorphology

Ölutamate dehydrogena

Urea nitrogen (Urea-N)* Total @ olester (Chol)*

Cotal bilirubin (T-Bili) Total protein (TP)*

Orum protein electrophoresis

Trigivcerides (Trig)

Öric Acid (Uric-A)

Albun 🖓 ní (ALB) *

Globalins (Gob)

Glucose (gluc)*

Creatinine (Creat)*

Mean corpuscular hermoglobin (MCH)*

Mean Orpuscular volume (MOV)*

Mean corpuscular hemogrobin conc.(MCHC)

Red Blood Cell Distrikation Width (RDW) Hemoglobin Distribution Whith (HOW)

4. Ophthalmoscopic examination

Following the acclimation period and prior to initiation of dosing, ophthalmic examinations were conducted on all animals. Ophthalmic examinations were also conducted on all animals prior to termination of the study.

5. Hematology and clinical chemistry

Clinical chemistry and a hematology, including differentials, were performed on all animals (days 28) and 29; prior to being euthanized). Animals were fasted overnight prior to the collection of blood from the orbital sinus of all rats, under anesthesia with Isoflurane. The parameters evaluated are marked@x) in the lists below.

- a. Hematology
- Hematocrit (HCT)* Х
- Х Hemoglobin (HGB)*
- Х Leukocyte count (WBC)*
- Х Erythrocyte count (RBC)*
- Х Platelet count* Blood clotting measurements*
- Х (Thromboplastin time) (Clotting time)
- (Prothrombin time) Х
- * Recommended for 28-day dermation view studies based in Guideline st b. Clinical Chemistry ELECTROLYTES
- b. Clinical Chemistry
- Х Calcium (calc)
- Х Chloride (Cl) Magnesium (Mg)
- Х Phosphorous (Phos
- Х Potassium (K)*?
- Х SodiunQ(Na)* ENZYMES (more than 2 bepatic enzymes)*
- Х Alkaline phosphatase (AR) Cholinesterase (ChE)
- Х Creatine phosphokinase (CKC
- Х Lactic acid tehydrogenase LDH
- Х Alanine aminotransferase (ALT/SGP)
 - Aspartate amiliøtransforase (ÅST/SGOT
- Х **Bile Acids** Х Gamma Glutamyl trensferase (GGD)
- A/G ratio $(A\backslash G)$ studies based on Guideline 870.3200 * Recommended for 28-day derma toxicity
- 6. Urinalysis

Urinalysis was not performed and is not required by the guideline

7. Sacrifice and pathology

Animals were eutoanized at the end of the study by asphyxiation with CO2. A complete gross examination was performed on all animals. The necropsy consisted of a systematic gross examination of each animal's general physical condition, body orifices, external and internal organs and tissues. A list of the tissues coeffected and weighed at necropsy is presented in the table below (marked with an x). Alkitssues were processed, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosi@(H&E) for examination under the light microscope. Histopathologic evaluation was conducted on all protocol-required tissues from the control and high-dose group animals and from the liver of lowand mid-dose group males and females. Where appropriate, all findings were assigned a severity score



of normal, 1 = minimal, 2 = mild or slight, 3 = moderate, and 4 = marked. The mean severity was determined by dividing the sum of the individual animal severity scores by the number of tissues examined in the group.

Tissues Collected at Necropsy, Organs Weighed, and Tissues Examined Microscopically **CARDIOVASC./HEMAT DIGESTIVE SYSTEM** GLANDULAR Х Cecum* Х Aorta* Х Adrenal gland*+# Х Colon* Х Bone marrow* Х xorbital/lacrimal gland Х Х Heart*+# Duodenum* Х (with parathyroid Х Esophagus* Х Lymph node, mesenteric* Х Ileum* Х Lymph node, @rvical* NEUROLOGIĆ Х Х Jejunum* Spleen*# Х Liver*+# Х Thymus* mebellum Х Pancreas* erebrum-Midbrain Х **UR@GENI** lulla/Pons Rectum* Х Х Salivary glands* Harderian glar Х verve, ôptic Stomach, glandular * Х Øervix a Clitoral glanda Х Stomach, non-Х Tongue@ Epididymides Pittertarv* Х Spinal cord, cervical* Tooth &idnev*®**+# Spinal cord, thoracic* Manunary gland* Spinal cord lumbar* RESPIRATORY Oŵarv*+ Х Larynx* Preputial gland Х Lung* OTHER rostate' Х Bone, femur@ Nasal struc Bone, sternum Х Nasophary Х Gross lesions* Oral structure IterWK Х Muscle, protocol@ aginaa Trachea Seminal Vesicle Physical Identifier Skin, treated* glanda Х Zymbal Х Skin, untreated* dav dermal doxicity studios Based on Guideline 870.3200. * Recommended for +Organ weight required @ a 21/28-Da #Organ weighed! @No histopathology performed II. Results and discus A. Observations and mortality 1. Clinical signs of toxicity There were no compound-refered ciprical observations for males and females at any dose level. ~O 2. Mortality No animals were found dead of sacrificed in-extremis during the study. Body weight and body weight gain R. There was no compound-related effect on body weight for males and females at any dose level. C. Food consumption There was no compound-related effect on food consumption



D. Ophthalmoscopic examination

There were no compound-related ophthalmic findings for males and females at any dose level.

E. Hematology, clinical chemistry, and urinalysis

1. Hematology

The only hematology parameter which was affected by compound administration was a statistically significant increase in prothrombin time for high-dose group males as compared to concurrent controls.

There was a statistically significant increase in large mistained cells (LUC) in high-dose group, males as compared to controls. This was considered not to be compound-related since it was within Baver laboratory's historical control range.

2. Clinical Chemistry

The only clinical chemistry parameter which was a statistically significant increase in total cholester values for high-dose group females as compared to the concurrent controls.

There was a statistically significant decrease in potassium (K) values in high-dose group demales as compared to controls. The significant decrease in potassium values was considered bot to be treatment-related for the following reasons; they were not dose dependent and the docreased values were within Bayer laboratory's historical control range (Appendix 1 of the report).

F. Sacrifice and pathology

1. Terminal body weight and organ weight

Terminal body weights were not affected by compound administration of males and females at any dose level.

Statistically significant increases in 4000 mg/kg/day male fiver weights (relative) and 1000 mg/kg/day female liver weights (absolute and elative) were considered to be compound-related. These findings were further substantiated by the presence of a minimation mild degree of hypertrophy in the liver in this dose group.

A statistically significant increase in mean kidney weights (retative) in 1000 mg/kg/day males and a statistically significant increase in mean uver weights (absolute and relative) in 100 mg/kg/day females were considered not to be compound related for one or more of the following reasons: this difference from concurrent compole vas not dose dependent; the relative weight increases were due to corresponding lower terminal body weights for males and females.

2. Gross pathology

There were no compound-related gross pathology findings for males and females at any dose level.

3. Microscopic pathology

In the liver, staristically significant increases in the incidence of hypertrophy (centrilobular and midzonal) in 1000 mg/kg/day males and females were considered to be compound-related. However, hypertrophy was not observed in livers of 300 and 100 mg/kg/day males and females. The liver hypertrophy is attributed to hepatic enzyme induction, as hepatic enzyme induction was observed in a 28-day dietary fat study with this compound. All other microscopic observations were considered to be incidental and/or background and not related to compound administration.

G. Deficiencies

No specific deficiencies are noted.



III. Conclusions

Compound-related findings were only observed in the high-dose group and consisted in an increased cholesterol concentration in females, an increased prothrombin time in males and effect in the liver (increased liver weights for males and females associated with hepatic hypertrophy). The increased liver weights and hypertrophy in the high-dose group are attributed to hepatic enzyme induction, based on findings in another study (see section 5.3.1) and thus are considered to be an adaptive response to AE C656948.

In conclusion, the NOAEL was 300 mg/kg/day, the mid-dose group in this study.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283. In conclusion, the NOAEL was 300 mg/kg/day

CA 5.4 Genotoxicity testing

Fluopyram (AE C656948) was tested in a standard battery of genotoxicity and mutagenicity tests *in vitro* and *in vivo* carried out according to the current OECD. European and US-EEA guidelines. The studies were performed in compliance with CLP requirements from 2005 to 2008. There was no indication of gene mutation either in the presence or absence of metabolic activation in both the bacterial reverse mutation and mammalian gene mutation tests. A supplementary Ameetest was conducted with a representative batch of the proposed specification for the technical AP C656948. The *in vitro* chromosome aberration test and the *in vivo* mouse micronucleus test were also both negative. These studies demonstrate that fluopyram has no genotoxic potential. In addition, an *in vivo* micronucleus test with fluopyram SC600 (600 g/L) solo formulation is also presented to provide further evidence that the bone marrow was reached in the *in vivo* micronucleus test with fluopyram and for which a letter of access has been provided by the current owner of the study report (M-708091-01-1).

Table 5.4-1	20
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Sommary of genotoxicity studies with Fluopyram 🖉

Study Study	Concentrations of	Result N	Reference
	[Substance] tested	KV , O	
In vitro assays 🔬		Å	
Ames Salmonella	16, 500, 500, 500, 500, 500, 500, 500, 50	Negative	2006;
microsome test - Plate incorporation and preincubation method	158\$ and 5000	-G	<u>M-269978-01-1</u>
incorporation and SOV	µ@plate 0 0	10°	
preincubation method			
Ames Salmonella	16, 50, 158, 500,	Negative	2008;
/microsome test - Plate	1585 and 5000 µ@plate 16, 50 158, 500, and 581 µg/plate		<u>M-298529-01-1</u>
incorporation and	ug/plate 30, c0, 120, 180 and 240 µg/mL		
preincubation method			
Chromosome aberration	30, 60, 120, 180	Negative	2005;
test with Chinese	and 240 µg/mL		<u>M-266066-01-1</u>
hamster V79 oells			
V79 / HPRA mampalian	§1.95 to 256 ~	Negative	2006
mutagenieity study	μg/mil		<u>M-268775-01-1</u>
In vivo assay >	~~~~		
Mouse (male)	250, 500 and 1000	Negative	2005;
Mouse (male) micronucleas assay	mg/kg bw		<u>M-263710-02-1</u>
	Fluopyram 600 (600 g/L) s	olo formulation	•
Mouse (male)	500, 1000 and 2000 mg/kg bw	Negative	2016;
micronucleus assay			<u>M-560911-03-1</u>



Data Point:	KCA 5.4.1/01
Report Author:	
Report Year:	
Report Title:	AE C656948 - Salmonella/microsome test plate incorporation and preincubation
	method & A A A
Report No:	AT02911
Document No:	<u>M-269978-01-1</u>
Guideline(s) followed in	OECD 471 (1997) EEC Commission Directive 2000/32/EC Method 913/1402000
study:	EEC Commission Directive 2000/32/EC Method 913/1402000
	OPPTS 870.5100 (August 1998)
	MAFF 12 Nousan No8628 (December 06, 2000) 0 0 4
Deviations from current	current guideline; $QECD = 71, 2020$ Q Q
test guideline:	Deviation: None X X X A A O X
Previous evaluation:	Yes, evaluated and accepted of the DAR (2007)
GLP/Officially	Yes, conducted under GLP Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes a the area of

Executive Summary

In this *in vitro* assessment of the mutagenic potential of AE C656948 (Batch-No.: Mix-Batch:08528/0002, 97% purity), instiding dependent appropriate mutants of Salmonella typhimurium, strains TA 1535, TA 1535, TA 98, TA 100 and TAOT02 were exposed to AE C656948 up to 5000 µg/plate, diluted in dimethyl sulfoxide (DMSQ). For each bacterial strain and dose level, triplicate plates were used in both the presence and absence of an Aroctor 1254 induced rat liver metabolic activation system (S9 mix). Diviso was also used as a negative control. Specific positive controls were used for each strain, After 48 hours of incubation at 370C, the numbers of revertant colonies were scored using ő an automated colony counter X) \bigcirc

There was no indication of bacteriotox effect of A& C656948 at any dose up to and including 5000 µg/plate. From 1581 µg/plate @nwards" the test substance precipitation was observed nevertheless assessment was possible up to the highest dose of 5000 µg plate.

AE C656948 did not cause any significant increase in the number of revertant colonies in either the , L L presence of metabolic activation.

All the positive control compounds produced expected increases in the number of revertant colonies, thereby demonstrating the sensitivity of the assay and the efficacy of the S9 mix. Additional supporting positive control data are presented in docupents Herbold, B.;2009; M-345002-01-1 (Check of S9 metabolizing capacity \$9 fraction, batch of September 13, 2005) and Herbold, B.;2009; M-345004-01-1 (Check of S9 metabolizing capacity S9 fraction, batch of March 20, 2007).

Therefore AE C6\$6948 was not mutagenic with or without S9 mix in the plate incorporation as well as in the pre-incubation modification of the Salmonella/microsome test.



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I. Materials and methods

A. Materials

	. Ç. Z
1. Test material:	AE C656948
Description	Light brown solid powder
Lot / Batch #:	Mix-Batch:08528/0002
Purity:	94.7 % A 6 20 0
CAS #	658066-35-4
Stability of test compound:	Stable for the duration of the stude \mathcal{O} \mathcal{O} \mathcal{O} \mathcal{O}
2. Control materials:	
Negative:	None (Culture predium was used as the negative control) \mathcal{O}^{\vee}
Solvent / final	
concentration:	AE C656948 Light brown solid powder Mix-Batch:08528/0002 94.7 % 658066-35-4 Stable for the duration of the study None (Culture medium was used as the negative control)
	Sodium azide (Segva) foo TA 1535 at 40 µg/phate, 🦄 🐃
	Nitrofurantoin Sigma for TA 100 at 0.2 µg/plater A
	4-Nitro-1,2-ppenylene diamone (Merck-Schuchardt) for TA
	1537 at 10 µg/plate and DA 98 at 0.5 µg-plates
Positive:	Matomycin C (Flaka) for TA 192 at 92 µg/pate only in plate
1 05101 v C.	meorporation Plate, S , S , S , S
A	Cumene hydroperoxide (Sigma) for TAC102 in pre-incubation
@n	* trials only at 50 pg/plates
	2 Aminoanthracene (Aldrich) for the activating effect of the S9
	χ mix in all strains at 3 µg/plate.
3. Activation:	
Source	The S9 fraction was isolated from the livers of Aroclor 1254
Source	Jinduced male oprague Davyey rate
Č. L. Š	
	$^{\circ}$ MgCl2 :6 H2Q $^{\circ}$ $^{\circ}$ $^{\circ}$ $^{\circ}$ $^{\circ}$ $^{\circ}$ $^{\circ}$ $^{\circ}$ 246.0 mg
S9 mix composition:	OGlucose-6-phosphate, disectium salt 179.1 mg
S9 mix composition:	OGlucose-6-phosphato, disochum salt 179.1 mg NADP disodium salt 315.0 mg
	In 100 mM sodium-ortho-phosphate buffer at pH 7.4
	Salmonella typhimutum LO2 strains: TA 1535, TA 100, TA
4. Test organisms	$\simeq 1535$ TA 98, and TA 102 \sim
5 Test concentrations	
 4. Test organisms 5. Test concentrations: Preliminary cytotoxicity assay (+/-\$9) and plate incorporation: 	16, 50 158, 500, 1581 and 5000 μg/plate
assay (+/=\$9) and nlate	$\sqrt{46}$, 50 158, 50, 158 and 5000 µg/plate
incorporation:	46, 50 158, 500, 1581 and 5000 μg/plate
Mutation assay – pre-	For all strains with or without S9 mix: 16, 50, 158, 500, 1581
incubation: 💊 🔗	and 5000 µg/state
B. Test performance	
1. In life dates 30 Nover	mbær 2005 – 19 December 2005
2. Bacterial maintenance	

2. Bacterial maintenance The S. type investigation of the second second



For both the plate incorporation and the preincubation assays reported here, the bacterial suspensions were grown for 17 hours in nutrient broth at 37°C with 90-rpm rotation. The bacterial count used for each assay was not standardized in either assay, but titers were measured in parallel to the actual assays to demonstrate that enough numbers of bacteria had been used in each assay. To determine the bacterial titer, the bacterial suspension was diluted at 1:1000,000, suspended in soft agaf containing historice at 5-fold greater concentrations than those used in the mutagenicity assay. Plates were then incubated for 48 hours and colonies were counted.

3. Plate incorporation assays

AE C656948 or the positive control material was dissolved in 0.1 mL of DMSO 0.1 mL containing AE C656948 or positive controls were added to glass Pessels with 0.1 mb of bacteriak cultures grown overnight, 0.5 mL of S9 mix or buffer and 2 mL of soft agar. The mixture was placed for a waterbath at 45°C for 30 seconds, shaken and a Gerlaid onto Retri dishes containing solid agar. After 48 hours of incubation at 37°C, the numbers of revertant colonies were soored using an automated colony counter. Three plates were used, both with and without S9 mix, for each strain and dose. The doses for the first trial were routinely determined on the basis of standard protocol with a maximum dose of 5000 µg/plate and at least 5 additional doses. If tess than three doses were used for assessment, at least two repeats were performed.

4. Pre-incubation assay

An independent repeat test was performed as pre-incobation of the previously described mixture in a water bath at 37°C for 20 minutes. At the end of the preincubation period, 2 mD of molten soft agar were added to the tubes, the content mixed and plated onto Petri dishes with solid agap After 48 hours of incubation at 37°C, the numbers of revertant colonies were also scored using an automated colony counter.

5. Statistics

statistical analysis of the data is not mandatory. guideline According to the OFCD Ş

6. Evaluation criteria

m A study is considered to be acceptable by the performing aboratory only if:

- a) The negative controls were within the range of either published data or the laboratory's own Austorical control data \bigcirc 21
- b) The positive control substances produced revettant counts within those previously seen in the k V laboratory and A «
- Determination of tite, showed sufferent bacteria density in the cultures used for the tests. c)

A positive test (revertant counts vere increased with est substance) in which criteria b and c were not met is accepted as positive but an independent repeat of the test is conducted.

A trial is considered to be positive of there is a reproducible and dose-related increase in mutants in at least one strain. In TA 1555, TA 500, and TA 98 the mutant count should increase to twice the mutant count seen in negative controls for a trial to be considered positive. In TA 1537 mutant count in a positive trial should be approximately 3 times that of negative control incubations. In a positive trial with TA 102 mutant count should reach & least 100 colonies.

Results and discussion

A. Analytical determinations

AEC656948 is stable in the solvent at room temperatureat concentrations ranging from 0.01 mg/ml to 420 mg/mil for at least 3 days, a time interval, which covers the time range from preparation of the formulation to last treatment.



B. Preliminary cytotoxicity assay

The preliminary cytotoxicity study was conducted by plate incorporation method at AE C656948 concentrations of 16 to 5000 μ g/plate, in TA 1535, TA 100, TA 1537, TA 98, and TA 102. The preliminary study was carried out both with and without S9, and three replicate plates were prepared for each concentration, strain, and condition. There was no indication of a bacteriotoxic effect of SE C656948 technical at doses of up to and including 5000 μ g per plate. From 1581 μ g/plate onwards, the test substance precipitated nevertheless assessment was possible up to the highest dose of 5000 μ g/plate.

Results are presented in the tables below. There was no relevant increase in mutant counts at any concentration of fluopyram compared to the negative controls both with and without metabolic activation (S9 mix).

C. Mutation assays

<u>The preliminary cytotoxicity</u> study was conducted by plate incorporation method at AE C656948 concentrations of 16 to 5000 µg/plate, in TA 1535, TA 100, TA 1537, TA 98, and TA 102. The preliminary study was carried out both with and without S9 and three replicate plates were prepared for each concentration, strain, and condition.

None of the five strains concerned showed in the plate incorporation dest adose-related and biologically relevant increase in mutant counts over those of the negative controls. These applied both to the tests with and without S9 mix and was confirmed by the results of the pre-incubation trials.

The positive controls caused the expected significant increase in the oumber of revertant colonies compared to the controls demonstrating the constitutive of the system.

Neither the plate incorporation nor the preincubation trials with AE C656948 produced any indication of mutagenicity in any strain, either with or withour metabolic activation. The esults from both the preliminary and the definitive studies are presented in the following tables.

	Summary of N	Alean Values ±Sta	dard Deviation M	vithout S9 Mix	
Test substance	~~ .O	O & &	Strain	<i>a</i> n	
and concentration (µg/PQate)	TAU535	TA 100	TA \$37	TA 98	TA 102
0 (DMSO)		<u>~~</u> ∠94€£1^~		27±2	190±5
16			0 6±3	25±5	144±20
50	\$ A ≢2 <u>\$</u>	84±6	591	22±5	167±8
158 🥡	$\begin{array}{c} 3 \\ 5 \\ 6 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7$	[∞] 84±6√ [∞] 93 4 17 0	∀ @4±1	20±5	198±21
506Q	0 8 <u>±</u> 30 ~	[∞] 9) ≠13 [∞] 35±6 (P0) [∞] 7±14 (P) [∞]	≫ 4±2	22±4	161±19
1 <u>58</u> 1	8±2@P)	,,,,,,,,,,,,,	↓ @ 4±2 (P)	18±8 (P)	134±6 (P)
5000	.100≠2 (P)	Ø7±14(0) 🗞	6±2 (P)	23±5 (P)	127±22 (P)
Na-azide	\$14±32		<i>v</i>		
NF NF		239±22			
4-NPDA			71±12	166±18	
MMC @			andard Deviation		550±23
()	Summary of	Sean <u>y</u> alues ±St	andard Deviation	with S9 Mix	
0 (D MS O)	× 7± ×	~12±2	8±2	32±2	248±17
A6		ľ10±34	5±2	28±2	247±6
S 50 S	6±2≈€	106±5	6±3	27±2	237±20
50 × 158 ×	5±20°	138±8	5±0	24±2	255±16
<u>~</u> 5690	642	99±5	8±2	28±2	215±38
× 2581	6 ± 2 (P)	95±5 (P)	6±2 (P)	19±8 (P)	219±23 (P)
٢٥٥٥ گ	5±1 (P)	102±15 (P)	4±1 (P)	26±5 (P)	209±21 (P)
2AA	74±7	1302±278	83±15	965±123	609±33
P= Precipitate					

Table 5.4.1-1Results from the plate incorporation assay in Salmonella typhimurium strains



	Summary of N	/lean Values ±Sta	ndard Deviation	without S9 Mix	a,°
Test substance	-		Strain		
and					
concentration	TA 1535	TA 100	TA 1537	TA 98	TACTO2
(µg/Plate)					
0 (DMSO)	20±3	120±3	8±3	26±5	209±29
16	24±5	111±6	6±1	≈ 25±5	248 6 8 x
50	17±3	106±6	⊘ ⊭2	√27±10	k√ 2Q4±6 ~S
158	21±8	105±19	\$6±1	24±6	200 ± 26
500	17±6	112±8	,≪, 6±1	_O [♥] 24±5 √	200±2\$ 0
1581	16±3 (P)	108±10 (P)	4 [℃] 6±1 (P)	22±10 (P)	, 174±8,(P)
5000	17±3 (P)	104±10 (P)	6±2 (P)	30±1.00)	[→] 177±14 (P)
Na-azide	537±18	- A			\$343±490
NF		309±26			× ×
4-NPDA		0.	82 17	∑9164±700	& A co
Cumene		A . C			0 349±40 0
	Summary of	Mean Values +St	tandard Decration	n with S9 Mix 🔬	
0 (DMSO)	10±4	Ø30±1,1%	7±3	0 43±12 5 0 38±1€	249≠18
16	7±1		r ∧¢±1 @	38±1€	© 236±16
50	8±3	🔬 1110£7 🏷	5±2 🔊	33≠10 5	247±25
158	9±4 "	≶ \$21±6_©	0° 7±25		≈252±53
500	10±2 🖉	\$¥18±110	∱¥ 7\$4″ <u>¢</u>	27±4	& 241±42
1581	6±1 (P)	100±€(P)	7±1 (P)	©30±4 (P)	© 241±28 (P)
5000		✓ 106 10 (P)	©±2 (P) *	29 ≇6 (P) Ø	205±12 (P)
2-AA	84+3	12984±49	£ 14] \$ 8 \$	\$\$6±98	436±22
2-AA = Precipitate	845	25		<u>↓ \$16±98</u>	430±22

Table 5.4.1-2 Results from the pre-incubation assay in Salmonella typhimurium strains

D. Deficiencies

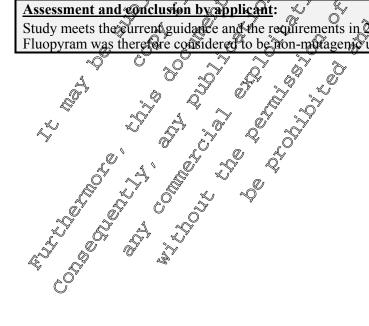
No deficience were identified.

Conclusions

L. III. Conclusions fluopyrant in this test. Finopyran was therefore considered to be non-mutagenic. 2 \bigcirc

 \bigcirc

Assessment and conclusion by applicant: Study meets the current guidance and the requirements in 283/2013. Fluopyram was therefore considered to be pon-moragen of under the conditions of this study.





Report Author: 2008 Report Year: 2008 Report Title: AE C656948 (project: Fluopyram) - Salmonella/microsome test - Plate incorporation and preincubation method Report No: AT04419 Document No: M-298529-01-1 Guideline(s) followed in study: OECD 471 (1997); EEC Commission Directive 200032/EC Method B13/12 Deviations from current test guideline: OECD 4712-C-98-247, OEPTS Series 870;5100 (August 1998) Deviations from current test guideline: Current guideline: OECD 471, 1997 Previous evaluation: Yes, evaluated and accepted in the DAR (2001) GLP/Officially recognised testing facilities: Acceptability/Reliability: Acceptability/Reliability: Yes	Data Point:	KCA 5.4.1/02
Report Title: AE C656948 (project: Fluopyram) - Salmonella/microsome test - Plate incorporation and preincubation method Report No: AT04419 Document No: M-298529-01-1 Guideline(s) followed in study: OECD 471 (1997); EEC Commission Directive 200032/EC Method B13/13 Deviations from current test guideline: current guideline: OECD 471, 1997); deviation: None Previous evaluation: Yes, evaluated and accepted in the DAR (2001) GLP/Officially recognised testing facilities: Yes, conducted under GDP/Officially recognised testing facilities Acceptability/Reliability: Yes	Report Author:	
incorporation and preincubation method Report No: AT04419 Document No: M-298529-01-1 Guideline(s) followed in study: OECD 471 (1997); EEC Commission Directive 200032/EC Method B13/12 Deviations from current test guideline: (2000); US EPA712-C-98-247, OEPTS Series 870 5100 (August 1998) Deviations from current test guideline: current guideline: OECD 471, 1997 Previous evaluation: Yes, evaluated and accepted in the DAR (2011) GLP/Officially recognised testing facilities: Yes, conducted under GDP/Officially recognise@testing facilities Acceptability/Reliability: Yes	Report Year:	2008
incorporation and preincubation method Report No: AT04419 Document No: M-298529-01-1 Guideline(s) followed in study: OECD 471 (1997); EEC Commission Directive 2000/32/EC Method B13/12 Deviations from current test guideline: (2000); US EPA712-C-98-247, OEPTS Series 870/5100 (August 1998) Deviations from current test guideline: current guideline: OECD 471, 1997 Previous evaluation: Yes, evaluated and accepted in the DAR (2001) GLP/Officially recognised testing facilities: Yes, conducted under GDP/Officially recognised testing facilities Acceptability/Reliability: Yes	Report Title:	AE C656948 (project: Fluopyram) - Salmonella/microsome test - Plate
Document No: M-298529-01-1 Guideline(s) followed in study: OECD 471 (1997); EEC Commission Directive 200032/EC Method B13/12 Deviations from current test guideline: (2000); US EPA712-C-98-247, ORPTS Series 870 5100 (August 1998) Deviations from current test guideline: current guideline: OECD 471, 1997 Previous evaluation: Yes, evaluated and accepted in the DAR (2011) GLP/Officially recognised testing facilities: Yes, conducted under GDP/Officially recognise testing facilities Acceptability/Reliability: Yes		incorporation and preincubation method
Guideline(s) followed in study: OECD 471 (1997); EEC Commission Directive 200032/EC Method B13/14 (2000); US EPA712-C-98-247, ORPTS Series 870 5100 (August 1998) Deviations from current test guideline: current guideline: OECD 471, 1997 deviation: None Previous evaluation: Yes, evaluated and accepted in the DAR (201) GLP/Officially recognised testing facilities: Yes, conducted under GDP/Officially recognised testing facilities Acceptability/Reliability: Yes	Report No:	AT04419
study: (2000); US EPA712-C-98-247, ORPTS Series 870,5100 (August 1998) Deviations from current current guideline: OECD 471, 1997 test guideline: deviation: None Previous evaluation: Yes, evaluated and accepted in the DAR (2011) GLP/Officially Yes, conducted under GDP/Officially recognised testing facilities: Acceptability/Reliability: Yes	Document No:	
study: (2000); US EPA712-C-98-247, ORPTS Series 870,5100 (August 1998) Deviations from current test guideline: current guideline: OECD 471, 1997 Previous evaluation: Yes, evaluated and accepted in the DAR (2011) GLP/Officially recognised testing facilities: Yes, conducted under GDP/Officially recognised testing facilities Acceptability/Reliability: Yes	Guideline(s) followed in	OECD 471 (1997); EEC Commission Directive 2000 32/EC Method B13/12
test guideline: deviation: None Previous evaluation: Yes, evaluated and accepted in the DAR (2011) GLP/Officially Yes, conducted under GDP/Officially recognised testing facilities facilities: Acceptability/Reliability: Yes Yes	study:	(2000); US EPA712-C-98-247, OPPTS Series 870 (August 1998) 5 (2000)
Previous evaluation: Yes, evaluated and accepted in the DAR (201) GLP/Officially Yes, conducted under GDP/Officially recognise@testing facilities facilities: Acceptability/Reliability: Yes Yes	Deviations from current	current guideline: OECD 471, 1997
GLP/Officially recognised testing facilities: Yes, conducted under GDP/Officially recognise testing facilities Acceptability/Reliability: Yes		
recognised testing g<	Previous evaluation:	Yes, evaluated and accepted m the DAR (200) $\sim \sim \sim$
recognised testing g<		
recognised testing g<	5	Yes, conducted under Gor Officially recognise desting facilities
Acceptability/Reliability: Yes		
	Acceptability/Reliability:	Yes y y y y y
Executive Summary VI 13 Star Star Star	Executive Summary	

Executive Summary

This supplementary test was performed with a representative termical Batch of the proposed specifications as there were a few changes in the importing profile of the test material compared to the one used in the initial test. In this in vitro assessment of the source potential of AE (656948 (Batch-No.: 2007-010986, 95.7% purify), histidine dependent auxotrophic mutates of Salmonella typhimurium, strains TA 1535, TA 1537, TA 98, TA 60 and TA 102 were exposed to AE C656948 up to 5000 µg/plate, diluted in dimethyl sulfoxide (BMSO). For each bacterial strain and dose level, triplicate plates were used in both the presence and absence of an Aroclor 254 and used rat liver metabolic activation system (S9 mix). DMSO was also used as a negative control. Specific positive controls were used for each strain. After 48 hours of incoration at 37°C, the oumber sof revertant colonies were scored using an automated colony counter.

There was interation of a bacterio oxic effect of AE Cos6948 starting at 500 µg/plate. Nevertheless assessment was possible up to 1581 µg/plate but not at \$000 µg/plate

AE C656948 did not cause any significant increase in the number of revertant colonies in either the presence of metabolic activation.

All the positive control compounds produced expected inexeases in the number of revertant colonies, thereby demonstrating the sensitivity of the assay and the efficacy of the S9 mix. O'

Therefore, ACC65948 was non-mutagenic with or without S9 mix in the plate incorporation as well as in the pre-incubation modification of the Salmonella/microsome test.

- Materials and methods I.
- A. Materials **ЖЕ СФॅ6948** 1. Test material: Description Fine white powder 2007-010986 Lot / Batch # 95.7 % Purify: 658066-35-4 CAS# Stabilit for test compound: Stable for the duration of the study 2. Control materials: Negative: None (Culture medium was used as the negative control) Solvent / final DMSO concentration:



Sodium azide (Serva) for TA 1535 at 10 µg/plate, Nitrofurantoin (Sigma) for TA 100 at 0.2 µg/plate, 4-Nitro-1,2-phenylene diamine (Merck-Schuchardt) for TA 1537 at 10 µg/plate and TA 98 at 0.5 µg/plate, Mitomycin C (Fluka) for TA 102 at 0.2 µgplate only in plate **Positive:** incorporation plate, Cumene hydroperoxide (Sigma) for TA 102 in pre-incubation trials only at 50 µg/plate, 2-Aminoanthracene (Aldrich) for the activating effect of the mix in all strains at $\Im \mu g/plate$. 3. Activation: Rat liver S9, from rats induced with phenobarbital and Aroclor 1254 Source The S9 mix was freshly prepared, kept on ice and used only on the same day. per 70 mk Mg@l2 KC **S9 mix composition:** Qucose 6-phosphate disodium salt .1 m⁄aź NADP disodium salt .Omg 🔬 In 100 mM sodium-ortho phosphate beffer at H 7.4 Salmonella typhimurium strains: TA@535, PA 100, TA 1537, 4. Test organisms: TA 98 and TA 102 5. Test concentrations: Preliminary cytotoxicity Focall strains with or without \$9 mix \$6, 50, 158, 500, 1581 and 5000 µg/plate assay (+/-S9): \bigcirc without S9 mix: For all strains with or 5, 16, 50, 158, 500 and **Mutation** assay 1580 µg/plate B. Test performan

12,De cember 2007 1. In life dates: 10 January

2. Bacterial maintenance

The S. Sphimurium bacteria used in this arsay have been maintained at the test facility since 15 August 1997 and were originally receiver from Dr. B.W. Ames. Bacteria were cultured as described in the original papers (omes et al., McCannet al and were stored frozen at -80°C. Stock cultures were generated as needed through Sulturing on Datrient agar followed by overnight incubation in nutrient broth at 37°C Stock cultures were then shecked for crystal-violet sensitivity and UV sensitivity, and were frozen at -80°C untiluse.

For both the plate incorporation and the preincubation assays reported here, the bacterial suspensions were grown for 17 hours in nutrient broth at 37% with 90-rpm rotation. The bacterial count used for each assay was not standardized in either assay but titers were measured in parallel to the actual assays to demonstrate that sufficient numbers of bacteria had been used in each assay. To determine the bacterial titer the bacterial dispension was diluted at 1:1000,000, suspended in soft agar containing histidine at Stold greater concentration than those used in the mutagenicity assay. Plates were then incubated for 48 hours and colonies were counted.

3. Plate incorporation assays

AE 0656945 or the control material was dissolved in 0.1 mL of DMSO. DMSO (0.1 mL) containing AE C656948 or controls were added to glass vessels with 0.1 mL of bacterial cultures grown overnight, 0.5 mL of soft agar. The mixture was placed in a waterbath at 45°C for 30 seconds, shaken and overlaid onto Petri dishes containing solid agar. After 48 hours of incubation at



37°C, the numbers of revertant colonies were scored using an automated colony counter. Three plates were used, both with and without S9 mix, for each strain and dose. The doses for the first trial were routinely determined on the basis of a standard protocol with a maximum dose of 5000 µg/plate and at least 5 additional doses. If less than three doses were used for assessment, at least two repeats were performed.

4. Pre-incubation assay

An independent repeat test was performed as pre-incubation of the previously describe mixture in a water bath at 37°C for 20 minutes. At the end of the prefocubation perfed, 2 mL of molten soft agar were added to the tubes, the content mixed and plated onto Petri dishes with solid agar. After 48 hours of incubation at 37°C, the numbers of revertant colories were also seored using an automated folony counter.

5. Statistics

According to the OECD guideline 471, statistical analysis of the data is not mandal ia Q

6. Evaluation criteria

A study is considered to be acceptable by the performing laboratory only

- a) The negative controls were within the range of sther published data or the taboratory's own historical control data,
- The positive control substances produced recertant counts within hose previously seen in the b) laboratory, and
- c) Determination of titer showed sufficient@acterial density in the cultures used for the tests.

A positive test (revertant counts were increased with test substance) in which cateria b and c were not met is accepted as positive but an independent repeat of the test is conducted.

A trial is considered to be positive if there is a reproducible and dose-related increase in mutants in at least one strain. In TA 1535, TA 100, and TA 98 the mutate cound should increase to twice the mutant count seen in regative controls for a trial to be considered positive. In TA 1537 mutant count in a positive trial should be approximately 3 times that of regative control incubations. In a positive trial with TA 102, mutant count should reach at least 100 colonies.

II. Results and discussion

Ø1

Concentrations of Sp to 138 µg/plate did not produce any indication of bacteriotoxicity. From 500 µg/plate onward AE \$656948 produced strain specific toxicity. Nevertheless, concentrations up to 1581 µg/plate could boursed for assessment.

The results are presented in the tables below. There was no relevant increase in mutant counts at any concentration of fluopyram compared to the negative controls both with and without metabolic activation (S9 mix). Those results were confirmed in the 2nd experiment with a pre-incubation step. The positive controls caused a significant increase in the number of revertant colonies compared to the controls demonstrating the sensitivity of the sostem. Ø

Table 5.4.1-3	Results from the pla	te incorporation assay in	n Salmonella typhimurium strains
---------------	----------------------	---------------------------	----------------------------------

	Summary of Mean Values ±Standard Deviation Mix without S9 Mix				
Test substance			Strain		
and concentration Ang/Plate)	TA 1535	TA 100	TA 1537	TA 98	TA 102
× , 6°, ×	25±5	111±14	8±1	22±4	189±10
96	22±3	95±2	8±1	20±4	194±8
50	26±7	90±4	6±2	18±7	217±8



Summary of Mean Values ±Standard Deviation Mix without S9 Mix					
Test substance			Strain		0
and					Q
concentration	TA 1535	TA 100	TA 1537	TA 98	TA 102
(µg/Plate)				<u>~</u>	
158	20±1	81±14	6±2	14±2	210±32 🔊
500	17±5	81±5	7±1	15 2	183±18
1581	16±1	47±20	8±1	<u>⊿1</u> ±2	⁵ √163 £ 6
5000	9±5	28±7	4±3	2±1	∑≫ 159 ⇔ 12
Na-azide	502±94	-	~~	<u> </u>	
NF	_	346±67	- *	<u> - 0</u>	J - Z
4-NPDA	-	-	85±18	156±28	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
MMC	-	-	A - 😵	6° - 6	" € 629±56 "
	Summary o	f Mean Values 🔊	andard Deviation		
0	14±3	113±5 🔬	° 9±5%	<u>35</u> €12 ℃	∘
16	13±4	93±110	0 742	23±7	221≠20
50	9±3	64±18		© 21±6	° 208 – 35 √
158	6±1	88 28 ~	~7±3~	A 2944	197±17
500	8±2	83±10	6±3	≫ 35/±11 ~~	≪_229±43
1581	6±2	Q 67±4	V M V	€ 28±5©	\$ 136±13
5000	3±2	27 ×	v ≪4±2 ~~	<u>~</u> 10±5° .<	1 € 4±11
2-AA	123±15	Q 1253±28	≥181±12	° 1480 ±233 ⊱	∿493±162



Summary of Mean Values ±Standard Deviation without S9 Mix @					
Test substance			🔬 Strain 🕺		
and					
concentration	A 1535	خت TA 100		× TA 98	TA 102
(µg/Plate)			N a.		
0		\$\$32±19		∕ 2 7±7	254±39
5	₽ % ±4 [·] *		\$ <u>7</u> ¹ 2	≪) [™] 27±5	244±8
16	9±2,0	O 130≠24 ∞	$\begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & & $	@_ 28±7	257±17
50	0 10±4 0	162±27 ×		✓ 31±4	266±45
158	12±4	@101±6	~ 74	24±8	232±64
500	09±3 0	[©] 112±19 Č	Š Kбl . Ó	25±5	222±20
1581		<u>96</u> 10	& 4±2,5×	20±5	208±23
Na-azide	N 757±44		o' 🔬	-	-
NF		~626±38		-	-
4-NPDA 🖉	ِنْ ^{حَ} رَّى - ^{حَ} رَّى		[∞] 100/3±12	181±29	-
Cumene			¢,		474±11
hydroperoxide	U 29		<u> </u>	=	4/4±11
Summacy of Mean Vatures ±Standard Deviation with S9 Mix					
ð	~~10±4	∽ 154⊊15 .∽	9±3	41±10	232±30
5	I.#⊒37° ∿, '	2 10 8±29	8±3	39±3	258±11
16	<u>√</u> 9₽2_0″	134±19	8±6	36±7	251±10
50	942 C 8±2 C	~~ 163 _0 0	5±2	40±8	278±58
158		✓ 138±11	7±1	37±1	273±12
500 🔊	x 9 6 x	~@30±9	7±1	34±3	267±26
1580 ×	§ <u>9</u> #2 ~	108±16	4±1	40±7	209±20
2, AA , , , , , , , , , , , , , , , , ,	_115±24	1958±176	158±27	1464±93	512±39
		•		•	•

A. Deficiencies &



III. Conclusions

In the absence of any increase in mutant counts, there was no indication of any mutagenic effect of fluopyram in this test. Fluopyram was therefore considered as non-mutagenic.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013. Fluopyram was therefore considered as non-mutagenic under the conditions of the as

Data Point:	KCA 5.4.1/03
Report Author:	
Report Year:	
Report Title:	AE C656948 (Project: AE C656948); In vitto chromosome oberration test with
-	chinese hamster V79 cells V O O O O O O O O O
Report No:	AT02798
Document No:	M-266066-01-4 ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~
Guideline(s) followed in	OECD 473 (5997); EEC 2000/32/EC Method B.10 (2000); OS EPA 712-C98- 223, OPPT Series \$70.5375 (August 1998)
study:	223, OPPTS Series \$70.53% (August 1998)
Deviations from current	Current grideline. OECD 473, 2016 5 6 5 5
test guideline:	Deviation. 200 anstead of 300 pretaphases were scored. This deviation is not
	considered to impact the integrity of the study.
Previous evaluation:	Yes evaluated and accepted in the DAR (2011)
GLP/Officially	Xes, conducted under QUP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability.	Yes 2 a s a s

Executive Summary

In this *in vitro* assessment of the clastogenic potential of AE C656948 (Batch-No.: Mix-Batch08528/0002, 94.7% purity), Chinese Hamster V79 cells were exposed to AE C656948 at 30, 60, 120, 180 and 240 μ g/mL, diluted in dimethyl sulfoxide (DMSO) in the absence and in presence of an Aroclor 1254-induced at liver metabolic activation system (S92mix). Cultures of all concentrations were harvested 18 hours after treatment start. In addition, cells treated at 120, 180 and 240 μ g/mL were harvested 30 hours after treatment start. Without S92mix, an additional experiment was performed using continuous treatment of 18 hours, harvest at the same time, and AE C656948 concentrations of 60, 120 and 180 μ g/mL. Concentrations were selected for metaphases reading on the basis of their cytotoxicity and precipitation in the medium Adequate positive controls (mitomycin C and cyclophosphamide) were also used in each experiment.

Without S9 mix, cytotoxic effects were observed at 120 μ g/mL and above after 4 and 18 hours treatment. With S9 mix, cytotoxic effects were observed at 180 μ g/mL and above. Precipitation in the medium occurred at 120 μ g/mL and above.

None of the cultures treated with AE C556948 in the presence and in the absence of S9 mix showed biologically relevant or statistically significant increased numbers of aberrant metaphases.

The positive controls into my cin C and cyclophosphamide induced clastogenic effects and demonstrated the sensitivity of the test system and the activity of the used S9 mix.

AE C656948 was considered not to be clastogenic for mammalian cells *in vitro*.



 \mathcal{O}

Materials and methods I.

A. Materials

1.	Test material:	AE C656948 Light brown solid powder Mix-Batch:08528/0002 94.7% 658066-35-4 Stable for the duration of the study Culture medium DMSO for AE C656948 and Hanks's balanced safe solution for positive controls (Seromed) Mitomycin C (Floka, baten 454188/244903156) without S9 mix
	Description	Light brown solid powder
	Lot / Batch #:	Mix-Batch:08528/0002
	Purity:	94.7%
	CAS#	658066-35-4
	Stability of test compound:	Stable for the duration of the stude \mathcal{O} \mathcal{O} \mathcal{O} \mathcal{O}
2.	Control materials:	
	Negative:	Culture medium 2^{4} 4^{4} 0^{4} 2^{4}
	Solvent:	DMSO for AE C656948 and Mank 2s balanced salt solution for
	Solvent:	positive controls (Seromed)
		Mitomycin, C (Fluka, batch 454488/2 44903196) without S9 mix
		at 0.1 µg/mL for a treatment period of 4 hours and 0.03 µg/mL
	Positive:	for a treatment period of 18 bours.
		Cyclophosphamide (Endoxan 100 mg infection vials of dry S
		substance, Baxter Oncology GmbH) with S9 mix at 2 µg/mL.
3.	Activation:	substance Baxter Oncology GmbH) with SS mix at 2 µg/mL. +254 X Rat X Liver arbitol 4 Hanster 4 Q Lung
<u>S9</u>	derived from	
X	induced V Amelor	state of the transferred to the
Λ		arbitolo & Mouse & & Lung
	non-induced Phenots	aronolo in avrouse in the Lung
	None [®]	arbitolo A Rat A Lung A Hamster A Other A Other
	🔬 Other	Querer & Contraction
	Composition of Somix-com	position (containing 40% \$9 fraction):
	Cofactor Solution per 25 ml	§9 mix: ディング 、 グ 、 デジ
	Sodium phosphate baffer (1	90 mM pH 7.4) _15.0 m
	MgCl ₂ x 6 H2O	1 2 40.7 mg
	KCP & S	6125 mg 0
	Sucose-6-phosphate disod	A Rat A A Live arbitol arbitol Hamster Other O
	NADP (disodnum sata)	78.8 mg
4. 7	Test cells: St A	item salt) 57 28.0 mg 78.8 mg
1170	a sella susan hain a funda in the	Chale Marshe B Delevate dt The cells arrived at the Terriceleast of

V79 cells were bained from Dr. Utesch, Merck AG, Datonstadt. The cells arrived at the Toxicology of Bayer Health Care AG, Wuppertal on November 8, 1993. Ì

Chinese hanster V79 cells can be kept in citizer, as established cell lines (Kao and Puck, 1967). The mean generation time of the used cell line is approximately twelve hours.

Priot to the start of the study Chinese honster 779 cells from a frozen permanent, which was stored in liquid nitrogen, were normally grown in 20 ml medium and 75 cm² flasks or under comparable conditions. Incubation of the cells was always performed at 37°C in a CO₂-incubator (5% CO₂). Unless conditions. Inequality of inequelity was always performed at $3/^{\circ}$ C in a CO₂-incubator (5% CO₂). Unless reported otherwise, cells were grown in medium containing 10% fetal calf serum [FCS = fetal bovine serum (FBS)].



5. Culture medium:

As medium, PAA Ready Mix was used. PAA Ready mix is a commercially available by PAA, Paching, Austria and consists of Eagle's minimal essential medium (MEM, Earle) and the following supplements:

PAA Ready Mix (10% FBS) 1% L-glutamine 1% MEM-vitamins 1 % MEM NEAA 1 % Pen/Strep 10%FBS(=FCS)

PAA Ready Mix (2% FBS) 1% L-glutamine 1% MEM-vitamins 1 % MEM NEAA 1 % Pen/Strep 2%FBS(=F

6. Test compound concentrations used:

Non activated conditions: Activated conditions:

30, 60, 120, 180 and 240 kg/mb 30, 60, 120, 180 and 240 μg/mL.

B. Test performance

ember 14 September 2005 1. In life dates:

2. Preliminary cytotoxicity assay

250 μg/mL with or without S9 mix for 4 hours or 78 hours (without S9 mix). The putotic index was determined for all cultures. The number of mitotic cells among a total of 1000 cells per culture was determined using a light microscope at a magnification of about 630. All cells which were not in ő interphase were defined as mitoric. L

In the main study, cultures with a total incubation period of 4 hours were additionally used to determine the cytotoxicity of AE C656948 at concentrations ranging from 30 to 240 pg/mL with a harvest time of 18 hours and at concentrations of 120 to 240 by/mL with a barvest time of 30 hours. Additional cultures were exposed without \$9 min for 18 hours to concentrations of AE Co56948 ranging from 60 to 180 µg/mL. These cultures were harvested at the end of the treatment period.

Concentrations of up to 4000 µg/mLAE C656948 did not change the pHin the medium. The osmolality in the medium of the pre-test was not charged up to a concentration of 250 µg/mL.

3. Cytogenetic assay

a.	Cell exposure time.		Test 🕅	aterial S	olvent Control	Positive Control
	Non-activated:		. Ó ^S , 18	.0 hr 🔊	18.0 h	18.0 h
	Activated:	Ĩ D a	× ~ 4.	0 kO″ _{>>}	4.0 h	4.0h
h.	Spindle inhibition		r s			

- Colcemid, 40 µg/ml water Administration time: 2 hours prior to harvest, respectively
- Test Material **Positive Control** c. Cell Marvest times Solvent Control Non-activated: t or 18 h 4 or 18 h 4 or 18 h Activated: 4 h 4 h

d. Details of slide preparation

After the removal of the medium from each flask, the cells were trypsinized, suspended in medium and centrifuged for approximately primites at 700 rpm. The supernatant was removed and 1 to 2 mL of a hypotonic solution (0.4% KCC 37°C) was added to each tube. Within 4 minutes, the volume was brought to 6 mL with additional hypotonic solution and cells were resuspended. The cells were centrifuged again and fixed with cold (4°C) fixative (ethanol/acetic acid 3:1) for 20 minutes at room temperature. Cells were pedeted and resuspended in fixative as before and centrifuged again. The pelleted cells were resuspended in a small volume of fixative and the suspension was dropped onto clean slides. The slides were allowed to dry for at least 2 hours. Thereafter, they were submerged in pure methanol for 3 minutes



and stained for 15-20 minutes in 3% Giemsa solution. Slides were rinsed twice in water and once in acetone and were then kept in xylene for about 30 minutes. The slides were allowed to dry completely and covered. At least two slides were generated per culture.

e. Metaphase analysis

No. of cells examined per dose:	200
Scored for structural?	Yes
Scored for numerical?	Yes: polyploidy, endoreduplication
Coded prior to analysis?	Yes Ö

4. Statistics

The statistical analysis was performed by pair-wise comparison of AE& 656948 reated and positi control groups to the respective solvent control group. ()

The mitotic index was statistically analyzed (provided that it was reduced compared to the mean of the corresponding solvent control) using the one-sided Chi2-test.

The numbers of metaphases with aberrations (including and excluding gapo and of metaphases with exchanges were compared (provided that these state superseded the respective solvent control). The oneside Chi2-test was used for the statistical evaluation

A difference was considered to be granificant, if the probability of

Evaluation criteria 5.

 \bigcirc An increased incidence of gaps of both types without concomitant increase of other aberration types was not considered as indication of a chastogenic effect. C

A test was considered positive if

there was a relevant and statistically significant increase in the aberration rate.

A test was considered negative

- there was no such increase a any time interval.
- _ there were spatistical significant values, which were, Doweyer, within the range of historical negative controls.

A test was considered equivocal if

- there was an increase above the range of historical negative controls which was statistically significant but not considered relevant, or if
- an increase occurred which was considered relevant, but which was not statistically significant

6. Assay Acceptance Criteria

An assay was acceptable, if there was a bologically relevant increase in chromosome aberrations induced by the positive controls and if the numbers of aberrations for the negative controls were in the expected range based on results from our laboratory and from published studies

Resolts and discussion П.

A. Analytical determinations

AE C656948 is stable in the solvent at room temperature at concentrations ranging from 0.01 mg/mL to 420 mg/ml for at least 3 days, a time interval, which covers the time range from preparation of the formulation to fast treatment.

B. Preliminary cytotoxicity assay

In the absence and in the presence of S9 mix Chinese hamster V79 cells were exposed for 4 hours to AE C656948 at concentrations of up to and including 240 mg/mL and, in addition, without S9 mix for 18



hours to AE C656948 at concentrations of up to and including 180 mg/mL. With and without S9 mix substance precipitation occurred in the medium at the concentrations 120 mg/mL and above.

Mitotic Index

The mitotic indices were determined in the main study following 4 or 18 hours reatment.

Mitotic index without S9 mix:

In absence of S9 mix, the mitotic index was only significantly reduced at 180 µg/mL af treatment

Mitotic index with S9 mix:

With S9 mix, there was no reduction of the mitosis rat

Survival Index

18 hours treatment The survival indices were determined in the main study following 4 or Ó

Survival index without S9 mix:

ards in the absence of So mix. Survival indices were significantly reduced from 20

Survival index with S9 mix:

in the presence of S9 Survival indices were significantlo from 1 mix.

C. Cytogenetic assays

for Microscopic evaluation of the metaphases structural chromosome oberrations was conducted following 4 or 18 hours treatment.

AE C656948 without \$9 mix

No biologically sevend and statistically significant increases of numbers of metaphases with aberrations were detected after 4 hours treatment and total culture times of 18 or 30 hours. The same was true for a reatment period and total culture time of D hours. Ø 1

The treatment with the positive control mitodycin Oresulted in Sclear and statistically significant increase of metaphases with aperrations and demonstrated the sensitivity of the test system.

AE C656948 with S9 mix:

No biologically relevant and statistically significant increases of numbers of metaphases with aberrations were detected after 4 hours treatment and total culture times of 18 or 30 hours.

The positive control cyclophosphamide induced statistically significant and biologically relevant increases of metaphases with aberrations and demonstrated the sensitivity of the test system and the activity of the used Somix

ind retrations and the first of the state of



O

Exposure, h	S9	Conc., µg/mL	Mitotic index, % control	Incl. gaps	Excl. gaps	Carrying ° exchanges
		Solvent - DMSO	100.0	4.0	3.5	0,ĝ× 4
		Mitomycin C: 0.1	112.4	73.5	Þ	
4	-	60	111.3	2.0	2.0	
		120	119.2	1.0	_« 1.0	Q 0.0
		180	133.9	3.5	3.5	
		Solvent -DMSO	100.0 💍	5.0	5.0 🕺	J ~2.0 \$
	+	Cyclophosphamide: 2	77.0* 🕉	53.5	52.5	2.0 2.0 $2.1.5$ 1.5 1.5 4
4		60	120,5	3.00♥	3.5 📞	$ \frac{1}{2} $ $ 1$
		120	108.7	D.	· 3.0	, [®] [®] , [®]
		180	Ø6.9	2.5	QO O	
		Solvent - DMSO	100.0	0 1.5 × 46.5	1.5	$ \begin{array}{c} $
		Mitomycin C: 0.03	89 9	46.5	45.0 3.0	12.0
18	-	60	408.8	Ø:5 冷	3.0°	
		120	s @18.4	. ×1.5 «		£0.0 Ø
		180	103.55	Ô 2.0€		1.0
p < 0.05		Į,		y _v oʻ		
D. Deficien	ncies		103/5y			Ŭ . Ŝ
None.		sions		$\tilde{\mathcal{S}}$		хар (
III.	Conclu	sions $\sqrt[9]{\sqrt[9]{2}}$	V S O			& ,

D. Deficiencies

III. Conclusions

Based on the results of this test, thiopyram is considered not to be elastogenic for mammalian cells *in vitro*.

Assessment and corclusion by applicant:

L'A Study meets the spirrent guidance and the requirements in 283/2018. Based on the results of this test, fluopyram is considered not to be clastogenic for mammalian cells in vitro. N.

Data Point: 0 KOX 5.4.1704 0 0 0	
Report Author:	
Report Year: $\sqrt{2006}$ $\sqrt{7}$ $\sqrt{7}$ $\sqrt{7}$	
Report Title: AE \$656948 - V79/HPRT test in zirro the detection of induced forward	
\mathcal{O} \mathcal{O} mutations \mathcal{O} \mathcal{O} \mathcal{O}	
Report No: $\sqrt[n]{0}$ $\sqrt[n]{0}$ $\sqrt[n]{0}$ $\sqrt[n]{0}$ $\sqrt[n]{0}$	
Document No: $M-268775-0447$	
Document No: M-263/15-0414 Guideling(s) followed.in? OEOD 476 (1997) EEC Commission Directive 2000/32/EC, Method B.17.	
study: (2000); OPPTS \$70.5300 EPA 712-C-98-221 (August 1998); MAFF 12 Nous	n
2000)	
Deviations from current Current guideline: QCD 476, 2016	
test guideline: Destation None.	
Previous evaluation: -> Kes, evaluated and accepted in the DAR (2011).	
GLP/Officially w Stes, conducted under GLP/Officially recognised testing facilities	
recognised testing O	
facilities a second sec	
Acceptability Reliability: Ves	



Executive Summarv

The purpose of the study was to assess the point mutagenic potential of AE C656948 (Batch-No.: Mix-Batch:08528/0002, 94.7% purity) at the hypoxanthine-guanine phosphoribosyl transferase (HCRT) locus in V79 cells.

AE C656948 was tested at concentrations ranging from 4 to 256 µg/mL with or without metabolic activation. Without S9 mix, AE C656948 induced decreases in relative population growth at 256 µg/mL. With S9 mix, AE C656948 did not induce decreases in survival or in relative population growth However AE C656948 was tested up to its limit of solubility under critture conditions. Precipitation occurred in the culture medium at 128 µg/mL and above. Adequate positive controls (ethyl methanesulfonate and dimethylbenzanthracene) were used for each experiment.

With and without S9 mix there was no biologically relevant increase in mutant frequency above that of the vehicle controls. Ethyl methanesulfonate and dimethylbenzanthracene induced clear mutagenic °∼ effects demonstrating the sensitivity of the test system and the activity of the S9 mR.

Based on these results, AE C656948 was considered to be non-prutagence in the V79/HPRF forward Based on these results, AE C650948 was considered to be non-mutagene in the vorticity mutation assay, both with and without metabolic activation.
 I. Materials and methods
 A. Materials

	: AE C656948
1. Test material	$: \qquad \qquad$
Description	
Lot / Batch #:	Mix-Batch; 98528/0002
Purity:	946%
CAS #	\sim 658066 $35-4$ \sim \sim \sim \sim
Stability of te	st compound: Stable for the duration of the study
2 Control moto	
2. Control mate	Culture medium Eagle's minimal essential medium supplemented with 1% L-gluamine, 1% MEM-vitamins, 1%
	Supplemented with 1% L-gluramine, 1% MEM-vitamins, 1%
	() () () () () () () () () () () () () (
Ô	$[\mathcal{O}]$ serium (F(S)) $[\mathcal{O}]$
	Demos In the contract of the c
Solvent:	
	o for environmethanesulfonate as it is a liquid.
	Exbyl methanesulfonate (EMS), a directly alkylating agent, used
-	Q S aff a final concentration of 900 μg/mL in non-activation trials.
Positive	Dimethylbenzanthracene (DMBA), promutagen requiring a
	metabolic activation, used at a final concentration of 20 ug/mL
à'	for trials with \$9 mix.
L.	
3. Activation:	A & A A
3. Activation:	exceeding 1% (v/v) in the culture medium. No solvent needed for ethyl methane sulfonate as it is a liquid. Ethyl methane sulfonate (EMS), a directly alkylating agent, used of a final concentration of 900 μg/mL in non-activation trials. Dimethylbenzanthracene (DMBA), promutagen requiring a metabolic activation, used at a final concentration of 20 μg/mL for trials with \$9 mix.

The \$9 fraction was isolated from the livers of Aroclor 1254 induced male Sprague Dawley rats. The preparation dated from April \$, 2004 (protein content 22.2 mg/mL) and September 13, 2005 (protein content 24.0 mg/mL) and was kept frozen at -80°C. The batch was tested for contamination and cytotoxicity prior to use in the first studo. Cofactors were freshly dissolved in sodium phosphate buffer (150 mM@pH 7.). Three parts of the cofactor solution were mixed with two parts of the S9 fraction giving rise to the following foral concentrations in the S9 mix:

8 mM MgCI₂ x 6H₂O 33 mM KCI 5 mM Glucose-6-phosphate 4 mM NADP 40 % (v/v) S9 fraction



60 % (v/v) Sodium phosphate buffer

The S9 mix was kept on ice until use and only used at the same day.

- 4. Test cells: V79 Chinese hamster lung cells
- Hypoxanthine-guanine phosphoribosyl transferase (HPRT) 5. Locus examined:

6. Test compound concentrations used:

A preliminary cytotoxicity test was conducted without and with metabolic activation asing A preliminary cytotoxicity test was conducted without and with metabolic activation alsing concentrations of AE C656948 ranging from 1.95 ug/mCo 250 ug/mL Concentrations of up to 250 pg/mL AE C656948 did not change the pH in the medium of the pre-test. The osmolatity in the medium of the pre-test was not changed by concentrations of up to 250 pg/mL AE C656948.
Clonal cytotoxicity assay: 1.95 to 250 µg/mL
Mutagenic assay: 4, 8, 16, 32, 64, 128 and 256 µg/mL
B. Test performance
I. In life dates: 04 November 2005 – 15 December 2005
Clell treatment Determination of cytotoxicity
Exponentially growing V79 cells. were plated in 20 mL culture medium in a 75 cm2 flask with a total

B. Test performance

- 1. In life dates:
- 2. Cell treatment

Exponentially growing V79 cells were plated in 20 mL culture medium in 275 cm2 flask with a total volume of 275 mL (4x106 cells per flasks). For each concentration, one oulture was available. After attachment (16 to 24 hours later), cells were exposed without Symix to vehicle along or to a range of concentrations of the test substance for 5 hours if 20 mb medium containing 2% FGS. In experiments with metabolic activation I mL of medium was replaced by 1 mL of S9 mix. Thereafter, each cell monolayer was washed with PBS, trypsinized and replated on 5 mL culture medium at a density of 200 cells into 3 Petri dishes (diaméter of 60 mm). These dishes were incubated for 6 to 8 days to allow colony development Thereafter, and ones were fixed with 95%, methanol, stained with Giemsa (Merck; stock solution diluted 1:50 ith deionized water) and counted automatically using an Artek counter, when there was no interference by precipitation of the plates or colouration of the plates. Cytotoxicity was expressed by comparison of colonies in treated contures versus chiclescontrol cultures (relative cloning efficiency).

Treatment without Metabolic Activation

Exponentially growing V79 cells were plated in 20 ml culture medium in two 75 cm² flasks per concentration (4x)06 cells per @ask) including all control groups. After attachment (16 to 24 hours later), the cells were exposed to vehicle along or to a range of concentrations of the test substance for 5 hours in 20 m culture medium with reduced serum content (2%). Thereafter, cell monolayers were washed with PBS, trypsingzed and replaced in 20 mI culture medium using 1.5x106 cells per 75 cm² flask and in 5 mL culture medium using 200 cells per Petri dish (diameter of 60 mm). One flask and 3 Petri dishes were used per culture. These dishes were incubated for 6 to 8 days to allow colony development and to determine, the cythroxicity associated with each test substance directly after treatment (survival to treatment)

Cells in 75 cm flasks were incubated to permit growth and expression of induced mutations. Cells were subcultured country, normally after 3 days) by reseeding 1.5x106 cells into 20 mL of medium in 75 cm² flasks. At the end of the expression period (=count 2, normally a total of 6 days), cultures were reseeded in Petry dishes (dianeter of 100 mm) at 3x105 cells per dish (8 dishes per culture) in 20 mL culture medition without hypoxanthine but containing 10 µg/mL 6-TG for selection of mutants. In addition, 200 cells per dish (diameter of 60 mm, 3 dishes per culture) were seeded in 5 mL culture medium to determine the absolute cloning efficiency for each concentration. After incubation for 6 to 8 days, the colonies were fixed, stained with Giemsa and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.



Treatment with Metabolic Activation

The activation assay was performed independently. The procedure was identical to the non-activation assay except for the addition of S9 mix. In these experiments 19 mL instead of 20 mL culture medium and additionally 1 mL of S9 mix was added to the flasks for the treatment period, resulting in a concentration of 5% S9 mix in the cultures. The number of 6-TG resistant mutants and viability were determined as in the non-activation assay.

Two trials were performed.

3. Statistics

The statistical analysis relied on the mutant frequencies which were submitted to a weighted analysis of variance as well as to a weighted recursive regression, both with Poisson derived weights.

The two mutant frequency values obtained per group were, although, somewhat related, considered as independent measurements thus increasing the power of the statistical tests applied. Since the protocol of the HPRT assay required at least two independent frais, the overall analysis without respectively with activation was the most important one for classifying substances into mutagens and non-mutagens. However, separate analyses were run for each trial in order to examine the consistency of the results.

All acceptable groups were included in the weighted analysis of variance followed by pairwise comparisons to the vehicle control on a nominal significance level of $\alpha = 0.05$ using the Dennett test. The regression analysis part was performed on the basis of the actual concentrations thereby omitting the positive, negative and vehicle controls. If there was a significant concentration related increase of the mutant frequency ($\alpha = 0.05$) in the main analysis the highes concentration was dropped and the analysis repeated. This procedure was repeated until p $\gg 0.05$. In that way eliminated concentrations were flagged correspondingly.

4. Evaluation Criteria

The mutant frequency at each concentration was used to determine whether the substance was mutagenic. $\int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \int_{-\infty}$

Mutant frequencies will only be used for assessment, if

- at least 5 dishes per culture were available and clative survival to treatment, relative population growth and absolute cloning efficiency were 10% or greater.

Q

A trial will be considered positive if

- a concentration-related and in parallel cultures reproducible increase in mutant frequencies is observed. To be relevant, the micrease in mutant frequencies should be at least two to three times that of the highest negative or vehicle control value observed in the respective trial. If this result can be reproduced in a second trial, the test substance is considered to be mutagenic.

Despite these criteria, apositive result will only be considered relevant, if

no significant charge in conolality compared to the vehicle control can be observed. Otherwise, unphysiological calture conditions may be the reason for the positive result (Scott et al, 1991).

A test substance will be judged as equivocalif

- there is no strictly concentration related increase in mutation frequencies but if one or more concentrations induce a reproducible and biologically relevant increase in mutant frequencies in all trials.

An assay will be considered negative if

my reproducible and relevant increases of mutant frequencies were observed.



Results and discussion П.

A. Preliminary cytotoxicity assay

In the absence and in the presence of S9 mix Chinese hamster V79 cells were exposed to AE C65 at concentrations of up to and including 256 µg/mL. With or without S9 mix, twopyram precipitation occurred in the medium at concentration of $128 \mu g/mL$ and above.

Good cloning conditions were demonstrated by the absolute cloning efficiency for the vehicle controls ranging from 52.6% to 67.0% and from 50.0% to 64.8% without and with metabolic activation respectively. T.

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B. Mutation assays

The test system proved to be sensitive on both experimental conditions (activation and non-activation since treatment with the positive controls caused biologically relevant increase in mutant frequencies as compared to the corresponding controls. L, Ò

There was no relevant increase in mutant frequencies after treatment with fluopyram at any concentration (up to the highest dose of 255 µg/mL) either with or without metabolic activation.

Table 5.4.1-6	Result	s of th	e HPRT-locu	s mammalia	n gene muta		ssay y	۵ ۵
			~ 0	Culture I	* >		Culture II 🔬	ĝ.
Treatment	Conc., μg/mL	S9	СЕ И , %	SPutant colonics 10° cells	Total Mutant colonies		Mutant colonies / 10 gells	Total Mutant colonies
DMSO	\$	ĝ,	49.8±300	A.7	2%	∠63.3 ±6 8	≪9.9	15
EMS	900.0	7	33.3±3.3	230.00	Ô ^r 84 «.	43.2≅9.4	437.3	453
	4	-	43 <u>4</u> 0 <u></u> ≇6.3	6,8	× 7 0 [×]	50,5±3.5°	6.6	8
	4 8		20.8±6.8	400.2	× 10,	56.7±5.4	1.5	2
	16	52	× - ×	~ - ,~9		√ 42.8±2.3	6.8	7
AE C656948	QŽ 🔊	Ĩ	∀ 47.&±0.6	7.00	~~~8 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		3.6	4
ð		×°	52ر5.3%	<u> </u>	$\sqrt[3]{8}$	65.0±6.2	7.1	11
~	12 8 0	\ll	\$3.8±4.6	×9.8		≪66.0±1.8	8.2	13
	256P 🛒	, ,	≪58.3± 5 .8	₩0.7		Ø 55.3±4.5	1.5	2
DMSO			51.222.9	· 4.6	\$ 5 O	75.3±3.3	7.2	13
DMBÅ [♥]	20		74,0±3.5		122	74.3±7.2	105.9	189
		~~	48.8±3.7	8.5	<u>م</u> 10	63.2±5.0	13.2	20
	× A		61.2+3.2	≪ 6.1 _∞	0 9	71.5±5.8	4.1	7
Q	16 🔗	+	65.© <u></u> ≝5.0 _©	26	7 4	68.7±2.9	5.5	9
AE C656948	32 Û	Õ	~55.8±4.6	<u></u> 2×.5 ~	, 6	60.7±5.4	8.2	12
A	64	0°	42.7±3€3∕		2	61.2±4.8	3.4	5
Q,	128P 🖏	ć	[©] 61.8 ⊈6 .0	4.7	7	49.5±8.0	2.5	3
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	256R	4	$\sim C \leq$	× .~	11	53.8±4.0	10.1	13
DMSØ	s,		<u>\$\$</u> 3±6	×3.8	5	70.7±10.6	2.4	4
EMŠ	900.0	e e	©40.2±3.4	©301.1	254	47.7±4.0	465.0	532
( (	¢4	Ô	61,3#6.2	2.0	3	56.0±9.3	8.2	11
Ó	8		53/2±2.8	[♥] 3.9	5	62.0±9.0	7.4	11
	16		≪64.7±209	3.9	6	56.5±8.0	5.9	8
AE C656948	\$ <u>7</u> 2 O	Í	℃42.0±3.6	6.0	6	50.2±7.8	5.8	7
	r 64 <u>s</u>		58.3±5.8	2.9	4	51.7±3.8	1.6	2
	1280	K) [®]	58.3±7.8	1.7	2	77.2±4.0	11.9	22
A D	256P 4	\$	70.5±17.3	0.0	0	72.0±4.9	0.0	0
DMSO 5	1	]	48.8±4.3	2.6	3	54.2±0.8	4.6	6
DMB	20	+	81.0±4.8	104.9	204	54.2±2.8	118.5	154
AE C656948	4		55.8±8.8	3.7	5	72.2±3.0	6.4	11
111 00000000	8		69.3±6.3	3.0	5	54.7±4.2	8.4	11

Table 5 4 1_6 Desults of the HDDT



				Culture I			Culture II	
Treatment	Conc., μg/mL	<b>S</b> 9	CE II, %	Mutant colonies / 10 ⁶ cells	Total Mutant colonies	CE II, %	Mutant colonies / 10 ⁶ cells	Total ° Mutant colonies
	16		43.2±3.3	8.7	9	61.7±3.8	10.8	16
	32		69.3±3.7	6.6	11	55.8±4.00°	4.5	
	64	_	56.0±1.5	6.7	9	63.5± <b>3</b> .5	9.8	
	128P	-	56.5±10.0	8.8	12	64.5±5.4	7.j~>>	×11
<ul> <li>precipitation</li> <li>Cells lost due to</li> <li>Deficiencie</li> </ul>	256P contaminat s	ion	С		<u> </u>	$64.5\pm5.4$ $5007\pm5.1$		
No defic	iencies w	ere no	oted.		·	N O	ð ×	Ĵ. Ĵ
III. Cor	nclusions			o v	× á	y jo	Ŝ.	4
Jnder the exp V79/HPRT forv	erimental vard muta	cond tion a	litions descr	bed, fluops	ram was	considered in	on mutage	spic in this
Assessment a	nd conclu	sion	<u>by applicant</u>	× ×		N N		Ŝ
Study meets t	he curren	t gui	dance and th	ne requirero	ents in 283	2013. There	e jêžno côr	icern for
induction of ge	ene mutat	ion in	the manimal	1an cells				
P= precipitation C: Cells lost due to C. Deficiencie No defic III. Con Under the expo V79/HPRT forv Assessment an Study meets t induction of ge CA 5.4.2	In sin	y vo stů	dies in som	Atic cells				
Data Point:			5.4.2/01		N K	y _x ş		
Report Author					d s			
Report Year:	O"	2011			¥ 0	×		
Report Title.	Å,	JAE (	2656948 Mi	cromucleus-te	st on the mal	Ømouse - 1st	amendment	to Bayer
Report No:	Ŭ		rt AT0\$753 o: 2758A		<u>, 2005 _ 0°</u>			
Document No:		( v ·	$\frac{2}{63\%10-02}$		<u></u>			
Guideline(s) fol	low ed in	OFC	171 (1007)	w o	ð			
study:		EEQ	2000032/EC EPA OPPTS S	Method B12 (	2000)			
~Ģ [®]	Ŭ,	PR 1	EPĂ ØPPTSS	eries 870.53	5 (August 1	998)		
^		ÔМА.	AD12 Ngusan	Nø 8628 Ø	ecember 06, 2	2000)		
Deviations from test guideline:	i currento	Dev mate	riatorn the Bo	ntoneat admin od, but signs	nistration ration for the systemic t	her than oral. I	at all doses t	ested.
· ¥	ų [°]	the	esults.	S.	t the outcom	e of the study	and interpre	tation of
Previous evaluation	tion: $A$ *	den th	evaluated and e DAR (2001)	)	·			
GLP/Officially recognized testin facilities:		Yes,	sönducteð un	der GLP/Offi	cially recogi	nised testing fa	cilities	
Acceptabilit R	eliability:	Xes						
	42	Ð						



#### **Executive Summarv**

The micronucleus test was conducted in male NMRI mice to investigate a possible clastogenic effect of AE C656948 on the chromosome of bone-marrow erythroblasts. Cyclophosphamide was used as positive control.

Groups of 5 male mice were administered AE C656948 twice via the intrapersioneal route at doses of 250, 500 and 1000 mg/kg bw, separated by 24 hours. Positive control animals received a single injection of cyclophosphamide (20 mg/kg bw). Bone marrow from at least one femur from each animal was sampled 24 hours after the last intraperitoneal injection. Sticles of bone marrow cells were prepared and scored for the occurrence of micronucleated polychromatic erythrogytes (micronucleated) PCEs), of micronucleated normochromatic erythrocytes and PCEttotal erythrocytes ratios.

micronucleated polychromat. All animals survived until the end of the study but showed symptoms of toxicity after administration starting at 250 mg/kg bw, which included apath@semi-anestherized state, roughened fur, weight bss, sternal recumbency, spasm, body stretching and difficulty in breathing. There was an alteration of the polychromatic to normochromatic erythrocyte ratio at all dose levels tested depionstrating a relevant systemic exposure to the test substance.

There were no biologically significant variations in the introdence of micronucleated polychromatic erythrocytes between the control and the AE C656948 treated groups

In conclusion, there was no indication of a clastogenic effect of C656948 in the micronucleus test on the male mouse

- Materials and methods I.
- A. Materials
- 1. Test material: AXE C6**56**948 Description igh@brown solid powder Mix-Batch:0852800002 Lot / Batch #; Q4.7 % **Purity:** 858060-35 CAS # Stability of test compound: CAS# Stable for the duration of the stud 2. Control materials: Negative: none  $\bigcirc$ 0.5% aqueous Cremophor emulsion Solvent: cyclophosphamide used in form of Endoxan 100 mg injection **Positive:** wals of dry substance Baxter Oncology GmbH) 3. Test animals: Species: moase Hsd/Win/NMR Strain-6-12 weeks approximately Age 36-43 g (males only) Weight at dosing Source: At least 5 days Acclimation period: Feed 3883 (10 mm cubes), produced according to specification Diet: by Provimi Kliba SA, CH-4303 Kaiseraugst, ad libitum Waters Tap water ad libitum Housing: Single housing type II cages with bedding of soft wood granules Environmental conditions: Témperature? 21-22°C à Humidity: Approximately 45-57% Air changes: Ten times per hours **Photoperiod:** 12 hours light, 12 hours dark



#### 4. Test compound concentrations used:

Range-finding test: 2 intraperitoneal injections of 1000 and 2000 mg/kg bw separated by 24 hours. @p

Micronucleus assay: 0, 250, 500 and 1000 mg/kg bw. The administered volume was 10 mL/kg in all of the dose groups.

#### **B.** Test performance

1. In life dates: 27 september 2005 – 21 October 2005

#### 2. Treatment and sampling times

Sampling took place 24 hours after the last intraperiteneal injection, The positive contra 24 hours after the single intraperitoneal injection.

#### 3. Tissues and cells examined

Bone marrow; 2000 polychromatic erythrocytes (PCEs) examined per animal: the number normochromatic erythrocytes (NCEs, more mature & BCs) per 2000 PCfo was noted.

#### 4. Details of slide analysis

At 24 hours after the second intraperioneal injection of AF C656948 of vehicle control, or 24 hours after the only one intraperitoneal injection of positive control, the appropriate groups of animals were sacrificed. Bone marrow smears were prepared from at least one infact femur for each animal. Cell smears were prepared and stained according to conventional cytological procedures.

Coded slides were scored for the presence of micronuclei in 2000 PCE sper animal. The ratio of PCEs to NCEs was also recorded for each animal, as an indication of cytotoxicity to the target tissue. The number of normochromatic erythrocytes showing micronuclei was also established.

#### 5. Evaluation criteria,

 $\bigcirc$ To determine whether a statistically significant response in MN PCE frequency was treatment related, the following criteria were applied:

us tibat - Whether or bot there were dose dependent effects were consistent with a treatment-induced ò response and

relation to both concurrent and bistorical vehicle and positive control - The degree of the response in data

#### 6. Statistical methods

The AE C656948 groups) with the tighest mean provided this superseded the negative control mean) and the positive control were checked by Wilcoxon's pon-parametric rank sum test with respect to the number of micronucleated polycoromatic erythocyte and the number of normochromatic erythrocytes.

The rate of normochromatic erythrocytes containing micronuclei was examined if the micronuclear rate for polychromatic erythrocytes was already relevantly increased. In this case, the group with the highest mean was compared with the negative control sing the one-sided Chi²-test.

#### Results and discussion II.

#### A. Dose Range-finding test

A. Dose range-tinding test in the range-finding study, groups of three males and three females received two intraperitoneal administrations 24 hours apart of 1000 or 2000 mg/kg AE C656948. Treated males showed the following compound-refated cirrlical signs: apathy, semi-anaesthetized state, roughened fur, loss of weight, staggering gait, lateral recumbency, sternal recumbency, spasm, twitching, periodically stretching of body, difficulty in breathing, slitted eyes and closed eyes. In addition, 2 of 3 males died in the 2000 mg/kg group. In females the following symptoms were recorded for up to at least 24 hours after the second application, startingat 1000 mg/kg: apathy, semi-anaesthetized state, loss of weight, lateral



recumbency, sternal recumbency, spasm, twitching, periodically stretching of body, difficulty in breathing, slitted eyes, closed eyes and reduced body temperature. In addition, 1 of 3 females died in the 2000 mg/kg group. These symptoms demonstrate relevant systemic exposure to AE C656948.

Based on these findings, 1000 mg/kg AE Based on these findings, 1000 mg/kg AE C656948 was chosen as the MTD for males. Based on the results of the dose range finder it is concluded, that there are no substantial differences between sexes in toxicity. Therefore, no females were used in the micronucleus assay.

#### B. Micronucleus assay

#### 1. Toxicity

There was no mortality throughout the study. Clinical signs including apathy, semi-anesthetized state, roughened fur, weight loss, sternal recumbency, spasm, body stretching and difficult, in breathing, were observed at all dose levels. This demonstrated a relevant systemic exposure of the animals to the test substance.

#### 2. PCE ratio

Table 5 / 2_1

The positive control cyclophosphamid caused a significant increase in the number of micronacleated polychromatic erythrocytes compared to controls which demonstrated the sensitivity of the test system. On the other hand, fluopyram did not cause any increase in this parameter at any dose level used in the study.

## 3. Micronucleated normochromatic and polychromatic erythrocytes

There was an increase in the number of normodiromatic erythrocytes in all fluopyram treated groups compare to controls although the difference was only statistically significant at the highest dose level. This demonstrated a relevant systemic exposure of animals to the test substance.

Table 5.4.2-1	is of the pricroauci			
Experimenta group	Number evaluated PCE	Number of NCE per 2000 CE	Number of MNNCE per 2000	Number of MNPCE per 2000 PCE
Negative control	100 <b>00</b> (5 x 2000)	377 <b>5</b> ≈± 737€		4.0 ± 1.6
Fluopyråm 250 mg/kg	10000 (5 \$ 2000)			4.4 ± 1.8
Fluopyram 🖗 500 mg/kg	10000 (5 x 2000)	• € 6090 ± 852 • € 6090 ± 852	1.5 ± 0.9	$3.2 \pm 2.3$
Fluopyram 🔊 🗘 1000 mg/kg	10000 (5 x 2000)	\$\$66* ±2018	$1.3 \pm 0.7$	4.2 ± 1.5
Positive control Cyclophosphamide	10000 (5 x 2000)	3248 ± 337	$1.7 \pm 0.8$	28.6*±6.1

*P < 0.01 in non-parametric Wilcoxon ranking test

#### C. Deficiencies

None.

In addition, analysis of lines of evidence as recommended by EFSA (EFSA, 2017b) suggest that there is sufficiently onvincing evidence of bone marrow exposure to fluopyram in this study:

The main lines of evidence of exposure to the bone marrow is indicated by:

• A depression of the immature erythrocyte ratio. The ratio of polychromatic to normochromatic erythrocytes (PCE:NCE) was altered by the treatment with fluopyram,



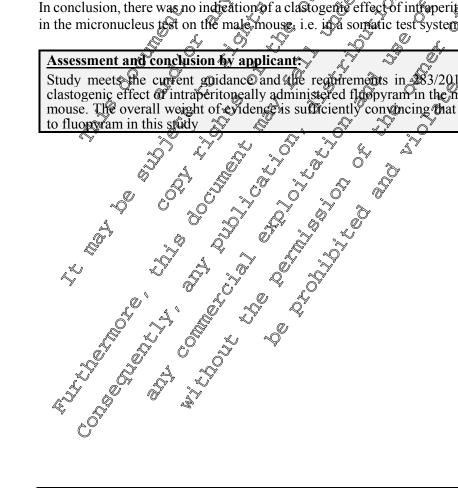
being 2000:3775 in the negative control, 2000:5309 in the 250 mg/kg group, 2000:6090 in the 500 mg/kg group and 2000:7866 in the 1000 mg/kg group.

- In the micronucleus assay, treated males showed the following clinical signs: apathy 0 semi-anaesthetized state, roughened fur, loss of weight, sternal recumbency spasm, periodically stretching of body and difficulty in breathing. These symptoms demonstrate relevant systemic exposure to fluopyram.
- In addition, an oral mouse micronucleus assay is available with Fluopy ram 600 (600 0 g/L) solo formulation, where 4000 PCEs per animal were examined which is summarized hereafter under data point KCA 5.4.2/02 showed a slight decrease (132%)in the number of PCEs in the 48 h high dose animals indicated a sight cotoxic effects on the bone marrow, whilst the marked clinical signs of toxicity reported in this study (CNS type effects) confirmed systemic exposure. This study provides further support for the exposure of the bone marrow to fluopyram.
- Additionally, two quantitative whole-body autoratiography (QWBA) studies the rat 0 (fluopyram is labelled with ¹⁴C in the pyridyl ring (M-29648 01-1) in one study and in the phenyl ring in the other (M29662202-1), showed exposure of the parent molecule to the bone marrow. Although these studies were performed via the oral gavage and were in the rat they are considered to contribute to the overall weight of evidence indicating exposure of the bone pharrow to fluopyran in the mouse micronucleus study

#### Conclusions III.

In conclusion, there was no indication of a clastogene effect of introperitoneally administered fluopyram in the micronucleus test on the male mouse, i.e. it a somatic test system in vise.

Study meets the current goidance and the regorierments in \$83/2013. There is no concern for clastogenic effect of intraperitoneally administered floopyram in the micronucleus test on the male mouse. The overall weight of evidence is sufficiently convincing that the bone marrow is exposed to fluopyram in this study





Data Point:	KCA 5.4.2/02
Report Author:	
Report Year:	2016
Report Title:	Fluopyram FS 600 (600 g/L): micronucleus assay in bone marrow cells of the
	mouse
Report No:	1761202
Document No:	<u>M-560911-03-1</u>
Guideline(s) followed in	OECD 474 (2014); EEC 2000/32/EC Method B12 (2000); US EPA OPPTS
study:	
	Series 8/0.5395 (August 1998); MAAF 12 Nousan No 8628 (December 06, 2000)
Deviations from current	OECD 474, 2014 ninth amendment
test guideline:	Deviation: No measurement of test material to the blood, but signs of system c
	toxicity noted at all doses tested. These deviations have no impact the outcome of
	the study and interpretation of the results
Previous evaluation:	Not previously evaluated so the second secon
GLP/Officially	Not previously evaluated Yes, conducted under GLP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes y y y y y y y

#### **Executive Summary**

In a preliminary dose range finder study, Fluopy ram K\$ 600,600 g/s) (formulateOin sterile water) was administered to a group of mice (2/sex/gp) orally *via* gavage at 2000 mg/kg bw, employing a dose volume of 10 mL/kg bw. Clinical signs of toxicity were reflective of CNS toxicity (*i.e.* including but not limited to hunched postere, squinted closed eye, ruffled fur, spontaneous activity). All clinical signs of toxicity resolved 30 hours post dosing, with all animals priving to the scheduled termination

From these results 2000 mg/kg by was deemed to be the maximum percommend dose, in accordance with current *in viva* genotoxicity test guideline, and was therefore selected as the maximum dose for the Main Experiment. Two lower doses of 500 and 1000 mg/kg bw were also selected.

As no substantial difference in toxicity was observed between male and female animals in the rangefinder test, male only were used in the main experiment.

A single and dose group animal was filled *in extremis* approximately 24 hours post dosing due to the severity of clinical signs. Other clinical signs of toxicity reported throughout the test article treated groups were again reflective of CVS toxicity and were comparable to those already reported in the range-finding test. These clinical signs of toxicity were spread access the test article dose groups, completely resolving at 6 hours post dosing for the low dose group, persisting to termination (24 hour time point) for the mid and high dose groups, with normality observed in the high dose group at the 48 hour termination. This demonstrated prelevant systemic exposure of the animals to the test substance.

All animals treated with Fluopyram FS 600 (600 g/L) exhibited both group mean and individual MN PCE (micronucleated polychromatic egythrocyte) values which were comparable with both the concurrent vehicle control and the laboratory chistorical solvent control data.

All animals treated with the positive control exhibited marked increases in MN PCE such that the frequency of MN PCE in the positive control group was significantly ( $p \le 0.05$ ) greater than the observed frequency in the concurrent vehicle control group, thereby demonstrating the sensitivity and specificity of the test system and the analysis ability to detect MN in the PCE population.

Animals dosed with Fluorytam FS 600 (600 g/L) at 500, 1000 and 2000 mg/kg bw and sampled 24 hours post dosing day exhibited group mean %PCE values that were comparable (group mean range 64.3 - 651%) to the concurrent vehicle control (64.5%). A marginal reduction in %PCE values in the 2000 mg/kg bw dose group, sample 48 hour post dosing was observed (56.0%), however this was not deemed evidence of marked toxicity in the bone marrow in accordance with OECD 474 (2016) as the



proportion of immature erythrocytes (PCE) among total erythrocytes in the bone marrow did not exceed 20%.

Whilst no concurrent target organ exposure assessment was undertaken in this study to confirm Fluopyram FS 600 (600 g/L) exposure to the systemic circulation, the marked clinical signs of Astricit reported in this study (CNS type effects) confirmed systemic exposure.

It is concluded that Fluopyram FS 600 (600 g/L) did not induce micronuclei in the polychromatic. erythrocytes (PCE) of the bone marrow of male mice treated at 500, 1000, and 2000 mg/kg w (the maximum recommended dose in accordance with current regulatory test guidelines for the in yes micronucleus assay). This methodology included a single dose of Fluopyram FS 600 (6002/L) erally via gavage, with bone marrow sampling 24 and 48 hours post dosing

Fluopyrant FS 600 (600)

Light-Deige liquid

Stable for the duration

cyclophosphamide

NK#3FX395

658066-35-4

none 🔊

Sterilewater

(~alternative name: Eleopyram (

7 (w/w) (no correction

- I. Materials and methods
- A. Materials
- 1. Test material:

Description Lot / Batch #: **Purity:** CAS#

Stability of test compound: Q 2. Control materials: **Negative:** 

Solvent: **Positive:** 

**Species:** 

Strain:

Age:

Diet.

Water: Housing:

3. Test animals: €mouse NMR 7-8 weeks approximately Weight at dosing? 32.6-41-8 g (males only) Source: Ö Acclimation period; At least 5 days

Ceklad Certifice Global 18% Protein Rodent Diet, produced by Envigo, ad libitum Tap water and libitum stugle housing type II cages with bedding of soft wood granules

mg/kg bw

Environmental condition Temperature: 🖒 Approximately 45-65% **Humidity:** Air changes: Ten times per hours Photoperiod: 12 hours light, 12 hours dark

# 4. Test compound concentrations used:

Range-finding (est (2 animals sex): Single of ose by oral gavage at 2000 mg/kg bw.

Micronucleus assay 7 mates/dose group? a single oral gavage dose at 0, 500, 1000 and 2000 mg/kg bw, with sampling at 24 hours post dosing. A further vehicle and high dose group (2000 mg/kg bw) were desed, with sampling 48 hour post dosing. The administered volume was 10 mL/kg bw in all of the dose groups.

B. Test performance

1. In life dates: 18 April 2016 – 2 June 2016



#### 2. Treatment and sampling times

For the micronucleus assay sampling took place at 24 hours (vehicle, 500, 1000, 2000 mg/kg bw and positive control) and 48 hours (2000 mg/kg bw) after a single oral gavage dose.

#### 3. Tissues and cells examined

Bone marrow; 4000 polychromatic erythrocytes (PCEs) examined per animal/for the incidence of micronuclei (MN). The ratio of normochromatic erythrocytes (NCE) to PCE were recorded for each animal until a total of 500 cells were scored, as an indication of cytotoxicity to the target these.

#### 4. Details of slide analysis

At 24 and 48 hours after the first oral gavage dose of fluopyram FS 600 (600 g/L) or vehicle control, or 48 hours, the appropriate groups of animals were sacrificed. Bone mappow spears were prepared from the intact femurs for each animal. Cell smears were prepared and stained with Gremsa?

Coded slides were scored for the presence of MN in 4000 PCEs per animal. The ratio of PCEs to NCEs was also recorded for each animal, as an indication of cytotoxicity to the target tissue. Scoring was undertaken under a light microscope using oil immersion

#### 5. Evaluation criteria

To determine whether a statistically significant response in MN PCE requerey was treatment related, the following criteria were applied:

- At least one of the treatment groups exhibited a statistically significant increase in the frequency of MN PCE compared with the concertent negative control.

- This increase was dose-related at least at one sampling time when evaluated with an appropriate trend test and

- Any of these results were outside the distribution of the historical negative control data (e.g. Poissonbased 95% control limits).

### 6. Statistical methods

Statistical significance of MNPCE frequency at ( $\beta < 0.05$  was evaluated by means of the non-parametric Mann-Whitney jest

# II. Results and discussion

### A. Range-finding test

In the range-finding study, after a single oral gavage administration at 2000 mg/kg bw Fluopyram FS 600 (600 g/L) both males and females showed the following compound-related symptoms, which were deemed evidence of CNS toxicity: hunched posture, abdominal posture, sunken flanks, squinted closed eye, ruffled fur, spontaneous activity. All clinical signs of toxicity resolved 30 hours

post dosing, with all animats surviving to the scheduled termination.

From these results 2000 mg/kg bw was deemed to be the maximum recommend dose, in accordance with current *in vivo* genotoxicity test guidenne, and was therefore selected as the maximum dose for the Main Experiment. Two fower doses of 500 and 1000 mg/kg bw were also selected.

As no substantial difference in Exicity was observed between male and female animals in the rangefinder test, make only were used in the main experiment.

# B. Micropycleus assay

1. Toxicity

A single mid dose group animal (#18) was killed *in extremis* approximately 24 hours post dosing due to the severity of clinical signs (spinning around, glued eyes). Other clinical signs of toxicity reported



throughout the test article treated groups were again reflective of CNS toxicity and were comparable to those already reported in the range-finding test. These clinical signs of toxicity were spread across the test article dose groups, completely resolving at 6 hours post dosing for the low dose group, persisting to termination (24 hour time point) for the mid and high dose groups, with normality observed in the high dose group at the 48 hour termination. This demonstrated a relevant systemic exposure of the animals to the test substance.

#### 2. PCE ratio

Animals dosed with Fluopyram FS 600 (600 g/L) at 50(%1000 and 2000 mg/kg bw and sampled % hours post dosing day exhibited group mean %PCE values that were comparable (group mean range 64.3 – 67.1%) to the concurrent vehicle control (64.5%). A marginal reduction in %PCE values in the 2000 mg/kg bw dose group, sample 48 hour post dosing was observed (56.0%); however this was not deemed evidence of marked toxicity in the bone marrow in accordance with OECD @74 (2016) as the proportion of immature erythrocytes (PCE) among total erythrocytes in the bone marrow did not exceed 20%.

#### 3. Micronucleated normochromatic and polychromatic erythrocytes

Male rats dosed with Fluopyram FS 600 (600 g/l) exhibited group mean MN PCE frequencies that were similar to and not significantly ( $p\leq0.05$ ) higher than those observed in the concurrent vehicle control groups for all doses analysed at both the 24 and 48 hour time points. For dose groups of 500/21000 and 2000 mg/kg bw at the 24 hour time point, the mean %AIN PCE values were 0.13% 0.13%, and 0.12% respectively compared to the vehicle control value of 0.13% MN PCE and historical vehicle control (95% reference) range of 0.043 $\approx$  0.198% MN PCE. For the 48 hour time point %MN PCE value for the 2000 mg/kg bw group was 0.12%

Individual MN PCE values for all Fluopyrand FS 600 (600 g/L) treated animals were consistent with historical vehicle control distribution ranges and similar to values observed within the concurrent vehicle control group. These data were considered to indicate no evidence of a test substance related effect on MN induction.

All animals treated with the positive control exhibited marked increases in MN PCE such that the frequency of MN PCE in the positive control group was significantly ( $p \leq 0.05$ ) greater than the observed frequency in the concurrent vehicle control group, thereby demonstrating the sensitivity and specificity of the test system and the analysis ability to detect MN in the PCE population.

Table 5.4.2-2		Ś	esult	s≁of t	he N	ficro	nme	leus	Kest	×	1										
Parameter	(	MN ACE/4000 PCE % PCE/NCE % PCE/NCE																			
		2. A A A A A A A A A A A A A A A A A A A																			
A	Ő	C C Vefriele control: sterile water																			
Animal no.	"1	2	3 (	4	5	<b>6</b>	Ŷ	1	<b>Q</b> 2	3 C	4	5	6	7	1	2	3	4	5	6	7
Individual	7	9	B	4		1	3	048	0.23	<b>Q</b> .00	0.10	0.30	0.03	0.08	64.9	63.5	66.6	59.7	67.2	64.2	65.4
score Group mean (±SD)		Ž	\$ 5.	1/ <b>4</b> 00	)0	8	Ŵ		L. Z	0.1	3 ±0	.11					64	1.5 ±2	2.5	<u> </u>	
Parameter		$\sqrt{2}$ $\sqrt{2}$ $\sqrt{2}$ $\sqrt{2}$ 500 mg/kg bw																			
Animal no.	8Ø	, °9	10	11	×12	13	14	8	9	10	11	12	13	14	8	9	10	11	12	13	14
Individual score	Ŏ¥	4	4	M.	8	Ĩ	4	0.10	0.10	0.10	0.25	0.20	0.05	0.10	61.6	67.2	74.7	60.2	62.5	59.7	64.2
Group mean (±SD)	, ¢	Ş	Ì	₹/400 (			Ŷ			0.1	3 ±0	.07					64	1.3 ±5	5.2		•
Parameter	R	4	1	<u>_</u>	7					1000	mg/k	kg bw									
Animal no.	¥15	16	17 s	18	19	20	21	15	16	17	18	19	20	21	15	16	17	18	19	20	21
Individual score	10	3	74	-	6	1	3	0.25	0.08	0.18	-	0.15	0.03	0.08	71.3	66.4	64.0	-	69.1	68.4	63.2
Groupmean	5.3/4000 0.13 ±0.08 67.1 ±3.1																				
(±SD)																					
Parameter		2000 mg/kg bw																			

Table 5.4.2-2 Results of the Micronucleus Rost



Parameter		Ι	<u>AN P</u>	CE/4	000 1	PCE				%	5 MN	PCE	2				%	PCE/	NCE		
		24 hour time point																			
		Vehicle control: sterile water																			
Animal no.	22	23	24	25	26	27	28	22	23	24	25	26	27	28	22	23	24	25	26	27	
Individual	6	5	9	4	6	1	3	0.15	0.13	0.23	0.10	0.15	0.03	0.08	62.0	62.5	75.6	67.7	62.3	63.5	62.0
score																Ć	7			Ũ	Ô
Group mean		4.9/4000 0.12 ±0.06																			
(±SD)		Positive control: CPA 40 mg/kg bw																			
Parameter		r		r	r													$\mathcal{C}$	)″	- Q	
Animal no.	29	30	31	32	33	34	35	29	30	31	32	33	34	35		30	31	32	33%		35,
Individual	98	122	150	111	93	87	132	2.45	3.05	3.75	278	2.33	2.18	3.30	58.8	67.8	58.5	50.2	<u>56</u> 4	64.2	Ø.5
score											ľ.	_*	L(	¥.					S.		
Group mean			11	3.3/4	000					28	$3\pm 0.5$	7*	Å	,			° ^{39.}	9±\$	8	»O	
(±SD)																					
Parameter		2000 mg/kg hw ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~																			
	26	25	20	20	40	41	12	200						×	N	× 27		200			ÿ 10
Animal no.	36	37	<b>38</b>	39	40	41	42	36%		380			41	942 0.02	36	37	38	<u>3</u> 9×	¥ 40	<b>4</b> ₩	42
Individual	4	6	8	7	3	3	1	0.10	0.15	0920		0.08	U WS	0.0	3 <b>40</b> .2	33M	·	j V	Ô	\$58.3	54.y
score Group mean				.6/40	00					<u>ار م</u>	 2∕≇0.0	<i>4</i>	ę	.4		Ś	56	<u>0</u> ±1.		<u> </u>	Û
(±SD)			4	.0/40	00		L		Š,		∠ æ0.\ ∦	°S	ı	Õ		)`	×°.	0 ±1.	9≫	- S	1
(±5D)						Hi	Ø.	- Non	ntrol		(/401	M P(	TEA O	)″	<del>S</del>	0	7,		1	0	
				1	Vehic	le 🕅	Hrol	(25 s	tudié	Se Se	pt 20	14 -	A Pr	sitin	e con	tra	/ (25 st		· Ser	ot 201	4_
					, cinc	Ś		ec 2		<b>, 50</b>	P S O			×		Х ^р г	lec 2	15)	,,	201	•
					đ	Ç,		MN		2	ř	R	R	Õ	.0	ر %	MÛN	nĩ5) PCE	Ý		
Mean	$(\%) \pm$	S.D:			Ø	\$						Ũ	Ó	<i>i</i>	<u> </u>	22		).476			
Min-N				<i>6</i>	Ş	° A	0.0	125 £ ¥6 –	0.20	5	×,		Ē,	Ĉa	)	1.6	50 – 1	30495			
	% RŘ			*	J	K,			0.19		(Tri)	2	1			Ô.6	05 🛼	3.507			
Individual ar	nimal	MN 1	angę	Ô	(	)	S	0 - 1	Ľ.	P	-0	~		Ş		3	74-20	3.507 )4.7			
* $p \le 0.05$				Ý	.1		-42	Å	,×	GP/A	: cyc	opho	spha	mide	K)		Ŵ				
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NCE: normod	hrom	atic g	Wthr	ocyte	Š .	Š			S	ĩ	Š,	(	)	- ×	1	Č¥.			-		
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#### C. Deficiencies

Whilst no concurrent targer organ exposure assessment was undertaken in this study to confirm fluopyram ES 600 (600 g/l) exposure to the systemic circulation, the slight decrease (13.2%) in the number of PCEs in the 38 h high dose animals indicated a slight cytotoxic effect on the bone marrow, whilst the marked clinical signs of toxicity reported in this study (CNS type effects) confirmed systemic exposure.

#### Conclusions III.

III. Conclusions State Conclusion for the polychromatic for the po erythrocytes (PCE) of the bone marroy of male mige treated at 500, 1000, and 2000 mg/kg bw (the maximum recommended dose in accordance with current regulatory test guidelines for the in vivo micronucleus assay), This methodology included single dose of Fluopyram FS 600 (600 g/L) orally via gavage, with bone marrow sampling 4 and 28 hours post dosing. Ô Ø O

### Assessment and conclusion by applicant

Study meet the current guidance and the requirements in 283/2013. There is no concern for clastogenic effect of orolly administered Fluopyram FS 600 (600 g/L) in the micronucleus test on the mal@mouse.



#### CA 5.4.3 *In vivo* studies in germ cells

Based on the results of the *in vitro* and *in vivo* studies as reported under points 5.4.1 to 5.4.2, no further studies in germ cells were triggered.

#### CA 5.5 Long-term toxicity and carcinogenicity

The long-term toxicity and the oncogenic potential of fluopyram (AE C656948) was assessed in both the mouse and rat. The studies were performed between 2005 and 2007 following the current OEGP, EU, USEPA and Japanese MAFF testing guidelines and in compliance with the GLP requirements. A summary of these results is presented in Table 5.5-1.

In the rat combined chronic toxicity and carcinogenicity study, groups of males and temales were fed diet containing 0, 30, 150 and 750 ppm and 0, 30, 150 and 1500 ppm, respectively. In males the dose level of 750 ppm was reduced to 375 ppm from week 85 onwards since there was higher mortality in this group. Overall, there was a statistically significant increase in mortality in males at 750/375 ppm after 24 months, though no clear cause for these premature deaths could be established. Body weights were reduced in females at 1500 ppm and males at 750/375 ppm at various times throughout the study. At the 12-month ophthalmology examination, abnormal color of the refinal fundus was observed in females at 1500 ppm, together with small refinal vessels at these dose levels and in males at 150 ppm. In addition, hyperreflectivity in the refinal vessels at these dose levels and in males at 150 ppm. In addition, hyperreflectivity in the refinal vessels at these dose levels and in males at 150 ppm. In addition, hyperreflectivity in the refinal vessels at these dose levels and in males at 150 ppm. In addition, hyperreflectivity in the refinal vessels at these dose levels and in males at 150 ppm. In addition, hyperreflectivity in the refinal vessels at these dose levels and in males at 150 ppm. In addition, hyperreflectivity in the refinal vessels at these dose levels and in males at 150 ppm. In addition, hyperreflectivity in the refinal vessels at these dose levels and in males at 150 ppm.

In females at 1500 ppm, higher mean cholester levels were observed throughout the study, higher mean triglyceride concentrations were noted up to month 6 and lower mean glueose concentrations at months 6, 12 and 18, orange to red colored under was observed throughout the study. In males at 750/375 ppm and 150 ppm a higher incidence and severity of cellular casts was noted in the urine at months 3 and 6. The target organs were the liver, Divroid gland, Kidne Cand eye. In females at 1500 ppm, at the 12-month sacrifice, liver and thyroid gland weights were increased. Mistological changes which included a higher incidence of altered hepatocytes (cosinophilic foci) and hepatocellular brown pigments, focal/multifocal/bepatocellular vacuofation increased number of mitoses, centrilobular to panlobular hypertrophy and hepatocellutar single cell necrosis in the ver. In the thyroid gland, follicular cell hyperprophy, in association with chigher incidence and severity of colloid alteration was observed. In the kidney a higher incidence of tubulat golden brown pigments and of hyaline casts was observed. At the 24-month sperifice, liver weights were increased. Histological changes correlated with those observed after 12 months. Macroscopically, liver nodules/masses (5/60 females) were noted and correlated histologically with neoplastic changes resulting in liver cell tumors. These findings were associated with non-heoplastic/prepeoplastic charges observed at 12 months and marked hepatocellular toxicity after 24 months. In the Kaney, marked degenerative changes resulting from exacerbation of the microscopic findings nated at the end of the chronic phase were observed, together with an increased incidence of tubular golden/brown pigments and collecting ducts hyperplasia. In the thyroid gland, exacerbation of the histological findings (follocular cell hyperplasia and/or hypertrophy and colloid alteration) noted at the end of the chronic phase was observed. In the eye, bilateral retinal atrophy was noted, together with a higher incidence of less degeneration and peripheral bilateral retinal atrophy.

In males at 550/375 ppm at the 12 month sacrifice, liver and thyroid gland weights were increased. Histologically, in the liver, a higher incidence of altered hepatocytes and centrilobular to panlobular hypertrophy was noted. In the kidney, chronic progressive nephropathy was noted. In addition, a higher incidence of ryaline droplets and of bilateral basophilic tubules was observed. In the thyroid gland, follocular call hypertrophy, in association with a higher incidence and severity of colloid alteration, was observed. At the 24 month sacrifice, liver weights were increased. Histologically, changes in the liver, kidney and thyroid gland which were exacerbations of changes seen at 12 months were noted. In



addition, changes were seen in the testis and stomach which were considered to be secondary changes and not directly treatment-related.

In males at 150 ppm, at the 12 month sacrifice, centrilobular to panlobular hypertrophy in the fiver, chronic progressive nephropathy and a higher incidence of hyaline droplets, follicular cell hypertrophy in the thyroid gland, was noted. At the 24 month sacrifice, male liver weights, bere increased, months in males, together with a higher incidence of colloid depletion of the thyroid gland in females. In males, secondary changes were also noted in the testis.

Neoplastic changes at the end of the carcinogenicity phase consisted of liver cell tumors (carcinoma and adenoma) in females at 1500 ppm (equivalent to 89 mg/kg/day).

Over a 12-month period of dietary administration with AE C656948 to the Wista rat, the NOADL was 30 ppm in males (equivalent to 1.37 mg/kg body weight/day) and the NOEL in femates was 150 ppm (equivalent to 9.6 mg/kg body weight/day).

Over a 24-month period of dietary administration with AE C656948 to the Wistor rat 30 ppm was the NOAEL in males (equivalent to 1.20 mg/kg/day) and females (equivalent to 1.68 mg/kg/day)

In order to clarify the mode of action for the liver cell timor observed in tomale cats after 2-years of treatment with AE C656948 at 1500 ppm (89 mg/kg/day), additional mechanistic work was conducted, details of which are presented in this section under

# Summary of Mechanism of Action and supporting data:

## Summary of Supporting data regarding female rat liver tumors

In summary, the 28-day rate study showed the reduction of cytochrome P450 (increased total hepatic cytochrome P450 content, increased BROD and PROD activities) consistent with a phenobarbital-like MoA. Further mechanistic studies performed in female rate for durations of 3, 7 or 28 days demonstrate that fluopyram at the dose levels tested in the carcinogenicity study resulted in the induction of cytochrome P450 Content, increased cytochrome \$450 and UDPGT@isoenzyme activities and corresponding changes in gene expression, bepatocyte proliferation and associated histopathological change. Findings were reversible and were similar to those induced by phenobarbital. A study performed in CARPAR wild-type (WT) and knock-out (KO) mice showed significant liver enlargement, hepatocyte hypertrophy, and liver enzyme induction in WT but not in KO mice, indicating that activation of CAR/PXR is the initiating event of the rodent for liver tumours in the rat (and thyroid tumours in the mouse). An in vitio comparative study examining the proliferative response in rat and human primary hepatocytes showed a clear concentration-related increase in proliferation in rat but not in human cells in response to wooyram and phenobarbital. Overall, the mechanistic studies together with the standard repeated dose toxicity Sudies clearly demonstrate that the MoA for the rat liver tumours following chronic exposure to thiopyram is via activation of the CAR/PXR nuclear receptors. Furthermore, the lack of proliferation in primary human hepatocytes (compared to rat hepatocytes), provides convincing avidence that the liver rumours seen in the rat are non-relevant to humans. Other MoA have effectively excluded. Consequently, the MoA demonstrated to be responsible for liver tumour formation in the female rat following chronic exposure to high dose levels of fluopyram is not relevant to humans.

More in deptodiscussion is presented following on from rodent carcinogenicity studies under:

Mechanism of Action and Supporting data

Supporting data regarding female rat liver tumors Mechanistic studies are summarized under data points 5.5/03 – 5.5/08



In the mouse carcinogenicity study, dose levels of 0, 30, 150 and 750 ppm were used for both sexes. There was no treatment-related effect on mortality or clinical signs in either sex. Body weights were reduced between weeks 30 and 58 and higher platelet counts were noted at months 13 and 19 in plates at 750 ppm. Liver weights were increased at both the 12-month and 18-month sacrifice in both secret at 750 ppm and 150 ppm, and at 18 months only in males at 30 ppm. At the high dose level, kidner weights were decreased in both sexes at 12 months and females only at 18 months. The target organis were the liver, kidney and thyroid gland. Histologically, after 12 months, the pre-neoplastic change of follocular, cell hyperplasia was observed in the thyroid gland of males at the two highest dose levels. After 18 months histological findings consisted of a higher incidence of the neoplastic change of follicular con adenoma of the thyroid gland in males at 750 ppm. Non-neoplastic charges were seen in the ther, kitney and thyroid gland. The principal change noted in the liver was centrilobular to particulation of the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the principal change noted in the liver was centrilobular to particulate the particulate th which was seen in both sexes at the two highest dose levels, Dogether with higher incidences of hepatocellular single cell degeneration/necrosis observed in males at the two highest dose levels. In the kidney, a higher incidence and/or severity of bilateral cortical basophilic tubules, hyaling easts and interstitial mononuclear cell infiltrates, fogether with a higher incidence of glomerular congestion/hemorrhage(s), associated with higher severity of amyboid deposition was obted in temates at 750 ppm. In the thyroid gland, a higher incidence of follicular cell hyperplasta was noted in both sexes at 750 ppm and males only at 150 ppm Ø

Dietary administration of AE C656948 over an 18-nonth period to the C5/BL/CF mouse, at a dose level of 750 ppm in males (equivalent for 105 mg/kg/day), resulted in a higher incidence of fellicular cell adenoma in the thyroid gland.

The NOAEL was 30 ppm in males (equivalent to 4.2 mg/kg/day) and females (equivalent to 5.3 mg/kg/day).

In order to elucidate the mode of action for the follicitar cell tumors observed in male mice exposed to AE C656948 at 750 ppm (105 mg/kg/day) for 18 months, additional mechanistic work was conducted, details of which are presented in the section under

## Summary of Mechanism of Action and supporting data;

# Summary of Supporting data regarding male nouse thyroid tamors

In summary, the thyroid effects seen to male mice were associated with marked liver effects and can therefore be assumed to be secondary to the induction of liver enzymes, specifically UDPGT. This CAR/PXR initiated MoA is further supported by a number of mechanistic studies. Administration of fluopyram to male mice resulted in cytochrome P450 induction (increased BROD/PROD activities), reduced T4 and increased TSE levels and a more topid clearance of T4. The pivotal CAR/PXR wild-type (WT) and knock out (KO) mice showed significant liver enlargement, hepatocyte hypertrophy, and liver enzyme induction in WT but nooin KO mice provides compelling evidence for CAR/PXR induction peing the initiating event for the MoA that eventually results in the formation of a low incidence of thyroid adenomas in the male mouse at the high dose in the cancer bioassay. Further supportive evidence is provided in an *in vitro* comparative study examining CYP and UGT induction in human and Wistar rat hepatocytes with fluopyram. Phase I and II liver enzymes were induced in both species, but critically UGT-T4 was induced to rat hepatocytes but not in human hepatocytes. This MoA (increased TSE secondary to fiver enzyme induction causing increased T4 clearance, resulting in thyroid follicular cell hyperplasia and carcinogenesis) is not of relevance to humans. Fluopyram was shown not to be an infibitor of thyroid peroxidase (TPO) activity, thereby discounting an alternative MoA.

Overall, comprehensive mechanistic data are available, which elucidate the MoA and demonstrate that neither tumbur type is of relevance to humans. Classification of fluopyram for carcinogenicity is therefore not triggered on the basis of the liver tumours seen in female rats. Similarly, classification of fluopyram for carcinogenicity is not triggered on the basis of the thyroid tumours seen in male mice. In the absence of any carcinogenicity of relevance to humans, fluopyram does not require classification for carcinogenicity in any category, according to the CLP criteria.



More in depth discussion is presented following on from rodent carcinogenicity studies under:

#### Mechanism of Action and supporting data

Mechanism of Action and	supporting da	ta			o s
Supporting data re	garding male	mouse thyroid	tumors		5 ³ , 5 ⁴ , 5
Mechanistic studies are s	summarized u	nder data poin	ts 5.5/09 – 5.5/22	ð	
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				A . (	
Table 5.5-1Summ	ary of long-ter	rm toxicity/carci	nogenicity with AR	C656948 🔬	
Study	NOAEL	LOAEL	Main findings	, Ø	Reference
Doses tested mg/kg bw/d	ppm mg/lvg bw/d	ppm mg/kg bw/d			
Rat – 104-week	<b>mg/kg bw/d</b> 1.20/1.68	6.0/8.6 0	Eye lesions in high c	on the set of the set	
Chronic Toxicity/		4D	m , 𝒴 1 ∞ ♥	arked liver	<u>M-298369-01-1</u>
Oncogenicity	()	(M/F)	toxicity, nephropath	win the 🔗	Y W
30, 150 & 750/375			kidney and follicular hypertropby in the th		A c.°
(males) 1500 females			Diver cell tumors (ca	arcinom giand	
ppm			adenoma) in high do	se/females –	
0, 1.20, 6.0, 29 M			Additional data supr	ort an 🖉 🔗	, O
0, 1.68, 8.6, 89 /F,	4		nhenoberbital-life m	XIC, S	
over 24 months	- V	6 9 J	action a		Ϋ́Υ
		20.9/26 20.9/26 2 2 3 4 4 5 4 5 4 5 4 5 4 5 4 5 4 5 4 5 4 5	Additional data supr indirect, ron-genoro phenobarbital-like m action		
Mouse - 78 week-	4.2/5.%	20.9/26.8	mephropatny in the	kiqney in	
Chronic/ Oncogenicity	¥.2/5.% (M/P)	(MAF)	high dose females.		<u>M-295688-01-1</u>
30, 150 & 750 ppm			Treatment-related for hyperplasta in the th		
0, 4.2, 20.9, 105 M	ð "N		ventrilobular to panl	obular	
0, 5.3, 26.8, 129 /F	L . Š .		hypertophy and hep	atocellular	
over 18 months			single cell degenerat	ion /necrosis	
			in the live with	ular cell	
	50		Adenomas in Kigh do	ose males –	
			Additronal data supp		
			indirect, non-genoto mechanism of action	xic threshold	
	4 5		to liver effect.	i secondur y	
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30, 150 & 750 ppm 0, 4.2, 20.9, 105 M 0, 5.3, 26.8, 129 /F over 18 months 0 0 0 0 0 0 0 0 0 0 0 0 0		v			
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Data Point:	KCA 5.5/01
Report Author:	
Report Year:	
Report Title:	AE C656948:Chronic toxicity and Carcinogenicity study of AE C656948 in the
	Wistar rat by dietary administration
Report No:	SA 04312
Document No:	<u>M-298339-01-1</u>
Guideline(s) followed in	OECD 453 (1981); EEC Directive 88/302/EEC Method B33 (1987); PA Health
study:	Effects Test Guideline (OPPTS 870,4300; 1998), M.A.F.F. in Japan notification
	12 Nousan N°8147 (2000) guidelines
Deviations from current	Current guideline: OECD 453, 2018
test guideline:	Deviation: None
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially	Yes, conducted under Gor Officially recognised desting facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes A A A A A A A A A A A A A A A A A A A
Executive summary	

Executive summary

AE C656948, (Mix-Batch 08528/0002, a beige powder, >94.5% parity) was administered by continuous dietary treatment to groups of 60 pale Wistar rats at 30, 150 and 750 ppm (due to the high mortality rate in the high dose group makes, this dose level was reduced to \$75 pion from Week 85 onwards), corresponding to 0, 1.20, 6.0 and 29 mg/kg/day, respectively, and groups of 60 female W istar rats at 30, 150 and 1500 ppm, corresponding to 1.68 9.6 and 89 mg/kg/day, respectively, over a 24-month period. Additionally, groups of 10 male and 10 male cats were treated at 30, 150 and 750 ppm (males) / 1500 ppm (females) AE C656948, corresponding to 1.37 6.9 and 35 mg/kg/day in moles and 1.88, 9.6 and 95 mg/kg/day in femeles, over a 12-month period to investigate chronic toxicity only. Mortality and clinical signs were checked daily Detailed physical examinations including palpation for masses were performed at least weekly throughout the study Body weight was recorded weekly for the first 13 weeks, then approximately every 4 weeks thereafter. Food consumption was recorded twice weekly for the first 6 weeks of the study, then weekly up to Week 13, then every 4 weeks thereafter. Ophthalmology examinations were performed on all animals during acclimatization and after approximately 12 and 24 months. Dematology and clinical chemistry determinations and urinalysis were performed during months 3, 6, 12, 18 and 24 of selected animals. At the scheduled chronic and carcinogenicity phase sacrifice, selected agans were weighed and designated rissues sampled and examined microscopically.

At 1500 ppm in females

Õ The overall incidence and percentage of mortality was low with no evidence of a treatment-related increase throughout the soudy. There were no reatment-related clinical signs during the first year of treatment whereas a higher incidence of hair loss and wasted appearance was noted during the second year, in comparison to the controls. Mean body weight or body weight gain parameters were essentially comparable to the controls throughout the first three months of treatment. Thereafter, mean cumulative body weight gain was lower between Weeks, ^{Q4} to 26 (-29%, p≤0.01), 26 to 54 (-15%, not statistically significant) and 34 to 79 (-59%, p≤0,01) in comparison to the controls, whilst mean body weight was lower by 3, 5014 and 12% respectively, at Weeks 26, 54, 79 and 102 (statistically significant for most time points); when compared to the controls. Mean food consumption was similar to the controls throughout the study.

Ophthatmological examinations revealed abnormal color (pale) of the retinal fundus in 4/67 animals compared to no cases in the controls after one year and a higher incidence of small retinal vessels, apprormal color (pale) of the retinal fundus and hyperreflectivity in retina after two years, in compatison to the controls.



There were no toxicologically relevant changes at the hematology evaluation throughout the study. At the clinical chemistry evaluation, the only consistent changes observed throughout the study were slightly higher mean total cholesterol concentrations (+31% to +38% in comparison to the controls). Higher mean triglyceride concentrations were also noted at Months 3 and 6 (+36%), and slightly lower mean glucose concentrations at Months 6, 12 and 18 (-9%, -13%, -16%, respectively). Uthalysis revealed higher incidences of abnormal color of urine (orange to red) throughout the study compared to the controls.

At the end of the chronic phase (12 months), mean terminal body weight was slightly lower by 10% (not statistically significant). Mean absolute and relative liver weights were 39 to 54% higher than the controls. This was associated with macroscopic changes (enlarged liver, dark liver and prominent lobulation) and with histological changes including a higher incidence of altered hep-tocytes (eosinophilic foci) and hepatocellular brown pignents, focal/multifocal hepatocellular single cell necrosis. In the thyroid gland, mean absolute and relative weights were 33 to 38% higher than the controls and follicular cell hypertrophy was noted at the histological examination in association with a higher incidence and severity of colloid alteration. Dark (adney was found in most animals compared to no cases in the controls, and a higher incidence of tubular governbrown pignents and of hyaline casts was observed at the histological examination.

At the end of the chronic phase, there was no evidence of a treatment related increased increase

tumors of any type in any organ. Q At the end of the carcinogeniety phase (24 months), mean terminal body weight was 11% lower (not statistically significant). Most pathological effects correlated with those seen at the end of the chronic phase: mean absolute and relative liver weight were 39 to 56% higher than the controls and were associated with a higher incidence of enlarged fiver, dark liver, prominent tobulation, red and white foci on the liver at the macroscopic observation, when compared to the controls. In addition, liver nodules/masses (5/66) females, compared to no saises in the controls) were noted and correlated with liver cell carcinome or adenome noted at the microscopic examination. Higological changes attributed to the treatment and indicative of marked liver toxicity were also noted, including metabolic, degenerative oppoliterative changes. They correspond to the exacerbation of the microscopic findings observed at the endoof the chropic phase. In addition in the liver minimal to slight extramedullary hematopoiesis was observed. In the knowney, a higher incidence of dark kidney, enlarged kidney and irregular surface on the kidney was observed, when compared to the controls. Marked degenerative changes resulting from exacerbation of the microscopic findings noted at the end of the chronic phase were also observed in this organ degether with an increased incidence of tubular golden/brown pigments and collecting ducts hyperplasia. In the thyroid gland exacerbation of the microscopic findings (follicular cell@ayperg@asia and/or hypertr@hy and collord alteration) noted at the end of the chronic phase was observed. In the eye, bhaterabretinal atrophy was noted, together with a higher incidence of lens degeneration and peripheral bilateral retinal atrophy. Retinal atrophy was characterized by degeneration of the order plexitorm layer, other nuclear layer and rod/cones lamina.

Treatment-related reoplastic charges were observed in the liver, where a higher incidence of liver cell tumors (carcinoma plus adenoma) was noted, in comparison to the controls. These findings were associated with non-neoplastic/preneoplastic changes and were seen at a dose causing marked hepatocellula toxicity.

At 750/375 ppm makes:

During the first year of treatment, 11/70 animals were found dead or were sacrificed prematurely for humane reasons, compared to 6/70 in the controls. The main clinical signs in these early decedent males consisted of soiled fur of anogenital region (3/11), focal swelling (2/11) and wasted appearance (1/11), together with the usual signs associated with morbidity (limited use of hindlimbs, reduced motor activity, general pallor). After 2 years of treatment, analysis of the survival rates showed that mortality

incidence was increased in the male high dose group, when compared to the controls. No clear cause of death could be established for these early decedent males.

There were no treatment-related clinical signs during the first year of treatment, whereas a higher $\sqrt{2}$ incidence of the usual signs associated with morbidity (prostration, general pallor and soiled an genital region) was noted during the second year, in comparison to the controls. Mean body weight or body weight gain parameters and mean food consumption were essentially comparable to the controls throughout the study, except for a slight reduction in mean food consumption by up to 7% (p<01 or p<0.05) between study Days 18 to 39.

At the ophthalmological examinations, there were no treatment-related changes after one year, whereas a higher incidence of corneal opacity, oedema of the cornea, nuclear opacity of lens small ctinal cossels and abnormal color (pale) of the retinal fundus was noted after two cars, in comparison to the control of the con

There were no treatment-related changes at the hematology and clinical chemistry, evaluations throughout the study. Urinalysis revealed a higher incidence and severity at cellular casts at Months 3 and 6, when compared to the controls. This change was found to be reversible, acho cellular casts were noted at Months 18 and 24.

At the end of the chronic phase (12 months), mean terminal body weight was unaffected by the treatment, whilst mean absolute and relative fiver weights were higher by between 17 to 18% and mean absolute and relative kidney weights by 38%, in comparison to the controls. At the macroscopic observation, enlarged liver was found in 1/10 animats and prominent lobulation on the liver in 3/10 animals, compared to no cases in the controls. In addition pale kidney, enlarged kidney or irregular surface on the kidney were found in some animals, compared to access in the controls. Histological examination revealed toxicologically relevant changes in the liver, kidney and thyroid gland, in comparison to controls. In the liver, the incidence of altered hepatocytes (comparise focial progressive nephropathy was noted. This change is a combination of thickfored basement membranes (tubular and glomerular), basophatic tubules and hyaline casts with a variable inflammatory cell infiltrate. In addition, a higher incidence of hyaline droptets and of bilateral basophatic tubules was noted. In the thyroid gland, follicular cell hypertrophy was noted, in association with a higher incidence and severity of colloid alteration.

At the end of the chronic phase, there was no evidence of a treatment-related increased incidence of tumors of any type in any organ.

At the end of the carcinogenicity phase 24 months) mean terminal body weight was 7% lower (not statistically significant) and mean absolute and relative diver weights were 5 to 12% higher, in comparison to the control group. At the macroscopic observation, a higher incidence of enlarged liver and white focion the liver were found, together with a higher incidence of enlarged kidney and irregular surface on the kidney, when compared to the controls. Histological examination revealed toxicologically relevant changes in the diver, know, dryroid gland, and secondary effects in the testis and stomach, in comparison to the courols. In the liver and thyroad gland, changes resulting from exacerbation of the findings noted at the end of the chronic phase were observed. In the kidney, marked degenerative changes resulting from exacerbation of the preroscopic findings noted at the end of the chronic phase were observed, were with higher incidence of tubular hypertrophy, collecting duct hyperplasia and hyaline droplets. In the tests, a higher incidence of arteritis and periarteritis was noted. This vascular change was solated (not found in sensative tissues like aorta, mesenteric arteries) and is most likely explained by secondar hypertensive changes due to increased severity and incidence of chronic nephropathy. Therefore, this change in the testis was considered not to be a direct effect of the treatment. In the stom to h, a higher incidence of regenerative non glandular hyperplasia was noted. This minor change was mainly observed in animals which died prematurely and was attributed to secondary stress due to porbidity. Therefore, its increased incidence was clearly linked to the increased mortality rate in this male high dose group.



No treatment-related neoplastic changes were observed.

<u>At 150 ppm</u>

There were no treatment-related clinical signs throughout the study in either sex. Mean body weight or body weight gain parameters and mean food consumption were unaffected by treatment in either sex over the two years of the study. At the ophthalmological examinations, there were no treatment-related changes after one year, whereas a higher incidence of corneal opacity, oedema of the cornea, nuclear opacity of lens and small retinal vessels was noted in males after two years, in comparison to the controls.

There were no toxicologically relevant changes at the hematology and clinical chemistry evaluations of throughout the study. Urinalysis revealed a higher incidence and severity of cell dar casts at Months 3 and 6 in males, when compared to the controls. This change was found to be reversible, as no cellular casts were noted in this male group at Months 12, 8 and 24.

At the end of the chronic phase (12 months), mean terminal body weights and mean organ weights were unaffected by treatment in either sex. No treatment-related changes were noted at the macroscopic observation. Histological examination revealed toxicologically relevant changes in the liver, kidney and thyroid gland in males only, in comparison to controls. In the liver, centrilobular to particular hypertrophy was observed. In the kidney, chronic progressive nephropathy was foted, together with a higher incidence of hyaline droplets in the thyroid gland, follicular cell hypertrophy was observed.

At the end of the carcinogenicity phase (24 month), mean terminal body weights and mean organ weights were unaffected by treatment in either sex, with the exception of a slightmore ase of between 8 to 12% in mean absolute and relative liver weights in males, when compared to the controls. At the macroscopic observation, a higher incidence of enlarged kidneys was noted in males from the unscheduled sacrifice group only, when compared to the controls. Histological examination revealed toxicologically relevant changes in the liver, kidney, the controls. Histological examination revealed toxicologically relevant changes in the liver, kidney, the controls. Histological examination revealed toxicologically relevant changes in the liver, kidney, a higher incidence and severity of chronic progressive nephropathy and a higher incidence of tubular hypertrophy and tubular dilatation were observed in males. In the hypoid gland, follicular cell hypertrophy was observed in males, whereas a higher incidence of colloid alteration was noted in females. In the testis, a higher incidence of arteritis and periarteritis was noted. This change was considered not to be a direct effect of the treatment.

No treatment-related peoplastic changes were observed at this dose level in either sex.

At 30 ppm:

No toxicologically relevant changes are noted throughout the course of the study in either sex for any of the parameters evaluated

In conclusion, there was no evidence of a treatment felated increased incidence of tumors of any type in any organ, with the exception of a higher incidence of liver cell tumors (carcinoma plus adenoma) in the female high dose group only at the end of the carcinogenicity phase, in comparison to the controls.

Over a 24-month period of dietary administration with AE C656948 to the Wistar rat, 30 ppm was the NOAEL in males (equivalent to 1.20 mg/kg body weight/day) and females (equivalent to 1.68 mg/kg body weight/day).

I. Materials and methods

A. Materials

14 Test paterial: Description Lot / Batch #: Purity:

AE C656948 beige powder Mix-Batch: 08528/0002 ≥94.5% a.i.



658066-35-4 CAS# Stable in rodent diet at 20 and 10000 ppm over a 105-day period **Stability of test compound:** at ambient temperature, checked in a previous study () 2. Vehicle and / or positive none control: 3. Test animals: **Species:** Rat Wistar Rj:WI (IOPS HAN) Strain: 6 weeks approximately (start of dosing) Age: 216 - 219 g mean group weight for the males Weight at dosing: mean group weight for the females Source: **Acclimation period:** 13 days A04CP1-10 (formerly reference) as A@C-1(PP1) from S.A.F.E. Scientific Animal Food and Engineering, Augy, aa Diet: libitum except at designated time periods Filtered and softened tap water from the municipal water Water: supply, ad libitum By sex in groups of 5, anless reduced by mortality or isolation. **Housing:** The cages were suspended, stainless steeDwire pesh. 🦃 Environmental conditions: **Temperature:** . 55 ± 45% **Humidity:** Air changes: 10-15 per høur Alternating 12-bour ght and dark **Photoperiod:** Study design 06 March 200 From 09 February 2005 1. In life dates: 2. Dose level selection The dose levels were selected based on the results from a previous 90-day dietary study in the rat (M-

The dose levels were selected based of the results from a previous 90-day dietary study in the rat (M-250946 91-1), where the NOAEL was established at 50 ppm for males and 200 ppm for females. The NOAEL was mained based on effects observed in the liver (increased weight in association with hepatocellular hypertrophy in both sexes) and thyroid gland (follicular cell hypertrophy especially in males) at 1000 and 3200 ppm At these dose levels, changes were also observed in some hematology and clinical elemistry parameters. In addition in males, a higher incidence of cellular casts was observed at urinalysis and higher incidence, seventy of hyaline aroplet nephropathy (related to the accumulation of alpha 2n-globulin in the proximal dibules) and of hyaline casts were observed in the kidney from 1000 ppm onwards and also at 200 ppm for most of these changes.

3. Animal assignment

All animals were weighed twice during the acclimatization phase. Their health status was checked on arrival. All animals were subjected to a detailed physical and ophthalmological examination once during the acclimatization phase. On the day oprandomization, animals were allocated to dose groups using a computerized randomization procedure that ensured a similar body weight distribution among groups for each sex. Selected animals were in a weight range from 183 to 244 g for the males and 131 to 181 g for the females at the start of exposure to the test substance, i.e., within $\pm 20\%$ of the mean body weight on the day of randomization. Any animal deemed unsuitable for selection based on weight, ophthalmological abnormalities or health status was not used for the study.



On study Days 2 and 3 of the acclimatization phase, each animal was identified by a micro identification implant.

Animals were assigned to the test groups noted in the following table. Control animals received of untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 5.5-2	Details of group size	es and treatmer	it,	U,	0°		
		Achieved dose to	Interim s	sacrifice	Main 104 w	study veeks ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	, ,
Test Group	Diet concentration (ppm)	animal (weeks 1- 104) (mg/kg/day	Sy Male	Pemale	Male Q	Female	
Control (1)	0	0	10 🕎	<u></u> , ¶∕Õ	× 60°	\$ 60 °	
	Animal identity	Š.	PT1M0697 to 0686	₽Ĵ1F0749 ∳to 0756	PT 000687 0746	PT1¥0757 to 0816	
Low (2)	M: 30 F: 30	M: 1.20 F: 1.68			60 0	60	
	Animal identity		PT2M0817 to 0826	872F0887 to 0896	PT2M0827	PT260897 to 0956	
Mid (3)	M: 150 F: 150	F: 8.6				J 60	
	Animal identity		PT3M9957 to 4966	♣T3F19€7 , to 1036	PTOM0967	PT3F1037 to 1096	
High (4)	M: 750/975* (F:2)500	ÀT: 29 €	@ 10 ^{\$}		69	60	
* 770	Animal identity		PT4M0097 to 9106	PT4F₩67 ✓ tocl176	PC#M1107 to 1166	PT4F1177 to 1236	

Table 5.5-2Details of group sizes and treatment,

* 750 ppm up to study Day 588 (study Week &), 375 ppm from study Day 589 onwards (study Week 85)

4. Diet preparation and analysis

The test substance was incorporated into the diet to provide the required dietary concentrations of 30, 150, 375, 750 or 1500 psin. The test substance formulations were prepared to cover the dietary requirements over 6 to 8 weekly periods apart from the thirtcenth formulation (F13) due to the decrease in dose to be used in the male high dose group and from the last formulation (F16) which covered the dietary needs until the end of the study. When not in use, the diet formulations were stored at ambient temperature.

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The homogeneity of the test substance in diet was verified at least from the first loads at all concentrations on the first formulation (F1) and on the first loads at 30 and 1500 ppm of formulations F2, F7 and F13, to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as the measured concentration.

The concentration was checked at beast for all loads at all dose levels for formulations F1, F2, F4, F7, F10 $\cancel{F13}$ and F16.

Homogeneity and concentration results of AF C656948 in the diet were within the in-house target range of 85 to 115% of maximal concentration, except for 14 out of 242 results, which however were considered acceptable for the current study.

5. Statistics

Mean and standard deviation were calculated for each group and per time period. The Bartlett test was performed to compare the homogeneity of group variances.

- If the Bartlett test was not significant (p>0.05), means were compared using the analysis of variance (ANOVA), which was followed by Dunnett test (2-sided) if ANOVA indicated significance.



- If the Bartlett test was significant ($p \le 0.05$) (for body weight change parameters, terminal body weight, absolute and relative organ weight parameters, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, % neutrophils, % lymphocytes, prothrombin time), group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-side() if the Kruskal-Wallis test indicated significance.

- If the Bartlett test was significant ($p \le 0.05$) (for body weight and average food consumption/days parameters), data were transformed using the log transformation. If the Bartlett test, on log transformed data was not significant (p > 0.05), means were compared using the ANOVA on log transformed data, which was followed by the Dunnett test (2-stoed) on log transformed data if ANOVA indicated significance. If the Bartlett test was significant ($p \le 0.05$) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided), if the Kruskal-Wallis test indicated significance.

- If the Bartlett test was significant ($p \le 0.05$) (for homatology parameters such as red blood cell count, platelet count, white blood cell count, neutrophil count lymphocyte count), data were transformed using the square root transformation. If the Bartlett test on square root transformed data was not significant (p > 0.05), means were compared using the ANOVA on square root transformed data, which was followed by the Dunnett test (2-sided) on square root transformed data, if ANOVA indicated significance.

- If the Bartlett test was significant (p 0.05) even after square root transformation, group means were compared using the non-parametric Kruskal-Walks which was followed by the Dumy test (2-sided), if Kruskal-Wallis test indicated significance.

For urine analysis (pH), group means were compared using the non-parametric Kruskal-Wallis which was followed by the Dunn test (2-sided), if Kruskal-Wallis est indicated significance.

If one or more group variance(s) equaled a means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance.

For survival analysis: 🗞

Adjusted mortality rates were estimated using Kaplan-Meier estimation procedures. Kaplan-Meier estimates were calculated separately for each sex and treatment group. Mortalities which were the result of animals dying following a cidents (accidental frauma or dired during anesthesia) or at scheduled sacrifice were considered to be censored observations.

Statistical significance of differences in survival rates between treated and control groups and doserelated trend in survival were assessed using Cox's and Farone's tests on life table data. Probability values presented were two fided for pairwise comparisons and trend test. Group mortality rates were compared at the 5% and 1% devels of significance. Survival analyses were performed on the carcinogenicity phase.

For neoplastic and non-neoplastic findings

When the incidences of the 30 and 150 ppm the deated groups were equal to 0, only the high dose group was compared to the control group and no trend test was performed.

When the number of design bearing animals was equal to 1 in one group and was equal to 0 in the other groups, no statistical analysis was performed.

Not adjusted analyses

Selected lessons were analyzed by Cochran-Armitage method for trend (1-sided) and the Fisher's exact test (1-sided) for control versus treatment comparisons. Cochran-Armitage trend significance is reported when there is existence of any monotone response in the incidence data (that is there is a relationship between the response and the dose represented by a regression line that is continually increasing (or decreasing), but perhaps not in a straight line).



Survival adjusted analyses:

Further survival adjusted analyses, considering any possible intercurrent mortality differences due to the competing toxicity among the treated groups, were performed on lesions. For non-palpable tumors, each tumor was categorized as fatal if the tumor was a factor contributing towards the death of the minal, incidental otherwise.

Incidental tumors and non-neoplastic lesions data were analyzed by logistic regression of unnor prevalence. Logistic regression analysis is based on the assumption that the diagnosed lesions were not directly responsible for the animal's death. Treated and control group lesion rates and dese-related trends were compared using the corrected score test. Fatal tumors were analyzed by the life able test. The life table test is based on the assumption that all lesions were fatal. Statistical significance of differences in incidences between treated and control groups and tose-related trends were investigated using Cox and Tarone's tests.

Trend tests were conducted firstly including all groups. When both the trend test including all the dose levels and only the high dose group were significant a second trend test social the high dose group was also done. The reported results reflect A side desting

Group incidences were compared at the 5% and 1% Evels of significance. All finding analyses were performed on the carcinogenicity phase.

C. Methods

1. Observations

Animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Animals were observed for clinical signs at least once daily. Detailed physical examinations including palpation for masses were performed weekly throughout the study.

2. Body weight

Each animal was weighed at least weekly during the acclimatization period then weekly for the first 13 weeks of study, approximately every 4 weeks. Additionally diet fasted animals were weighed prior to scheduled necropsy. Body weights recorded prior to necropsy are referred to as terminal body weights.

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3. Food consumption and compound intake

Food consumption was $\frac{1}{2}$ corded twice weekly during the first 6 weeks of treatment, then weekly up to Week 13, and once approximately every $\frac{1}{2}$ weeks thereafter.

The weekly mean achieved dos get intake in my kg body weight/day for Weeks 1 to 13, then 1 week per month thereafter was calculated as follows:

Test substance interes Test substance intere

Test substance intake Groupmean body weight (g) at the end of the food consumption period

The monthly and overall mean achieved do sage intake for the 24 months of treatment were derived from the weekly data.

4. Ophthalmogopic examination

During the acelimatization phase, all animals were examined by indirect ophthalmoscopy. During the treatment period, unduscopic (indirect ophthalmoscopy) and biomicroscopic (slit lamp) examinations were performed on all surviving animals after approximately 12 and 24 months of treatment with AE C656948. Each eye was examined by direct ophthalmoscopy in the first instance, and then after instillation of an adopinic agent (Mydriaticum, Merck Sharp and Dohme), each eye was re-examined by means of a slit lamp and an indirect ophthalmoscope.

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5. Hematology and clinical chemistry

Blood was sampled from Isoflurane anesthetized animals by puncture of the retro orbital venous plaxus after overnight diet fasting. Blood was collected in tubes containing EDTA for hematology, lithium heparin (for plasma) and clot activator (for serum) for clinical chemistry and sodium citrate for coagulation. At terminal sacrifice, blood smears were prepared for all animals not sampled for hematology. When possible, a blood smear was prepared for the moribund animals, just before sacrifice.

Blood analyses were performed on all surviving animals of the interim sacrifice groups of Week 12 or 13, 25 or 26 and 51, and on the first ten suitable surviving cats of the terminal sacrifice groups of Weeks 12 to 13, 25 or 26, 51, 78 and 103 or 104.

a. Hematology

Parameters were measured using an Advia 120 (Bayer Diagnostics, Puteaux, France) and an ACL 3000 (months 3, 6, and 12) or an ACL ElitePro thereafter (Instrumentation Laborator) Paris, Frances for blood clotting measurements. Leukocyte differential count* Hematocrit Hemoglobin concentration Mean corpuscular HGB Leukocyte count Mean corpuscillar HOB concentration Erythrocyte count Mean corpuseular volume Platelet count Reticuloevte count Blood clotting measurements: Prothrombin time Minimum required for carcinogenicity studies (Control and high dose fours miless prects are observed) * based on Guideline 870.4300 & OECD 453 A blood smear was prepared and tained with Wright (Months 3, 6, 12, and 18) or May-Grünwald-Giemsa (Month 24) stains? It was examined only when the results were aboorman For moribund and terminal socrificed animals, the blood smear were stained with Wright stain (until October 05, 2006) May Grünwald-Giensa stam (from October for possible differential white blood cell determination but were not examined). b. Clinical Chemistry Parameters overe measured using an Advia 1650 (Bay Diagnostics Puteaux, France). **OTHER** Calcium Albumin Chloride* Cřeatinine Magnesium* Urea 🏠 Inorganic phosphorus Total Cholesterol* Globulins* Potassium* Sodium* odium* ENZYMES (more than 2 bepatic enzymes Gucose (fasting)* Total bilirubin Alkaline phosphatase* Total protein* Chofinesterase L. Triglycerides Creatine phosphokinas Serum protein electrophoresis Lactic acid defedrogenase Alanine amotransferase Aspartate aminotransferase* Gamma@lutanayItransferase* Sorbitor dehydrogenase Glutamate dehydrogenase * Recommended for combined chronic and carcinogenicity studies based on Guideline 870.4300. Any significant change in the general appearance of the plasma and the serum was recorded. Globulin concentrations and albumin/globulin ratio values were calculated.



6. Urinalysis

Urinalysis was performed on all the surviving animals of the interim sacrifice groups during on Weeks 12 or 13, 24 or 25 and 52 or 53, and on the first ten suitable surviving rats of the terminal satisfice of groups during Weeks 12 or 13, 24 or 25, 52 or 53, 77 and 104.

Diet and water were withdrawn during the overnight (approximately 16 hours) collection period. Any significant change in the general appearance of the urine was recorded. The urine volume was measured. pH was assayed using a Clinitek 200+ and Ames Multistix dipsticks. Urmary refractive index was measured using a RFM320 refractometer. ő

- The following semi-quantitative parameters were assayed using a Chritek 200+ and Antes Multistix dipsticks: Appearance* Volume* Specific gravity / osmolality / refractive index* Bidirubins pH* Sediment (microscopic) Protein* Any significant change in the general appearance of the urine was recorded. **a. Quantitative parameters** Urine samples were weighed to determine trinary solume. pH vas assayed using a Clinitek 200+ and Multistix dipsticks (previously referenced as Ames Multistick for the protocol) 200+ and Multistix dipsticks (previously referenced & Ames Multistick for the protocol) (Bayer Diagnosties, Puteaux, France).
 - Urinary refractive index was measured using a RFM 320 refractometer. (Problock Scientific, Ő Illkirch, France).
 - b. Semi-quantitative parameters
 - \bigcirc Glucose bilirubin, ketone bodies, occult blood, protein and utobilinogen were assayed using a cliniter 200 and Multistix dipsticks (previously referenced as Ames Multistick in the protocoly (Bayer Diagnostics Puteaux, France). 🖉 1
 - c. Missroscopic examination of the sediment
 - Microscopic examination of the urinary sectiment was performed after centrifugation of the urine. The presence of red flood cells, white blood cell, epithelial cells, bacteria, casts and crystals was graded. \bigcirc

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8. Sacrifice and pathology

On study Days 366 to 368 for the 12 month chronic phase and on study Days 730 to 743 for the 24month carcinogenicity phase all surviving mimals dedicated to chronic phase and carcinogenicity phase groups, respectively, were sacrificed by exsanguination of moder deep anesthesia (inhalation of Isoflurane). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day of sacrifice. Animals were diet fasted overnight prior to sacrifice.

All animals were nearopsied. The neuropsy included the examination of external surfaces, all orifices, all major organs, tissues and body cavities. Al@ignificant macroscopic abnormalities (including masses and their regional lymph nodes when possible) were recorded, sampled and examined microscopically.

The CHECKED (X) tissues were collected for histological examination. The organs CHECKED (XX) were also weighed fresh at scheduled sacrifice only. Paired organs were weighed together

Digestive system		Cardiovasc. / Hemat.		Neurologic
$X \xrightarrow{\sim} 1 \text{ ong} (0) \xrightarrow{\sim} y \xrightarrow{\sim} \sqrt{y}$	X XX	Aorta, thoracic* Heart*+	XX	Brain (3 sections)*+
a glavid	Х	Bone marrow*, sternum	Х	Sciatic nerve*
X Stomach*	Х	Lymph node*, mesenteric	Х	Spinal cord (cervical, thoracic, lumbar)*
X Duodenum*	Х	Lymph node, submaxillary	Х	Eyes (retina)*



					- 10°PJ - 111
	Digestive system		Cardiovasc. / Hemat.		Neurologic
Х	Jejunum*			Х	
X	Ileum*	XX	Spleen*+		
X	Cecum*	XX	Thymus		Optic nerves* Glandular Pituitary gland* Adrenal gland*+ Parathyroid gland*
X	Colon*	2121	Thymus	XX	Pituitary gland*
Х	Rectum*		I hogonital	XX	Adrenal gland*+
л XX	Liver*+	XX	Urogenital	Х	Papathyroid gland*
			Kidney*+	Λ	Papathyloid gland
Х	Pancreas*	X	Urinary bladder*	VV	 Thyroid gland (weighed with parathyroid gland) Lacrymatexorbital gland Lacrymatexorbital gland Other Bone (stermin) Skeletal muscles
		XX	Testis*+		 with parathyroid gland Lacrymatexorbital gland Harderian gland Other Book (stermin) Skeletal nuscle
	Respiratory	XX	Epididymis*+	Ű	
Х	Trachea*	XX	Prostate gland*	Q	Lacrymatexorbital gland
Х	Lung*	Х	Seminal vesicle*	X.	Harderian gland
\wedge	Pharynx*	XX	Ovary*+	Ŵ,	• Other
^	Larynx*	XX	Uterus (woh cervix)*+	X	Bong (stermin)
^	Nasal cavities*	Х	Mammary gland*	X	Skeletal muscles
		Х	Vaginka ~	X	≪Skin* [™] [™]
				a s	All gooss lestions and
			A R. U A	XU	masses (ncluding their
				Å	Winph prodes if possible ?
		(Å.	Articular surface
		Ő		X X	(fenestotibishioint)
*	required for carcinogenicity	studies base	d op US EPA Suideling 870 43	x	
+	organ weights required for r	odent studies		Ŭ, Ŏ	O S . W
Х	tissues were collected for hi	stological ex	ininations O 6	£	
Х	organs were weighed fresh a	at s@redule&	Prostate gland* Seminal vesicle* Ovary*+ Uterus (with cervix)*+ Mammary gland* Vagina d or US EPA guideline 870 A3 minations sacrific only, with paired organ pathologic examination only prepared from sacrifice ed with May Grünwald Ga y or bone marrow histo	weighed	Articular surface (femorotibial joint) Gogether and collected for
	organs were preserved for m	siblemicro	nathalogic examination only	U' . Q	
	organs were preserved for p	ossiolomilier		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	°∼° ©
wo f	emoral bone marrow s	means we	be prepared from sacrifice	ed∡anima	de (except on weekends and
ublic	holidays), one of which	n was stain	ed with Max Grünwald Gr	emsa, bì	it not examined as no relevant
nang	es were observed in k	Ematalao	v or bone marrow histo	oov th	it not examined as no relevant as second smear was stored
nstai	ned			0 ⁵ ,0 ¹	
Istal	neu.			al a	Ø.
ampl	es were fixed by imme	rston in ne	eutral buffered 10% Forma	u kin with	The exception of the eye and $\mathbb{R}^{\mathbb{Z}}$
tic r	herve Harderian aland	enididami	s and testis that were fixe	in Dav	idson's fixative
		Phone			145011 5 11AULIVE.
	a. Histotechnology	, Ô,		Ś	
	- All the above s	amples A	sted in the above Table	excent	exorbital lachrymal gland,
	larvny/nh@vny.a	and nasal	avities) were embedded in	naraffin	wax
			Duithatamaterilia	parain	i wun.
	- Histological sect	ions		eosin, w	rere prepared from all organs
	and tyssue sample	es usted in	the above table from all a	nımals.	
	b. Histopathology	S e			
	- Anstopathology	aminatic	ms were performed as foll	ows (int	erim sacrifice):
	- a all organs and	tissue sa	wales from animals sacri	ificed or	dying during the treatment
	period, &				aying during the treatment
		Q, V			
	\simeq all organs and	tissue sam	ples from animals of contr	fol and h	ligh dose groups,
L.	🧹 – liver, Jung, kud	ney and tag	get organs detected at mic	croscopi	c examination (thyroid gland)
Ý	from animals of	of the inter	media dose groups,		
	- Histonathology	aminalic	ons were performed as foll	ows (ter	minal sacrifice) [.]
		ticon	ples from all animals,		Succession Succession
		Lissue Saill	Pros nom an annais,		
	- 🖉 gross abnoona	IIIIes Irom	gan animals.		
		õ .			

For all onscheduled sacrificer or dead animals on study, the cause of death was determined when possible. The dragnoses presented in the report represent the consensus opinion of two pathologists.



II. Results and discussion

A. Mortality

Chronic phase, all animals

Within the first year in the male high dose group, 11/70 animals were found dead or were sacrificed prematurely for humane reasons compared to 6/70 in the control group. The main clinical signs in these early decedent males consisted of soiled fur or anogenital region (3/11) and focal swelling (2/11), together with usual signs associated with morbidity (limited use of hindlinds, reduced motor activity general pallor, wasted appearance). In view of the low mortality rate in the male control group and in other male treated groups during the first year of the study, the early deaths in the male high dose group were considered to be treatment-related. No clear factor contributing to the death of these animals could be established at the microscopic examination.

No effect on mortality was noted in females.

Table 5.5-3:Morta	lity rate in ma	ale and female	rats – weeks	J052 (ŐY .	O A
Sex		Males	\sim \sim	A) O Fem	ales 🕺	
Diet concentration of	0	30 × 150 ×	750	, Q [×]	×30 5	150	1300
AE C656948 (ppm)	U					Ŵ	<u>.</u>
Group size	70	70 70 70	%7 0 ~	70	* 7 0 07	ZØ _	9 70
Mortality	6 Q	E of	> 11	10	Ê	,5 🏹	3
(% mortality)	(8.6)	(4.3) (2.9)	(15.)	(4.4)	Q2.9) QO(7.1	(4.3)

After 2 years of treatment, analysis of the survival rates showed that the mortality incidence was increased in the male high dose group and in the female low dose group (p 9.05). Higher mortality in the male high dose group was considered to be treatment related as the trend test was statistically significant in males, whereas in the female low dose group it was considered to be incidental and not related to treatment as the trend test was not statistically significant. No clear factor contributing to the death of these animals could be established.

Carcinogenicity phase

		" %)		v	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	2	\bigcirc
Table 5.5-4:	M	ortality	ratean	male and	female	ranš –	weeks
1	1.1	or evening .			~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		

		S M						
Sex S		🔊 N	lales	Y LY		Fem	ales	
Dose level of AE		×20	\$150 X	750(375		30	150	1500
C656948 (ppm) 🔊 🤇		\$30 <u></u>	⊃ [×] 150√, [×]	\cap^{\prime}		50	150	1300
Number of animats		60%	60	60 0	60	60	60	60
Killed for humane				§ 20°	12	24	19	23
reasons 🔊 Õ	.0.		γ		12	24	19	25
Found dead	020 🔬	29 🕰	28	28	7	8	4	6
Dead during anesthesia		0	Ĩ,	~ 1	0	0	2	1
Total mortality	37 📎	~44	£34 Q	o [™] 49	19	32	25	30
(% mortality)	(677)	Ø3.3)	D (56.7)	(81.7)	(31.7)	(53.3)	(41.7)	(50.0)
Adjusted survival rate $(\%)^{a}$	37.8% [©]	26.7%	22.3%	19.9%*	68.3%	46.7%*	60.4%	51.0%

a: Kaplan Meier estimated survey al rates at the end of the study after adjusting for censored animals $*: p \le 0.05$

B. Clinical observations

Chrome phase

During the first year of treatment there were no treatment-related clinical signs



Carcinogenicity phase

During the second year of treatment, the following treatment-related clinical signs were observed, in animals allocated to the carcinogenicity phase:

Sex		Μ	ales			Fen	nales 🔊	
Diet concentration (ppm)	0	30	150	750/875	0	چ 30		×1500 \$
Number of animals examined	55	57	58	ر ب ل	5.90	58	56Q	AT \$
Hair loss	nc	nc	nc 🖉	nc ©° බ	8 13.6% [374]	10 (17.2%) [3771]	(8.9%) (8.9%) (37 b)	17 (29.8%) [371]
Prostration	0 (0%)	1 (1.8%) [724]		(6.1%) (6.1%) (6.1%)	Qnc	nc nc	Önc (
General pallor	2 (3.6%) [543]	2 (3.5% [624]	(1.7%) (1.7%) (445]	(8.2°) (8.2°) (44)		e nc		^O nc
Wasted appearance	nc			anc of) 10 (16,4%) (16,4%)	60.7%) [553]	2 ¹¹ (19,6%) [483]	14 (24.6%) [504]
Soiled anogenital region	2 (3.6%) [704]	(D8%) (108%)	で 1 よ 3 (1.7%) [641]	(8.2%)	nçş		o nc	nc

[]: first day of appearance during the second year of treatment

In the female high fose group (1500 ppm), a higher incidence of heir loss and wasted appearance was noted, in comparison to the controls.

In the male high dose group (750/375 ppm), a slightly higher ncidence of the usual signs associated with morbidity (prostration, general pattor and soiled anogenital region) was noted, in comparison to the controls, reflecting the higher mortality observed in this group?

No treatment-related dinical sign were wied at the mid and low dose levels in either sex.

C. Body weight

In the female wigh dise group (1500 ppm) mean body weight or body weight gain parameters were essentially comparable to the controls throughout the first three months of treatment. Thereafter, mean cumulative body weight gain was lower than in the control group between Weeks 14 to 26 (-29%, $p \le 0.01$) to 54 (-15%, not statistically significant) and 54 to 79 (-59%, $p \le 0.01$), whilst mean body weight was lower by 3, 5, 44 and 12% at Weeks 26, 54, 79 and 102 (statistically significant for most time points), when compared to the controls.

In the male high dose group \$750/375 ppm mean body weight or body weight gain parameters were essentially comparable to the controls throughout the study. The few changes in mean body weight gain/day (reaching statistical significance) observed were considered to be incidental as they corresponded to both transient increases and decreases.

At the mid and low dose levels (150 and 30 ppm), mean body weight or body weight gain parameters were unaffected by the treatment in both sexes over the two years of the study. The few changes (reaching statistical significance) observed were considered to be incidental as they were noted in isolation and with no dose-relationship.



Table 5.5-6

Group mean(± SD) body weights (BW) and cumulative body weight gains (BWG) (g)

Males				<i>Q</i> [°]
AE C656948 dosage level (ppm)	0	30	150	750/375#
Initial BW (Day 1) (%C)	219±11	219±12 (100)	216±12 (99)	218±12 (100)
BW Week 2 (Day 8) (%C)	277±14	278±16 (100)	278474 (100)	279\$#15 (10)
BW Week 14 (Day 92) (%C)	527±37	526±42 (100)	529±41 (100)	\$26±41(100)
BW Week 26 (Day 176) (%C)	604±45	600±51 (99)	608±49 (101)	609±47(101)
BW Week 54 (Day 372) (%C)	697±60	699±74 (100)	704±61 (104)	624-61 (100)
BW Week 78 (Day 547) (%C)	719共78	728±86 (01)	733±76 (102)	902±64(98)
BW Week 102 (Day 708) (%C)	67 <u>2</u> ¥89	639±86 (95)	686±68 (102)	627 ±6 4 (93)
BWG Weeks 1-2 (Days 1 to 8) (%C)	69±5	59±7 (100)	62£) (1050) ×	61*±6 (103)
BWG Weeks 1-14 (Days 1 to 92) (%C)	308±34	307±36.(100)	308±40 002)	~308±37(100)
BWG Weeks 14-26 (Days 92 to 176) (%C)	⊃ 7 <i>7€</i> 16 ≿	₩74±1 6 (96)	79±17(103)	82±17 (106) 。
BWG Weeks 26-54 (Days 176 to 372) (%C)	₹8±32 ©	97±95 (110)	93, 31 (106)	81234 (92)
BWG Weeks 54-78 (Days 372 to 547) (%C)	~~25±34	<u>30</u> ±33 (124)	∘28±47 (\$¥2)	23±30(92)
BWG Weeks 79-102 (Days 547 to 708) (-48+57 🌾	-67±49 (143)	₩-42±55 (89)	>-60±4\$(128)
Overall BWG (Days 1 to 708) (%C) \mathcal{A}	453±91℃	421 #86 (93)	470569 (104)	4 1@ ±65 (91)
Females Q &	<u>\$ \$</u>	<u>S</u>	$\sim \sim$	\$~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
AE C656948 dosage level (ppm) 🦉 🔩		Q Q	° 380 %	1500
AE C656948 dosage level (ppm) Image: Cost of the second	155±10	156±11 (104)) 157±10 (101)	157±10 (101)
BW Week 2 (Day 8) (%C) \bigcirc \bigcirc \bigcirc	@183±13	186±14 (102)		· · · ·
$DWWWCCKIT(Duy)2)(70C) \cong$			283=23 (101)	
BW Week 26 (Day 176) (%C)	310±25	316 25 (102)		()
BW Week 54 (Day 32) (%C)	~ ³⁵⁰⁺⁴⁹	@61±40 (103)	351±42 (100)	
BW Week 79 (Dag) 540) (%C)				356**±45
	A12±75	422\$67 (102)	408±59 (99)	(86) 374**±56
Final BW (Day 708)/0%C) 🔍 💦 👔 🕺	425978	452±92 (106)	425±68 (100)	
BWG Weeks 1-2 (Days F to 8) (CoC)	27±7 ~	× 30*±7 (111)		27±7 (100)
BWG Weeks 1-14 (Days 1 to 92) (%C)	×124±17	31*±16	126±18 (102)	122±16 (98)
BWG Weeks 14-26 (Days 92 to 176) (% 🕸 🐇	Ĵ 31±10 (> 30±10 (97)	30±9 (97)	22**±9 (71)
BWG Weeks 26-54 (Dass 176 to 372) CC	9±33	43±24 (110)	37±24 (95)	33±20 (85)
BWG Weeks \$4-79 (Days 37@to 547) (%C)	61± 4 2	60±42 (98)	56±32 (92)	25**±23 (41)
BWG Week, 79-102 (Days 547 to 708) (%C)	\$ 2 ₂ 1 <u></u> €44	22±52 (105)	25±34 (119)	20±39 (95)
Overall BWG Weeks 1-102 (Days 1 to 708)	269±73	296±89 (110)	269±64 (100)	216**±53 (80)

75% ppm up to study Day 58% Rtudy Week 84,0575 ppm from study Day 589 onwards (study Week 85)

 $\overline{\#} 75\% \text{ ppm up to stars} = 0$ C = control; nc = not calculated* Statistically different (p<0.05) from the control.
** Statistically different (p<0.01) from the control.

D. Food and water consumption

Overall, Snean food consumption was similar to the controls throughout the study in both sexes and at all dose level, with the only exception of a slight reduction by up to 7% ($p\leq0.01$ or $p\leq0.05$) in the male high dose group between study days 18 to 39. The few other minor differences (reaching statistical significance) from controls were considered to reflect inter-individual variations but not treatmentrelated offects.



Table 5.5-7	Group mean fe	ood consumption	(g/animal/day)

	Mal	es			a,°
Dose Group (ppm)	0	30	150	750/375	1500
Week period 1 to 13 (%C)	26.4	25.9 (98)	26.1 (99)	26.1 (99)	87 0
Week period 14 to 26 (% C)	25.3	25.2 (100)	25.8 (102)	29.7 (102)	<u>0</u> - 5
Week period 27 to 52 (% C)	25.5	24.8 (97)	25.3 (99)	25.6 (101)	× - ,
Week period 53 to 78 (% C)	24.8	24.2 (98)	24.9 (101)	25.7 (104)	
Week period 79 to 104 (% C)	24.5	24.1 (98)	24.5 (100)	25.6 (105)	
	Fema	ales 🖉	a y	Å,	
Week period 1 to 13 (%C)	19.0	19.5 (103)	19.@103)	.0	S18.7 (99)
Week period 14 to 26 (% C)	18.1	18.4 (102)	18,9 (104)		17.697)
Week period 27 to 52 (% C)	18.2	18.8 (103)	48.6 (1002)	L - L	18.0 (99)
Week period 53 to 78 (% C)	19.3	19.6 (102)	9 //	<i>¶</i> ₹ <i>₹</i> 0″	\$8.6 (9 6)
Week period 79 to 104 (% C)	20.5	21.8 (10.0)	20,8 (101)		20.9 (102)
Percentage from control in parentheses.	<u> </u>			<u>A</u>	, <u> </u>

E. Achieved intake

Compound intake is shown in Table 5.5 **2**-6 for beriod Varıou

The mean achieved dietary intakes of AE 656948 eived by the animals during the study were as follows:

Table 5.5-8	Mean achieved	dietary intake o	of AE C656948	(mg)kg/day)
-------------	---------------	------------------	---------------	-------------

(Ca

		Y al	\sim	~~ ()		- O	
Sex	×.		Males	~ ~		> Females	
Dosage level (ppm)	, ,	300	AP50 (, 750/3,75*	× 30 ×	150	1500
Week period 1 to 13	S.	1.84	O 9.20	× 46 0	2 .35	12	117
Week period 1 to 52	, X %	§1.37	6.9	S 35	1.88	9.6	95
Week period 1 to 104	Ś	1.20	×6.0 ×	29	2 [×] 1.68 [×]	8.6	89

* 750 ppm up to and Day 588 (stody Wee 084), 325 ppm from stude Day 588 onwards (study Week 85)

F. Ophthamoscopic examinations

atment, the following treatment-i@lated ophthalmological findings were At the end of the first ar of the observed: A O

Incidence of reatment-related ophthalmerogical findings noted at the first year **Table 5.5-9** exomination (animals allocated the coronic and carcinogenicity phases) Ø

Sex A A A A A A A A A A A A A A A A A A A					Females			
Dosage lever (ppm) 💦	, and a second sec	3 0	∞‴150∞	750	0	30	150	1500
Number of animals	65 ~	68	× 68	59	69	68	66	67
examined	$\beta $							
Retina fundus abnormal		Ø₽∕	1	0	0	1	0	4
color: pale	(0%)	@0%)	§ ∕(1.5%)	(0%)	(0%)	(1.5%)	(0%)	(6%)
%): incidence 🔏 🔥	Q° j	Ş K	\$					

In the female high dose group, abnormaticolor (pale) of the retinal fundus was observed in 4/67 animals, compared to no sase in the compols.

No treatment related ophthal mological findings were noted at any dose level tested in males or at the mid and low dose lovels in females at the end of the first year of treatment.



At the end of the second year of treatment, the following treatment-related ophthalmological findings were observed:

Sex		Μ	ales		🖉 🕺 🖉 🕺 🖉			
Dosage level (ppm)	0	30	150	750/375	0	30	150 🖉	1590
Number of animals	25	21	31	14	43	\$31	37	<u>,</u> ⊗32 ≪
examined				Ğ	Ő	\$∕ ∕	N.	
Corneal opacity	2	2	7	°\$*3	2 Q	0		₽ ¢v
	(8.0%)	(9.5%)	(22.6%)	(21.4%)	$(4.7\%)^{*}$	(0%)	× 2.7%	(6%) 🕵
Oedema of the cornea	1	1	4 2	3	Ŵ.	0 0	k.	0 0
	(4.0%)	(4.8%)	(12,9%)	(21.4%)	(4.7%)	\$ (0%R	(20%)	e (0%)
Nuclear opacity of lens	12	11	27	。12 《	r″35°≫	2%	≈\31 ×	30,8
	(48%)	(52.4%)	(87.1%)	(85.7%)	(81,4%)	(89.9%)	Q(83.8%)	(93.8%)
Small retina vessels	1	1	3 🔊	<u>گ</u>	<u></u> 2 '	6 ⁰ 2 ⁽	ř 25	A 14 °
	(4.0%)	(4.8%)	(9.7%)	Q.4%)	(4.7%)	(6.5%)	(5.4%)	(43.8%)
Retina fundus abnormal	2	L.		⁶ 6 🕅	′ 3Ç>	s.S.	≪ 2	15
color: pale	(8.0%)	(4.8%)	& (6.5%)	(42.9%)	(7%)	×(9.7%)	(5.4%)	(45.9%)
Hyperreflectivity in retina	1	<u> </u>	v 0.		@1 .	V 1 S	Ľ	õ 3
· - ·	(4.0%)	$\sqrt[6]{(0\%)}$	(0%)	(7.1%)	2.3%	(3.2%)	(\$7%) ≼	(9.4%)
%): incidence		1 0	Ô (ð á	, o	<u>_0</u>	<u> </u>	

In the female high dose group a higher incidence of small retinal ressels, abnormal color (pale) of the retinal fundus and hyperreflectivity in retina was hoted, in comparison to the controls.

In the male high dose group, a higher incidence of correal opecity, oedema of the correat, nuclear opacity of lens, small retinal vessels and abnormal color (pare) of the retipal fundus was noted, in comparison to the controls.

In the male mid dose group, a higher incidence of corneal opacity, ocdema of the cornea, nuclear opacity of lens and small retinal vessels was noted, in comparison to the controls

No treatment-related ophthalmological findings were observed at the stid dose in females or at the low dose in either sex at the end of the second year of treatment

G. Hematology, clinical chemistry, and urinaly

1. Hematology

A tendency towards tower sythrogyte parameters (herooglobin concentration, mean corpuscular volume, hemaforrit and/or no an corpuscular hemoglobin was observed in the female high dose group throughout the study. In view of the low magnitude of these variations, they were considered not to be toxicologically relevant. ŵ

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No treatment-related indings were noted at the instatology evaluation at any dose level tested in males or at the mid and low dose levels in females.

2. Clinical Chemistry

Slightly higher mean total cholester of concentrations were observed in the female high dose group throughout the study. In addition a slightly higher mean total cholesterol concentration was observed in the female nod dose group at Month 3, but as this change was observed in isolation and as its magnitude was fow, it was considered not to be toxicologically relevant.



Table 5.5-11	Total cholesterol concentrations in females (mmol/L): mean value ± standard
	deviation (% change when compared to controls)

		Females		
Dose level of AE C656948 (ppm)	Control	30	150	1500
Month 3	1.76 ± 0.33	1.69 ± 0.37 (-4%)	2.03 ± 0.2	2.38 ± 9.32 ***
Month 6	2.07 ± 0.37	2.01 ± 0.34	2.32 0.27	$2.19 \pm 0.566 ** $
Month 12	1.98 ± 0.33	2.01 € 0.49 √(+2%)	299 ± 0.27 (+11%)	2.74 0.76 *
Month 18	2.22 ± 0.31	(+25%)	2.21 ± 0.31 ° (0%)	2.92 ± 0.76 (+32%)
Month 24	2.44 ± 0.57 - &		$2,32 \pm 0.51$ (- 5%)	3.30 0.46
: p≤0.05, **: p≤0.01	0*		A D O	L A

In addition in the female high dose group, higher mean triglyceride concentrations were observed at Months 3 and 6 (+36% at both time points, $p \le 0.01$), and slightly lower mean glueose concentrations were noted at Months 6, 12 and 18 (-9%, -13% and <16%, respectively, $p \le 0.01$ @ $p \le 0.05$).

No treatment-related findings were boted at the clinical chemistry evaluation of any cose level tested in males or at the low dose level in females. Slightly lower mean total of lirubin concentrations were seen in all female treated groups on one or more occasions. However, since the variations were not consistent throughout the sampling periods and were observed with no dose relationship, they were considered not to be treatment-related.

3. Urinalysis

In the high dose females, abnormal color of urine was noted at Month 6 (red color in 9/18 animals), Month 12 (orange to dark brange color in 14/20 animals) and Month 18 (orange color in 6/8 animals).

In the male high and mid dose groups, a dose related increase in incidence and severity of cellular casts was observed at Months 3 and 6, in Comparison to the controls

	0					
			🔊 🔊 Maleş			
Dose level of AE	CG\$6948 (ppm) 🖉 🔹	🖉 Control 🖉	/ ~30	150	750
Month 3	~~ a	Slight 🕺	//19	<u>ک</u> 2/19	4/20	0/19
	\mathcal{O}	Moderate,	>>> 0/19	0/19	4/20	6/19
		Markedy	~ 0/49 ~	0/19	2/20	5/19
4	ð	Sevene		0/19	0/20	6/19
	Ô	total 🖉	í syl/19,≪	2/19	10/20	17/19
Month 🚱		_Singht	0/26	0/19	5/20	6/20
L.		Moderate	Ø <u>0</u> 20	0/19	1/20	9/20
	Ô	Marked	∞ ∂⁄20	0/19	1/20	4/20
l c	, ` .	Severe 🖉	0/20	0/19	0/20	0/20
4	A	total 🏹	0/20	0/19	7/20	19/20

Table 5.5-19 Incidence and severity of the presence of cellular casts in urine in males

This effect was temporary seen only after 3 and 6 months), since cellular casts (moderate severity) were observed in only 20 high dose males at month 12, compared to no case in the controls and not observed anymore in this group at months 18 and 24.

No treatment-related findings were noted at the urinalysis at the mid dose level in females or at the low dose level in either sex.

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H. Sacrifice and pathology

1. Terminal body weight and organ weight

Chronic phase

In the female high dose group, mean terminal body weight was lower than the controls ($\sqrt{40\%}$, but statistically significant) but was unaffected at all dose levels tested in males are at the mid and low dose levels in females.

In the high dose groups, mean absolute and relative liver weights were higher by betwee females and by 17 to 18% in males, when compared to the controls.

**	nen compa			9	\sim . (V 💜	\ ⁰	Q U
Sex		Ma	ales 🖉			(Fen	nades 🔊	
Dose level of AE C656948 (ppm)	0	30	150	⁷⁵⁰		8 ⁷ 30 0	150	Å500
Mean absolute liver weight (g)	12.21 ± 1.33	12.70 ± 1.50 (+4%)	(1.05 √ 1.05 (42%)	14.35 * ± 1.92 4(+17%)	7.51±1 0.99	7.35 ±~1.01	7.67 ± 1.43 (+2%)	10.44 ± 1.61 (-99%)
Mean liver to body weight ratio	1.86 ± 0.12	1.90± 618 (+4%)	0.15 (+1°p)	2.19 **± 0.18 (517%)	2.07± 	2.195 ± 0.002	256 0.20 (+4%)	
Mean liver to brain weight ratio	540.35 ± 61.69	552.56 59.71 (62%)	569.79 ± 55.10 (+5%)	€ 5.39 * 87.57 (+18%)	367 \$5 ±	354.69© 2±43.06 (-3%)	378 $1/2\pm 62.80(+3%)$	517.51 ** ± 61.47 (+41%)

Table 5.5-13 Liver weight changes (± SD) at scheduled sacrifice of the chronic phase (% chan when compared to controls)

*: p≤0.05; **: p≤0.01

In the male high dose group mean absolute and relative kidney weights were 28% higher than the controls.

In the female high dose group, mean kidney to body weight ratio was 22% higher than the controls, but this change was attributed to concomitant lower mean terminal body weight and was judged not to be relevant.

Table 5.5 A Kidney weight changes (± SD) at scheduled sacrifice of the chronic phase (% change when compared to controls)

Sex 🔊	· . *	Nh:	rles 淤	Ő A		Fem	ales	
Dose level of A C656948 (ppm)		300	×¥50 €	\$ 75 0	0	30	150	1500
Mean absolute kidney weight (g)	0.92	$364 \pm$ 0.33 (+4%)	∀3.78,±7 0.25 (±8%) ×	4.48,**± Ø.70 (+28%)	2.39 ± 0.24	2.40 ± 0.37 (0%)	2.48 ± 0.37 (+4%)	2.64 ± 0.36 (+10%)
Mean kidney to body	1 ali	$0.55 \pm$ 0.04 (+2%)	0.08 0.08 (+0%)	0.69 ** ± 0.13 (+28%)	$\begin{array}{c} 0.70 \pm \\ 0.08 \end{array}$	0.71 ± 0.09 (+1%)	$0.73 \pm 0.08 \ (+5\%)$	$\begin{array}{c} 0.85 ** \pm \\ 0.08 \\ (+22\%) \end{array}$
Mean kidney to brain weight ratio	155.15 ± 13.34	158.68/± 15,77 (+2%)	17.58 ± 18.90 (+12%)	198.22 ** ± 25.86 (+28%)	117.11 ± 11.71	$115.46 \pm 14.64 \\ (-1\%)$	$122.15 \pm 16.97 \\ (+4\%)$	$131.11 \pm \\ 13.48 \\ (+12\%)$

In the tenale high dose group, mean absolute and relative thyroid gland weights were 23 to 38% higher than the controls. This effect was associated with follicular cell hypertrophy at the microscopic examination.



Table 5.5-15	Thyroid gland weight changes (± SD) at scheduled sacrifice of the chronic phase (%
	change when compared to controls)

Sex		Ma	lles			Fem			
Dose level of AE C656948 (ppm)	0	30	150	750	0	30	150	4500 Č	
Mean absolute thyroid gland weight (g)	0.0272 ± 0.0059	$0.0280 \pm 0.0036 + 3\%$	0.0244 ± 0.0066 (-10%)	0.0295 ± 0.0059 (+8%)	0.0201 ± 0.0052	0.0196 ± 0.0063 √(-2%)	0.0213 ± 0.004 (+@%)	0.0248/± 0.0058 (+23%)	
Mean thyroid gland to body weight ratio	0.0042 ± 0.0009	$\begin{array}{c} 0.0043 \pm \\ 0.0006 \\ (+3\%) \end{array}$	$\begin{array}{r} 0.0038 \pm \\ 0.0012 \\ (-10\%) \end{array}$	0.0045 ± 0.0008 	0.0058 ± 0.001	0.0057 ± 0.0015 (-1%)	0.0063 ± 0.0013 0.0013	0.0080 ± 0.0019 $(\pm 8\%)$	
Mean thyroid gland to brain weight ratio	1.2025 ± 0.2522	1.2226 ± 0.1797 (+2%)	0.3036 (-7‰)	(\$ 9 %) *	0.2479	0.9423 0.2958 (4%)	1.051/2 ± 0.2287, (+7%)	1.2358 - 0.2815 (+26%)	
**: p≤0.01			0,			ST O		A s	•

Carcinogenicity phase

In the high dose groups, mean terminal body weight was 11% lower in females and 7% lower in males, compared to the controls (not statistically significant). Mean terminal body weight was an affected at the mid and low doses in both sexes.

In the female high dose group, mean absolute and relative liver weights were 39 to 56% higher than the controls (statistically significant). This effect was associated with microscopic hepatocellular hypertrophy.

In the male high and mit dose groups, mean absolute and/or relative liver weights were also slightly higher by between 5 to 12%. This charge was also considered to be treatment-related as it was associated with hepatocellular hypertrophy at the microscopic examination.

In the male low dose group, mean absolute and relative liver weights were 6 to 12% lower, but these changes were judged not to be treatment-related in view of their low magnitude and in the absence of any associated change at the gross observation of microscopic examination.

Table 5 5-46	Liver weight changes (± SD) at scheduled sacrific of the carcinogenicity phase (% change when compared to controls)
	above where a manual to contract to a contract of the cartering context phase (//
R.V.	cusulte with combared forcourture)

Sex	- N	<u> </u>	Iale O		A A	Fe	male	
Dose level					<i>(</i> Q)			
of AE C656948))) ~~	C 156	\$\$0/375 %	y 0	30	150	1500
(ppm)	0							
Mean	12.56	11.09*	14.02 *	13,24	9.46	9.69	9.89	13.16 **
absolute	± 1.71	±\$12	± 2.06	\$⊉∕2.25	± 2.19	± 2.12	± 2.12	± 2.87
liver weight	~~	<u>(</u> -12%)	(+12%)	××(+5%)		(+2%)	(+5%)	(+39%)
∠~~(g)	\sim	S" N	Q ^					
Mean liver	2.06	1,94	Q, 2.22	2.32	2.37	2.31	2.52	3.70 **
to body 🍃	$\mathcal{O} \pm 0.33$	±Ø.26 *	$\cancel{9}^{\vee} \pm 0.3 \cancel{9}^{\vee}$	± 0.43	± 0.37	± 0.30	± 0.35	± 0.59
weight ratio		A -6%) [*]	(+8%)	(+12%)		(-3%)	(+6%)	(+56%)
(%)	× .	d v	~9					
Mean lever	\$36.35 ^C	482.19	590.42	573.01	447.20	458.65	466.02	656.49**
to brain	℃±78.%7	⊴© 5.17	± 91.06	± 90.66	±100.55	± 96.36	± 95.77	± 151.35
weight ratio		(-10%)	(+10%)	(+7%)		(+3%)	(+4%)	(+47%)
(%) Q	10	ČÝ.	, ,	``		` '	```	` '
*: p<0.05	< 0.01		•					



2. Macroscopic findings

Chronic phase

a. Unscheduled deaths

Two animals from the chronic phase were found dead before scheduled sacrific

- One control male (PT1M0678) was found dead on study Day 246, with blood at the brain surface and a mottled red thymus.

- One mid dose female (PT3F1032) died during anesthesia on study Day 81, with dark kidneys thymus, dark liver, white foci on spleen and a dilatation of uterine hors.

b. Terminal sacrifice:

Treatment-related findings were found in the live and kidney

In the female high dose group, enlarged liver was found in 9/10 animals, dark liver in 8/10 animals and prominent lobulation on the liver in 4/10 animals, compared to no case in the controls

In the male high dose group, enlarged liver was found in 1/10 animals and pointment lobulation on the liver in 3/10 animals, compared to no case in the controls.

Table 5.5-17	Incidence of mag	ekoscopioc	hanges in th	e liver, sche	diffed sacr	fice of the o	bronic phase

Sex	Males		Females
Dose level of AE	0 30 30 ~	150 750 Q	30 150 1500
C656948 (ppm)			
Obviously large	0/8 0/10 5 0	0/10 1/10 0/1	0,
Dark	0%9 🔬 0/10 🥰 0	(10 ° 0/1	0 0/10 0/9 8/10
Prominent lobulation	≪_0/9 < [™] 0/1@ (₽10 5 3/HQ 654	0 0/10 0/9 4/10

In the female high dose group, dark kidneys were found in \$/10 animals, compared to no case in the controls.

In the male high dose group pale kidneys, enlarged kidneys or fregular surface on the kidneys were found in some animals, compared to no case in the compole.

 Table 5.508
 Incidence of macroscopic changes in the ridney scheduled sacrifice of the chronic phase

Sex N	a V	No Ma	íles 🧷	O s		Fem	ales	
Dose level of AP C656948 (ppm)		30	€ ^{7¥50} €	750	0	30	150	1500
Dark 🖓 🤇	0,0	×110	× 0/10×	€}10	0/10	0/10	0/9	8/10
Pale	0/9	\$0/10 <i>~</i>	0/40	% /10	0/10	1/10	1/9	0/10
Obviously large	, ©0/9 .Q	0/10	@ /10 >	3/10	0/10	1/10	0/9	1/10
Irregular surface 🔌	0/9	0%10	\$1/10\$	3/10	0/10	0/10	0/9	0/10

Carcinogenicity phase

Treatment-related findings were noted in the liver and kidney.

a. Unscheduled deaths: ",

Two hundred and seventy animals died before the end of the study.

In the temale fligh dose group, a higher incidence of enlarged liver, dark liver, white foci or red foci on the there was observed, when compared to the controls. In addition, a liver nodule/mass was noted in one animal and correlated with a hepatocellular carcinoma noted at the microscopic examination.

In the male high dose group, a higher incidence of enlarged liver and white foci on the liver was noted, when compared to the controls.



Incidence of macroscopic changes in the liver-unscheduled sacrifices of the Table 5.5-19 carcinogenicity phase

Sex		Ma	ales			Fen	nales		ð,
Dose level of AE C656948 (ppm)	0	30	150	750/375	0	30	150	Ø\$500	Y
Nodule(s)/masse(s)	0/37	1/44	0/34	0/49	0/19	0/32	0/25 🔌	1/30	
Obviously large	1/37	4/44	1/34	6/49	1/19	₄ 1/32	1/250	230	
Dark	2/37	0/44	0/34	1/49	0/19	1/32	1/23		y .
Focus (i), white	2/37	6/44	4/34	X4 9	3/19	3/32	<i>≸</i> €/25 ∧	9/30	,Ø
Focus (i), red	6/37	9/44	9/34	\$/49	5/1.0	6/32	0 ^{7/25} 5	1430	\$

In the female high dose group, a higher incidence of dark kidneys, enlarged kidneys and irregular Surface on the kidneys was observed, when compared to the controls.

In the male high dose group, a higher incidence of enlarged kidneys and irregular surface on the kidneys was noted.

In the male mid dose group, a higher incidence of onlarged kidneys was also observed Ó

Incidence of macros opic eftanges if the kidney, wischeduled sacrifices of the Table 5.5-20 carcinogenicity phase 47 Ô \approx N

	0 1	. 6		s k		Ň		2
Sex	d	O [×] Ma	ales			Û Fena	ales 🔊	
Dose level of AE	0_0	× ~ ~	0150	750/3275	Å	20 0	\$150	1500
C656948 (ppm)		. 30 °	·0'	130 343		° 30°°	XQU O	1300
Dark	1797	<u>ک</u> 0/44 ک	v 0/3(4,	1/49	§ 0/19	2032	0/25	5/30
Obviously large	j 👰/37 🔍	D" 2/44	694	5/49	0/19	× 2//32 ×	0/25	6/30
Irregular surface	≈3/37	2/44	£4/34 û	× 8/49	1/19	©"2/32©	1/25	7/30
	K. QY	<u> </u>	0 %	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	× 4.	~~ <u>~</u> ~		

b. Terminal saerifice?

A Contraction In the female high to se group, a higher incidence of entarged fiver, dark liver, prominent lobulation and white foci on the liver was found when compared to the controls. In addition in this group, liver nodules/masses were poted and concellated with liver carcinoma or adenoma noted at the microscopic examination. The overall incidence (unscheduled deathsplus terminal sacrifice) of liver nodules/masses was 5/60 high dose females, compared to no case in the controls. Ś

Table 5.5-21	Incidence of macroscopic changes in/the liver scheduled sacrifices of the	
	arcinogenicits phases	

	1	r-w	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~								
Sex Q S D Males & S						Fem	ales				
Dose level of AE C656948 (ppm)				350/375	0	30	150	1500			
Nodules/masses	0/23	0,16	~~0/26~	1/11	0/41	1/28	2/35	4/30			
Obroously large	1/23/	0/16	2/26	0/11	6/41	1/28	7/35	28/30			
Dark 🗸	AQ23 (y 0/1@	ſ% 26	2/11	0/41	1/28	1/35	19/30			
Focus (i), white	~4/23~~~	1/16/	*/26	3/11	14/41	7/28	9/35	22/30			
Prominent lobulation	1/28	Ø /16	∮ 2/26	0/11	2/41	3/28	3/35	13/30			

In the female bigh dose group, a higher incidence of dark kidneys, enlarged kidneys and irregular surface on the kidneys was observed, when compared to the controls. In the male high dose group, a higher rate of enlarged kidneys or inegular surface on the kidneys was noted.



Table 5.5-22	Incidence of macroscopic changes in the kidney - scheduled sacrifices of the
	carcinogenicity phase

Sex		Ma	ales			Fem	ales		Ş
Dose level of AE C656948 (ppm)	0	30	150	750/375	0	30	150	\$ 500	"
Dark	0/23	0/16	1/26	1/11	4/41	1/28	1/35 🐔	17/30	
Obviously large	3/23	0/16	4/26	3/11	1/41	1/28	1/35	~ 6 30	
Irregular surface	5/23	0/16	7/26	8/11	1/41	2/28	3435	 €/30 	
				(CA)	Å	1	sti a		_

3. Microscopic findings

Chronic phase

a. Unscheduled deaths:

One control male was found dead on Day 246; meningeal herborrhage was considered to be the cause of death. One mid-dose female died during anesthesia on Day 81 without any significant microscopic findings explaining the cause of death findings explaining the cause of death.

b. Terminal sacrifice:

Treatment-related non-neoplastic charges and thyroid gland.

Non-neoplastic findings

In the liver of high dose temales, a higher incidence of altered hepatocytes (ecomophilic foci), focal/multifocal hepatocellular vacuolation, increased number of mitoses, bepatocellular single cell necrosis and hepatocellular brown pignonts was noted, when compared to the controls. In addition in this group, centrilobular to panlobular hypertrophy and centrilobular to not zonal hepatocellular macrovacuolation were observed. Hepatocellular macrovacuolation or vacuolation was considered not to be an adverse effect, since it is a coversible change and as no clear associated hepatocellular degeneration (only two animals with minimal to slight single cell necrosis) was established.

In the male high dose group, a high incidence of altere hepatoeytes (eosinophilic foci) was observed. In addition, centrilobular to paplobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were observed. In the male mid dose group, centerlobular to panlobular hypertrophy and centrifobular to midzonal repato ellular macrovacuolation were noted. In the male low dose group, centrilobular to midzonal hepatocellular macrovacuolation was observed. Hepatocellular vacuolation or macrovacuolation poted in males at the three dose levels was also considered not to be adverse, as it was

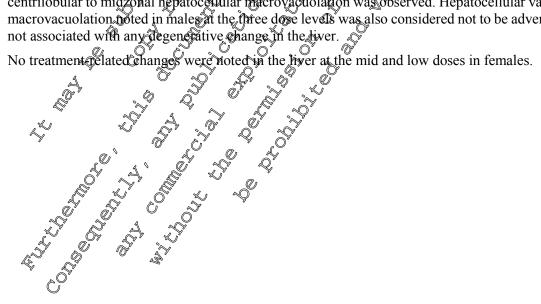




Table 5.5-23 Incidence and severity of microscopic changes in the liver, all animals of the chronic phase

phase								<u> </u>
Sex		Ma	les			Fen	nales	
AE C656948	0	20	150	750	0	20	150	A500 0
Dose level (ppm)	0	30	150	750	0	30	150	01200
Number of examined animals	10	10	10	10	10	ÂÎ O	10 🗳	10
Focus(i) of hepatocellular alter	ation: eos	sinophilic	: focal/mu	ultifocal	4		Q	
Minimal	2	1	2	4		> 0	\$ <u></u>	\$\vee2_
Slight	0	0	0	Ĉ5 1	0	0	$\swarrow 0 \sim$	> 1
Total	2	1	2 🔨	5	ð	0 @	₽ 0 ,⊘°	S /
Centrilobular to panlobular he	patocellu	lar hyper		liffuse	,0×	d d		
Minimal	0	0	<u>s</u>	5	<u>ه کې</u>	QO QO	0>	
Slight	0	0	<u></u>	5	× 83	Ŵ.		2
Moderate	0	0 4	Ø 0	0 5 ⁷	<u>`</u> ~0	~ 0		~87
Total	0	0 🖇	30	10	× 0 ×		Ĩ)	¥10
Hepatocellular macrovacuolati		<u>ilobular t</u>			<u>y</u> 20	Ø,	<u> </u>	
Minimal	0		@ 4 _ (V 5 🖓	0	\$ ⁰		<u>2</u>
Slight	0	$\sqrt{1}$	y 2~y'	<u></u>		0.0 %	<u> </u>	S.Z
Moderate	0	$\mathcal{V} = 0$		<u>~</u> 1	\bigcirc 0 \swarrow		Ĵ,	<u>3</u>
Total	0 0		<u></u> 6	<u>, ° 9</u>	0,0	J.	Į O 🖉	7
Hepatocellular vacuolation: for	al/multif		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		<u> </u>	<u>Č</u>	<u>, v</u>	
Minimal	- Se		00	<u>B</u>			Ž>	3
Slight	$\bigcirc 0$		- Br		$ $	<u>b</u>	<u>%</u> 0	1
Total 🔬	<u> </u>		0		<u> </u>	2	© 2	4
Increased number of mitoses		<u> </u>	jy o	° °)	
Present N	0		<u>Q</u>	0	× 2 💭		1	6
Total 🔬 🦼	\$> 0 Ø	6	~ 9		2		1	6
Hepatocellular brown pigment	(s): focal	/multifoca	k K	<u>, 0</u>		L.Y	1	
Minimal	<u> </u>	0 ×		Ø	0	0	0	1
Total O	<u>``0 ``</u>		<u>`</u>	<u></u>		0	0	1
Hepatocellular single cell necro				- 49	i W	1	1	
Mi@rmal S O	0	% <u>0</u>		x 6-2	Ø	0	0	1
Slight "O"	<i>\$</i> 0		0	0	$\swarrow 0$	0	0	1
Total 🖧 🤿	<u>ک</u> 0 ک	0	O		0 0	0	0	2

In the kidney of high the females, a higher incidence of tubular golden/brown pigments and of hyaline casts was noted, when compared to the controls Ô

In the male high and mad dose group chronic progressive nephropathy was observed, together with a higher incidence of hyaline droplets. Chronic progressive nephropathy is a combination of thickened basement membranes (tubular and glopperular) basophilic tubules and hyaline casts with a variable

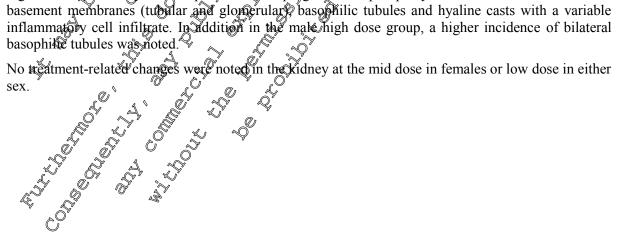




Table 5.5-24Incidence and severity of microscopic changes in the kidney, all animals of the chronic
phase

-								<i>a</i> .°	~
Sex		Ma	ales			Fen	nales		Č,
AE C656948 Dose level (ppm)	0	30	150	750	0	30	150	3 500	7
Number of examined animal	s 10	10	10	10	10		10 🖄		
Chronic progressive nephrop	athy: focal	/multifoca	al		4	0	<i>C</i>		°.
Minimal	0	0	3	1		0		Ø ^v 0 🗸	Q
Slight	0	0	0	Ô 1	04	0	_≪″0 ∧		a
Total	0	0	3	[™] 2	, S	0	0.0	, A	Ś
Intratubular golden/brown p	igments: fo	ocal/multi	focal 🔊		,0×	Ŕ			Õ.
Minimal	0	3	<u></u>	0	× 1 。	20	, l∛	õ 4 ø	1
Slight	0	0	66	0,	× QQ	Q.		1	
Total	0	3 '	1	0	૾ૺૼૼૼૼૻ			Ś	
Hyaline droplets: proximal t	ubules	s and a second s		2		, Q	°∼≯	Ŵ	
Minimal	0	1°	Z.	õ 1 0	v 08	Ø	×0 .		
Slight	0	Á	Ø1 🔪	P 5 🖓	0	~ 0			
Moderate	0	ji i a		A		° 0 🔬	0	S.	
Total	0	V 167	8	×10	O´ 0 🔬	0	ð	00	
Basophilic tubules: bilateral:	focal/mat	ifocat		\$ ®			N N		
Minimal	25	0 4	× 0 ×	3.	Š,		§ 0 🗸	2	
Slight	₩¥	6 0 Q		ð		00	ð≫	0	
Total	3×	4 0	Ŕ	_©6 _	2°2 ⁽¹⁾	A	<u>%</u> 0	2	
Hyaline cast(s): focal/multifo	cal /			×	/* <u>`</u> Q		0		
Minimal			00	1			2 1	4	
Total 🍾	2	Ø 1 🔊	<u> </u>	1	$\sqrt[6]{1}$		1	4	

In the thyroid gland of high dose females and male follies ar cell hypertrophy was noted together with a higher incidence and severity of colloid attention, when compared to the controls.

In the male mid dose group, only follicular cell hyperprophy was observed

No treatment related changes were ooted in the thyroid chand at the mid dose in females or at the low dose in either sex.

Table 5,5 25 Incidence and severity of microscopic changes in the thyroid gland, all animals of the chronic place &

		j 🔊					
Sex Sex	1. NIA	ales 🔍	ð		Fen	nales	
Sex 2 (AE C656948 (Dose level (ppp) (Č 30	ð 150	750	0	30	150	1500
		100	10	10	10	10	10
	ollicular cell	hypertrop	hy: diffu	se			
Minimal V P		2∛∑	2	0	0	0	4
Slight Sight		0	2	0	0	0	1
Total 50	Q 0 S	2	4	0	0	0	5
	Colloi	d alterati	on				
Minimal N D A	× 4	3	2	1	0	0	3
Shight N & 0	_@ 1	1	2	0	0	0	1
	~\$P0	0	3	0	0	0	0
Total 4	5	4	7	1	0	0	4
Total 4							



Neoplastic findings

There was no evidence of a treatment-related effect on the incidence of neoplastic findings in animals of the chronic phase.

Carcinogenicity phase

a. Unscheduled deaths:

Increased mortality observed in the male high dose group was mainly associated with mimals dead (28/49). No clear cause of death could be established for most of them.

b. All animals (terminal sacrifice plus unscheduled deaths):

Major treatment-related effects of AE C656948 were found in the over, kidney, thyroid gland and Ö

Non-neoplastic findings

In the liver of high dose females, marked effects of the treatment indicative of toxicity were noted, including metabolic, degenerative and proliferative changes. They correspond to the exace thation of microscopic findings observed after the chronic phase.

Proliferative changes like altered hepatocytes (eosthophibe foco, clear cell foci, multing leated hepatocytes with anisocaryosis or increased number of mitoses were associated with metabolic morphological changes: centrilobular to panlobular hyperfrephy, Jocal/pultifocal hepatocellular vacuolation, centrilobular to midzonal depatocollular macrovacuolation, bown organity in Kupffer cells or hepatocellular brown plgments. Degenerative change (hepatocellular single cell necrosis) was also noted. This change most likely results from the long term effect of hepatocellular hypertrophy. In addition, minimal to slight extramedullars hematopoiesis was observed in the liver, in this group.

In the male high and mid dose groups, treatment-related effects (seen also in the ternale high dose group) were noted. In a similar way these changes result from exacerbation of findings noted at the end of the chronic phase. In the male high dose group, a higher inchance of altered hepatocytes (eosinophilic foci) was observed. In addition, centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular pracrovacuolation were noted. In the male mid dose group, only eosinophilic foci and centrilobular to panlobular hypertrophy were observed in the absence of any associated degenerative No treatment-related changes were noted in the twer at the mid dose in females or at the low dose in either sex. change in the liver, centrilobular & midzonal hepatocebular macrovaeuolation was considered not to be



Table 5.5-26Incidence and severity of microscopic changes in the liver, all animals of the
carcinogenicity phase

carcinogeni	city phase	2						@°^
Sex		Ma	les			Fen	ales	
Dose level (ppm)	0	30	150	750/375	0	30	150	2500
Number of examined animals	60	60	60	58	60	ð	60	0 ⁷ 59 🔊
Centrilobular to panlobular he	patocellu	lar hyper	trophy: d	liffuse				
Minimal	1	1	14	15	0 4	0	00	
Slight	0	0	0	15	0 🐇	> 0	~ <u>9</u>	\$21 x
Moderate	0	0	0	Ô 0	04	0	×0 ~	21
Total	1	1	14**	30**	ð	0 @	0.0	48**
Focus(i) of hepatocellular alter	ation: cle	ar: focal/	multifoca	1	08	Ŕ	, a l	
Minimal	8	6	JO L	11 🗸	0.	20	3 [♥]	[*] @ 8 گ
Slight	2	2	<u>ð</u> ľ	5	1 <u>1</u>	QÍ,	0 ⁹ 1 a	3
Total	10	8 '	∛ 7 ৢ	1.6	્રેઓ	∞ 4 ∾	<u>4</u> %	} ¶\$**
Focus(i) of hepatocellular alter						<u>í q</u>	~	×
Minimal	12	200	≪ J8	<u>ک</u> 15 ۵	r 23⊖	10	<u>المجامع</u>	25 °
Slight	4		010	12 🖓	₄ 6	~7	^O 15	12
Moderate	0	$\sqrt{1}$	/ 2	ð		° 2 🔊	1.	ST.
Marked	0	$\mathcal{V} = 0$, l	°~~~0 √	O`0 🔬		Ĩ	3
Total	16	24	ુ 🖓 1 * ્ર્	Ş28**ô	29	26	©30 🍦	48**
Hepatocellular vacuolation: for	cal/multif	ocal	× ×		ð		<u>)</u>	·
Minimal	Ň0	Ôg 4 Ôg	130	<u>ð</u>	2 ⁰ 5 2		\uparrow	13
Slight	0 🔊	2 "0"	Ĩ	¢ů á	Q*1 U	Ð	<u>&</u> 2	9
Moderate 🔬	° Q		0			0 ھ	$\bigcirc 0$	0
Total	ÂØ.	ۍ 7 (y 16 🖉	7	~6	~J4**_Q	9	22**
Increased number of mitoses		9 . S	Ś				-	
Present		Ю [°]	~ ⁹⁰ .	\bigcirc 0 $\&$	6	<u> </u>	5	33
Total 🖉 🖉		0	S 0 K	× 0 ○	<u>s</u>	<u>الاي</u>	5	33**
Multinucleated hepatocytes wi		nçyösis 🏾 ^		<u> </u>		, V	r	
Present	γî ×	<u> </u>	N.	590	4~~	2	6	38
Total	1%		<u>_%1</u>	0	4∜`	2	6	38**
Hepatocellular single cell need					<u></u>	1	1	
Minimal	<u></u> \$2		10	1	$\swarrow 0$	3	1	25
Slight 2		° 0 °	J.	0°	0	1	0	12
🕂 🔍 Total 🔍 🔍	<u> </u>		<u> </u>	$ \downarrow 1 0 $	0	4	1	37**
Hepatocellular brown pigment				A "				
Minima	Ø0 K	× 0 0	00°	\mathbb{O}^0	1	1	2	22
Slight		<u>Q</u>	<u>0</u>	\mathcal{O} 0	0	0	0	2
Tetral O' O'				¥ 0	1	1	2	24**
Accumulation of brown pigme			r focalom		0	-	0	27
Minimal	36			5	8	7	9	27
Slight				2	4	3	2	4
Moderate			> 0	0	0	0	0	1
<u>S</u> Total <u>S</u>			10		12	10	11	32**
Hepatocellular macrovacuolati						0	0	5
Minmal ()		~Q,″	0	9	0	0	0	5
Sight Sight	U V	$ \bigcirc 0 $	2		0	0	0	4
Aroderate O		₽ 0	0	0	0	0	0	2
	0	0	2	10**	0	0	0	11**
Extramedullary hematopoiesis			10	15	17	24	21	20
Slight	16	7	10	15	17	24	21	30
Si Siigint 🔍	1	3	1	1	2	1	3	3
Moderate	10	0	0	0	0	0	0	0
Total	18	10	11	16	19	25	24	33**
**: p≤0.01								



In the kidney, marked degenerative changes resulting from exacerbation of the microscopic findings noted at the end of the chronic phase were observed in both sexes at the high dose and in the male at the mid dose. These changes were mainly characterized by an increased incidence and severity of chronic progressive nephropathy. Specific findings (tubular hyperplasia, tubular dilatation or renal cysts) were judged to be associated with chronic nephropathy.

In addition in the female high dose group, an increased incidence of tubular golden/brown pigments and collecting ducts hyperplasia was noted. Brown pigments were also previously noticed in the liver in this group.

In the male mid dose group, a higher incidence of tibular hypertrophycollecting durch hyperbolasis and hyper In the male high dose group, a higher incidence of tubular hypertrophy collecting ducts hyperplasis and hyaline droplets was also noted hyaline droplets was also noted. a,



Table 5.5-27Incidence and severity of microscopic changes in the kidney, all animals of the
carcinogenicity phase

carcinogeni	city phas	e						@-° ^
Sex		M	ale			Fen	nale	
Dose level (ppm)	0	30	150	750/375	0	30	150	1500
Number of examined animals	60	60	60	60	60	ð	59	2 ⁷ 60 🔊
Chronic progressive nephropa	thy: focal	/multifoca	al			- Star		
Minimal	17	24	16	11	17 4	12	110	
Slight	7	8	10	18	3 4	> 7	×Ω,	Q13 X
Moderate	5	2	11	<u>گ</u> 15	04	3	×1 ~	7.8
Marked	1	1	4	8	<i>A</i>	2 @	0 0	3, L
Severe	1	2	14	0	٥Ő	0 🔊	ø	
Total	31	37	.492	52** (20 .	24	° 23	<u>642**_</u> ٢
Tubular hyperplasia: focal/mu			<u></u>	<u>ه</u>		Q,	Ő »	
Minimal	5	1	5	8	^∼2	∞ Š ∧	3	~@*
Slight	0	1 🖇	30	, Â	× 0 ×		Ĩ	\sim_1
Moderate	0	00	~ (°	õ 1 9	7 08	Ø	£0 4	
Total	5	Å.	8	11*	2	£75	0'4	50
Collecting ducts hyperplasia: u		: focal/mu	lifocal			O K	· · · · · ·	
Minimal	2	V 16 Y	30	8.	<u>0 1 %</u> ,	16.5	- Å	6 4
Slight	3	<u>s</u>		∇ 1 \sim	1		ĴÛ 0	2
Moderate	ø	- 00 ⁻	× <u></u> 0 ×		Ŵ			2
Total	- Qi	© 2 ©	50	Â	⁰ 2 °		<u> </u>	8
Tubular hypertrophy: focal/m	ul#ifocal [%]	<u> </u>	- A		$\frac{1}{2}$ - $\frac{1}{2}$		×,	Ũ
Minimal «	5	~&	e 18	17 17 C	10	<u>~</u> 13	O_{11}^{*}	8
Slight	×		6 0	2			2	4
Total	. 9		24*	19**	× 9 ×	16	13	12
Intratubular golden/brown pig	4 -	lken .		O &	<u> </u>		10	
Minimal Q 0	40		£ 4 v	70	251	x 16	17	17
Slight S	- AS			æ		3	7	32
Moderate 0	20 2	$\sqrt[n]{0}$	°,0,°	~ ⁹⁰		0	0	8
Total	4%	3	.44	8	32	19	24	57**
Hyaline droplets: proximal the					 	17	- •	01
Minimal	©0	4 1 0	1	5	№ 1	1	0	1
Slight	S' i	$\begin{array}{c} 1 \\ 7 \\ 7 \\ 0 \end{array}$			$ \begin{bmatrix} 0 & 1 \\ 0 & 0 \end{bmatrix} $	1	1	1
Moderate . O		 0%	0°0 ×	$ \begin{bmatrix} y \\ 1 \end{bmatrix} $	1	0	0	0
Marked	1 %0/		× 0 %	A A	0	0	0	0
Total Total			10	<u>~10</u>	2	2	1	2
Cortical tubular dilatation: for	M/multit		î©		-	4	1	2
Minumal O	<u>an mutu</u>		09	17	4	0	6	7
Stight S	~2		50	11	0	4	6	6
AJoderate			, JO	0	0	1	0	3
Total	2^{10}		<u>~</u> 15	28**	4	5	7	16**
Medullary tubular datation:		tifoxol o	<u>~из</u>	20	4	5	Ι	10
Minimal Solution		$0, 12 \infty$	8	21	11	4	10	9
Slight	0^{3}	γ_{2}^{12}	13	17	2	3	2	11
Moderate ()	× 4 ⊾∼		13	2	0		0	2
Dotal A		<i>Q</i> , 15	1 22*	<u> </u>		1 0		<u>2</u> 22*
		10,15	22~	40^^	13	8	12	22"
Renal cyst(s): focal/multiocal	Ñ 7	× 1	6	12	2	2	2	2
Present U	7	3	6	13	2	2	3	3
*: p<0.905, **: p20.01	7	3	6	13*	2	2	3	3
$\cdot P - v \cdot q \omega$, $\cdot p - v - v \cdot q \omega$, $\cdot p - v - v - v - v - v - v - v - v - v -$								

In the thy foid gland, in the male and female high dose groups, exacerbation of the microscopic findings noted at the end of the chronic phase was noted: follicular cell hypertrophy together with a higher incidence of colloid alteration and a slightly higher number of follicular cell hyperplasia.



At the mid dose, follicular cell hypertrophy was observed in males and a higher incidence of colloid alteration was noted in females.

No treatment-related changes were noted in the thyroid gland at the low dose in either sex.

Incidence and severity of microscopic changes in the thyroid gland, all animals of the Table 5.5-28 carcinogenicity phase

Sex							d X	
			ale	- I			naleÔ	<i>l</i> o ^v
Dose level (ppm)	0	30	150	7,50/375	0	30	× 150 ~	¥1500
Number of examined animals		59	57 4	54	60	60		58
Follicular cell hyperplasia: foc			- A		, Ô [¥]	<u> </u>		£C.Y
Minimal	0	0	<u></u>	2	× 1	10	0×	
Slight	0	0	<i>i</i>	1		Â.	<i>∱</i> √1	2
Moderate	1	0	0 0			N N	0,0	
Marked	0	0 🌾	100°	3		$\frac{0}{2}$	υÿ	~~0
<u>Total</u>	1	00"	Ŵ			201		
Follicular cell hypertrophy: di					Â	0		j I P
Minimal	0							¥4
Slight Moderate								$\bigcirc \frac{3}{1}$
Total				7**		<u>~~0</u>		12**
Colloid alteration			* * 4			0		12**
Minimal	Ø, 18 , «	\$ 5 0			5 0		<u>%</u> 10	17
Slight		/ <u>) //</u>	Ŷ6	20		$-\phi$	\bigcirc 5	17
Moderate			× 4 0	⁴ √ 20 € ≫ 9 ⁴ √			1	3
Marked		\sim \sim						1
Total	A 28 ~	15		Q 39** ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	7	<u>, 0.</u>	16*	36**
: p≤0.05, **: p≤0.01		10			<u> </u>		10	50
f lens degeneration and peri trophy was characterized by od/cones lamina.								



Table 5.5-29 Incidence and severity of microscopic changes in the eye, all animals of the carcinogenicity phase

								<u> </u>	F
Sex		M	ale			Fen	nale) D
Dose level (ppm)	0	30	150	750/375	0	30	150	1500 0	Ť
Number of examined animals	60	60	60	59	60	ð	60	Ø ⁷ 60 🔊	
Retinal atrophy: bilateral: diff	use						Ž	.\$	
Minimal	0	0	0	0	0 4	0	00		2
Slight	0	0	0	0	0 🏑	> 0		Ø14 🗸	2
Moderate	0	0	1	Ĉ 0	0	0	,≪″0 ~	× 19	
Marked	0	0	0	0	ð.	0 @	$\bigcirc 0 $	× 19.5 2	1
Total	0	0	14	0	၂၀၇	0 🖉	b	27** 0	C
Lenticular degeneration : focal			Å	L	<u>)</u>	L.	.ſ	Ô, Ő	ÿ
Minimal	2	0	ÓÓ	0,	2	Ŵ.	0^{1}	2	
Slight	0	0 `	∞ 0 ⊗	201	<u>`</u> ~0	a 2 a			
Moderate	0	0 📡	10		$ \sum_{i=1}^{\infty} 0 \ll $		27	~3	
Marked	0	10	×)		r 18	()) [×]			
Total	2	A .	Ø1 🔍	2 🖓	₄ 3	<i>2</i> 3	⁰ 4 🖉	98	
Peripheral retinal atrophy: bila	ateral	2. ~		, D	\mathcal{L}	O K		S.	
Minimal	1	$\mathcal{V} = 0$			O`3 🔬		Ĵ,	3	
Slight	0 🔗	10, V	. \$0 <i>;</i>	S 0 @	0,0			2	
Moderate	Ø	Ø	<i>∞</i> 0 <i>∞</i>		ð		\$°0,≪°	2	
Total	٣¥.	\$ 0 \$	0,0	ð,	⁰ 3 ⁽¹⁾		1×	7	
*: p≤0.05, **: p≤0.01	\$ `*	Ĵ O	- P		<u>5, 0</u>	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	×		

Some indirect effects were noted in the tests and stomach:

In the testis, a higher incidence of arteritis/perior teritis was noted in the matchingh and mid dose groups, when compared to the controls (15/59) and $\mathcal{B}/60$, respectively, kersus 8/60 in controls, p ≤ 0.05). This vascular change was solated not found in sensitive tissues like aorta mesenteric arteries) and is most likely explained by secondary hypertensive changes due to increased severity and incidence of chronic nephropathy. Therefore, this change was considered not to be a direct effect of the treatment.

In the stomach of high doce males, a kigher incidence of regenerative non glandular hyperplasia: focal/multifocal (10958 versus 6/58 in controls), non grandular erosion: focal/multifocal (7/58 versus 3/58 in controls), submucosal edema (00/58 versus 4/58 in controls, (p≤0.05) was noted. These minor changer were mainly observed in animals found orematurely dead and were attributed to secondary stress due to morbidity. Therefore, their increased incidence was clearly linked to the increased mortality rate in this male high dose group

Neoplastic findings

At the end of the carcinogenicity phase, a higher incidence of tumors in the liver (carcinoma and adenoma) was noted in the female high dose group only, in comparison to the controls.

These findings were associated with non neoplastic/preneoplastic changes and were seen at a dose causing marked hepatocellular toxicity.

There was no evidence of a treatment-related increased incidence of tumors of any type in any other organ.



Incidence of microscopic neoplastic changes in the liver, all animals of the Table 5.5-30 carcinogenicity phase

	, F	-						e ° `	
Sex		М	ale			Fen	nale		
Dose level (ppm)	0	30	150	750/375	0	30	150	1500	
Number of examined animals	60	60	60	58	60	ð	60	Ø ⁷ 59 🔥	
M-Hepatocllular carcinoma							~		
Incidental	0	0	0	0	0 4	0	20		
Total	0	0	0	0	0	0	~ 2		
B-Hepatocellular adenoma			-	Ò	a la		× ×		, ,
Incidental	2	1	2 1	1	ð.	2 @	$\bigcirc 0 $	& 4	<i>y</i>
Total	2	1	24	1	°2	2 🖉	ø	9 * 0 ⁰	
Hepatocellular adenoma + car	cinoma		A C	Å	Ĵ	d'	.ſ	Č "Q [*]	
Total	2	1	Ĩ Ž	1	2	Q.	õ [×] 2 💦	11+	
+ One animal had both adenoma and	carcinoma		×	<i>W</i>	, N			ŝ	
*: p≤0.05, **: p≤0.01		s.	, Q		S &	ĭ "Ö	"M	*	
		O	, Ø	en la companya da companya d	.y ~0	1 and the second se	ſ	4	

I. Deficiencies

None

III. Conclusions In conclusion, there was a treatment-related increased incidence of lover cell tumors (careinoma plus adenoma) in the female high dose group only at the end of the carcinogenicity phase, in comparison to Ľ 0 the controls. Õ Ŗ °

Over a 24-month period of dietary administration with AE C656948 to the Westar pat, 30 ppm was the NOAEL in males (equivalent to P20 mg/kg body weight/day) and temales (equivalent to 1.68 mg/kg body weight/day). Ó \bigcirc

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 383/2003.

In conclusion there was a treatment-related increased incidence of liver cell tumors (carcinoma plus adenoma) in the female high dose group only at the end of the carcinogenicity phase, in comparison Ø Over #24-month period of detary administration with AE C656948 to the Wistar rat, 30 ppm was

the NOAEL in makes (equivalent to 1.20 mg/kg body weight/day) and females (equivalent to 1.68

the second secon



Data Point:	KCA 5.5/02
Report Author:	
Report Year:	2007
Report Title:	AE C656948, Carcinogenicity study of AE C656948 in the C57BL/6J mouse by
-	dietary administration
Report No:	SA 05094
Document No:	<u>M-295688-01-1</u>
Guideline(s) followed in	OECD 451 (1981); EEC Directive 88/302/EEC – Annex V - Method B.32.
study:	(1987); EPA Health Effects Test Guideline (OPPT \$ 870.4200; 1998); M:AF.F.
	in Japan notification 12 Nousan 38147 (2000) goldelines.
Deviations from current	current guideline: 0ECD 451, 2018
test guideline:	Deviation: No significant deviations.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2921).
GLP/Officially	Yes, conducted under GD Officially recognise desting facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes A A A A A A

Executive summary

Groups of 60 male and 60 female C\$7BL/67 mice were fed diet containing 0, 39, 150 ppm of AE C656948 (Mix-Batch 08528/0002) for 52 weeks. After 52 weeks, 10 male and 10 females from each group allocated to the chronic plase of the study were necropsied ache schedules interim sacrifice. The remaining 50 animals/sex/group, allocated to the carcinogenicity phase of the study, continued treatment until the scheduled final sacrifice of the study after at least 78 weeks of treatment, the mean intake of AE C656948 over 18 months was 0, 42, 20, and 105 mg/kg/day in males and 0, 5.3, 26.8 and 129 mg/kg/day in females, at, 0, 30, 430 and 750 ppm, respectively. Mostality and cline al signs were checked daily. Additionally, setailed physical examinations including palpation for masses were performed weekly throughout reatment. Body weight and food consumption were measured weekly for the first 13 weeks of the study, then monthly thereafter. Hematology determinations were performed at approximately Q and Q months from designated animals. Where possible, blood smears were prepared from moribund animals just before sacrifice. All animals were subjected to necropsy, with selected organs weighed at scheduled interim and final sacrifice. Designated tissues were fixed and examined [°] microscopically. S Ô

There was no treatment-related effect on $\hat{\mathbf{D}}$ or taking, clinical signs or food consumption at any dose level tested.

At 750 ppm

Mean body weight in males was comparable to controls from Week 1 to 26. Between Weeks 30 (study Day 204) to 58 (study Day 400) of the study mean body weight was reduced by up to 5%, and mean cumulative body weight gain by up to 13% over this period from the start of treatment. The effect was statistically significant at most time point during this period ($p \le 0.05$ or $p \le 0.01$). Thereafter, mean body weight and mean cumulative body weight gain were comparable to controls until the end of the study. Body weight parameters were not affected in females at 750 ppm. Slightly higher mean platelet counts were noted in males at Moran 13 ($\pm 25\%$, $p \ge 0.01$) and Month 19 ($\pm 22\%$, $p \le 0.01$), when compared with the controls

At the chronic phase sacrifice 12 months), mean absolute and relative liver weights were increased by between 17 to 25% (p \leq 0.01) in males and 28 to 31% (p \leq 0.01) in females, whilst mean absolute and relative kidney weights were decreased by between 11 and 17% (p \leq 0.05 or p \leq 0.01) in both sexes. At the macroscopic examination, enlarged liver was observed in 1/10 males and 2/10 females. A microscopic examination was performed on the thyroid gland of males, as a higher incidence of follicular cell adenomas was observed in males at this dose level, to determine if there were any



precursory neoplastic changes in the thyroid gland after 12 months of treatment. Thyroid gland follicular cell hyperplasia was seen in 2/10 males.

No treatment-related cause of death was established for the few animals allocated to the carcinogeneticity phase (18 months) of the study which died or were humanely sacrificed before the end of the study.

At the 18-month terminal sacrifice of the carcinogenicity phase of the study, mean absolute and relative liver weights were increased by between 27 to 31% ($p \le 0.01$) in males and 35 to 38% ($p \le 0.01$) in females. In addition, mean kidney to body weight ratio in females was 5% ($p \le 0.01$) hower than the controls. At the macroscopic examination, enlarged liver was observed in 3/42 males and 30/38 females, and dark liver was noted in 14/42 males and 4/38 females. These findings were correlated with relevant histopathological findings. At the microscopic examination of animals allocated to the carcinogenicity, phase (decedents and terminal sacrifice animals), treatment-related changes were seen in the liver, kidney and thyroid gland. The only neoplastic charge consisted of a higher in dence of follicular cell adenoma in the thyroid gland in males (7/50), compared with the control group (150), this effect was statistically significant (p≤0.05). Non-neoplastic charges were seen in the liver, kidney and thyroid gland. In the liver, centrilobular to panlobular hypertrophy was observed in both sexes. Centrilobular hepatocellular vacuolation decreased markedly with concomitant minimal to moderate hepatocellular hypertrophy in males. In addition in males, higher incidences of repatocellulat, cholestasis, hepatocellular single cell degeneration necrosis, interstitial mixed cell infiltrate cosinophilic inclusion bodies and multinucleated hepatocytes were noted. In fetrales, a higher incidence of cosinophilic foci of altered hepatocytes was observed. In the kidney, a higher incidence and/or severity of bilateral cortical basophilic tubules, hyaline casts(s) and interstitial mononuclear cell infiltrate, together with a higher incidence of glomerular congestion/hemogrhage(s), associate@ with higher seventy of amyloid deposition (mainly observed within glomerular interstition) was noted in females. In the thyroid gland, a markedly higher incidence of follicular cell hyperplasia was noted in both sexes

At 150 ppm

At the chronic phase sacrifice (12 months), mean absolute and relative liver weights were increased by between 11 to 15% ($p \le 0.05$ or 0.01) in males and 17 to 20% ($p \le 0.05$ or 0.01) in females. At the macroscopic examination, enlarged fiver was observed in 2/10 males. At the microscopic examination of the thyroid gland, follicular cell hyperplasia was seen in 2/9 males.

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At the 18-month terminal sacrifice, mean absolute and relative liver weights were increased by between 14 to 15% ($p\leq0.01$) in males and 15 to 17% ($p\leq0.01$) in females. At the macroscopic examination, enlarged liver was observed in 7/40 females. At the microscopic examination of animals allocated to the carcinogenicity phase, treatment related changes consisted of centrilobular to panlobular hypertrophy observed in the liver of both spices and a higher incidence of hepatocellular single cell degeneration/necrosis in males, together with a higher incidence of follicular cell hyperplasia observed in the thyroid gland of males.

At 30 ppm

No adverse treatment related effects were observed at this dose level. The only treatment-related finding was an increase in mean absolute and relative fiver weight of between 6 to 8% ($p \le 0.05$ or 0.01) in males at the 18-month terminal sacrifice. In the absence of additional related microscopic changes, this finding was considered to be non-adverse at this dose level.

In conclusion, dietary administration of AE C656948 over an 18-month period to the C57BL/6J mouse, at a dose level of 350 ppir (equivalent to 105 mg/kg/day in males and 129 mg/kg/day in females) resulted in a higher incidence of folk calar cell adenoma in the thyroid gland in males.

Non-neoplastic changes were observed in the liver and thyroid gland in both sexes and in kidney in females at 750 ppm. At 150 ppm (equivalent to 20.9 mg/kg/day in males and 26.8 mg/kg/day in females), non-neoplastic changes were seen in the liver in both sexes and in the thyroid gland in males.



The NOAEL was 30 ppm in males (equivalent to 4.2 mg/kg/day) and females (equivalent to 5.3 mg/kg/day)



The dose tevels were selected based on the results from previous 28-day dietary study in the mouse (M-088486-01-1) and 90-day dietary study in the mouse (M-251136-01-1). In the 28-day study, a dose level of 5000 ppm clearly exceeded the Maximum Tolerated Dose (MTD) as all males and 3/5 females were sacrificed for humane reasons. At 1000 ppm, hypertrophy of the zona fasciculata was seen in the adrenal glands of females and minimal to moderate hypertrophy of centrilobular hepatocytes was observed in all animals in both seves. At 150 ppm, only minimal to slight hypertrophy of centrilobular hepatocytes was considered to represent the NOAEL. In the 90-day study, a dose level of 1000 ppm induced minimal to moderate hypertrophy of centrilobular hepatocytes in all animals, whilst a dose level of 150 ppm induced minimal to slight hypertrophy of centrilobular hepatocytes in all animals to slight hypertrophy of centrilobular hepatocytes in all animals of some females. At 30 ppm, there were no adverse effects observed in either sex. Therefore, a dose level of 150 ppm was considered to represent the NOAEL.



3. Animal assignment

On the day of randomization, animals were allocated to dose groups using a computerized randomization procedure that ensured a similar body weight distribution for each sex. The acceptable body weight range for each sex was $\pm 20\%$ of the mean body weight on the day of randomization. Any animal defend unsuitable for selection based on weight, clinical findings or health status was not used for the study

Animals were assigned to the test groups noted in the following table, Control animals received untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Group (ppm) (mg/kg/day) Male • Female Male • Female Male • Female • Female </th <th>Table 5.5-</th> <th>31 Study</th> <th>y design and</th> <th>group sizes</th> <th></th> <th>Š^e do se se</th> <th>\$ 5° \$</th>	Table 5.5-	31 Study	y design and	group sizes		Š ^e do se	\$ 5° \$
$\begin{array}{c c c c c c c c c c c c c c c c c c c $		Concentration	dose	28 °m	veeks 🥎 .	Sa w	eeks Q
(1) 0 0 0 50 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	Group	(ppm)	(mg/kg/day)	Male	° Female 🔬	Male 🔊	· Female
Animal identity 2448 2508 2398 2458 Low (2) 30 M: 4.2 F: 5.3 50 50 10 10 Animal identity PT2M2519to2568 PT2F2579 to PT2M2509to2578 PT2F2569 to 2578 Mid (3) 150 M 20.9 F: 26.8 50 50 10 10 Animal identity PT2M26264 to PT3F2699 to PT3F2629 to PT3F2689 to 2578 Mid (3) 150 M 20.9 F: 26.8 50 50 50 10 10 Animal identity PT2M26264 to PT3F2699 to PT3F2629 to 2638 2698 High (4) 750 M 405 F: 129 50 50 10 10 Animal identity PT4M2759 to PT4F2809to2868 PT4M2749 to PT4F2809to2818		0	0	50			
Low (2) 30 F: 5.3 20 30 F: 6.8 70 70 710 70 710 Animal identity PT2M2519to2568 PT2F2579 to PT2M2509to PT2M2509to PT2M2509to 2578 Mid (3) 150 M.20.9 50 50 50 10 10 Animal identity PT2M2624 to PT3F2699 to PT3M2629 to PT3F2689 to 2698 High (4) 750 M.405 50 50 50 10 10 Animal identity PT3M2624 to PT3F2699 to PT3M2629 to 2638 2698 2698 High (4) 750 M.405 50 50 50 10 10 Animal identity PT4M2759 to PT4F289402868 PT4M2749 to PT4F2809to2818		Animal identit	у	PT 1 1 2399 to			
Mid (3) 150 M 20.9 50 50 10 10 Mid (3) 150 F. 26.8 (F. 26.8 (Constraint)) PT3M263 to 268 (Constraint) PT3F2699 to 268 (Constraint) PT3F2689 to 268 (Constraint) PT3F2689 to 2698 (Constraint) PT3F2689 to 2698 (Constraint) PT3F2689 to 	Low (2)	30	E. 5 2	8 59 . S	\$50,00		
Animal identity PTPM2624 to 2688 PT3F2699 to 248 PT3F2629 to 2638 PT3F2689 to 2698 High (4) 750 M 905 F. 129 50 50 10 Animal identity PT4M2759 to PT4F289 to2868 PT4M2749 to PT4F2809to2818		Animal identit	y Q	PT2M2519to2568		₱T2M2\$09to2\$18	
Animal identity 2685 748 2635 2698 High (4) 750 M 05 50 750 750 10 Animal dentity 905 50 750 905 10 Animal dentity 905 904 904 904 905 904 <t< td=""><td>Mid (3)</td><td>150</td><td>M:≪20.9 F: 26.8 ‰</td><td></td><td></td><td></td><td>10</td></t<>	Mid (3)	150	M:≪20.9 F: 26.8 ‰				10
Animal Sentity PT4M2759 to PT4F2859to2868 PT4M2749 to PT4F2809to2818		Animal identit			ρ FIJFZQ99 $IQ_{/}$		
Animal Sentity PT4M2759 to PT4F28, 9to2868 PT6M2749 to PT4F2809to2818	High (4)	750			50 0	or to	10
		Animaljuentit		PT4M2759 to 2808	DI 4F28 9to2868	PT#M2749 to	PT4F2809to2818

Table 5.5-31	Study design and group sizes
1 abic 5.5-51	Study design and group sizes

4. Diet preparation and analysis

AE C656948 was incorporated into the diet or provide the required dietary concentrations. The test substance formulation were prepared approximately every were weeks. When not in use, the diet formulations were stored at ambient temperature?

The stability of the test substance in the diet was demonstrated in a previous and recent 28-day rat study (M-085510-01-1), where AE \$6569 W was found to be stable in the diet at 20 and 10000 ppm over a 105-day period at ambient temperature, and also for 95-days when stored frozen and then kept for 10 days at ambient temperative.

In the present study the homogeneity of the test substance in diet was verified at 30, 150 and 750 ppm on the first formulation and at 30 and 750 ppm on the sixth formulation, to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as measured concentration. In addition, the concentration of each dietary level was verified prior to administration to the animals for the following dorm fations: \$1, F3, F6, F9 and F10 (at 30 and 750 ppm only).

5. Statistics

Means and standard deviation were calculated for each group and per time period. The Bartlett test was performed to compare the bomogeneity of group variances.

If the Bartlettest was not significant (p>0.05), means were compared using the analysis of Dariance (ANOVA), which was followed by Dunnett test (2-sided) if ANOVA indicated significance.

- If the Bartlett test was significant ($p \le 0.05$) (for body weight change parameters, terminal body weight, absolute and relative organ weight parameters, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, % neutrophils, % lymphocytes), group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test p^2 -sided) if the Kruskal-Wallis test indicated significance.

- If the Bartlett test was significant ($p \le 0.05$) (for body weight and overage food consumption/day parameters), data were transformed using the log transformation. If the Bartlett test on log transformed data was not significant (p > 0.05), means were compared using the ANOVA on log transformed data, which was followed by the Dunnett test (2-sided) on log transformed data if ANOVA indicated significance. If the Bartlett test was significant ($p \le 0.05$) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunnet test (2-sided), if the Kruskal-Wallis test indicated significance.

- If the Bartlett test was significant ($p \le 0.05$) (for kematology parameters such as red food cell count, platelet count, white blood cell count, neutrophil count, lymphocyte count) data were transformed using the square root transformation. If the Bartlett test on square root transformed data was not significant ($p \ge 0.05$), means were compared using the ANOVA on square root transformed data, which was followed by the Drunett test (2-steed) obsquare root transformed data, if ANOVA indicated significance.

- If the Bartlett test was significant $(p \le 0.95)$ exer after square root ransformation, group means were compared using the pon-parametric Kruskal-Wallis which was followed by the Dunn test (2-sides), if Koiskal-Wallis est indicated significance $\sqrt{2}$

If one or more group variance(s) equaled 0 means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance statistical analyses were carried out using Path/Tox system V4.2.2 (Module Enhanced Statistics).

For survival analysis:

Adjusted mortality rates were estimated using Raplan Meier estimation procedures. Kaplan-Meier estimates were calculated separately for each sex and treatment group. Mortalities which were the result of animals dying following accidents (accidental mauma died during anesthesia or killed at Study Director request) or at scheduled sacrifice were considered to be censored observations.

Statistical significance of differences in survival rates between treated and control groups and doserelated trend in survival were assessed using Cox's and Farone's tests on life table data. Probability values presented were two-aded for pairwise comparisons and trend test. Group mortality rates were compared at the 5% and 1% toyels of significance. Survival analyses were performed on the carcinogeneity phase.

For neoplastic and non-neoplastic findings

When the number of lesion-bearing animals was less or equal to 1 in one group and equal to 0 in the other groups, no statistical test was performed.

When the inordences of the 30 and 50 ppm treated groups were equal to 0, only the 750 ppm treated group was compared to the control group and no trend test was performed.

Not adjusted and yses;

Selected lesions were analyzed by Cochran-Armitage method for trend (1-sided) and the Fisher's exact test (1-sided) for control versus treatment comparisons. Cochran-Armitage trend significance was reported when there was existence of any monotone response in the incidence data (that is there was a relationship between the response and the dose represented by a regression line that was continually increasing (or decreasing), but perhaps not in a straight line).



Survival adjusted analyses:

Further survival adjusted analyses, considering any possible intercurrent mortality differences due to the competing toxicity among the treated groups, were performed on lesions. For non-palpable tumours, were performed on lesions to an intercurrent mortality differences due to the animal, incidental otherwise.

Incidental tumours and non-neoplastic lesions data were analyzed by logistic regression of tumour prevalence. Logistic regression analysis was based on the assumption that the diagnose lesions were not directly responsible for the animal's death. Treated and control group lesion rates and dose-related trends were compared using the corrected score test. Fatal tumours were analyzed by the life-table test. The life-table test was based on the assumption that all lesions were fatal. Statistical significance of differences in incidences between treated and control groups and dose-related trends were investigated using Cox's and Tarone's tests.

The reported results reflect 1-sided testing.

Group incidences were compared at the 5% and 1% levels of significance. All finding analyses were performed on the carcinogenicity phase.

C. Methods

1. Observations

Animals were checked for moribundity and mortality twice daily once daily on weekends or public holidays). Animals were observed for chinical signs of least once daily. Detailed physical examinations including palpation for masses were performed weekly throughout the study.

2. Body weight

Each animal was weighed at least weekly during the acclimatization period ther weekly for the first 13 weeks of study, approximately every 4 weeks thereafter and prior to becropsy. Body weight recorded prior to necropsy are referred to asterminal body weight.

3. Food consumption and compound intake

Food consumption was recorded weekly during the first 73 weeks of the atment, and once approximately every 4 weeks thereafter. The weekly mean achieved dosage atake immg/kg body weight/day for Weeks 1 to 13, then 1 week per month thereafter was calculated as follows:

Test substance intake = $\frac{\sqrt{2}}{3}$ Dose level (ppm) × Group mean food consumption (g/day) (mg/kg/day) Group mean body weight (g) at the end of the food consumption period

The monthly and overall mean achieved do sage intake for the 18 months of treatment were derived from the weekly data.

4. Hematology

Henatology was performed on all the serviving animals of the interim sacrifice groups and on the first ten surviving animals of the terminal sacrifice groups on Weeks 53 or 54.

Hematology was performed on the first twenty surviving suitable mice of the terminal sacrifice groups on Weeks 55 or 54 and prior to necrops on Week 79 or 80. The following parameters were measured: hematocrif hemoglobin reukocyte count, erythrocyte count, platelet count, leukocyte differential count, mean coopuscular hemoglobin mean corpuscular hemoglobin concentration, mean corpuscular volume.

A blood smear was prepared and stained with Wright stain or May-Grünwald-Giemsa stain. It was examined only when the results of Advia 120 determinations were abnormal.

For moribund and terminal sacrificed animals, the blood smears were stained with Wright stain or May-Grünwald-Giemsa stain for possible differential white blood cell determination.



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5. Clinical Chemistry

Not evaluated in the present study.

Not required for carcinogenicity studies based on Guideline 870.4200 & OECD 451.

6. Urinalysis

Not evaluated in the present study.

Not required for carcinogenicity studies based on Guideline 870.4200 & DECD 451

7. Sacrifice and pathology

Contraction of the second seco On study Days 366 to 368 for the 12-month internal kill, and on study Days 549 to 562 for the carcinogenicity phase, all surviving animals dedicated to the interim sacrifice group and carcinogenicity phase group, respectively, were sacrificed by oxsanguination under deep anesthesia (foofluratie). Animals were fasted overnight prior to sacrifice,

All animals, including animals at scheduled sacrifice, found dead or sacoficed during the course of the study, were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. All significant macroscopic findings were recorded

The following organs or tissues were sempled and/or weighed at accrons

I ne I	ollowing organs or tissues v	vere sa	mpled and/or weighed at he	cropsy:	
	Digestive system	Å	Martin Andrew Weighed at act Cardiovasc. / Hemat. Aorta thora act Heart*+ Bore marrow*, sternum Lymple node*,	ð	Neurologic 🗸
Х	Tongue	×	Aortagthoracite* 6	© √xx %	Brain B sections)*+
v	Submandibular	XX	Heart + 🕉 🖉 🤤		Julians Sections)
Λ	(salivary) gland 🛷	X ″	Bone marrow*, sternum	ХŶ	Sciatic nerve*
Х	Esophagus* 🔊	X	"Jymplenode*,"	X	Spinal cord (cervical,
Х	Stomach*	(mesenteric 🗸 💦	~~~ √	thoracić, lumbar)*
Х	Stomach* Duodenum* Jejunum*	v	Lymph node,	X	Eyes (retina)*
Х			submax aray 🔊	X	Optic nerves*
Х	Ileum* 🖉 🖌 🧏	ØXX,	Spleen*+	C,	
Х	Ileum* Cecum* Colon*	X XX XX XX	Vrogenital Kidney*+ Urinary baddet Epidadymist	, "Ş	^y Glandular
Х	Colon O	Ő		X	Pituitary gland*
Х	Rectum* 🔗 🗸	Ča.	Urogenital S	XX	Adrenal gland*+
XX	Rectum* 7 Liver*+ Gall bladder* Pancreas*	۶ XX	Kidney*+ Urinary Hadder	ЪХ	Parathyroid gland*
Х	a 🖓 🖓 🖓	X	Urinary Badder	X	Thyroid gland*
Х	Pancreas* 5	«ХХ	Festis**	Х	Harderian gland
		XX	~EpidiaTymis	\wedge	I commol avarbital aland
	Respiratory Trach@*	X	Prostate gland*		Lacrymal exorbital gland
Х	Trach@* 0°	.X	Sominal		
Х	Gall bladder* Pancreas* Respiratory Trachæ* Lung*+ Pharynx*		Övary 4 🗞		Other
\wedge	Pharynx*	XX	Uterus (with cervix)*+	Х	Bone (sternum)
\wedge	Larynx* 🔍 📿	X	Mammary gland*	Х	Skeletal muscle
^	Lung*+ Pharynx* Earynx* Nasal cavitres*	X	Mannary gland*	Х	Skin*
A		`0' ∀ '		Х	All gross lesions and
		, Ø		Λ	masses*
	A A Q'	, S	Q°.	v	Articular surface
		·~~ ./	Ø	Х	(femorotibial joint)
*					· · · ·
+	organ weights required for roder	nt studies	5		
Х	tissues were collected for histol	ogical ex	aminations		

organs were weighed fresh at scheduled sacrifice only, with paired organs weighed together, and collected for histological examination

- organs were preserved for possible micropathologic examination only



Two femoral bone marrow smears were prepared from sacrificed animals (except on weekends and public holidays), one of which was stained with May-Grunwald Giemsa, but not examined. The second smear was stored unstained.

Samples were fixed by immersion in neutral buffered 10% formalin with the exception of the second and optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson fixative.

II. Results and discussion

A. Mortality

There were no treatment-related mortalities at any dose level in either set during the students at any dose level in either set during the students at any dose level in either set.

During the first 53 weeks of the study, the mortality rate was low with no indication of a treatment related effect.

These animals are included in the following table but were consored in the statistical analysis of the survival rate.

During the whole study period (at least 38 weeks), the mortality rate in animal allocated to the carcinogenicity phase of the study was very similar between the treated and control groups, with no indication of a statistically significant effect.

Table 5.5-32	Mortality - Uns	cheduledates	aths

Dose group (ppm)	First 53 weeks (np to study Day 370) All animals (n=60)	phase animals 50)
	Male Male Male Male	Female
0	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	11 (22.0%)
30	3 3 3 3 3 3 3 3 3 3	13 (26.0%)
150	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	10 (20.0%)
750	₹ (3.3%) (3.3%) (8.3%) (8.3%) (16.0%) (16.0%)	12 (24.0%)

Percentage prortality in pare theses at the set of the

B. Clinical observations

There were no treatment-related clinical signs observed of any dose level in either sex throughout the course of the Gudy.

C. Body weight

At 750 ppm in males, mean body weight was comparable to controls from Week 1 to 26. Between Weeks 30 (study Day 204) to 58 (study Day 400) of the study mean body weight was reduced by up to 5%, and mean cumulative body weight gain by up to 13% over this period from the start of treatment. The effect was statistically significant at most time points during this period ($p \le 0.05$ or 0.01). Thereafter, mean body weight and mean cumulative body weight gain were comparable to controls until the end of the study.

Body weight parameters were not affected in females at 750 ppm or in either sex at 150 or 30 ppm.



Table 5.5-33	Mean body weight (± SD) (BW) and cumulative body weight gain (BWG) (g)
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Dose group (ppm)	0	30	150	750 _ 🖉 °
Males	-			
Initial BW (Day 1) (%C)	20.8 ± 1.1	21.1 ± 1.0	20.8 + 0.9	20.7 1.1
		(101)	(100)	~(100) ~
BW Week 2 (Day 8) (%C)	21.9 ± 1.1	21.9 ± 0.9	22.0 ± 1.1	21.7 ± 121
		(100)	(100) 🗞	0° (99) «
BW Week 14 (Day 92) (%C)	27.4 ± 1.5	🗳 27.9 ± 1.3 🧳	≠ 27.9 *± 1.1	275 ± 1.1
	ÿ	(102)	(102)	\$(100)
BW Week 26 (Day 176) (%C)	28.8 ± 3.8	29.4 ± 1,6	29.1 + 1.5	Q28.4⊕1.3
		(102)	<u>(</u> 691) ((99)
BW Week 54 (Day 372) (%C)	34 ± 1.9	31.5 ± 1.4	30.8 ± 1.7	29 8 ** + 9.3
		S(101)	K) (98)	× (95)
Final BW Week 78 (Day 540) (%C)	931.8-1.7	32.2 ± 1.6	31.6@ 1.8 L	314 ± 1.8
			(499) Qalaz	
BWG Weeks 1-2 (Days 1 to 8) (%C)	$\gamma = 0.0$		• 4.2 ± 0.7	
	6:5,≇0.9 <	°~7 (84) 7 6,8 ≇0.8 ~ ~		
BWG Weeks 1-14 (Days 1 to 92) (%C	0.2 + 0.3 %		$(109)^{-1}$	× (105)
	@1.5 ± 1	(103) (105 + fin		$0.8 ** \pm 0.9$
BWG Weeks 14-26 (Days 92 to 176) (%		(190) C	(73)	(53)
DWC Weeks 26 54 (Dave 176 to 2722) () ()	25 ± 0.80		*L**±008	$1.3^{**} \pm 0.9$
BWG Weeks 26-54 (Days 176 to 372) (%C)		(84) [×]	× (72)	(52)
BWG Weeks 54-78 (Days 372 to 340) (%C)	0.3 0.8 .	0° 0.7 4 1 1	08 0.9	1.3 ** 0.9
		(233)	S(267)	(433)
Overall BWG (Days 1 to 540) (%)	10.9 ± 04	A.1 ± 1.4	@10.8 ± 1.6	10.4 ± 1.5
		^{مَ} (102) لَ	S (99)	(95)
Females & A O O &		× 2° a,		
Initial BW (Day 1) (%C)	17.9 ± 0.9	17.7 ± 0.8	17.7 ± 0.9	17.6 ± 0.9
		(99)	(99)	(98)
BW Week 2 (Day 8) (%)	18.9± 0.8*	18.5 ± 0.8	18.1 ± 0.9	18.1 ± 0.9
		~(101)	(99)	(99)
BW Week 14 (Daý2) (%)	22.5 ± 1.0	22.9 ± 1.1	22.7 ± 0.9	22.7 ± 0.9
		(102)	(101)	(101)
BW Week 26 (Day 176) (%)	24.2 ± 1.3	24.5 ± 1.7	24.3 ± 1.1	23.7 ± 0.9
		(101)	(100)	(98)
BW Weth 54 (Day 372) (%C)	26.7 ≠ 2.0	26.8 ± 2.3	26.7 ± 1.9	25.8 ± 1.3
		(100)	(100)	(97)
Final/BW Week 78 (Day 590) (%C)	27.1 ± 1.8	27.8 ± 2.1	27.6 ± 1.7	27.0 ± 1.4
		(103)	(102)	(100)
BWG Weeks 52 (Daves 1 to 89 (%C)	0.4 ± 0.4	$0.8 **\pm 0.6$	0.4 ± 0.7	0.5 ± 0.7
	4.6 ± 0.7	(200) 5.2 **± 1.0	(100) 5.0 * ± 1.0	(125) $5.1 ** \pm 0.9$
BWG Weeks 1-16 (Days \$40 92) (%C)	4.0 ± 0.7			
	1.7 ± 0.9	(113) 1.7 ± 1.1	(109) 1.6 ± 0.6	(111) 1.0 **± 0.6
BWG Week 24-26 Days 92 to 176) (%C)	1.7 ± 0.9			
	2.7 ± 1.3	(100) 2.3 ± 1.1	(94) 2.5 ± 1.2	(59) 2.1 ± 0.9
BWG Weeks 26-54 (Days 176 to 372) (%C)	2.7 ± 1.3			
		(85)	(93)	(78)



Dose group (ppm)	0	30	150	750
BWG Weeks 54-78 (Days 372 to 540) (%C)	0.6 ± 1.3	0.9 ± 1.4	1.1 ± 1.0	1.0 ± 1.1 °
		(150)	(183)	(1672)
Overall BWG (Days 1 to 540) (%C)	9.4 ± 1.6	10.0 ± 1.9	9.8 ± 1.6	9.4 1.4
		(106)	(194)	(400) Ó

%C: % vs control; *: p≤0.05; **: p≤0.01; nc: not calculated. Week numbers quoted represent the start of the week

D. Food consumption

Food consumption was not affected by treatment at any dose level in other sex, of the study.

E. Achieved intake

expre The mean achieved dietary intakes of AE C656948 the animals during the study were as follows: Ì

Table 5.5-34	Mean achieved intak	e (mg/kg/day)
--------------	---------------------	---------------

Sex	Q V	W ales &			Females	Č ⁱ
Dose group (ppm)	30	@ [*] 1509	∜750	~30	159	\$ 750
Week periods 1-13	\$.1 ⊗	25,5	⇒ 12 8	0 6.4	<u>82</u> .0 ×	v 156
Week periods 1-52		QŽ.2 🍣	jaž ,	6 ^y 5.7 ^o	28.6 <u>%</u>	138
Week periods 1-80 🔬	× 4.2×	20.9	Å¥05 €	× \$3	26. © ″	129

F. Hematology

1. Hematology

 \sum_{m}^{1} in males, a Month 13 (+25%, p≤0.01) and Slightly higher mean platelet Month 19 (+22%, 20.01)

G. Sacrifice and pathology

weight 1. Terminal body weight and organ

12-month sacrifice

Ô Ó^Ç in terminat body weight of treated animals when compared to control There was no relevant change animals. Ô

At 750 ppm and 150 ppm, mem absolute and relative liver weights were statistically significantly higher when compared to controls in both sexes viver weight changes were found to be dose-related. No effect was seen on liver weight a 30 ppn.

SD) of scheduled sacrifice, chronic phase (% change when Table 5.5-35 žver weight changes 😭 × 1 omnated to control

	« Company							
Sex	Ĩ,	🔊 Mấ			Females			
Dose group (ppm)	0	ا% 30 [™]	150	750	0	30	150	750
Mean absolute	A26±	1.24€	1.40*±	$1.47^{**} \pm$	$1.09 \pm$	$1.07 \pm$	$1.31^{**} \pm$	$1.40^{**} \pm$
liver weight (g)	J0.10	0.09	0.15	0.13	0.13	0.18	0.12	0.14
di a		(52%)	♥(+11%)	(+17%)		(-2%)	(+20%)	(+28%)
Mean liver to body	4,53 ±	Q4.52 ±	$5.20^{**} \pm$	5.68**	4.7 ±	$4.61 \pm$	5.51**±	$6.12^{**} \pm$
weight ratio (%)	JV366 K	v ^v 0.232	0.503	±0.235	0.404	0.688	0.365	0.325
		(0%)	(+15%)	(+25%)		(-2%)	(+17%)	(+30%)
Mean livecto brain	271.96±	$269.65 \pm$	8.59**±	324.60**	$235.58 \pm$	$226.49 \pm$	$276.48* \pm$	307.67**
weight ratio (%)	17.276	18.272	30.464	± 23.541	33.240	39.824	26.136	± 22.751
		(-1%)	(+13%)	(+19%)		(-4%)	(+17%)	(+31%)
* .0.05 ** .0.01								

*: p≤0.05; **: p≤0.01



At 750 ppm, mean absolute and relative kidney weights were statistically significantly lower when compared to controls in both sexes. No effect on kidney weights was seen at 30 or 150 ppm in either sex.

Table 5.5-36 Kidney weight changes (± SD) at scheduled sacrifice, chronic phase (% change when compared to controls)

Sex		Ma	ales		Females of S				Ì
Dose group (ppm)	0	30	150	75	0	2 ⁷ 30	ð 50	750	s.
Mean absolute	$0.553 \pm$	$0.538 \pm$	$0.554 \pm$	0.460*±	0.398	$0.385 \pm$	× Ø.388 ×	$0.342 * \pm 0$	0¥
kidney weight (g)	0.064	0.061	0.063	\$0 77	0.056	0.028	0.037%	0.032	1
		(-3%)	(0%)	(17%)		©(-3%)6 ⁵⁴	(-3%)	(-14%)	
Mean kidney to	1.99 ±	$1.96 \pm$	2.06 ± 🖉	1.78* ±	1.72 ±	1.67 ±	₹¥.63 ±	1.49*±	
body weight ratio	0.173	0.195	0.168	02191	S~0.243€√	Q. 3 4	0.13	0%083	
(%)		(-2%)	(+3))))	A 1%)		Q°3%)	(-5%)	<u>(</u> -13%) 。	
Mean kidney to	$119.69 \pm$	$116.80 \pm$	12 <u>1</u> .96 ±	101.38	85091±	81.45±	81068±	@75.03*≠	
brain weight ratio	11.654	12.203	\$40.907 °	± 14.739	×12.823	5.380	<i>"</i> 7.124 [«]	5.046	
(%)		(-2%)	×(+2%)	(25%)	6 de la	(-5%)	× (-5%)	(43%)	
*: p≤0.05; **: p≤0.01		Q			/ <u>k</u> j	R R	í ás	0	

18-month sacrifice

There was no relevant change interminal body weights of treated names and females when compared to control animals.

Mean absolute and relative liver weights were statistically significantly higher, when compared to controls in both sexes at 780 ppm and 190 ppm

Mean absolute and relative liver weights were slightly higher when compared to controls in males at 30 ppm but no concompant microscopic hepetocellular hypertrophy was noted.

Therefore liver weight changes were considered to be advecse at 760 and 950 ppm only.

Table 5.5-37 When compared to controls

Sex 2		S M	<u>.</u>					
Sex 🖉	. V .	Ój M	lales 、	Ô [°] V		Fen	nales	
Dose group (ppm)	\$0_ \$	30 7	×950 ×	, 12a *	Â [°] 0	30	150	750
Mean absolute	1,57	\$.26* J	1.34**	d.49**	1.26	1.31	1.45**	1.70**
liver weight (g)	±0.12	$5 \pm 0.11^{\circ}$	± @13 、	$O' \pm 0.160'$	± 0.17	± 0.16	± 0.21	± 0.23
~Q		(+8%)	(15%)	♥ (+2♂%)		(+4%)	(+15%)	(+35%)
Mean liver to body	4.23	450**	44.83**	5.\$3**	5.24	5.36	5.92**	7.14**
weight rate (%)	±_0.958	€0.352 [©]	± 0.358	≥ 0.363	± 0.473	± 0.387	± 0.602	± 0.957
	~~ .1	(+6%)	(±4%)。	Q(+31%)		(+2%)	(+13%)	(+36%)
Mean Hver to brain	×257.14	272.91*	294.43*	328.69**	265.02	272.46	310.63**	366.05**
weight ratio (%)	± 26.290	± \$5.272	$\pm 28.2 0^{\circ}$	± 33.960	± 35.514	± 31.668	± 47.983	± 48.110
Ĩ.	×	~~+6%)~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	(+15%)	(+28%)		(+3%)	(+17%)	(+38%)
*: p≤0.05;**: p≤0.01	à A	s d'						

Mean absolute and relative kidney weights were statistically significantly lower when compared to controls in male at 750 ppm. These changes were considered not to be treatment-related in males since there were no relevant microscopic findings. Mean kidney to body weight ratio was statistically significantly lower when compared to controls in females at 750 ppm. This change was considered to be treatment-related as it was associated with relevant microscopic findings in the kidney.

Û



Kidney weight changes at terminal sacrifice, carcinogenicity phase (% change when Table 5.5-38 compared to controls)

Sex		Μ	lales	28			Females			
Dose group (ppm)	0	30	150	750	0	30	, 150	9 50		
Mean absolute	0.542	0.557	0.556	0.491**	0.432	0.44	0.435	∀ 0.40 8 √	1	
kidney weight (g)	± 0.0501	± 0.063	± 0.058	± 0.055	± 0.053	± 0.050	± 0.038	±0.043	A.	
		(+3%)	(+3%)	(-9%)		((7 3%)	(+1.%)	<u>_</u> (\$6%) √	L.	
Mean kidney to	1.95	1.99	2.01	1.82**🏷	1.80	<i>√</i> 1.83	1× 2 ∕8 .	¥.71**Ş	<i>a</i>	
body weight ratio	± 0.111	± 0.181	± 0.155	$\pm 0.156^{\circ}$	± 0.131	± 0.144	±∯.107≪	2 ± 0.146		
(%)		(+2%)	(+3%)	(-7%)	Ó	☞ (+2%)	≪J(-1%)	(-\$%)	,0	
Mean kidney to	118.59	120.57	122.10	108.17**	90.25	92.93	[©] 92,96 [⊗]	87.80 Q	,¥ ¦	
brain weight ratio	± 10.248	± 11.792	± 11.328	A 11.754	± 10.841	🍳 9.69D	± 8 219	± 9.171		
(%)		(+2%)	(+3%) 4	Ø (-9%)		VI CONV	<u></u> ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	(-3.6%)	I	
**: p≤0.01			×,			×,		s s s s s s s s s s s s s s s s s s s		

Mean absolute and relative heart weights, were statistically significantly higher when compared to controls in females at 750 ppm (+ 17 to 19%, $p \le 0.01$). Mean heart to body weight ratio was statistically significantly higher when compared to controls in males at 750 ppm (48%, p\$ 0.05). These changes were considered not to be adverse since there were no relevant associated microscopi@findings.

Mean absolute and relative adrenal and weights were statistically significantly higher when compared to controls in females at 750 ppm (* 23 to 26%) ≤ 0.00). Mean adrenal gland to body weight ratio was statistically significantly higher when compared to controls in males at 750 ppm (24% p ≤ 0.05). These changes were considered not to be adverse spice there were no relevant associated microscopic findings.

2. Macroscopic findings

12-month sacrifice

One animal from the control group died prematurely before the end of the 12-month chronic phase, due to an accidental tranma on Day 301. No relevant macroscopie changes were observed except for a blood clot on the submaxillary glands? Ŵ

 \bigcirc

At 750 ppm, enlarged liver was found in 1/10 makes and 2/10 females

s found in 2/10 males At 150 ppnr, enlarged live

18-month sacrifice

Seventy-eight animals died prematurely before the end of the study. No treatment-related findings were Ô , O ô^s Ś noted.

At 750 ppm, enlarged liver and dark liver was found in some males and females.

At 150 ppf enlarged liver was Yound in some females.

These findings were correlated with relevant histopathological findings.

At 750 ppm, prominent building in liver was found in some males and females but since this gross Lidted W observation was not correlated with delevant histopathological findings, it was considered not to be adverse.



Table 5.5-39 Incidence of macroscopic changes in the liver, terminal sacrifice of the carcinogenicity phase

Sex		Μ	lales			Fem	ales		
Dose group (ppm)	0	30	150	750	0	30	150	Ø750	
Obviously large	0/44	0/41	0/41	3/42	1/39	2/37	7/40	× 30/3	
Dark	1/44	0/41	0/41	14/42	1/39	0/37	0/40	438	
Prominent lobulation	1/44	0/41	1/41	5/42	0/39	<i>5</i> 0/37	° O	×8/38	

3. Microscopic findings

12-month sacrifice

Due to an increased incidence of follicular cell adenomas observed in thyroid gland of the high cose group males (750 ppm), a microscopic examination was performed on the male thyroid gland, to establish in there were any pre-neoplastic changes in this tissue after 12 months of treatment.

Follicular cell hyperplasia was noted in 2/10 and 2/9 mates at 750 and 150 ppm, respectively.

Table 5.5-40 Incidence and severity of microscopic changes in the theroid gland, all animals chronic phase

	· ~	"0"	1	O	r éi si	≪j`
Sex	Ą	ĝ	ð		Males	°
Dose group			ð,	¢ _30	₽° 750 %	750
Follicular cell hyperplasia	a: focál/multi	focat				
Number of animal			¥ 9 Ø	10	× 9.0	10
Minima		Ø Ø	Q,	A W		1
Slight			~~ . C)		1
Total	The second			0 0	2	2
Ű			y sy	<i>a</i> .	Y Y	

18-month sacrifice

No treatment-related course of death was established for the few wimals which died or were humanely sacrificed before the end of the study.

a) Non-neoplastic

Treatment-related effects of AE C656948 were found in the liver kidney and thyroid gland.

In the liver, a higher incidence of eosimophilic foci of altered hepatocytes was observed in females at 750 ppm. Centril bular to pant bular hypertrophy was observed in both sexes with a dose-related effect at 750 and 150 ppm. Depatocellular cholestasis was noted in males at 750 ppm. Higher incidences of hepatocellular single cell degeneration/necrosis were noted in males at 750 and 150 ppm, together with interstitial hixed cell infiltrate posinophilic inclusion bodies and multinucleated hepatocytes in males at 750 ppm only.

At 750 ppm, there was a parkedly lowe fincidence of mainly centrilobular hepatocellular vacuolation with concomitant minimal to moderate hepatocellular hypertrophy in males. At 30 ppm, there was a higher incidence of mainly centrilobular hepatocellular vacuolation in males. However, in the absence of an effect at the higher doer level of 150 ppm, this finding was considered to have occurred by chance at 30 ppm.



Table 5.5-41	Incidence and severity of microscopic changes in the liver, all animals,
	carcinogenicity phase

carcinoge	menty pn	ase						a,°
Sex		Ma	les			Fem	nales	
Dose group (ppm)	0	30	150	750	0	30	150	6750
Number of animals examined	49	49	49	50	48	.	50 🦿	U 50 O
Eosinophilic focus(i) of altered	hepatocy	tes: focal	/multifoc	al	•	- Or		
Minimal	1	0	0	0	0 4	1	28	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Slight	0	0	0	<u> </u>	0 🔊	» 0	°∼0 «	\$ 2 ×
Moderate	0	0	0	$\bigcirc 0$	67	0		207
Marked	0	0	0	0	<u>A</u>	0 0		N.
Total	1	0	0	2		1	Ð,	.05*
Centrilobular to panlobular h	epatocellı	ılar hyper		liffuse 🦨	ç Ş	Å	Å,	C Q
Minimal	0	0	0^{016}	3~		Å	O' 18 🗞	240
Slight	0	0 "	≥ 22, ∘	L.O	× 70	$\bigcirc 0 \bigcirc$	l Ox	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Moderate	0		Ø.,	36	$\sqrt[6]{0}$	0.5	0 [™]	^ه 0
Total	0	0	38**	_C50**_	00	0	Å 18** Å	26*≵ °
Number of animals	49	×79	× ⁴⁰ 49. ∖	5.Q ×	<u>4</u> 8	\$50	[♥] 50 📚	5ØÚ
Hepatocellular cholestasis: foo	al/multife		Y av			y _i o		AN .
Minimal	0	04	Ŵ	<u>~</u> 29 🐇	$\int 0 $	<u></u>	-Q0	\bigcirc 0
Slight	0,0	0×	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	× 2_0		Ś	S 0 S	0
Total	ð	<u> </u>	<u> </u>	31	Ø) 0 ~ (0
Hepatocellular single cell dege	eneration/	necrosis;			<u> </u>			
Minimal	S 1 %	2	Ø,	L 28	V 1	Ø	×0	1
Slight	Ý 🍭	Ĩ, Î	$ $	12 🎸	°Q?	©0	0	0
Moderate 👸	0″	NO 0		0	, SÓ	$\gamma 0 $	0	1
Total 🖄	1	2	7	QO ^{**}	¹ 1 %		0	2
Interstitial mixed cell infiltrat	e; focal/m				<u> </u>		1	1
Minimal 🖉 🖤	18	Ø 5	چ [×] 19	<i>39</i>	(S) ^v	~y [′] 8	10	8
Slight &	<u> </u>	$\gamma 1$	<u></u>	<u>s</u> e	<u>, 2</u>	, 3	2	0
Total	ر»18	<u> </u>	ÌY [*]	~340 **_(ຽ [™] 10∕ີ 🤇	11	12	8
Eosinophilic in Ousion Dodies:	focal/mu		<u>j</u> v	× 5	/ ·v	1	1	1
Minimal 👸 🛷	2 -	×3 °	<u> 9 5 5</u>	100		0	0	0
Slight	L. I	$\rightarrow 0 $	0.0%	_@ ¹	\widetilde{O} 0	0	0	0
Total 🖉	S 2 Š	3	\$	\$19**	Ø	0	0	0
Multinucleated hepatocytes;	ocal/multi	ifocal		<u> </u>	1	1	1	1
Minimako 🥤 🎸	<u></u>		ړ 3	25	1	0	0	0
Slight Slight			10	<u></u> 2	0	0	0	0
<u> </u>	3 20		A	\$27**	1	0	0	0
Hepatocellular vacuolation:				~				
Minimal	~21	Q 15	<u>13</u> 0°	0	3	4	1	0
Slight	N 6 0	19	<u> </u>	2	2	5	0	1
Moderate Noterate	¥ Q	<u>A</u>	×3	1	0	1	0	0
Total 🖉 🐴	27	38 *	y ^v 27	3**	5	10	1	1
*: p≤0,05; **: p≤0.01	×.	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	v					

In the kidney, higher incidence, and/or severities of bilateral cortical basophilic tubules, hyaline casts(s) and interstition mononuclear cell infiltrate were noted in females at 750 ppm. A higher incidence of glomerular congestion/hemorrhage(s), associated with higher severity of amyloid deposition (mainly observed within gromerular interstitium) was noted in females at 750 ppm.



Table 5.5-42	Incidence and severity of microscopic changes in the kidney, all animals,
	carcinogenicity phase

8	pines	-						a,°
Sex		Ma	les			Fen	nales	
Dose group (ppm)	0	30	150	750	0	30	150	750
Number of animals examined	50	50	50	50	48	50	50 🤘	V 50 🔊
Cortical basophilic tubules: bil	lateral					ð	~	
Minimal	27	33	30	20	17 .4	11	180	~d7
Slight	9	2	2	1	2 🏑	2	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	Q14 ĸ
Moderate	0	0	1	Ö 1	0	1	× 0 ~	× 3
Total	36	35	33	22**	.09	14 🥥	21\$	34***
Glomerular congestion/hemor	rhage(s):	focal/mul	tifoca∯∕			×)	, Q,	Å ¢
Minimal	0	0	<u> </u>	0 4	<u>ه</u> کې	L.	3 ^v	
Slight	0	0	~~~Č	1		-Q`,	Ö ^v 0 Ø	30
Moderate	0	0	× 0	<u>00</u>	<u>^</u> >0	@ 1 💫) <u>0</u> %	
Total	0	0 🚿	8 2	L I	ູ 2 👗		37	27**
Amyloid deposition: focal/mult			<u>v</u>		<u> </u>	-		i de la construcción de la const
Minimal	13	<u>_</u> 45	<u>16</u>	19 🖗	27	\$23	15	\mathcal{D}^{\vee}
Slight	18	<u>ک</u> 4 ^	y 7 🌱	Ś	<u> </u>	[©] 15 ∞	17	S 4
Moderate	1	<u> 16</u>	<u></u>	× <u>7</u>	<u>ې 2 پ</u>	<u>2</u> 0	8	Õ [°] 27
Marked	0_0			$\mathbb{V} 0_{0}$		<u></u>	0 Ø	8
Total	32	20	¹ ⁹ 23	29	<u></u>	ر 40 ر	\$° 40≪°	41
Hyaline cast(s): focal/multifoca	11 ×			<u></u>	á à		· ¥	1
Minimal	<u>ي 3 کې</u>	1	- B	$\sqrt[6]{0}$	R 0 ~	ð	×1	8
Slight 🔍	<u>v Q</u>	<u> </u>		i 1 🗸	~ <u>0</u> ?	l j	0	3
Moderate 🦓	<u>Ø</u> *	2°0 (₽́1 ^{°0}	0	~Ç0	<u>~1</u>	2 0	0
Total 🦄	3	Q 1 S	3	<u></u>	[∞] 0 _≪	<u>2</u>	1	11**
Interstitial mononuclear, cell in					4		1	1
Minimal 🖓 🛛	<u> </u>	27	\$ ⁷ 25 K	y [♥] 25 [©]	ð	£27	26	16
Slight of the second seco	<u> </u>	~~~~ <u>2</u>	<u> </u>	<u>v</u>	9	, 11	12	25
Moderate	×0	~ 0.2	_ Å∳ ^v	5 ⁹⁰ ($v 0 \sim v$	0	1	1
Total O	32 %	29,″	2 9	27	42 [°]	38	39	42
**: p≤0.01 0 0 0	~	×~ ~	Q Q		, Ø			

In the thyroid gland, a higher incidence of follicalar cell hypoplasia was noted in both sexes at 750 ppm and as year in males at 50 ppm.

Table 5.5-43 Dicidence and Severity of microscopic changes in the thyroid gland, all animals,

Sex of the Males							Females					
Dose g	roup (ppm)	*	1	Q ⁷ 30 ¢	1500	750	0	30	150	750		
Number of	inimals exag	nined	S 50 <i>"</i>	50	-50	50	48	50	50	50		
Follicular ce	ll hyperplasi	a: foc	l/multifo	cal 🖉	²					-		
N N	finimal 🔊	L	Q	\$2 ×	10	18	11	4	10	10		
\sim	Shant	S.	<u>^</u> ~~3 ·	Q 2 🛇	5	6	5	2	5	12		
М	oderate	0 -	$^{\circ}_{10}$	24	2	5	1	2	3	6		
Ν	latked 1 N	Q	, 9	Â,	3	3	0	0	1	4		
(evere 🥎		$\widetilde{0}$	0	1	0	0	0	0	1		
Į.	Total	\bigcirc	≫4 ~	6	21**	32**	17	8*	19	33**		
*: p≤0.01	à s											

b) Neoplastic findings

In the thyroid gland, a higher incidence of follicular cell adenoma was noted in males at 750 ppm, when compared to controls.



Table 5.5-44Incidence of neoplastic microscopic changes in the thyroid gland, all animals,
carcinogenicity phase

0	• •								
Sex		M	ales			Females			Ő,
Dose group (ppm)	0	30	150	750	0	3 0	150	A750 Ø	F
Number of animals examined	50	50	50	50	48	50	50	J 50 m	
Follicular cell adenoma						ð	4	¥ . Ç	
Incidental	1	1	3	7	3 4	1	30		à
Total	1	1	3	7*	3	' 1	°~3		2
*: p≤0.05				Ö	a l		N.	Y 6	Ŵ

H. Deficiencies

No deficiencies are identified in this study which complied with the requirement of tg oecd 45 km for a when the study was carried out.

III. Conclusions

In conclusion, dietary administration of AE C656948 over an 18-month period to the C57BL/5 mouse, at a dose level of 750 ppm (equivalent to 105 mg/kg/day in males and 129 mg/kg/day in females) resulted in a higher incidence of follicular cell adenoma in the pyroid gland to males.

Non-neoplastic changes were observed in the liver and thyroid gland in both sexes and in kidney in females at 750 ppm. At 150 ppm (equivalent to 20.9 mg/kg/day in males and 26.8 mg/kg/day in females), non-neoplastic changes were seen in the giver in both sexes and in the thyroid gland in males.

The NOAEL was 30 ppm in males (equivalent to 4.2 forg/kg/day) and females (equivalent to 5.3 mg/kg/day).

Assessment and conclusion by applicant

Study meets the current guidance and the requirements in 283/2013

Dietary administration of AE Co36948 over an 18 month period to the @7BL/6J mouse, at a dose level of 750 ppm (equivalent to 105 mg/kg/day in males and 129 mg/kg/day in females) resulted in a higher incidence of follicular cell menomo in the thyroid gland in males.

Non-neoplastic changes were observed in the liver and thyroid gland in both sexes and in kidney in females at 750 ppm. At 150 ppm (equivalent to 20.9 mg/kg/da in males and 26.8 mg/kg/day in females), non-neoplastic changes were seen in the liver in both sexes and in the thyroid gland in males.

The NOAEL was 30 ppm in males (equivalent to 0.2 mg/kg/day) and females (equivalent to 5.3 mg/kg/day).

Mechanism of action and supporting data

Supporting data regarding female rat hver tumors

In the rat carcinogenicity study (M-298339-01G), a higher incidence of liver cell tumors (hepatocellular adenoma + carcinoma), was observed in females after 2-years of treatment with AE C656948 at 1500 ppm (89 mg/kg bw/d). No peoplastic changes were observed in males (highest dose = 29 mg/kg bw/d).

This neoplastic change was observed together with a number of proliferative changes (including altered hepatocytes (eosinophilic foci), clear cell foci, multinucleated hepatocytes with anisocaryosis or increased number of initoses) in association with metabolic morphological changes (centrilobular to panlobular hypertrophy, focal/multifocal hepatocellular vacuolation, centrilobular to midzonal hepatocellular macrovacuolation, brown pigment in Kupffer cells or hepatocellular brown pigment).

In the 28-day study in the rat by oral administration (<u>M-085510-01-1</u>), hepatotoxicity testing showed that AE C656948 was able to induce total cytochrome P-450 together with BROD and PROD activities



and therefore was considered to be a moderate phenobarbital-like cytochrome P-450 inducer. Consequently, a "phenobarbital-like" mode of action (MoA) was anticipated for liver cell tumor formation, especially as phenobarbital is known to stimulate cell proliferation in normal hepatocytes in rodents resulting in a liver tumor promotion. In addition to its ability to induce liver enzymes and to increase liver cell proliferation, phenobarbital responses that are key for its tumorigenic effect include among others, hypertrophy and development of altered hepatic foci (Whysner et al., 1996¹) which were all effects observed in the rat carcinogenicity study with AE C656948. These data support a similar MoA for AE C656948 and phenobarbital regarding the liver tumors observed in the female ration the carcinogenicity study. As for liver enzyme induction, these effects are mediated through an activation of nuclear receptors, in particular the constitutive androstane receptor (Car) and pregnane X receptor (Pxr). Although Car and Pxr are expressed in human hepatocytes (Moore et al. 2005), liver celk proliferation would only occur in rodents and not in humans, thicles a necessary precursory event for liver tumor formation, via this MoA. In addition, there are convincing data showing that patients receiving phenobarbital for many years do not show evidence of tumorigenic effect (IARC, 2001). For these reasons, the mode of action for phenobarbital-like P-450 induction is considered not to be relevant to humans (Holsapple et al., 2006⁴).

In order to test this hypothesis, an intial explanatory study was conducted with AEC656948 in tenale rats at a single high dose level of AE C656948, which was twice the high dose level used in the rat carcinogenicity study, administered for 7 days to assess its ability to cause liver cell proliferation under in-house experimental conditions. For comparison, a similar study was conducted with phenobarbital. Two further explanatory studies were subsequently conducted in the female rat to further characterize the profile of AE C656948 and to establish a dose and time concordance for activation of the key precursory events i.e. Car/PXF nuclear receptor activation, leading to liver cell proliferation, which in the female rat ultimately results in liver tumors following chronic exposure to AE C656948 at a high enough dose level. The range of dose levels tested included the dose levels used in the rat carcinogenicity study, plus intermediate dose levels, which were administered to female rats for 3, 7 or 28 days. The following parameters were assessed, induction of the Car/Pxr receptor, fiver cell proliferation together P-450 enzyme activities and UDPGT species isoenzome profiles and corresponding gene transcript expression, which acted as markers for activation of the Car/Pxr receptor, fiver cell proliferation together with histopatheogical changes in the liver. Reversibility of changes seen following 28 days of treatment with AE C656948 was also assessed. In both studies, prenobarbital was included as a positive control. These explanatory studies are symmatrized hereafter.

Overall, these studies showed that AE C656948 exhibits a similar pattern of effects as phenobarbital in our experimental system in terms of liver enzyme induction and in its capability to induce liver cell proliferation compared to untreated animals. A dose and time concordance was established for each key event and changes observed after up to 28 days of treatment with fluopyram were found to be reversible following cessation of treatment for 28 days.

In addition, a pivotal mechanistic study conducted to demonstrate Car/Pxr receptor activation as the first key event was a 28-day mouse study using both the wild-type (WT) C57BL/6J mouse and a genetically modified mouse that does not have functional. Car or Pxr receptors (Pxr-Car KO), this study is summarized in the section pertaining to house mechanistic studies. Mice were exposed to AE C656948 at the tumorigenic dose (750 ppm) in the mouse cancer bioassay and above (1500 ppm), equivalent in terms of mg/kg/d to the rat tumorigenic dose level. In this experiment, a significant induction of liver enzymes, liver enlargement and hepatocellular hypertrophy was seen in the WT mouse, but was not observed in the Pxr-Car KO mouse.

In order to deponstrate the non-relevance of rat liver tumors to humans, an in-vitro comparative study was conducted to examine the proliferative response in rat and human primary hepatocytes exposed to AE C656948 at a range of dose levels up to a cytotoxic dose, details of this study are summarized hereafter. In a similar manner to phenobarbital, used as a positive control, the rat cells exposed to AE C656948 showed a dose-response increase in proliferation, whereas human cells did not. Exposure to epidermal growth factor, a positive control, resulted in a marked proliferative response in both rat and



human cells. This *in-vitro* experiment clearly demonstrates that one of the crucial key events for progression of liver tumors (hepatocellular proliferation) would not take place in humans.

An in vitro CYP and UGT induction study in human and Wistar rat hepatocytes with AE (2020), M-759019-01-1, KCA 5.5/22), was conducted to address the T-roodality $^{\circ}$ C656948 (as part of the endocrine disruption assessment, which also provides additional widence for the species differences between rat and humans for the the tumor assessment. This study demonstrated AE C656948 was a strong CYP3A at >10 μ M and to a lesser extent a CYP1A2 inducer in Wistar rat hepatocytes. AE C656948 consistently induced UGT2B1 and increases UGT-T4 activity in Wistar rat hepatocytes. At >10 µM AE C656948 was a CYP1A2, CYP2B6, CYP3A and UGT1A linducer in human hepatocycles, but does not increase UGT-T4 activity in human hepatocytes.

Other plausible MoAs for liver tumor formation that are likely to be relevant to humans have effectively been excluded. Several MoAs have been identified for liver carcinogenesis in both rotents and huntans Antity (Cohen, S.M. (2010). Evaluation of Possible Carcinogenic Risk to Humans Based on Liver Tumors in Rodent Assays: The two-year bioassay is no longer necessary. Texicol Pathol 38: 487-501, 2010), KIIA 5.5.4 /36; Cohen, S. M.; 2010; M-367547-021) those highlighted in bold in the list below are likely to be relevant to humans:

- I. DNA reactivity
- II. Increased cell proliferation

DNA reactivity is the first brood category of a Mo Sfor hepatoce Qular carcinogens. A battery of *in-vitro* genotoxicity studies; the bacterial reverse mutation test (Ames test), mammalian chromosome aberration test, and a mampalian cell gene mutation test, together with an in-vivo mouse micronucleus assay showed that AE C656948 does not have a genotoxic potential. Thus, DNA reactivity can be excluded as a potential MoA for the induction of turnors in Pats or inice.

For non-DNA-reactive rodent Over carcinogens, several MoAs have been identified that act by stimulating hepatocellular prohieration through either a receptor- or non-receptor-mediated mechanism. The MoA studies in tats and mice with AE \$56948 clearly demonstrate a specific, dose-related increase in the Cyp2b/Car-associated and Cyp3a Pxr-associated gene and enzyme activity. Furthermore, the absence of such a response in Pxr-Car-KO mice supported the specificity for AE C656948-induced activation of Car and Pxr and excluded other receptor (Ppara, AhR, ER, statins, and cytotoxicity) and non-receptor mediated (cytotoxicity, infections, iron overload, and increased apoptosis) involvement in the key events leading to TE C656948-Induced liver tumors.

Overall, the mechanistic studies together with the standard repeat dose studies, clearly demonstrate the MoA for the Pat liver tumors following a life-time exposure to AE C656948 is via activation of the Car/Pxr nuclear receptors Furthermore, the lack of proliferation in primary human hepatocytes exposed to AE C6\$6948 as compared to rat hepatocytes, provide convincing evidence that the liver tumors in the rat are a not action demonstrated to be responsible for



liver tumor formation in the female rat following chronic exposure to high dose levels of AE C656948 is considered not to be relevant to humans.

Further detailed argumentation regarding mode of action and relevance to humans of the rat liver temors following exposure to fluopyram (AE C656948) are presented in the following Expert Summary® documents:

Position Paper

Ó Fluopyram: Mode of Action and Human Relevance Analysis of Rodent Liver and Thyroi MIIA Sec 3 /03: 2013; M-465168-01-2

Expert Summary Report

Fluopyram: Mode of Action and Human Relevance Framework Analysis for Fluopyram-Induce Rodent Liver and Thyroid Tumors, MIIA Sec /02 ;2013; M-454439-02-1 ° Ľ L

a.

1 Whysner J., Ross P.M. and Williams G.M. (1996) Phenobarbital mechanistic data and risk assessment enzyme induction, enhanced cell proliferation and tumor promotion. Pharmacol@Iher.74(1/2), 153-191. KIIA 5(5:4 /06; Whysney J.; Rogs, P. M.; Williams, G. M.; 1996; M-300847-01-1

2 Moore J.T., Moore L.B., Maglish J.M. and Knewer S.A. (2007) Functional and structural comparison of PXR and CAR, Biochem. Biophys. Acta, Feb 17, 1919 (3) 25 238. KAIA 5.59 /07; Moore, J. B. Magliel, J. M. Kliewer, S. A.; 2003; M-300852-01-1 Ô

3 IARC (2001) (Anon.), Phenobarbital and its sodium sats in Some Hypotropic agents Summary of data reported and evaluation", IARC Monograph Vol 79, IARC Press Lyon-France pp 167-288. KHA 5.5.4 708; Aron.; 2001, M-300830-01-1

4 Holsapple M.P., Pitot H.C., Cohen S.H., Boobis ACK., Klashnig J.E, Pastoor V., Dellarco V. Land Dragan Y.P. (2006) Mode of action in relevance of rodent ther tumors to human capter risk. Toxicol. Sci. 89(M, 51-56, KIIA, 5, 3.4 /09; Holsapple, M.; Pitot, H. C.; Cohen, S. H.; Boobis, A. R.; Klanig, J. E.; Pastoor, J.; Dellaço, V. L.; Dragao, Y. P. 2005; <u>M-300828-01-1</u> . K

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Data Point:	
Data Point:	
Report Author:	$\begin{array}{cccccccccccccccccccccccccccccccccccc$
Report Year:	2008 0 6 6 2
Report Title:	Ruopyram (AE C 656238): 7-6ay mechanistic study in the female Wistar rat by
<u> </u>	, dietary administration " @ @
Report No.	SA 97323 & Y Y
Document No:	M299274-01-1 ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~
Guideline(s) followed in	No specific guideline
study:	
Deviations from current	Cuivent guideline none
test guideline	
Previous evaluation:	Yes, evaluated accepted in the DAR (2011).
a va	
GLP/Officially	Yesyconducted under GLP/Officially recognised testing facilities
facilities:	
Acceptability/Reliability:	Yest Q
	Yest U L
Executive Simmary	

Executive Sammar

AE C656948 (Bytch number Mix-batch:08528/0002, 94.7% w/w purity), was administered continuously in the det to proups of female Wistar rats (15/group) for 7 days at the concentration of 3000 ppm (equivalent to 193 mg/kg body weight/day). A similarly constituted group received untreated diet and acted as a control group.

Animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. Body weight and food consumption were recorded at the end of the 7-day treatment period.



Hepatic cell proliferation was assessed by administration of 5-bromo-2'-deoxyuridine (BrdU, an analogue of thymidine) to all animals in the drinking water for 7 days before sacrifice. Water consumption was measured during the BrdU administration period. All animals were subjected to necropsy. Brains and livers were weighed. Selected portions of the liver were fixed for conventional histopathological examination and cell proliferation measurement. The remaining portions of the liver of 10 females from each group were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and cytochrome P-450 isoenzyme and UDPGT activities.

AE C656948 at 3000 ppm in the diet had no effect on body weight parameters. There was no evidence of a treatment-related effect on clinical signs, food or water consumption.

Mean absolute and relative liver weights were increased by between 40 to 43% when compared to the controls (statistically significant: $p\leq 0.01$). This increase was associated with macroscopic undings (enlarged and dark livers) in 13/15 females compared to no incidences in the controls Q

At histological examination, minimal to slight centribobular to pantobular hepatocellular hypertrophy was found in all treated animals. A markedl decreased incidence of periportal vacualation avas also noted.

Assessment of cell proliferation in the liver revealed a four told increase in mean BrdU tabeling index in both the perilobular and centrilobular areas of the hepatic lobule in treated and als, when compared to the controls.

Assessment of total cytochrome P450 content and nucrosonal profeins revealed a slight increase in total P-450 content and in mean EROD activity, a moderate increase in mean PROD and BROD activities and a marked increase in mean ODPGT activity (all statistically different from the control group ($p \le 0.01$)). These data showed that AE C656948 has the ability to induce moderately phenobarbital-inducible hepatic enzymes (total cytochrome P-450, BROD and PROD and UDPGT) as well as liver hypertrophy and cell proferention in the liver.

	I. Materials and methods	
A.	I. Materials and method	
1.	Test material: or 🦑 🖉	AE C656948 Light beige powder Mix-Batch:08\$28/0002 94.78
	Description 🔬 🔬	Light beige powder $@$
	Description	Mix-Batch:08528/0002
	Test material: Description Lot Batch #: Purity:	94.78
		658066-354
	Stability of test compound:	Stable in todent diet for a period covering the study duration.
2.	Vehicle and / or positive	noney by a
co	ntrol: 🔍 🔍 🔊 😒	
3.	Test animals:	
	Species:	Kat &
	Strain:	Wistor Rj: WI (IOPS HAN) – Female only
	Age:	11 weeks approximately at start of treatment
	Strain: Age: Weight at dosing: Source: Acclimation period:	228 -254 g
	Source: 4 A	
	Acclimation period:	12 days
	Acclimation period:	Certified rodent powdered and irradiated diet A04C-10P1 from
	Diet,	S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-
		Orge, France), ad libitum
/	Water O S	Tap water, ad libitum
4		Animals were caged individually in suspended stainless steel
	Housing:	wire mesh cages.
	Environmental conditions:	-



Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 air changes per hour
Photoperiod:	12 hours light, 12 hours dark

B. Study design

- 1. In life dates:
- 19 November 2007 26 November 2007

2. Animal assignment and treatment

AE C656948 was administered in the diet for 7 days to a group of 15 demales at the dose of 3000 ppm (193 mg/kg/day). The dose level was selected on the basis of other studies conducted with the test substance. A negative control group with the same number of animals received plain diet Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, MIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Control animals were fed control diet for # days, Test animals were fed diet Containing fluopyram (AE C656948) at 3000 ppm for 7 days. A solution of BrdU at 80 fig of BrdU/100 mL of drinking water was administered to all animals during 7 days to allow ther cell proliferation evaluation

3. Diet preparation and analysis

AE C656948 was incorporated into the diet by dry mixing to provide the required concentration. There was one preparation for the whole study. The stability was demonstrated during the course of the study at concentrations of 20 and 10000 pm for a time which covered the period of usage and storage for the study. Homogeneity of test substance in diet was verified on the formulation to demonstrate adequate formulation procedures. The mean value obtained in homogeneity check, was used as measured concentration.

The second secon	es) Animals assigned
Test group Concentration in diet (ppm) Female (mg/kg bw/day)	Female
	15
	15

4. Statistics

Table 5.5-45

• - Body weight change parameters.

Study design

- Terminal body weight absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

The F test was performed to compare the homogeneity of group variances.

If the F test was not significant (p < 0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant (p20.05), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

• - Body weight and average food consumption/day parameters

- Total ytochrome P450 content

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.



The F test was performed to compare the homogeneity of group variances.

If the F test was not significant (p>0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant (p≤0.05), data were transformed using the log transformation. If the F test on log transformed data was not significant (p>0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data,

If the F test was significant (p≤0.05) even after log transformation, mean of the exposed grou compared to the mean of the control group using the modified t-test (2-orded).

If one or more group variance(s) equal 0, means were compared using non-parametric procedur

• - Enzymatic activities and cell proliferation parameter

Ó Ø Mean of the exposed group will be compared to the mean of the control group using the exact Mann-Whitney test (2-sided).

Group means will be compared at the 5% and 1% hovels of significance

(Module Enhanced Statistics) Statistical analyses were carried out using Path/Tox, System 22 except for liver enzyme parameters and cell proliferation parameters which were analyzed using SAS programs version 8.2.

C. Methods

1. Observations

The animals were observed twice daily for moribandity and mortality once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least week during the treatment period. The Plature, onset, severity, reversibility, and duration of any chinical signs were recorded.

2. Body weight

Each animal was weighed on study by 1 and 7. Additionally, der fasted animals were weighed before scheduled necropsv

3. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals foring the treatment period. Any food spillage was noted. From these records, the weekly mean achieved dosage intake in mg/kg/day was calculated.

4. Water consumption

Drinking water bottles containing Brdl were weighed on the first day of BrdU administration. Empty water bottles were weighed on the day of scheduled sacrifice (study Day 8).

5. Blood sampling

On the day of necropsy, blood camples were taken from all animals in all groups by puncture of the abdominal aorta. Animals were diet asted ernight prior to bleeding and anesthetized by inhalation of Isoflurane. Bood was collected into tubes containing lithium heparin.

6. Sacrifice and pathology

Necropsy procedure Organ sampling

On study Day 8, all animals from all groups were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane (Baxter, Maurepas, France). Animals were diet fasted overnight prior to sacrifice



All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Brain and liver were weighed fresh at scheduled sacrifice only.

Duodenum and two central sections of the liver taken in the left and medial lobes were f_{0} d by $\tilde{0}$ immersion in neutral buffered 10% formalin. The remaining portions of the liver from ten females from each group were kept for microsomal preparations and determination of total stochrome P-450 convent and isoenzyme activities.

Histotechnology - Histopathology – Cell proliferation & sessment

Duodenum and the two central sections of the liver were embedded in paraffin way

Histological sections, stained with hematoxylin and cosin, were prepared and examined from all the animals.

For cell proliferation assessment, an immunohistochemical stanning demonstrating theineorporation of BrdU and the determination of the labeling index were performed to assess hepatocytic cell cycling on all study animals. The immunohistochemical reaction included incubation with a monoclond antibody raised against BrdU, amplification with a secondary biotinylated antibody and streptavidin-horse adish peroxidase complex, detection of the complex with the chromogen diamino-benzidine (DAB) and nuclear counterstaining with hematogylin.

The zonal labeling index, expressed as the number of BrdU positive hepatocytes per thousand, was measured separately on random fields comprising at teast 1000 centrilobidar and 0000 periportal cells using an automated image analysis system. The mean labeling indexes (periportal, centrilobular and combined) and standard deviation were calculated for each zone and each group.

The immunohistochemical staining for BrdU and determination of the labeling ordex were performed on all animals showing sufficient BrdU incorporation (estimated by water consumption or duodenum BrdU labeling).

7. Hepatotoxicitotesting

At final necropsy, the temaining portions of the liver from all surviving animals were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isochzyme profile (including FROD, DROD and PROD activities) to check the hepatotoxic potential of the test substance Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with chitrophenol as substate. Results were compared to those generated with well-known reference compounds.

II. Results and discussion

A. Clinical signs and mortality

1. Clinical signs of toxicity

There were no treatment-related clinical signs observed in any group.

2. Mortality

There was no mortality in an group during the course of the study.

B. Body weight and body weight gain

There was no receivant changeon mean bodyweight in treated animals compared to controls.

C. Food consumption and compound intake

There was a slight reduction in food consumption in the treated group compared to control, however this was essentially due to a single animal. No effect was observed on water consumption.

The achieved dose in the treated group was 193 mg/kg/day.



D. Sacrifice and pathology

Higher liver weights (+ 40% approximately) were observed in animals treated with AE C656948 compared to controls. This was associated with enlarged liver observed at the macroscopic examination in nearly all animals and with hepatocellular hypertrophy in all animals at the microscopic examination.

Table 5.5-46 Liver changes after 7 days of treatment with AE C656948 at 3000 ppm (mean±SD

Dosage level (ppm)	٥ گ	<u>م</u> 3000 م م م م
Liver weight Absolute (g)	5.61 ± 0.49	Q 7.86 ± 0.93 (+402)
Bodyweight-relative	2.53 ± 0.13	$3.63 \pm 0.320 ** (+42\%)$
Brain-relative	299.50 ± 30.26	\$21.3 ± \$0.62**\$(+41%)
Microscopic pathology		
Diffuse centrilobular to panlobular hepatocellular hypertrophy		
Diffuse mainly periportal hepatocellular vacuolation		
**: p≤0.01; Figures in par	entheses an % differences from cont	rolo and the second

1. Cell proliferation

Cell proliferation was assessed separately in the centrilobular and the periportal zones of the hepatic lobules. In the centrilobular and periportal areas, the mean Brou labeling indexes were found to be approximately 4 times higher in treated animals $(p \le 0.01)$, when compared to controls.

Ø1 Mean Brd Glabeling indern the liver after 7 days of treatment with AE C656948 at Table 5.5-47: Ø 3000 ppm (mean SD) 🖉 - 5 \bigcirc

	<u> </u>
Dosage level (ppm)	3000
Number of mimals	5 1 4
BrdU positive cells in the 44.54 ± 22.31	<i>Q</i> 179.68 ± 95.18**
centrilogular zone	, W
BrdU positive cells in the periportal zone $28.55 \pm 16.80^{\circ}$	112.94 ± 58.19**
periportal zone in the second se	Ý
Overall BrdU positive cells 3654 ± 18.70 **: $p \le 0.01$	$146.31 \pm 70.26^{**}$
**: p≤0.01 Q A Q Q Q	

2. Hepatotoxicity testing

Changes that were observed at described in Table 5.5.4-4. Assessment of total cytochrome P-450 content and microsomal proteins revealed a slight increase in total P-450 content and in mean EROD

content and microsoma@proteins revealed a slight increase in total P-450 content and in mean EROD activity, a moderate increase in mean PROD and BROD activities and a marked increase in mean UDRCT activity.



Table 5.5-48 Results of the hepatotoxicity testing after 7 day of treatment with AE C656948 at 3000 ppm

ppm		
Dosage level (ppm)	0	3000
Number of animals	10	10
Total P-450	0.91 ± 0.17	Or.23 ± 0.20 **
(nmol/mg prot.)		(+35 %) × ×
EROD (pmol/min/mg prot.)	47.99 ± 3.73	103.18 ± 13 4 **
		(+115%) $(+115%)$
PROD (pmol/min prot.)	6.65 ± 0.70	
	T C	
BROD (pmol/min/mg prot.)	£6.39±1.12 €0¥	7451 ± 50389 ** 57 0°
UDPGT (nmol/min /mg prot.)	6.42 ± 0.61	Q30.69 \$1.94 **
		(+378 %) ² ~ ~
**: p≤0.01		
(%) as compared to control		
	~ _ ~ _ ~	

E. Deficiencies

No specific deficiencies were noted in the stud

III. Conclusions

These data showed that ae c65694 has the ability to induce moderately phenobarbital-inducible hepatic enzymes (total cytochrome P-400, BROD, PROD and UDPGT) as well as liver hypertrophy and cell , L proliferation in the liver. \bigcirc Ø

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These data showed that AE C636948 has the ability to induce moderately phenobarbital-inducible hepatic enzypes (totel cytochrome 7-450, BROD, PROD and BDPGT) as well as liver hypertrophy

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Data Point:	KCA 5.5/04
Report Author:	
Report Year:	
Report Title:	Phenobarbital 7-day mechanistic study in the female Wistar rat by gavage
Report No:	SA 07325
Document No:	<u>M-299491-01-1</u>
Guideline(s) followed in	No specific guideline
study:	A O' S' P
Deviations from current	Current guideline: none
test guideline:	
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011) vertices and accepted in the DAR (2011) vertices and vertices a
GLP/Officially	Yes, conducted under GLP officially recognised testing familities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes O O V V V V V

Executive Summary

Phenobarbital (batch: 06100228, white crystalline powder purity 99.6%, was administered once daily by oral gavage to groups of female Wistar rats (15/group) for 7 day at a concentration of 80 mg/kg body weight/day. A similarly constituted group received unbreated diet and acted as a control group. Animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. Body weight and food consumption were recorded at the end of the 7-day treatment period. Hepatic cell proliferation was assessed by administration of 5-bromo-2-deoxyaridine (BrdU, an analogue of thymidine) to all animals in the drinking stater for 7 days before sacrifice. Water consumption was measured during BrdU administration period. All animals were subjected to necropsy. Brain and liver were weighed Selected portions of the liver were fixed for conventional histopathological stamination and cell proliferation measurement. The remaining portions of the liver of 10 females from each group were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and oytochrome P450 intenzyme and UDPGT activities.

Administration of phenobarbital at 80 mg/kg/day for 7 days caused reduced activity in all animals tested. There was a slight effect on body weight with an overall mean absolute body weight gain of 0 g compared to 7 g in the control group. At necropsy, there was no relevant change in mean terminal body weights when compared to controls. Mean absolute and relative liver weights were increased by between 19 and 22% when compared to the controls (statistically significant: $p \le 0.01$). At macroscopic examination, dark liver was found in 5/14 formales and entarged liver in 3/14 females compared to no incidences in the controls. At historogical examination, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was found in all reated animals. A decreased incidence of periportal vacuolation was also noted. Assessment of cell profiferation in the liver revealed a 2 fold increase in mean BrdU labeling uddex in the perilobular area and in the centrilobular area in the treated animals compared to the controls.

Assessment of total cytochrome P-450 content and microsomal proteins revealed a moderate increase in total P-450 content and in mean PROD, BROD and UDPGT activities (all statistically different from the control group $p \le 0.01$). These data indicate that phenobarbital has the ability to induce hepatic enzymes like total cytochrome B 450, PROD, BROD and UDPGT activities as well as liver hypertrophy and cell proliferation in the liver.



a.

I. Materials and methods

A. Materials

1. Test material:	Phenobarbital
Description	White crystalline powder
Lot / Batch #:	06100228
Purity:	99.6%
CAS #	50-06-6
Stability of test compound:	Phenobarbital White crystalline powder 06100228 99.6% 50-06-6 Stable in rodent diet for a period covering the study duration. Methylcellulose 400 Rat Wistar RK WI (IQPS HAN) – Female only 11 weeks approximately at start of teatment 226 - 263 g
2. Vehicle and / or positive	Mathulaellulaca 400
control:	Weinvicentulose 490
3. Test animals:	
Species:	Rat Q A A A A
Strain:	Wistar RK WI (IOPS HAN) – Femaleconly 🖉 🦄 💞
Age:	11 weeks approximately at start of reatment of a A
Weight at dosing:	226 - 2 63 g 0
Source:	
Acclimation period:	12 days y y y y y y y
	Sertified rodest powdered and irradiated thet AQUC-10P1 from
Diet:	S.A.F.E. (Scientific Animal Food and Engineeting, Epinay-sur-
₹ Ø1	Orge, France), addibitum
Water:	Tap water, ad Poitume
Housing:	Animal's were caged individually in suspended stainless steel wire mesh cages
	wire mesh cages
Environmental conditions;	20-24°C 40-70% 10-75 air changesper hour 12 hours light J2 hours dark
Temperature: 🖕 🖉	
Humidity:	40-70% 5 10-15 air changesper hour 5 0
Air changes:	10-15 au changes per hour
Humidity: Air changes: Photoperiod	(12 hours light 12 hours dark 2
Humidity: Air changes: Photoperiod B. Study design 5	
1. In life dates: ×19 Novem	ber 2007 26 November 2007 2
2. Animal assignment and reatm	ber 2007 26 November 2007 6
Phenobarbital was administered onc	e daily by oral gavage for 7 days to a group of 15 females at a dose

Phenobarbital was administered once daity by oral gacage for 7 days to a group of 15 females at a dose of 80 mg/kg/day in 0.5% aqueous solution of methycellulose 400. The dose level was selected on the basis of previous studies conducted with planobarbital. Anegative control group with the same number of animals received the vehicle only (0.5% aqueous solution of methycellulose 400). Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

A solution of BrdU at 80 mg of BrdU/100 of L of drinking water was administered to all animals for 7 days to allow over cell problemation.

Table 5.549 Studydesign

Test group	Treatment	Dose level (mg/kg bw/day)	Animals assigned
	Control	0	15
, cor	Phenobarbital	80	15



Both Phenobarbital and BrdU concentrations were checked and found to be acceptable for use on the study, being in the range of 97 to 98 % of nominal concentration. Homogeneity of phenobarbital solution was also checked and found to be acceptable for use on the study.

3. Diet and water

Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France) and filtered and softened tap water from the municipal water supply in individual bottles were available ad libitum, except prior to sacrifice when animals were diet fasted overnight. Routine analyses of food and water indicated that there was to contamination which could have compromised the study

4. Statistics

- - Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight charge parameters.

The F test was performed to compare the homogeneit of group variance

If the F test was not significant (p>0.95), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant (pC0.05), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-stated).

- - Body weight and average food consumption day parameters
- Total cytochrome P450 content

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.

The F test was performed to compare the homogeneity of group variances.

If the F test was not significant (p=0.05), mean of the exposed group was compared to the mean of the control group using the stest (2 sided)

If the F test was significant $p \le 0.05$), data were transformed using the log transformation. If the F test on log transformed data was not significant (p ≥ 0.05), mean of the exposed group was compared to the mean of the control group, using the t-fest (2-sided) on log transformed data.

If the F test was significant $(p \le 0.95)$ even after by transformation, mean of the exposed group was compared to the mean of the control group using the predified t-test (2-sided).

If one or frore group variances) equal 0, means were compared using non-parametric procedures.

• - Enzymatic activities and cell proliferation parameter

Mean of the exposed group will be compared to the mean of the control group using the exact Mann-Whitney test (2-sided).

Group means will be compared at the 5% and 1% levels of significance.

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics) except for live enzyme parameters and cell proliferation parameters which were analyzed using SAS programs version 8.2.



C. Methods

1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

2. Body weight

Each animal was weighed on study Day 1 and 7. Additionally, diet fasted animals were weighed before scheduled necropsy.

3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. Any food spillage was noted.

4. Water consumption

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration. Empty water bottles were weighed on the day of schequiled sacrifice (study Day 8).

5. Sacrifice and pathology

5.1 Necropsy procedure - Organ sampling

On study Day 8, all animals from all groups were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane (Baxter, Manrepas, France). Animals were diet fasted overnight prior to sacrifice.

n

All animals were necropsied, the necropsy included the examination of the external surfaces, all orifices and all major organs assues and body cavities. Brain and liver were werghed fresh at scheduled sacrifice only.

Duodenum and two central sections of the liver taken in the seft and medial lobes were fixed by immersion in neutral buffered 10% formalin. The remaining portions of the liver from ten females from each group were kept for microsomal preparations and determination of total cytochrome P-450 content and isoencyme activities.

5.2 Histotechnology Histopathology - Cell proliferation assessment

Duodenum and the two eentral sections of the liver were endedded in paraffin wax.

Histological sections Stained with hematoxylin and eosin, were prepared and examined from all the animals.

For cell proliferation assessment, an immunolistochemical staining demonstrating the incorporation of BrdU and the determination of the labeling index were performed to assess hepatocytic cell cycling on all study animals. The impunohistochemical reaction included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of the complex with the chromogen diamino-benzidine (DAB) and nuclear counterstaining with hematoxyling.

The zonal labeling index, expressed as the number of BrdU-positive hepatocytes per thousand, was measured separately on random fields comprising at least 1000 centrilobular and 1000 periportal cells using an outomated image analysis system. The mean labeling indexes (periportal, centrilobular and combined) and standard deviation were calculated for each zone and each group.



The immunohistochemical staining for BrdU and determination of the labeling index were performed on all animals showing sufficient BrdU incorporation (estimated by water consumption or duodenum BrdU labeling).

6. Hepatotoxicity testing

At final necropsy, the remaining portions of the liver from all surviving animals were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) to check the hepatotoxic potential of the test substance. Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate. Results were compared to those generated with well known reference compounds.

II. Results and discussion

A. Clinical signs and mortality

1. Clinical signs of toxicity

All animals receiving phenobarbital had reduced potor activit

2. Mortality

One animal was found dead on day 3 of the study. The animal was discarded without necropsy. No cause of death could be established

B. Body weight and body weight gain

Overall there was no mean body weight gain in the treated group compared ton g in the control group.

C. Food and water consumption

Food and water consumption were not affected by the treatment with phenobarbital.

D. Sacrifice and pathology

Higher liver weights 20% approximately were observed in animals treated with phenobarbital compared to controls. This was associated with enlarged (3/14) and dark (5/14) liver observed at the macroscopic examination and with hepatocellular hypertrophy in all animals at the microscopic examination.

Table 5.5-50	Diver changes	after	davs of	treatm	ent with	phenobarbital	at 80 mg/kg bw/d
	(mean±SD)	s,	ر ش		ð	1	00

(mean±SLa or	
Dosage level (ppm)	80
Liver weight Absolute (g)	6.63±0.98** (+19%)
Bodyweigherelative	3.02±0.36** (+22%)
Brain-relative 296 St=20.3	358.66±49.18** (+21%)
Microscopic pathology	
Diffuse centrilobular to 0/45 panlobular hepatocellular hypertrophy	14/14
Diffuse mainly periportal 7/15 hepatocellular vacuolation 7/15	3/14
**: $p \leq 0$ (1) $p = 1$ $p \leq 0$ (1) $p = 1$	

1. Cell prosiferation

Cell propreration was assessed separately in the centrilobular and the periportal zones of the hepatic lobules. In the centrilobular and periportal areas, the mean BrdU labeling indexes were found to be 2



fold higher in treated animals, when compared to controls ($p \le 0.01$). Centrilobular index was higher than periportal index in treated animals.

Table 5.5-51Mean BrdU labeling index in the liver after 7 days of treatment with phenobarbital at
80 mg/kg bw/d (mean±SD)

00 mg/ Kg	bw/d (mean=5D)	
Dosage level (ppm)	0	80
Number of animals	15	A 14 5 2 1
BrdU positive cells in the centrilobular zone	21.73 ± 16.34	55.21 ± 43.2)** ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
BrdU positive cells in the periportal zone	16.70 ± 10.02	
Overall BrdU positive cells	19.22 ± 12.52	44:20 ± 270 9** 0 0 1
**: p≤0.01	4. 6° 5	

2. Hepatotoxicity testing

Changes that were observed are described in Table 5.54-8. Assessment of total cytochrome P#50 content and microsomal proteins revealed a moderate increase in total P 450 content and in mean PROD, BROD and UDPGT activities (all statistically different from the control group p\$0.01).

Table 5.5-52	Results of the hepatotoxicit	v testing after	7 day of treat	tment with pl	enobarbital at 80
	mg/kg bw/d (mean±St)		S L		×

			S. O	Å Ö	»° «.	
Dosage level (ppm		\sim	° × 0 ć	N B	80	
Number of animals		O A	⊘~ 10 [™]		ا ک	
Total P-450			0.95 ± 0.20		$49 \pm 0.38 **$	
(nmol/mg prot.)	Á.			Ũ	(+ 57 %)	
EROD (pmol/min/r	ng prot.)) 38/25 ± 64	2 🐇	47.56 ± 9.75 *	
		<u>Ç Î</u>		0. 4	✓ (+ 24 %)	
PROD (pmol/min p	(köt.) 🖓 🖓 🗸	× ~	<u></u> 4.89 0 0.61		26.36 ± 17.55 **	
Ô		\sim		× × ×	(+ 439 %)	
BROD (pmol/min/1	ng prot.) 🔿 🔿	V X	v ^v ∂591 ± 0370) T	94.43 ± 62.94 **	
			S O		(+ 1823 %)	
UDPGT (nmol/min	/mg prot.)	» °ð"	6.9 % ± 0.52	200~	13.47 ± 1.66 **	
				Y	(+ 93 %)	
* : p≤0.05)***: p≤0.01		S' v				

(%) as compared to control

E. Deficiencies

No specific deffciences were noted in the study

III. A Conclusions

These data indicate that phenobarbital has the ability to induce hepatic enzymes like total cytochrome P-450, PROD, BROD and UDPGT activities as well as liver hypertrophy and cell proliferation in the liver.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides preliminary information on the effects and target organs of AE C656948 in the rat

These data indicate that phenobarbital has the ability to induce hepatic enzymes like total cytochrome PATS0, PROD, BROD and UDPGT activities as well as liver hypertrophy and cell proliferation in the liver



Data Point:	KCA 5.5/05
Report Author:	
Report Year:	2011
Report Title:	2011 Fluopyram (AE C 656948): Mechanistic investigation in the female rat by distant S
	administration for up to 7 days
Report No:	SA 10240
Document No:	<u>M-408029-01-1</u>
Guideline(s) followed in	No specific guideline
study:	
Deviations from current	Current guideline: none
test guideline:	
Previous evaluation:	Not previously evaluated of A S
GLP/Officially	Yes, conducted under GLP Officially recognised testing favilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes V V V V

Executive Summary

Fluopyram, (batch number: Mix-batch 08528/0002 a light beige solid, 94.7% (w), was administered daily in the diet to groups of adult female Wistar rats (30 rats dose level) for 3 days (Subgroup 1; n = 15/group) or for 7 days (Subgroup 2; n = 15/group) at dose byels of 30, 79, 150 600 and 1500 ppm. These doses equated to 2.4, 62, 120, 46.1 and 17.6 mg/kg/day, respectively for those animals sacrificed after 3 days of treatment. For those animals sacrificed to 10wing 7 days of treatment the doses corresponded to 2.3, 5.6, 14.6, 441 and 18.5 mg/kg/day, respectively. A group of animals dosed by oral gavage with 80 mg/kg/day phenobarbital (a CAR//XR nuclear receptor activator) acted as a positive control for the parameters investigated in this pretent study. A group of eceived untreated diet. Animals were observed daily for nortality and sinical signs. Physical examinations were performed weekly. Body weight and food consumption were recorded at the start and end of the treatment period for each subgroup. All animals were necropsied in the morning following three or seven days of treatment, the fiver was weighed and sampled for investigation of several parameters. Specifically, samples were fixed and examined microscopically.

Additional slides were stained for Ki67 for cell proliferation determinations. The duodenum was also sampled and used as apositive control tissue for the cell proliferation investigations. Small portions of the liver were frozen in liquid nitrogen and used for gene expression investigations. The remaining portions of the liver from 5 randomly chosen females/group that were sacrificed after 7 days treatment were homogenized for increasing preparations in order to determine cytochrome P-450 and UDPGT isoenzyme profiles.

There were no clinical signs and no effects on food consumption or body weight parameters for any of the groups treated with fluopyram (both secrifice times). Clinical signs consisting of reduced motor activity were, however, recorded for all females dosed with phenobarbital and some of these females had ocular discharge in one or both eyes. In addition, mean absolute body weight gain was reduced by 23% (not statistically significant) for those females dosed with phenobarbital for 7 days.

Fluopyram induced theatment-related changes in all liver parameters investigated. The number of parameters affected and also the magnitude of the responses were dose related as described below.

At 30 ppm, there were no treatment-related changes in any of the parameters measured at either of the sacrifice times

At 3° ppm a marginal but statistically significant increase in the expression of Cyp3a3 was recorded both after three (+48.4%; p \leq 0.05) and seven days (+95%; p \leq 0.01) of treatment. This increase in gene expression was, however not associated with any increase in enzyme (BROD) activity following seven days of treatment.



At 150 ppm, a slight, though significant, increase in hepatic cell proliferation (centrilobular and perilobular) was observed. This effect was similar in magnitude at both sacrifice times. In addition, increased gene expression of Cyp2b1 as well as Cyp3a3 was recorded at this dose level and for both sacrifice times. Following 7 day treatment the gene expression of Cyp1a1 was statistically significantly increased. These increases in gene expression for the Phase I enzymes were not associated with increased PROD, BROD or EROD enzyme activity following seven days of treatment.

At 600 ppm, the increased cell proliferation was more marked than that already observed at 15% ppm. For example, the % change (compared to controls) in global cell proliferation at 3 days was 266.6% compared to 86.0% at 150 ppm and at 7 days it was 194.7% compared to 106% at 50 ppm? A slight but statistically significant increase in liver weight (absolute and relative to body weight) was recorded of following 3 days treatment and in one female minimal centrilobular to panlobular hepatocellular hypertrophy was observed following 7 days treatment. Statistically significant increases were recorded at both sacrifice times for the gene expression of Phase I (Cyp2b1; Cyp3a3) and Phase II enzymes (Udpgh2; Gstm4 and Ephel). Gsta2 was significantly increased following 7 days treatment. Cyp1a1 gene expression was statistically significantly increased at both sacrifice times but was not associated with any enzyme activity at this dose level.

At 1500 ppm, the effects on cell prolitaration were even more marked than those recorded at 600 ppm. Specifically, the % change (compared to controls) in global cell proliferation at 3 days was 551.2% compared to 266.6% at 600 ppm and at 7 days it was 256.6% compared to 1917% at 600 ppm. In addition, an increased number of mitoses was observed in 465 females dosed for 9 days treatment with fluopyram. Furthermore a slight, though statistically significant increases in the expression of Tacstd1 (a marker for cell proliferation; 3 day treatment only) and Gadd45b (a marker for apoptosis, following 3 and 7 days treatment) was recorded. Significant increases in mean absolute and relative liver weight were recorded at both, time points, which could be associated with the centrolobular to panlobular hypertrophy observed in 6/6 females following 3 day treatment (minimal) and in 14/15 females following 7 days treatment (minimal to stright). The increases in enzyme activity observed at 600 ppm were more marked at 1500 ppm with statistically significant increases in EROD as well as total P450 content also being recorded at this dose level.

Phenobarbital was used as a positive control for the various parameters measured in the present study and as such induced charges in the liver pertinent for a compound that activates the CAR/PXR nuclear receptors. Thus, increased cell proliferation was observed following both 3 and 7 days treatment, with the effects being more apparent in the centrilobular region (+247.7% and +603.4% increases compared to controls at 3 and 7 days, respectively) than in the perilobular region (+58.0% and +54.7% increases compared to the controls at 3 and 7 days, respectively). In addition an increased number of mitoses was observed in 3/D5 females at both time points. Furthermore Gadd45b (a marker for apoptosis, following 3 and 7 days treatment) gene expression was statistically significantly increased at both time points. Liver weight (absolute and relative to brain and body weight following 3 days treatment; relative to body weight only following 7 day treatment) was statistically significantly increased following phenobarbital treatment, which could be associated with the centrilobular to panlobular hypertrophy observed in 3/15 females following 3 day treatment (minimal) and in 9/15 females following 7 days treatment (minimal to slight), BRCD, PROD and UDPGT-nitrophenol activity were statistically significantly increased due to 7 days treatment with phenobarbital. Furthermore, statistically significant increases in the gene expression of Cyp2/1, Cyp3a3 and Udpgtr2 were recorded (at both time points).

Overall. Grear and statistically significant changes in the liver (cell proliferation, hypertrophy and enzyme activity as well as associated changes in gene expression) were observed following fluopyram treatment. These changes were recorded as early as following 3 days of treatment and starting from 150 ppm. The dose of 75 ppm was considered as a NOAEL; based on the increased gene expression of Cyp3a2 at this dose level) and 30 ppm as a NOEL.



I. Materials and methods

A. Materials

1.	Test material:	AE C656948
	Description	Light beige solid
	Lot / Batch #:	Mix-Batch: 08528/0002
	Purity:	94.7%
	CAS #	658066-35-4
	Stability of test compound:	AE C656948 Light beige solid Mix-Batch: 08528/0002 94.7% 658066-35-4 Stable in rodent diet for a period covering the study duration Phenobarbital (positive control) White crystalline powder Lot No. 06100228 99.6% 50-06-6 Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration
2.	Vehicle and / or positive	
	ontrol:	Phenobarbital (positive control)
•••	Description	Phenobarbital (positive control) White crystalline powder Lot No. 06100228 99.6% Stable in 0.5% aque tas solution of methylcellulose for a period covering the study duration
	Lot / Batch #:	Lot No. 06190228
	Purity:	99.6% &. 6° 57 57 70 5° 57 57
	CAS #	50-06-60
		Stable in 0.5% aque was solve on of metholcellul se for preriod
	Stability of test compound:	covering the study duration
3	Test animals:	
5.	Species:	
	Strain:	Kat Wistar Rj: WI (IQPS HAN) – Feorale only 5
		10 Charles Control Con
	Age:	(Rat Wistar Rj: WI (IQPS HAN) – Febrale only 10 weeks approximately at start of treatment 218 - 263 g
	weight at dosing:	2)8 - 263 g 0 4 6 6
	Source: Acclimation period: ©	
	Acclimation period: 🖉 🛛 🛇	12 days
		Certified sodens powdered and irradiated dies A04C-10P1 from
	Diet:	S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-
	Ĩ, Š	Orge France), ad Libitum
	Water:	Certified roden, powdered and irradiated diet A04C-10P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur- Orge France), ad libitum Tap water, ad libitum Animals were caged individually in suspended stainless steel
	Housing:	Animals were caged individually in suspended stainless steel
	Water:	Wire mesh cages.
		Wire mesh carges. $26-24^{\circ}C^{\circ}$
	Environmental conditions: Temperature: Humidity: Air changes: Photoperiod:	26-24°C ^O
	Humidity:	\$10-70% ° * * ° . O
	Air changes:	10-16 air changes per hour
	Photoperiod	12 Nours Bight, 10 hours dark
D	Photoperiod: S Study design	
	Study design	$\mathcal{O}' \mathcal{O}' \mathcal{O}' \mathcal{O}'$
1.		10 - 2 Julo 2010 0
2.	Animar assignment and treatm	

Animals were assigned to dose groups using a randomization procedure by weight.

Seven groups of female rats were dosed for up to 7 days with the appropriate compound by the appropriate route of administration. Each group consisted of 30 female rats, 15 of which were sacrificed the morning after three days of treatment (Subgroup 1). The remaining 15 animals were sacrificed the morning after seven days of treatment (Subgroup 2).

Six groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the remaining five groups received fluopyram at the appropriate dietary level (30, 75, 150, 600 and 1500 ppm) at a constant level. The dose levels for fluopyram were set after evaluation of the results from previous studies conducted with fluopyram and were set after evaluation of the results from previous studies conducted with fluopyram and following discussions with the US (EPA) and Canadian (PMRA) authorities. The top dose level of 1500 ppm used in the present study represents the



top dose level used in the rat cancer bioassay in which an increase in liver tumors was observed in the females. $^{\circ}$

The seventh group was dosed by oral gavage with 80 mg/kg/day phenobarbital suspended in \$.5% aqueous methylcellulose 400 using a dosing volume of 5ml/kg bodyweight. The volume administered to each rat was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose is known to clearly induce liver cytochrom®P-450 activity and cell proliferation in the rat.

Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH public from N 86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

1 abic 5.5-	55 Study design	&, Ö	° 5° 5° 60		y W
		Dose per animal	(study averages)	Animals	assigned
Test	Concentration in diet	Femates Subgp 1 »	Females Subg 2	O' Fomolos	Famal
group	(ppm) of AE C656948	Days 1-3 (mg/kg	Dave 1-7 (mg/kg	5 ⁹ Females	Subgp 2
		🖉 bw(day) 🔬	bw/day) 🔬	Subgh 1	Subsp 2
1	0			15 °	۵ ا
2	30 🔍	2.4	2.3		15
3	75 🕡	<u> </u>	J 5,6 O		15
4	150	× ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~	A 16 Q	15 O [*]	15
5	600	46.1	0 44.k ž	× 19	15
6	750 📎 🕺	11.28	\$ 118.5 D	~~15	15
7	Phenobarbital 80 mg/kg			× 15	15
	bw/day by gavage 🔌			s,	
C 1		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	Ĩ (//)		

Subgp = Subgroup

3. Diet preparation and analysis &

Fluopyram (AE C656948) was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation for each concentration used in the study. The stability was demonstrated in an earlier study at concentrations of 20 and 10000 ppm for a time which covered the period of usage and storage for the current study. Homogeneity of test substance in diet was verified on the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean value obtained in homogeneity check was used as measured concentration. For the remaining dietary levels, concentration was checked.

A single formulation of phenobarbital was prepared by suspending the test substance (w/w) in a 0.5% aqueous solution of methylcellulose. The stability of phenobarbital at 8 g/l was demonstrated in an earlier study, which evered the period of usage and storage for the current study. Homogeneity of phenobarbital in aqueous methylcellulose was verified to demonstrate adequate formulation formulation procedures. The mean varie obtained in nongeneity check was used as measured concentration.

4. Statistics 🔍

4.1 Variables analyzed

- Body weight parameters
- Body reight change parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- $-\widehat{\mathfrak{G}}$ erminal body weight, absolute and relative organ weights parameters
- Total cytochrome P450 content and liver enzyme activities



- Cell proliferation parameters
- Gene transcript analysis

4.2 Statistical methods

Group means were compared at the 5% and 1% levels of significance.

With the exception of those used for the cell proliferation data, all statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). SAS programs (version 9) were used for the cell proliferation data.

Fluopyram Treatment

- · Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

The Bartlett test was performed to compare the homogeneity of group ariances.

If the Bartlett test was not significant (p>0.05), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant (p>0.05), the group means were considered to be homogeneous and no further analysis will be performed. If the ANOVA was significant ($p\leq0.05$), means of the exposed groups were compared to the mean of the control group using the Dunnet test (2-sided).

If the Bartlett test was significant ($p \ge 0.05$) group means were compared using the non-parametric Kruskal-Wallis test of the Kruskal-Wall's test was not significant ($p \ge 0.05$), the group means were considered to be homogeneous and no urther analysis was performed. If the Kruskal-Wallis test was significant ($p \ge 0.05$), means of the exposed groups were compared to the mean of the control group using the Durn test (2 sided).

- · Body weight and average food consumption/day parameters
- Total Cytochrome R450 content and liver enzyme activities
- Gene transcript analyses

Mean and standard deviation were calculated for each group and per time period for average food consumption day parameters.

The Bartlett test was performed to compare the homogeneity of group variances.

If the Bartlett test was not significant (p>0.05), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant (p>0.05), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant (p=0.05), means of the exposed groups were compared to the mean of the control \sim group using the Dupnett test (2-sided).

If the Bardett test was significant ($p \le 0.05$), data were transformed using the log transformation.

- If the Bartlett test on log transformed data was not significant (p>0.05), means were compared using the ANOVA on log transformed data. If the ANOVA on log transformed data was not significant (p>0.05), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log transformed data was significant (p \leq 0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (2-sided) on log transformed data.

 $\Box \tilde{f}$ the Bartlett test was significant (p \leq 0.05) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not



significant (p>0.05), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant (p \leq 0.05), means of the exposed groups were compared to the mean of the control group using the Dunn test (2-sided).

Cell Proliferation analyses

The Levene test was performed to compare the homogeneity of group variances.

If the Levene test was not significant (p>0.05), means of the exposed groups were compared too the mean of the control group using the Dunnett test (1-sided).

If the Levene test was significant (p≤0.05), data were transformed using the log transformation

If the Levene test on log transformed data was not significant (p>0.05), means of the opposed groups were compared to the mean of the control group using the Dunner test (1-sided) on log transformed data.

If the Levene test was significant ($p \le 0.05$) even after log transformation, becaus of the exposed groups were compared to the mean of the control group using the Dunn test (1-steed).

If one or more group variance(s) equal 0, means were compared using 10n-parametric procedures.

Phenobarbital Treatment

- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

The F test was performed to compare the homogeneity of group variances

If the F test was not significant (p-0.05), the mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ($p \le 0.05$), the mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

· Body weight and average food consumption/day parameters

• Total cytochrome P450 content and liver of zyme activities

• Gene transcorpt analyses 🖉 🐁

Mean and standard deviation were calculated for each group and per time period for average food consumption/deviation were calculated for each group and per time period for average food

The <u>F</u> test was performed to compare the homogeneity of group variances.

If the F test was not significant (p > 0.05), the mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ($p \le 0.05$), the data were transformed using log transformation. If the F test on log transformed date was not significant (p > 0.05), the mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.

If the F test was significant ($p \le 0.05$) even after log transformation, the mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

Cell@rolifesation analyses

The F test was performed to compare the homogeneity of group variances.

If the F test was not significant (p>0.05), mean of the phenobarbital exposed group was compared to the mean of the control group using the t-test (1-sided).



If the F test was significant ($p \le 0.05$), data were transformed using the log transformation. If the F test on log transformed data was not significant (p > 0.05), mean of the Phenobarbital exposed group was compared to the mean of the control group using the t-test (1-sided) on log transformed data.

If the F test was significant ($p \le 0.05$), even after log transformation, mean of the exposed group was compared to the mean of the control group using the exact Mann-Whitney test (1-sided)

If one or more group variance(s) equaled 0, means were compared using por-parametric procedures.

C. Methods

1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded to least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were fecorded.

2. Body weight

Each animal was weighed during the accilimatization period. Body valghts vere also measured of study Day 1 and 3 for Subgroup 1 and on study Day 1 and 7 for Subgroup 2. Additionally, dia fasted animals were weighed before scheduled nectopsy (terminal body weight).

3. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded on study Day 3 for all animals in Subgroup 1 and on study Day 7 for all animals in Subgroup 2. Any food spillage was noted. From these records, the mean achieved dosage intake in mg/kg/day for each Subgroup was calculated.

4. Sacrifice and pathology

4.1 Necropsy procedure - Organ sampling

On study Day 4 (Subgroup 1) and study Day 8 (Subgroup 2), all animals were sacrificed by exsanguination under deep anesthesia by inhalation of soflurane (Baxter, Maurepas, France). Animals were diet fasted overnight prior to sacrifice.

All animals were necropsied. The necrops sincluded the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Brain and liver were weighed fresh at scheduled sacrifice only.

Duodenum and two central sections of the fiver taken from the left and medial lobes from each animal were fixed by immersion in neutral buffered 10% formalin. In addition, a piece of the median and the left lobe of the liver from each animal were collected and flash frozen in liquid nitrogen, were stored at approximately -74° C $\sim 10^{\circ}$ C until used for qPCR investigations. From the Subgroup 2 animals, the remaining portions of liver from each of 5 randomly selected females per group were weighed and homogenized for microsofial preparations.

4.2 Histotechnology

4.2.1 Conventional Histopathological examination

Duodenus and two central sections of the liver were embedded in paraffin wax.

Histological sections, stained with hematoxylin and eosin, were prepared for each animal in all groups.

4.22 Ki67 staining for Cell proliferation assessment

For each animal in each group a section of a formalin-fixed paraffin-embedded block containing 2 liver samples and one sample of duodenum was prepared. The duodenum was included to serve as a positive



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control for staining as it has a high rate of cell proliferation. The immunohistochemical reaction included incubation with a monoclonal antibody raised against Ki67, amplification with a secondary biotinylated antibody and a streptavidin-horseradish complex, detection of the complex with diamino-benzium (DAB) and nuclear counter staining with hematoxylin.

4.3 Histopathology

Histopathological examinations were performed on the liver samples from all animals in all groups.

4.4 Cell proliferation assessment

The immunohistochemical staining for Ki67 and determination of the tabeling index was proformed on all surviving animals showing sufficient Ki67 staining (estimated by duodenal Ki67 labeling) to assess cell cycling in the liver. The zonal labeling index, expressed as the number of Ki67-positive heratocytes per thousand cells, were measured separately on random fields comprising of a least 1000 centrilobalar and perilobular cells using an automatic image analysis system. The mean and standard deviation were calculated for each group.

4.5. Hepatotoxicity testing

At final necropsy (ie Day 8), the remaining portions of the liver from were five randomly chosen tenales per treatment group (Subgroup 2) were weighed and bomogenized for microsomal preparations in order to determine total cytochrome P 430 content and specific cytochrome P 450 isoenzyme profile (including EROD, BROD and PROD activities) and UDPGT specific soenzyme profiles.

4.5.1 Total cytochrome P-450 content

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry (Cary Win UV version 3.0 (182)) using a reduced CO differential spectrum A single quantification was performed for each sample.

4.5.2 Enzymatic activities

Cytochrome P-450s and typical inducing agents					
Family	Enzôpmatic activity	Activity	Typical inducing agents		
CXXP 1A1		activation of mutagens	β-naphtoflavone		
1A2		stord carcológens			
CYP 2 2B1	\mathcal{L}' \mathcal{N} \mathcal{O}' \mathcal{L}'	detoxication of drugs and Chemicals activation of	Phenobarbital isoniazid		
2B2	PROD O	nutrosamines			
CYP 3A1 & 3A2		toxication of drugs and	Pregnenolone 16 α		
		chemicals	carbonitrile		
		<i>i</i>	phenobarbital		

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry (SAFAS SP2000 version 6.10.7.4) using the following substrates:

	Å	_ A	- etho	xvre	sørufi	n (ER	OD)
d		Ľ	R	2		fin (PF	,
		<u> </u>	<u> </u>	õ		,	,
K, ^v	- Si	A.		goxyi		IIIII (D	ROD)

Ethosyresorphin is a highly selective substrate for the isoform 1A, the isoform 2B metabolizes preferentially the O-dealkylation of pentoxyresorufin, while the benzoxyresorufin O-debenzylation is mainly metabolized by the isoform 3A. Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37°C.



Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate (method adapted from Zakim and Vessey. The enzymatic kinetic (disappearance of the colored 4-nitrophenol) was followed at 405 nm during 3 min. at 30°C. Three replicates from each sample were assayed. UDPGT with bilirubin as substrate was also determined using a spectrophotometry method (adapted from Heirwegh et al.) consisting in the determination of conjugated bile pigments after its conversion into azo-pigment derivatives. Absorbance was measured at 530 nm. Three replicates from each sample were assayed.

4.6 Quantitative PCR Analyses

4.6.1 Total RNA purification

Total cytoplasmic RNA was isolated from the liver of all surviving individuo control and reated animals using RNeasy Midi kits (Qiagen). RNA quality controls were performed based on the ribosontal RNA electrophoretic profiles using a Bioanalyset Agilent Technologies).

4.6.2 Quantitative PCR

Ten µg of total RNA was used for Reverse transcription (QT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on depand, Applied Biosystems), 1/50 diluted first strand cDNA Fast Start Universal Probe Master mix (Roche) on an ABI prism 7900 HT machine (Applied Biosystems), for each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA.

			<u> </u>
Gene	Abbreviation	Refset ID	∑∑Taqman assay ID
(Major function)		I Ô k. V	(Applied
X			Biosystems)
Ū [†]			
P450	POR S	NM_@\$1576.¶@	Rn00580820_m1
(cytochrome)oxidoreducta			
se <u>o</u>			
Cytochrome P450 12	Kutal	012540.2	Rn00487218_m1
Cytochrom@P450 2b1	Cyp212	¹⁰ 275205*	Cyp2b1_tc5
Cytochronne P450 3a3 🛈	Cxp3a3 (Cxp3a23)	\$ NM 013103.2	Rn01640761_g1
Cytochrome P450 4at	xxp4al S	NM_175837.1	Rn00598510_m1
<u> </u>	🔶 🔗 METABOLIS		
Glutathione S-trapsferase	Gstate &	NM@917013.4	Rn00566636_m1
A2 ~ ~		Y AY	
Glutathione Sorransferase	Sstm4 ~ ~ ~	NM_020540.1	Rn01789233_m1
mu3 v			
UDP 🔗		M_173295.1	Rn00756519_m1
glucorocosyltransferase 2		✗ NM_057105.3	
family, polypeptide			
Epoxide hydrolase 1,	February Contraction of the second se	NM_012844.2	Rn00563349_m1
microsomal			
Epoxide hydrolase 2, 🔨 🕚	Kohx2 × ×	NM_022936.1	Rn00576023_m1
cytoplasmic			
Sulfotranterase family 1E		NM_012883.1	Rn00820646_g1
member 🖉 🖉			
	CELL PROLIFERA		
	Tacstd1	NM_138541.1	Rn01473202_m1
molecule			
Growth@rrest and DNA-	Gadd45b	NM_001008321.1	Rn01452530_g1
damage-inducible 45 beta			
Retinoblastoma 1	Rb1	XM_344434.3	Rn01753308_m1

Table 5.5-55The list of Taqman assays used is as follows :



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*: Transcript made to order

Beta-microglobulin (B2m; Refset ID: NM 012512.1; Taqman assay ID: Rn00560865 m1) was selested as reference gene for the quantitative calculations of transcripts in the liver. The relative quantity (RO) value of each test transcript was calculated using the following formula:

$\Delta\Delta Ct = (Cttest - CtB2m)_{treated} - (Cttest - CtB2m)_{control}$

 $RO = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. As a Ct of ≥35 indicates that a gene is poorly expressed in the tissue investigated, any subsequent RQ data generated from such a Ct are considered a non-relevant due to an increased risk of contamination.

II. **Results and discussion**

A. Clinical signs and mortality

1. Clinical signs of toxicity

Three day treatment (Subgroup 1):

Fluopyram: There were no treatment related clinical signs during the three day reatment period

Phenobarbital: All females displayed reduced, motor activity starting from Day 2 of treatment. In addition, three females had ocular discharge in either one eye or both eyes on Dav3 of treatment.

Seven day treatment (Subgroup 2):

Fluopyram: There were no greatment-related clinical signs during the seven day treatment period.

Phenobarbital: All females displayed reduced more activity starting from Day 1 of treatment. Two females had ocular discharge starting from Day 2 of treatment.

2. Mortality

There was no nortalitoin any group during the course of the stu

B. Body weight and body weight gain

Three day treatment (Subgroup 1)

Fluopyram: There were no treatment-related changes to any of the body weight parameters between Day 1 and Day 3. Ø

Phenobarbital: There vere no relevant changes in any of the body weight parameters between Day 1 and Day 3 cooppared to the controls?

Seven day treatment (Subgroup 2):

<u>Fluopyram</u>: There were no relevant changes in any of the body weight parameters between Day 1 and Day compared to the controls.

Phenobarbital: Between Days and There was a reduced mean absolute body weight gain compared to the control group (10g compared to 13g in the control group; -23%, not statistically significant).

C. Food and water consumption

There were no reatment-related effects on food consumption for either Fluopyram or Phenobarbital following treatment for either 3 or 7 days

The mean achieved dietary intakes of Fluopyram expressed in mg/kg/day received by the females during the stud were as follows:



e d

Mea	n achieved dietary intake of fluor	oyram a bar a b
Diet Concentration (ppm)	Subgroup 1 Days 1 – 3 (mg/kg/day)	Subgroup 2 Days 1 – 7 (mg/kg/day)
30	2.4	© 2.3
75	6.2	5.6 5.6
150	12.0	11.6 2 2
600	46.1	44.1
1500	117.6 🖏	118.50 ~ 4
	No.	

Table 5 5-56 Mean achieved intake of fluonyram

D. Sacrifice and pathology

1. Terminal body weight and organ weight

Three day treatment (Subgroup 1): the controls. The mean absolute and mean liver to body weight ratio were statistically senificantly higher from 600 ppm when compared to controls. In addition, the mean liver to brain weight ratio was also statistically significantly higher at 1500 ppm when compared to control These liver weights changes were considered to be toxicologically relevant. L.Q

Phenobarbital: There was no change in mean terminal body weighten treated anithals when compared to the controls. Mean absolute and relative liver worghts were statistically significantly higher when compared to controls. These fiver weights manges were considered to be toxicologically relevant. à Ô

Table 5.5-57	Mean absolute and relative fiver weight changes following 3 day treatment with fluopyram or phenobarbio	h
	fluopyram.or phenobarbital	

Martin all the Spectra		D	(0/ .1	<u> </u>	1 4	(
Mean liver weight ± SD at sc	beanied :	Day sacringe			pared to cor	itrois)
Sea Sea		~ ~	Fen	ales 🦉		
Dose grôup (ppn) 😽	% 0			× 600	1500	PB
	Ő «	P 🔊 🖒	, 2			(80
			0			mg/kg)
Mean absolute liver weight (g)	5 84	©.93 ⁶ 5.9		6.23*	6.84**	6.36**
	.30	±0.35 ±0.4	1 9 ≠ 0 ,26	±0.49	±0.38	±0.57
		(+2%) (+2%)	%) (* 5%)	(+7%)	(+17%)	(+9%)
Mean liver to both weight ratio	2.69	2. 9 0 2.7	5 2.79	2.83*	3.15**	2.93**
(B) A. S	±608	±0.15 ±0.1	30 ±0.10	±0.17	±0.17	±0.22
		(0%) (+2°	(+4%)	(+5%)	(+17%)	(+9%)
Mean liver to brain weight ratio	313.30	318.52 321.	00 331.36	5 333.10	365.96**	341.02**
	±17.26	±18.34 £25.	37 ±18.03	3 ±25.17	±32.54	± 28.83
		°≈(<i>≠</i> 2%)≈(+2%	(+6%)	(+6%)	(+17%)	(+9%)

* p≤0.05©** p≤0.01

Seven day treatment (Subgroup 2):

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls. Mean absolute and relative liver weights were statistically significantly higher at 1500 ppm when compared to controls. These liver weight changes were considered to be toxicologically relevant.

Phenobar Gital: There was no change in mean terminal body weight in treated animals when compared to the controls Mean-liver to body weight ratio was statistically significantly higher when compared to controls. The change was considered to be toxicologically relevant.

Æ,



Table 5.5-58 Mean absolute and relative liver weight changes following 7 day treatment with fluopyram or phenobarbital

Sex				Fema	les		N R
Dose group (ppm)	0	30	75	150	600	© 1500	OPB S
					Ő	¢″	× (80 ~
					4		⊳ mg/kǧ)
Mean absolute liver weight (g)	6.18	5.88	5.96	5.96	6.29	7.17**	666 6 🔬
	±0.91	±0.53	±0.#2	±0.35	⊈ Ø.49	±0.54	≈ ≇0.37 <i>s</i>
		(-5%)	(-4%)	(-4%)	(+2%)	(+1.6)	
Mean liver to body weight ratio	2.67	2.52	<i>2.59</i>	2.61 0	∛ 2.76	36,6**	2.95
(%)	±0.34	±0.13 a	©±0.14	±0,201	±0,16	_Q=0.16	≠0.17 ¢
		(-5%)	(-3%)	(-2%)	Ø3%) "	(+18%)	(+11%)
Mean liver to brain weight ratio	332.98	317.58	321.09	320.04%	336.49	392.26**	361.85
(%)	± 53.94	±27.19	@2 ³ .25 ∝	D#23.7\$J	±22.32	±\$9.24 °≈	±20.36
		©5%),	Q(-4%)	(-4%)	(+ D %)	\$ ⁴ 18%;	(+9%)

2. Gross pathology

Three day treatment (Subgroup 1) Enlarged liver was found in 2/15 females at 1500 ppm fluopy no macroscopic changes

Table 5.5-59 Macroscopic changes in the liver following 3 days treatment with fluopyram or phenobarbital O

Incidence and severity of	macrosco	pic changes in	n Gr e liver- sched	luled 3 day	sacrifice
Sex 🖉 🧳	s i		<u> </u>	s 🏹	
Dose group (ppm)		30 750	159 600	1500	PB
	′ ≪ [¥] .	\sim		<u> </u>	(80 mg/kg)
Number examined	¢ 15	M5 & 15	~15 15 15 15	15	15
Splarged 0	0 061		y 0 2 0	2	0

Seven day Greatment (Subgroup 2):

Enlarged liver was found in 3055 females at 1500 ppm fluopyrand There were no macroscopic changes recorded for the phenobarbital treated females. j" **K**

\bigcirc Ø Macroscopic changes in the liver following 7 days treatment with fluopyram or Table 5.5-60 phenobarbital \bigcirc

Incidence and severits of macroscopic changes in the liver- scheduled 7 day sacrifice									
Sex 🔊	Ô		Ú. N.	W Females					
Dost	0	30	15	× 150	600	1500	PB		
дкоир		A W					(80 mg/kg)		
(ppm)			~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~						
Number	۵۴۵ _	A5 .0		15	15	15	15		
examined	× 1°		- Q						
Enlarged 🔊	O O Y		0	0	0	3	0		
	× v	Ô V	~Q						

3. Microsopic pathology

Three day treatment (Subgroup 1):

Flugpyram Minimal centrilobular to panlobular hepatocellular hypertrophy was found for 6/15 females at 1500 pm. In addition, an increased number of mitoses in hepatocytes was found at the same dose level for 4/15 females.



Phenobarbital: Minimal centrilobular to panlobular hepatocellular hypertrophy was found for 3/15 females. In addition, an increased number of mitoses in hepatocytes was found for 3/15 females.

Table 5.5-61 Microscopic changes in the liver following 3 days treatment with fluopyram or phenobarbital

Ind	I donoo ond		ianagaania aha	ngag in the li	ver- scheduled	day agait	
Sex	idence and	severity of m	icroscopic cha	Females	ver- scheduled		$\frac{2e}{\sqrt{2}}$
Dose group (ppm)	0	30	75	150 T	600 x	1500	198 (80 mg/kg)
Number examined	15	15	15	£ 15	Q3 Q4		
Hepatocellula	r hypertroph	y: centrilobula	ar to panlobula			Q	
Minimal	0	0	0 ~	.0 @	<u>``</u>		Ŭ [™] 3,© [™]
Total	0	0	0 🐇				/ 3
Increased nun	nber of mitos	ses	. 0	L Û		° 4	A co
Present	0	0	AQ . (∞ 0	~~ 4 [°]	3 0
Total	0	0		~ 0 \circ		×4, ,	38
leven dev tr	ootmont (S	uharoun 2).	C. LY	L ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		<u> </u>	Ő

Seven day treatment (Subgroup 2):

Fluopyram: Minimal to slight centrilobular to particular hepatocellitar hypertrophy was tound from 600 ppm (1/15 females at 600 ppm, 14/15 females at 900 ppm).

Phenobarbital: Minimal toglight centrilobular togranlobular hepatocefular hypertrophy in 9/15 females was found as well as an increased number of phoses in hepatocytes in 3/45 females.

Table 5.5-62 Meroscopic changes in the liver following 7 days treatment with fluopyram or

Inc	idence and seve	erity of microsco	prc changes in the l	iver-schedule	l 7 day sacrif	fice
Sex	8 .C .C		Females	S a.		
Dose			′5 <u>`</u> ~`	×600	1500	PB
group	, A			20°		(80 mg/kg)
(ppm)				\circ		
Number	15	รั 15 🔬 ล์ฑ์	5 3 4 15	15	15	15
examined				9		
Hepatocellula	r hypertrophy: co	entolobular to pa	nføbular 🖉 🖉			
Minimal		NO O	V V V	1	6	6
Slight 🔌			0 🖉 🔊 0	0	8	3
Total 🔬	0 0,			1	14	9
Increase	nber of mitosses					
Present		¥0 ~ ~ ~	0	0	0	3
Total	6 , 2		0	0	0	3

4. Cell proliferation

Three day treatment (Subgroup 4):

Fluopyrame Dose related increases in the centrilobular, perilobular and, therefore, the global proliferation increases were observed, which were statistically significantly higher from 150 ppm when compared to control s



Mean cell pro	liferation index	± SD following thr cont	ee days treatment rols)	(% change when	compared to °
Test Substance	Dose	Metric	Centrilobular	Perilobular	Total O
Control	0 ppm	N	15	15 0	ð s
		Mean \pm SD	14.6 ± 7.4	11.1 + 3.1	12.8 ± 5.8
Fluopyram	30 ppm	Ν	14	₄ 14	S 14 S
		Mean \pm SD	11.8 ± 8.0	107 ± 5.8	<u>~</u> 11.2,±∞.4 √
		% Change	<u></u>	-3.5%	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
	75 ppm	Ν	₩15	Q 15	A
		Mean \pm SD	£13.4 ± 5.9	O [♥] 15.4 ± 8.2≪	04.4±58 0
		% Change	<u>ج</u> -8.2% ج	39.3%	12.8%
Γ	150 ppm	N d	p [*] 15 🚬 *	2 15Q , (
		Mean ± SD 💖	25.1 ± 1	≥22.6 +13.4**	23 8 ± 10 6**
		% Change	\$ 71 .9%	104.2%	≥ 86.0%
	600 ppm	N U s		8 15 °	
		Mean SD.	57, 3 , 4 20.1	36.6±25.7**	47.0 15.8
		% Change	<u>∽</u> ∕293.8©r	€ 230.7% ≪	266.6%
	1500 ppm	[©] N ⊗		×15 ×	2 15
		Mean & SD &	≫ 99.5 ₽ 62.3**	6.4 ± 3.00**	Ø83.4 ± 38.3**
		🔗 % Change 🚿	583.2%	\$ 509.9%	\$\$1.2%
p≤0.05; ** p≤0.01		× & &			\sim

Table 5.5-63	Mean cell proliferation index following 3 days treatment with fluopyram
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In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences. $\int_{\partial Y} \int_{\partial Y} \int_{$

<u>Phenobarbital:</u> Centrilobular, perilobular and, therefore the global proliferation indexes were statistically significantly higher, when compared to control. The magnitude of the response was greater in the centrilobular region (217.7% increase compared to the corresponding controls) than that observed in the perilobular region (38% increase compared to the corresponding controls).

Ø

Table 5.5-64 O Mean cell proliferation index following 3 days preatment with phenobarbital

Mean cell proliferation index SD following three days treatment % change when compared to									
Test Substance	Dose &	Metric	Centrilobular	Perilobular	Total				
Control			(15 ×	15	15				
	3 . ⁴ 69	$Mean \pm Sp$	014.6 ± 7.4	11.1 ± 5.1	12.8 ± 5.8				
Phenobarbital	80 pros/kg/dats	N N	A4	14	14				
Ø	, or or .	©Mearo∉ SD ⊙	406.25 ±	$17.48 \pm 8.95*$	$31.87 \pm$				
~~			>36.24**		19.80**				
A	° 29'	Se Change	0 217.7%	58.0%	148.8%				

* $p \le 0.05$; * $p \le 0.0$ In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Seven day treatment (Subgroup 2): Q

<u>Fluopyram</u>: Centrilobular, perhobulal and, therefore, the global proliferation indexes were statistically significantly higher from 150 ppm when compared to controls.

ruopyram: Centrilobular, persobular and, therefore, the global significantly higher from 150 ppm when compared to controls.



Mean cell pro	liferation index	± SD following sev cont	en days treatment rols)	t (% change when	compared to
Test substance	Dose	Metric	Centrilobular	Perilobular	Total 🔗
Control	0 ppm	Ν	15	15 0	5 0
		Mean \pm SD	8.3 ± 5.3	10.5 + 5.1	9.4∞± 5.1√
Fluopyram	30 ppm	Ν	15	₄ 15	\$ 15 K
		Mean \pm SD	11.2 ± 7.6	15.3 ± 11.2	s_13.2,±\$\$.6 √
		% Change	2 5%	45.6%	K 40,7% S
	75 ppm	N	₩15	Q 15	2 ¹⁵ 2 1
		Mean \pm SD	12.0 ± 6.1	O [♥] 10.3 ± 5.1 ≪	M.1±M 0
		% Change	ي 43.5% <u>م</u>	。NC	18.5%
	150 ppm	N Q	p ^y 15 🔊	2 15Q (
		Mean \pm SD \approx	20,4 ± 13%**	≈√18.4 +8.1**	1964 ± 9.68*
		% Change	2 144, 5%	\$ 3.2% &	≫106.0%
	600 ppm	N U s		8 15 °	
		Mean SD.	27.8 ± 12.1**	427.0±4€.0** (27,4 £ 9.8*€
		% Change	>234.1	C 159.6% X	191.7%
Ī	1500 ppm	₩ N & Y	<u></u>	×15 Å	L 15
		Mean & SD	> 32.2€19.6*	34.9 ± 172**	©33.5 ± 15.0**
		% Change	286.3%	<u>کَ 232</u>	\$\$6.6%

Table 5.5-65	Mean cell proliferation index following 7 days treatment with fluopyram	
--------------	---	--

NC: no change; ** $p \le 0.01$

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: Centrilobular and the global poliferation indexes were statistically significantly higher when compared to controls. The magnitude of response was greater in the centrilobular region (603.4% increase compared & controls) compared to that recorded for the perilobular region (54.7% increase S. O.S. ¢ compared to controls). \bigcirc

Mean cellprolifePation and ex following days treatment with phenobarbital Table 5.5-66

Test Substance	Dose 2	Metric	Centrilobular	Perilobular	Total
<u>z</u> Ç		ф.	ST IS O		
Control	0 ppnx 🛛		15	15	15
		Mean ± SD	$0^{8.3} \pm 5.3$	10.5 ± 6.1	9.4 ± 5.1
Phenobarbital	80, mg/kg/day	Nº		15	15
~~					
*		Mean ± SD	\$.6 ± 33.9**	16.2 ± 10.2	37.4 ± 19.6**
		Chairse	603.4%	54.7%	297.9%

* p≤0.05;[®]¥ p≤0.01

* $p \le 0.05$; ** $p \le 0.01$ In the above table the presented & change do not@ways aculate exactly from the presented mean data. This is due to rounding-up differences.

5. Hepatotoxicity testing

Investigation of hepatotoxicity was only conducted on 5 randomly chosen females/group from those animals dosed for days Subgroup 2).

Fluopyram: No statistically significant changes in total P450 content or enzyme activity was observed up to 450 ppm. Statistically significant increases in PROD (p ≤ 0.05 at 600 ppm and p ≤ 0.01 at 1500 ppm), BROD and both UDPG isoforms (p≤0.01) were observed from 600 ppm. At 1500 ppm, total P450 content \Im as also statistically significantly increased (p ≤ 0.05). In addition, EROD activity was statistically significantly increased ($p \le 0.01$) at 1500 ppm, however the increase (63%) was considered



marginal in comparison with the positive control, beta-naphthoflavone, which induced EROD by 800% compared to controls (9).

Phenobarbital: A statistically significant increase ($p \le 0.05$) in total P450 was recorded as well as statistically significant increases BROD (p≤0.05), PROD (p≤0.01) and UDPGT-nitrophenol (p≤0.01) activity.

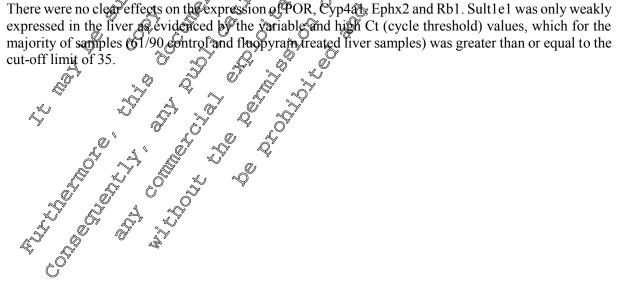
Table 5.5-67	Mean cytochrome P-450 content and enzymatic treatment with fluopyram or phenobarbital	activities in the liver	[.] followi	ing Adays &	
	treatment with fluopyram or phenobarbital	× ×	×		

			()	~	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	
Fluopyram	Μ	lean Increase C	Compared to C	ontrols (Statist	ical Significano	ce) of a line of a
level (ppm)	Total P450	EROD	BRQD	PROD [™]	UDPGI-4-	ÚDPGT- 0'
	Content		40 ⁷	á l	nitrophenol	💆 bilir ubin 🧷
30	x 0.93	x 1.05	x 1.09	x 1.34 Q	A 1.05	x 1.05
75	NC	x 0.94	🆓 1.17	X 1.46	x 1.10	x 1.28
150	x 1.11	x 1.02	≰_ x 1. 4 4 [°]	⇒x1,442 ·	x 1,29	≫ x 1.¥4
600	x 1.02	x 1.12 (∑ x,2,2,42 ≿	⊻ x216 _) x (b).52	x⊈.91 °°
		A	. 🚓**), 🖉	Q(*)	(**) O*	Q(**)
1500	x 1.34	x 1.62	× 5.89	≫ x 4.56	[∞] x 3.01	× 2.60×
	(*)	(**)	چ [♥] (*®/ ۰.	~~ (*ð [×] ~	× (**)	(***)°
Phenobarbital	x 1.65	x-Q12 (x x 7.7 ~	x 10.59 Ö	x 8.86	x 1.37
80 mg/kg/day	(*)		`~~~~(*) ≪		X9.86 X(**) X	<i>2Q</i>
C: no change; * p	≤0.05; ** p≤0.0)1Q'				

6. Gene Transcript Analyses

Three day treatment (Subgroup 1) & <u>Fluopyram</u>: At 30 ppm, no statistically signaticant changes in the expression of any of the genes investigated were observed. AC75 ppfa a marginal but statistically significant increase in the expression of Cyp3a3 (+48.4%; 20.05) was ecorded From 150 ppm, clear dos related increases were recorded for the expression of the Phase I genes (pp2b1 (+230%) at 150 ppm up to 24270% at 1500 ppm) and Cyp3a3 (+159.1% at 150 ppm/up to +2051% at 1500 ppm) From 600 ppm, genes coding for Phase II enzymes (i.e. Gaa2, Gam4, Udpgtr2 and Ephx1) were statistically significantly increased. In addition, Cyp1a1 was statistically significantly increased (+631, P%; p=0.01) from this dose level. A marginal, though statistically significant increases 45% \$20.05) in the marker for apoptosis (Gadd 45b) was also observed Finally, at 1500 ppm, marginal, though statistically significant increases in those genes associated with cell proliferation/apoptosis (Tacster 1, Gadd 45b) were recorded.

There were no clear effects on the expression of POR, Cyp4a, Ephx2 and Rb1. Sult1e1 was only weakly





Mean Kelati	ve Quantity ±			e transcripts (3 o	lay treatment)	(% change _{@,} °
compared to control mean values)						
Gene	Control	30 ppm	75 ppm	150 ppm	600 ppm	1500 ppm
transcripts					<u>```</u>	Ũ A
			TABOLISM: 1			
POR	0.87 ± 0.24	$0.82 \pm$	0.96 ± 0.37	0.71 ± 0.21	0.95 ± 0.30	0.98±0.39
		0.29	(+10.3)	(-18.4)	(+9.2)	(+126) «
		NC		Ò.	Ś _ *	$\nu' \sim \gamma \sim \varphi$
Cyp1a1	1.35 ± 1.14	$1.12 \pm$	1.51 ± 1.61	2.3 ± 2.3	9.87 ± 💭	84:0± 51.6**
		0.68	(+11.9)	(+70.4),©♥	8.29**	~(+616\$) (
		(-17)	<u> </u>	<u> </u>	(+631.9)	
Cyp2b1	1.27 ± 1.87	$0.81 \pm$	1.44 + 0.86	4.2 ± 3.38**	Ø 63@±	> 309.5 ±
		0.56	(403.4)	(+230.7)	71.2**	J76.7*
		(-36.2)	k g		<u>∢(</u> #4861)Q	°≫(+242¶Ø)
Cyp3a3	0.93 ± 0.49	$1.01 \pm$	©1.38,₽	2.41	_0°7.64 g ¥	£ 20.0 ±6.45** ₀
		0.50	0.68*	© 0.8 9 **	[©] 2,23** €) (#2 1051) / (
		(+8.6) 🖑	(+4/8.4)	(1,59.1)	<u>(</u> C ^{21.5})	<u> </u>
Cyp4a1	1.46 ± 0.33	1.37	\$1 ± 0₫	3 ± 0	$1,43 \pm 0.96$	¥.14 ±4€.27
		0.33	(+3,4)	(-11,09)	U (NC)	(-21.9)
		(.6.2)		$V \sim \delta$		Ľ "Ç
		Q' MET				_°>y [™]
Gsta2	0.51 ± 0.45	0.58¢	0052 ± 0.04	\$3 ± \$54	0~1.02€	$2.35 \pm 1.31 **$
		0.60	(NC)	~~ (+62 <i>2</i>) ~	0.67	(+360.9)
	··•	(+13.7) <u>(</u>)				
Gstm4	0.83 ± 0.35	0.05 ± 0.69	1.24 ± 0.78	1.01 ± 0.99	2.15 ±	3.86 ± 2.09**
		0.69	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	Q ^(+21.7)	× 1.23	(+365)
111 . 0		(+26.5)			(+159.0)	
Udpgtr2	$10^{77} \pm 0.93^{77}$		2.0 0.89	$3.0 \pm 2.13 \bigcirc$	″ 4\$27±	$6.66 \pm 3.68 **$
\$			\sim (+13) \circ	(469.5)	2.03**	(+276.3)
<u> </u>		(-2.1)			(+141.2)	2.12 + 0.02**
Ephx1	0.80 ± 0.24		0.84 ± 0.31	1.0 ± 0.36 (⊕7.6) , ©	[∞] 1.73 ±	$3.12 \pm 0.93 **$
[®]	\mathcal{O}_{λ} \ll	0.30		(⊕7.6)_©	0.44^{**}	(+267.1)
Enhol	1 1 481 0 21	(+/.19)			(+103.5)	0.95 ± 0.32
Ephy2	1.14 0.3 1	× 0.929±	0.94 ± 0.21 ($_{5}10.5$)	7.02 ± 0.26 (-10.5)	1.18 ± 0.33	
47	\sim	0.29	$(\varepsilon, \psi, \varepsilon)$	° (<u>-</u> ,(0.5)	(NC)	(-16.7)
Q-141 - 1		19.3) (2 ND			ND	ND
Sult1e1	P∕ND			ND	ND	ND
T				ASPOPTOSIS	1.26 + 0.22	1 ((+ 0.24**
Tacstd1		$1\% \pm 0.16^{\circ}$	1.1 ± 0.21	$0^{\circ} 1.12 \pm 0.23$	1.26 ± 0.22	$1.66 \pm 0.34^{**}$
C. IIA		(-7.6)	Q-7.6) Or	(-5.9)	(+5.9)	(+39.5)
Gadd 43b	0.8 ± 0.26	0.8 ± 0.29	• 0.84 ± 0.36	0.9 ± 0.23	$1.16 \pm 0.45^{*}$	$1.34 \pm 0.57^{**}$
Rb1		(INC)		(+12.5)	(+45)	(+67.5)
KDI	401 ± 0.15	0.15 (-7,9)	0.96 ≠ 0.16 S(-5)	1.05 ± 0.3 (+4)	1.04 ± 0.26 (+3)	0.82 ± 0.14 * (-18.8)

NC: no change; * p = 0.05; ** p = 0.07, ND: not detected In the above table the presented & changes to not always calculate exactly from the presented mean data. This is due to rounding-up differences,2 ~Ć ٧,

Phenobarbital: The gene expression data indicated that three days treatment with phenobarbital leads to dose-related increased expression of Phase 1 and Phase 2 enzymes. In particular, Cyp2b1, Cyp3a3, both isoforms of glutatorione (Gstm4 and Gsta2), Ephx1 and Udpgt were all statistically significantly increased in contrast there was a statistically significant (p≤0.01) down-regulation of Cyp4a1 and Ephx2 pression. The marker for cell proliferation (Gadd45b) was also marginally but statistically significantly increased. There were no clear effects on the expression of POR, Cyp1a1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values,



which for the majority of samples (9/15 phenobarbital treated liver samples) was greater than or equal to the cut-off limit of 35.

Mean Relative Quantity ± sta	andard deviation of gene transc	y treatment with phenobarbital
- •	compared to control mean val	cripts (3 day treatment) (% chailge lues) Phenobarbita (80 mg/kg/day)
Gene transcripts	Control	A Phenobarbital A
		$\sqrt{2}$ (80 mg/kg/day) $\sqrt{2}$
	METABOLISM: Purase I	
POR	$0.87 \pm 0.24\%$	Q 0.7 ± 0.24 Q 4
	Ś	<u> </u>
Cyplal	1.35 <u>*</u> 4.14	0^{2} 105 ± 2.05^{2}
		Q^{+} $Q^{+}(+22Q)$
Cyp2b1	1.27 ± 1.87	962.8 89 2.5 **
Cyp3a3	0.93 0.49 0	0° 0° $10.02 \pm 7.48^{**}$
	A 0 0	
Cyp4a1	4.46 ± 0.33	Ø0.52 ≠ 0.16**
		<u> </u>
	METABOLISM: Phose I	
Gsta2		2.22 ± 63**
Gstm4	. ↓ 00.83 # 0.35 ©	$362 \pm 3.82*$
X		(+336 ⁽¹⁾)
Udpgtr2		(+330) (+330) (+342.4)
		<u>√</u> <u>√</u> <u>(</u> #342.4)
Ephx1	$0^{-0.85}$	30 ³ ±2.3**
Eph	$\begin{array}{c} 5 \\ 5 \\ 5 \\ 5 \\ 5 \\ 5 \\ 5 \\ 5 \\ 5 \\ 5 $	$0.53 \pm 0.23 **$
Lunition (ND ND
	EIO PROLIFERATION APO	
Tacstall to a		1.08 ± 0.18 (-9.2)
		(-9.2)
Gadd45b	0.8 0.26	1.31 ± 0.67**
		(+63.75)
	\$ \$ 01 ± 6 Y5	$\begin{array}{c} 0.82 \pm 0.15^{**} \\ (-18.8) \end{array}$

Table 5.5-69:	Mean gene transcript analyses following 3 day treatment with phenobarbital	
	······································	1

NC: no change; * $p \le 0.05$; * $p \le 0.01$ ND: not detected In the above table the presented % changes to nor always calculate exactly from the presented mean data. This is due to rounding-up differences.

Seven day treatment (Subgroup 2):

Fluopyram:

A similar gene expression profile was bserved following 7 days treatment with fluopyram as that observed following 3 days treatment Q,

At 30 ppm, as significant manges in the expression of any of the genes investigated were observed.

At 75 ppt, a marginal but statistically significant increase in the expression of Cyp3a3 (+95%; $p \le 0.01$) was recorded ~?

From 150 ppm, clear dose-related increases were recorded for the expression of the Phase I genes Cyp2b1 \$1336% at 150 ppm up to +143300% at 1500 ppm), Cyp3a3 (+262.6% at 150 ppm up to +2756 (at 1500 ppm) and Cyp1a1 (+356.2% at 150 ppm up to +22192% at 1500 ppm).



From 600 ppm, genes coding for Phase II enzymes (i.e. Gsta2, Gstm4, Udpgtr2 and Ephx1) were statistically significantly increased.

Finally, at 1500 ppm, a marginal, though statistically significant increase (+82.5%; $p \le 0.05$) in Gaca45b was recorded. Cyp4a1 (-37%; $p \le 0.01$) and Ephx2 (-39.5%; $p \le 0.05$) were marginally though statistically significantly down-regulated at this dose level.

There were no clear effects on the expression of POR and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which for the majority of samples (59/90 control and fluopyram treated liver samples) was greater than the cut-off limit of 35 \$

Mean Re	Mean Relative Quantity ± standard deviation of gene transcripts (7 day treatment) 1% change					
~			to control mea			
Gene	Control	30 ppm	% 75 ppm °	JS0 ppm	600 pm	°∼_1500 ppm
transcripts			loʻ <u>@</u> `		Ø Å	<u> </u>
-			ABOLISM: P			
POR	0.85 ± 0.35	0.81 ± 0.2	0.81 ± 0.26	0.91 ± 0.3	1.03 ± 0.42	6.99 ± 0.56
		(-4.7)	×(-4.7)	(+7.1)	°∼ (+21,2)	(+163)
Cyplal	2.26 ± 1.3	3.07 ± 3.87	%√4.0 ±%√87	≫ 10.30 ±	~ 14307 ±	50 <u>3.</u> 8 ±
		(+39.8)	× (±¥7) «J	× 11.01**	\$\$.0** S	\$ \$0.5**
			à à	356.2	Q+6258	s (+22192)
Cyp2b1	0.95 ± 0.74	2.43 ± 1.83	2,93 ± 3,87	₩13.64	≥ 310,2 ±	"1362.3 ±
		(+155,8)	(+20204)	(12 .9 **	3220**	1422.2**
	*		r' Ly ar	(#1336)	(#32553)	(+143300)
Cyp3a3	0.99 ± 0.5	1.40 ± 0.84	@4.93 ±	_3.59 ⊉ ©″	12.32,₽	$28.27 \pm$
	1 N	(+47.5)	\$ 0.79 **	\$1.65**	3.75**	10.15**
	≪)	Ċ [,] Q	O (+395) 🧞	<u>) (262.6) (</u>	(+9044)	(+2756)
Cyp4a1	0.73 0.26	0.70¥0.22	0.62 ± 0.14	$0.64 \pm 0.26^{\circ}$	$0_{4}64 \pm 0.19$	$0.46 \pm$
		<u>,</u> 05∕4.1)^S	(-15.1)	@(-12.3)	(-12.3)	0.11**
	N° NO				Ç	(-37.0)
			ABOLISM: <u>Ph</u>) [°]	
Gsta2 🐧	2.08€1.7	1.37 ± 0.56 √	1.957 ± 1.45	2 3 ± 1.78	$3.6 \pm 1.83^{**}$	$7.03 \pm$
, Ôj	-0 -0	Q-34.1)	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	(+31,7)	(+73.1)	4.57**
	Č,					(+238)
Gstm4	2.02 1.02	Ď2.42 ±°1.76	∿ 1.8ू1⊕"0.97≪	2.78°±1.54	$4.2 \pm 1.82*$	$11.3 \pm$
·		(4 49.8) 💍	_x (710.4) <u>x</u>	≟ (¥37.6)	(+108)	19.5**
	, 3 ⁷ , 4	N IN	NO O	∧		(+459.4)
Udpgtr2	2.53 ± 28	.58 ±∂.32 γ	3.91,± 4	3.46 ± 1.5	7.33 ±	$12.01 \pm$
	¢ _s ò ^v é	(+44.5) C	⁷ ,1.06** 0	(+36.8)	3.22**	4.25**
ÂÇ,			d,¥54.5)		(+189.7)	(+374.7)
Ephx 1	1.2 ± 0.4	3946 ± 0.23	0.34 ± 0.32	1.2 ± 0.39	2.12 ±	4.21 ±
Cpixi2		Q (+21.9)	(+41.7)	(NC)	0.69**	2.34**
			* <u></u>		(+77)	(+250.8)
Ephx2	1.19/± 0.4	$1,18 \pm 0.25$	1.09 ± 0.48	1.03 ± 0.43	0.95 ± 0.38	0.72 ± 0.35 *
Ÿ	, O'	$\mathcal{O}^{\vee}(\mathrm{NC})^{\times}$	° (-8.4)	(-13.4)	(-20.2)	(-39.5)
Sult1e1	¢ ND (ND ND	ND	ND	ND
	CELL PROLIFERATION/APOPTOSIS					
Tacstd1	1.41 ± 0.3	1.55 ± 0.57	1.36 ± 0.23	1.1 ± 0.26	1.38 ± 0.45	1.51 ± 0.34
Ő,	S O	× (+10)	(-3.5)	(-22.0)	(-2.1)	(+7.1)
Gad@43b	≫ 1.2 ±0.45 ~	1.41 ± 0.43	1.13 ± 0.34	1.25 ± 0.65	1.55 ± 0.53	$2.19 \pm 1.27*$
		(+17.5)	(-5.8)	(+4.2)	(+29.2)	(+82.5)
Rb1 6	0.90 ± 0.48	0.98 ± 0.15	0.89 ± 0.13	0.83 ± 0.17	0.87 ± 0.14	0.79 ± 0.15
		(+8.9)	(NC)	(-7.8)	(-3.3)	(-12.2)
NC: no change; *	p≤0.05; ** p≤0.01					

 Table 5.5-70 Mean gene transcript analyses following 7 day treatment with fluopyram

 Mean Relative Quantity ± standard deviation of gene transcripts (7 day treatment) 4%

NC: no change; * p≤0.05; ** p≤0.01; ND: not detected

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.



Phenobarbital:

A similar gene expression profile was observed following 7 days treatment with Phenobarbital as that observed following 3 days treatment, although for some genes the expression was more marked after this prolonged treatment. Thus, increased expression of Phase 1 (Cyp2b1; Cyp3a3) and Phase 2 obymes (Gstm4, Gsta2, Ephx1 and Udpgtr2) was recorded after 7 days with the magnitudes of induction for Cyp2b1 and Cyp3a3 being almost 3-fold and 1.6-fold respectively greater than those observed at the earlier time point. Similarly the induction of Gstm4 was 5.1-fold greater after 7 days compared to that recorded after 3 days.

As with the earlier time point, there was a statistically significant ($p \le 0.01$) down-regulation of Cyp4a1 and Ephx2 expression and the marker for cell proliferation (Gadd45b) was also marginally but statistically significantly ($p \le 0.05$) increased.

There were no clear effects on the expression of POR, Cyp at and Rb1. Sult le was only weakly expressed in the liver as evidenced by the variable and high of (cycle threshold) alues, which for the majority of samples (8/15 phenobarbital treated liver samples) was greater than or equal to the cut-off limit of 35.

Table 5.5-71	Mean gene trans	crip@analyses fol	llowing 7 day ti	reatment with	phenobarbital (

Moon Doloting	Operative standard description Bra				
(7 day treatm	Mean Relative Quantity ¥ standard deviation of gene transcripts (7 day treatment) (% change compared to control mean values) Gene transcripts Gene transcripts (80 mg/kg/day)				
Conc transcripts	Control (70 change compared to Control	Dhenaharhital			
Gene transcripts		(10 mg/la/day)			
Gene transcripts	$\begin{array}{c} \bullet \\ \bullet $	(av mg/kg/uay)			
POR 6					
POR O	0.85 ± 0.35	0.69±0.20 (-22.4)			
Com la l	Q 02.26 1.30 Q	√ √ √ (-22.4) √ √ √ (-22.4)			
Cyplal L G		(-19.0)			
Cyp2	0.95 ± 0.74	2776.8 ± 1842.7**			
		(+292195)			
	0.99 0.59 0.59 0.59 0.59 0.59	16.26 ± 10.45**			
	0 0.99 ₽0.59 0 0.99 ₽0.59	♥ (+1542.4)			
© Cyp4al	$\mathbf{A} \mathbf{A} $	$= 0.40 \pm 0.08 **$			
Cop3a3 C Cyp4a1 Cyp4a1 Gsta2		(-45.2)			
	[™] METABQLØŠM: Phase IL				
Gsta2	$\begin{array}{c c} & \textbf{METABOLISM: Phase II} \\ & 2.08 \pm 1.90 \\ & & & & \\ & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & \\$	4.8 ± 2.3**			
Gstm4		(+130.8)			
Gstm4 💭 🔊		$18.3 \pm 11.8 **$			
		(+805.9)			
Vdpgtr2	2253±128	13.1 ± 7.8**			
A S		(+418)			
Ephx1	1.2 \$0.41	$4.76 \pm 2.43 **$			
		(+297)			
Ephx2 6	1.2 ± 0.41	0.81 ± 0.15 **			
		(-31.9)			
Suffle1	ND	ND			
Ephx2 $1.15**$ Suffle1 2 1.41 ± 0.32 $1.13 \pm 0.18**$ Tacstel 2 1.41 ± 0.32 $1.13 \pm 0.18**$					
Tacster of the	~♥ 1.41 ± 0.32	1.13 ± 0.18 **			
Gaidd45ba Rb1 Korrection Korection Korrection Korrection Korrection Korrection Kor		(-19.9)			
Gadd45b	1.2 ± 0.45	2.07 ± 0.89**			
		(+72.5)			
Rb1 &	0.90 ± 0.18	0.71 ± 0.12**			
		(-21.1)			

NC: no thange; * $p \le 0.05$; ** $p \le 0.01$; ND: not detected

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.



E. Deficiencies

No specific deficiencies were noted in the study.

III. Conclusions

Overall, clear and statistically significant changes in the liver (cell proliferation, hypertrophy and enzyme activity as well as associated changes in gene expression) were observed following fluon treatment. These changes were recorded as early as following 3 days of treatment and starting from 150 ppm. The dose of 75 ppm was considered as a NOAEL (based on the fricreased gene expression of cyp3a3 at this dose level) and 30 ppm as a NOEL.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2003 as it provides mechanised information on the effects and target organs of fluopyram in the rate of the current state of the current

Overall, clear and statistically significant changes in the liver feell profiferation, hypertrophy and enzyme activity as well as associated changes in gene expression) were observed tollowing fluopyram treatment. These changes were recorded as earloas following days of treatment and starting from 150 ppm. The dose of 35 ppm was considered as a NOAEL (based on the increased gene expression of cyp3a3 at this dose level) and 30 ppm as a NOEL.

Data Point:	
Report Author:	
Report Year:	
Report Title:	Flugtyram (AE C 656948) Mechanistic investigations in the liver of female rats
	for δ in δ in δ in δ in δ in δ in δ
Report No:	SA 11104 ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~
Document No:	M-427431-0K
Guideline(s) followed in	No specific guideline
study:	
Deviations from current	No specific guideline
test guideline:	
Previous Evaluation:	Non-previously evaluated \mathbb{O}^{\times} \mathbb{Q}^{\times}
GLP/Officially	Yes, conducted ander GLP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability	Yes Conducted and China Polycellary recognised testing facilities

Executive Summary

The objective of the present study was to investigate the mode of action by which fluopyram gives rise to liver tumors in the female rat following chronic exposure. The effects of different doses of fluopyram on the liver were determined following continuous dietary administration for at least 28 days. In addition, the reversibility of any effects observed at the highest dose level was assessed following a recovery period of one month on untreated control diet. Liver cell proliferation, hepatic total cytochrome P-450 as well as enzymptic activities and gene expression profiles for inducible P-450 and UDPGT enzymes were assessed. Liver histopathology was also evaluated. The CAR/PXR nuclear receptor activator, pherobarbital, was administered by oral gavage and was used as a positive control for the liver effects. The eversibility of any effects induced by phenobarbital was also assessed following a recovery period of one month on untreated control diet.

Consequently, groups of adult female Wistar rats were exposed to fluopyram (batch number: Mix-batch: 08528/0002: a light beige powder, 94.7% w/w) for at least 28 days in the diet at dose levels of 0, 30, 75,



150, 600 and 1500 ppm, which equated to 2.2, 5.6, 11.3, 44.5 and 111.4 mg/kg/day respectively. A group of animals dosed with 80 mg/kg/day phenobarbital by gavage acted as a positive control for the parameters investigated in this present study. Each group consisted of 15 females, apart from the negative control and 1500 ppm fluopyram group, together with phenobarbital group, where an additional 15 animals per group following the 28 days of treatment were maintained for a 1 month recovery period on untreated control diet.

Treatment Phase:

There were no relevant changes in any of the body weight parameters during the treatment phase for either fluopyram or phenobarbital. Fluopyram treatment caused ocular discharge and red conjunctive in some females of Groups 3 (75 ppm), 4 (150 ppm) and 5 (600 ppm), in addition, food consumption was statistically significantly reduced for females treated with 1500 ppm fluopyram during week 3 ($p \le 0.05$) and week 4 ($p \le 0.01$) of treatment.

All females dosed with phenobarbital displayed reduced motor activity and some females had ocular discharge and/or lacrimation in either one or both eyes. Other clinical signs included increased salivation and soiling around the mouth or the head. Food consumption was increased during treatment with phenobarbital ($p \le 0.05$ for weeks 1 and 4; $p \le 0.01$ for week 2).

Fluopyram had no effect on terminal body weight but indiced treatment-related charges in all liver parameters investigated. The number of parameters affected and also the magnitude of the responses were dose-related as described below.

At 30 ppm, a statistically significant $(p \le 0.01)$ increase in the expression of Cyp3a3 was recorded (+81%), which increased with increasing dose up to +4943% at 1500 ppm. This increase in gene expression was, however, not associated with any statistically significant increase in corresponding enzyme (BROD) activity until 600 ppm.

At 75 ppm, enlarged fiver was observed in 3/15 females and centritobular cell proliferation was marginally, but statistically significantly ($p \le 0.05$), increased ($p \le 47\%$) compared to the controls.

At 150 ppm, slight but statistically significant increases in mean fiver weight relative to body weight $(+7\%; p \le 0.01)$ and brain weight $(+01\%; p \le 0.05)$ were (corded) This increased liver weight could be associated with enlarged fiver in 5/15 females. Centrilobular and global hepatic cell proliferation (+106% and +70% respectively) were statistically significantly ($p \le 0.01$) increased compared to the controls in addition, statistically significant ($p \le 0.05$) increases were recorded for the expression of the Phase I genes Cyp1a) ((+71%), Cyp2b ((+990%)) and for the Phase II genes Gstm4 ((+136%)) and Udpgtr2 ((+64%)). Statistically significantly ($p \ge 0.01$) increased hepatic enzyme activity was, however, only observed for UDPGT-bilifabin (corresponding to Udpgtr2) and EROD (corresponding to Cyp1a1) starting from this dose level

At 600 ppm, marked changes to the liver were observed as evidenced by statistically significantly increased absolute and relative fiver weights (+10 to +15%), coupled with enlarged liver in 4/15 animals and minimal hepatocollular hypertrophy in 6/15 females. However, despite these macroscopic and microscopic changes, hepatic cell profileration was similar to that observed at 150 ppm with centrilobular and global proliteration being Otatistically significantly increased (+106% and +67% respectively; p. 90.01). Genes coding for additional Phase II enzymes were statistically significantly increased at this dose level (Gsta2: +53% and Ephx1: +116%). A marginal, though statistically significant (*75% p<0.05), increased expression of the marker for cell proliferation (Gadd 45b) was also observed. Increased activity of BROD (p<0.01), PROD (p< 0.05) and UDPGT-4-nitrophenol (p<0.01) were recorded from this dose level.

At 1500 ppp, the hepatic effects were more marked than those recorded at the lower doses. Specifically, for cell proliferation the % increase (compared to controls) in global cell proliferation was 188% compared to 67% at 600 ppm. Statistically significant increases in mean absolute and relative liver weight (+29 to +33%) were also recorded at this dose level as well as enlarged liver in 14/15 animals, which were associated with the centrilobular to panlobular hypertrophy observed in 14/15 females.



Changes in gene expression and enzyme activity observed at the lower doses were also more marked at this top dose level.

Phenobarbital was used as a positive control for the various parameters measured in the present study and as such induced changes in the liver pertinent for a compound that activates CAR/PXRAnclear receptors. Liver weight (absolute and relative to brain and body weight) was satisficantly significantly increased following phenobarbital treatment and enlarged liver was noted in 10015 animals, which sould be associated with the centrilobular to panlobular hypertrophy observed in 12/15 females and ano increased number of mitoses was observed in 1/15 females. Increased cell proliferation was observed with the effects being more apparent in the centrilobular region (+335% compared to controls) that in the perilobular region (+23% compared to controls). Furthermore, the gene expression of Gadd456 was O statistically significantly increased (+68%; p≤0.01), Statistically significant (p≤001) increases in the gene expression of Cyp2b1, Cyp3a3 and Udpgtr2 were recorded, which could be associated with the statistically significantly increased hepatic enzyme activity of PROD (#8.44-fold; p < 0.01), BROD (#8. fold; p≤0.01) and UDPGT-bilirubin (+1.38-fold; p≤0005) respectively. Enzyme activity of the second isoform of UDPGT (UDPGT-4-nitrophenol) was also statistically significantly increased (+1481-fold; p≤0.01) and increased expression of genes coding for additional Phase II enzymes (Ostm4 Osta2 and Ephx1) was also recorded following phenobarbital treatment. Ś

Recovery Phase:

Ô For the females previously treated with 1500 ppm fluopyram, there were no plevant changes in any of the body weight parameters during the recover phase A statistically significant reduction (p≤0.05) in food consumption was, however, recorded during the second and third week of the recovery phase. There was no effect on terminal body weight or any organ weights at the end of the recovery phase nor were there any macroscopic or mcroscopic changes recorded in the liver Centrobular and global hepatic cell proliferation were still statistically significantly prereased compared to the controls (+81%, p≤0.01 and +51%, p≤0.05, respectively); however these observed increases were lower than those observed immediately following treatment with 1500 pptr fluopyram (DI 5% and 188% for centrilobular and global cell proferation, respectively). Hepatic molecular and enzymatic changes were still apparent at the end of the recovery phase as evidenced by increased by activity/gene expression for BROD/Cyp3a² PROP/Cyp3b1, EROD/Cyp1a1 and ODPGT bilirubin. Marginal increases in the expression of Gstm4 Ephx2 and Gadd45b were also recorded. As with the cell proliferation parameters, the magnitude of these molecular and enzymatic changes was much lower than those recorded immediately following treatment. \bigcirc 21

A similar profile was observed for the females previously treated with 80 mg/kg/day phenobarbital. In these animals a loss or areduction in body weight gain warecorded during the first two weeks of the recovery phase, but there was no effect on terminal body weight or any organ weights at the end of the recovery phase nor were there any macroscopic or microscopic changes recorded in the liver. Hepatic cell proliferation was still statistically senificantly increased compared to the controls; however the magnitude of the increases were generally lower than those observed immediately following treatment with phenobarbital. Expatic molecular and enzymatic changes were still apparent at the end of the recovery phase as evidenced by increased enzyme activity and/or gene expression for, PROD/Cyp2b1, EROD, Cyp3a3 and UDPGT-bilirubin. Marginal increases in the expression of Cyp4a1, Gstm4 and Ephx1 were also recorded. As with the cello roliferation parameters, the magnitude of these molecular and enzymatio changes was generally lower than those recorded immediately following treatment.

Overall, treatment with thopyram for at least 28 days induced clear and statistically significant changes in the liver (cell proliferation hypertrophy and enzyme activity as well as associated changes in gene expression). The dose of 30 ppm was considered as a NOAEL Based on the increased gene expression of Cyp3a3, with no other correlated findings at this dose level. The hepatic changes appeared to be reversible as evidenced by the reduced hepaticresponses recorded in females previously treated with 1500 ppm fluopyram following the recovery period.



I. Materials and methods

A. Materials

1	Test material:	AE C656948 Light beige powder Mix-Batch: 08528/0002 94.7% 658066-35-4 Stable in rodent diet for a period overing the study duration Phenobarbital (positive control) White crystalline powder Lot No. 09050075 99.6% 50-06-6 Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration But Wistar Rj: WI (IOPS HAN) – Febrale only 10 weeks approximately at start of treatment 235 - 261 g
1.	Description	AE C030946
	Lot / Batch #:	Mix Batch: 08528/0002
	Purity:	
	CAS #	658066-35-4
	Stability of test compound:	Stable in rodent diet for a period overing the stady duration
2	Vehicle and / or positive	Studie in rodent dietwor a period covering the study diamon a
	ontrol:	Phenobarbital (positive control)
CU	Description	White crystalline powder
	Lot / Batch #:	Lot No. 09050075
	Purity:	99.6% & 6° 5° 4° 4° 6° 5° 4°
	CAS #	50-06-6° , , , , , , , , , , , , , , , , , , ,
		Stable in 0,5% aque bus solution of methylcellulose for period
	Stability of test compound:	covering the study duration 2 0 4
3.	Test animals:	
	Species:	Bat & A A A A A A
	Strain:	Wistar Rj: WI (IQPS HAN) – Febrale only
	Age:	10 weeks approximatel pat start of treament
	Age: Weight at dosing: Source:	2)5-261 g 0 4 9 0 0 0
	Source:	
	Acclimation period: ゆ の [*]	6-8 days
	Diet: \swarrow \swarrow	S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-
		Orge France, ad libitum
	Water:	Tap water, ad libuum
	Housing:	Animals were caged individually in suspended stainless steel
	Water: Housing: Environmental conditions:	Animals were caged individually in suspended stainless steel
		wire mesh cages. 26 $24^{\circ}C^{\circ}$
	Temperature:	$20,24 \cdot C = 20$
	Humidity:	40-70%
	Air changes:	10-10-air changes per nom
	Air changes: Photoperiod	12 yours to ark
B.	Study design	
1.	Photoperiod	10-10 air changes per hour 12 Yours toght, 12 hours dark riod 10 May 2011 0 09 June 2011
	A	
	Recovery j	period: 09 June 2011 – 08 July 2011
2.	Animal assignment and treatm	ent of the second se

Animals were assigned to dose groups using an andomization procedure by weight.

Seven groups of female rate were bosed for at least 28 days with the appropriate compound by the appropriate of administration.

Six groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the temaining five groups received fluopyram at the appropriate dietary concentration (30, *1*5, 150, 600 and 1500 ppm) at a constant level. The dose levels for fluopyram were set after evaluation of the results from previous studies conducted with the compound and following discussions with the OIS (EPA) and Canadian (PMRA) authorities. The top dose level of 1500 ppm used in the present study represents the top dose level used in the rat cancer bioassay in which an increase in liver tumors was observed in the females.



The seventh group was dosed by oral gavage with 80 mg/kg/day phenobarbital suspended in 0.5% aqueous methylcellulose 400 using a dosing volume of 5ml/kg bodyweight. The volume administered to each rat was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose is known to clearly induce liver cytochrome P-450 activity and cell proliferation in the rat.

Each group consisted of 15 female rats with the exception of the control group, the highest dose fluopyram group and the phenobarbital group, where 15 additional females were fed control or test diets (1500 ppm fluopyram) or were orally dosed (80 mg/kg/day phenobarbital) for at least 28 days and were then allowed one month (at least 28 days) of recovery daying which they were maintained on intreated control diet.

All animals were sacrificed in the morning after the last day of the recovery phase.

Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, MH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Europennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Table 5.5-72		D' 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
Group	Test substance 🖓	Dose level Number of mimals per group
1	Control 🖉	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
2	L &	\$\$0 ppm
3	à O'	275 ppm
4	Fluopyram 🔬	150 ppm () ((135)
5		600 pppa 27 0 4 245
6	Thenel Printed &	
7	a nendouronui	^{\$2} 80,g] \$2/day \$2 \$2 \$2 \$15+15*
*Animals al	located to recovery phase	

*Animals allocated to accovery phase

3. Diet preparation and analysis

Fluopyrand (AE C656948) was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation for each concentration used in the study. The stability was demonstrated in an earlier study of concentrations of 50 and 10000 ppm for a time which covered the period of usage and storage for the current study. Homogeneity of test substance in diet was verified on the lowest and highest oncentrations to demonstrate adequate formulation procedures. The mean value obtained in homogeneity check was used as measured concentration. For the remaining dietary levels, concentration was checked.

Two formulations of phenobabital were prepared by suspending the test substance (w/w) in a 0.5% aqueous solution of methylcellulose. The stability of phenobarbital at 8 g/l was demonstrated in an earlier study, which covered the period of use and storage for the current study. Homogeneity of phenobarbital in aqueous methylcellulose was verified to demonstrate adequate formulation formulation procedures. The mean value obtained in homogeneity check was used as measured

4. Statistics

Bodyweight parameters

- Boby weight change parameters calculated according to time intervals

- Werage food consumption/day parameters calculated according to time intervals
- Terminal body weight, absolute and relative organ weights parameters



STOP

- Total cytochrome P450 content and liver enzyme activities
- Cell proliferation parameters
- Gene transcript analysis
- 4.2 Statistical methods

Not Significant p > 0.05

Mean and standard deviation were calculated for each group.

Group means were compared at the 5% and 1% levels of significance.

The statistical analyses of the cell proliferation data were carried out by the statistician using the methods described in the appropriate paragraphs.

All other statistical analyses were carried out using ath/Tox System Enhanced Statistics).

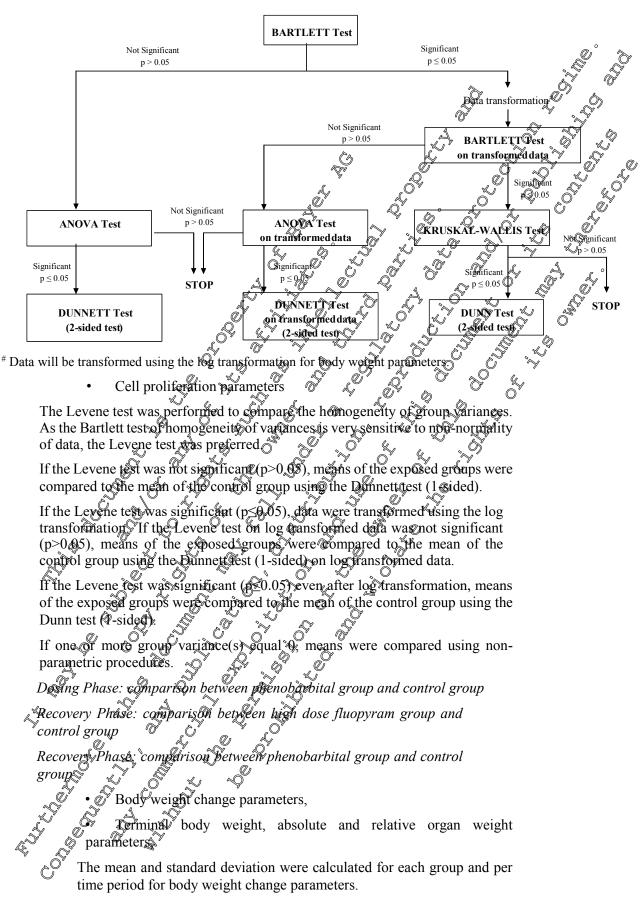
Dosing Phase: comparison between fluopyram groups

carried out by the statistician
 carried out by the statistician
 carried out using Path/Tox System V4.2.2. (Module)
 inparison between fluopyran groups and control group
 Body weight change parameters,
 Terminal body weight, absolute and relative organ weight parameters
 The mean and standard deviation will be calculated for each group and pertime period for body weight change parameters.

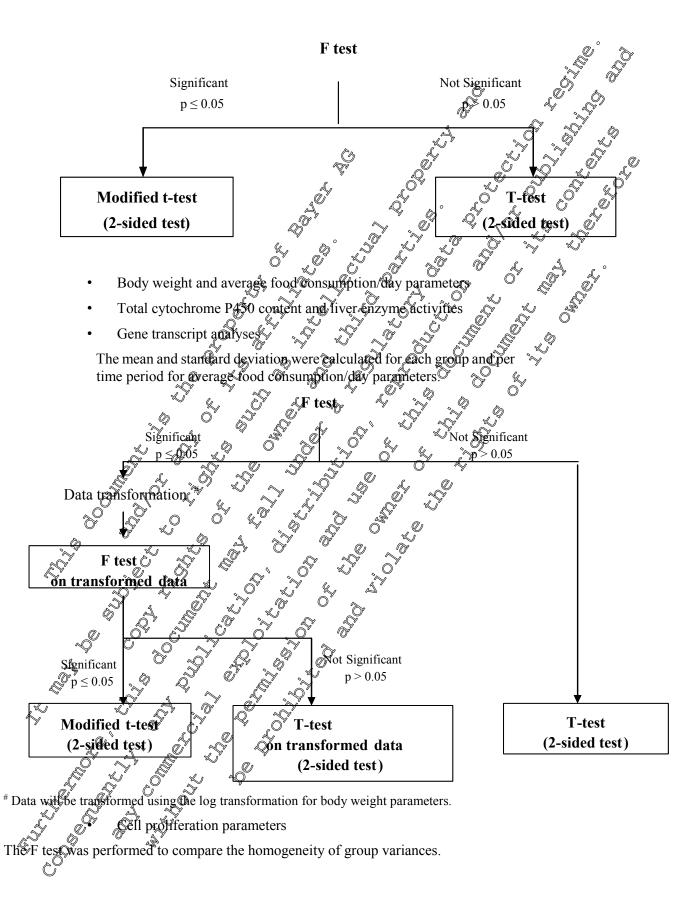
DUNNETT Tes STOP 🆄 **DUNN Test** (2 wided tes (2-sided test) & 0 weight and average food consumption/day parameters Total cytochrome P459 content and liver enzyme activities

The mean and standard deviation were calculated for each group and per time











If the F test was not significant (p>0.05), mean of the test group (high dose fluopyram or phenobarbital) was compared to the mean of the control group using the t-test (1-sided).

If the F test was significant (p < 0.05), data were transformed using the log transformation. If the F test on log transformed data was not significant (p 0.05), mean of the test group (high dose fluopyram or phenobarbital) were compared to the mean of the control group using the t-test (1-sided) on log transformed data.

If the F test was significant ($p \le 0.05$), even after log gransformation, mean of the test group were compared to the mean of the control group using the exact Mapri-Whitney test (1-sided).

If one or more group variance(s) equal 0, the means were compared using the non-parametric procedures.

Group means were compared at the 5% and 1% levels of significant

With the exception of those used for the cell prohiferation data, all statistical analyses were carried out using Path/Tox System V42.2. (Module Enhanced Statistics). SAS programs (version 9) were used for the cell proliferation data.

C. Methods

1. Observations

1. Observations The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

2. Body weight

Each animal was weighe during the acclimatization period Body weights were also measured on Day 1 and at least weekly during treatment and the recovery phase. Additionally, diet fasted animals were weighed before scheduled necropsy (terminal body weight). $\tilde{\bigcirc}$

3. Food consumption and compound intake

The weight of food supplied was measured on Day and weekly thereafter. Empty feeder weights were measured weekly. The weight of food supplied to each animal and that remaining at the end of the food consumption period was recorded weekly. Any food spillage was noted. From these records, the weekly mean achieved dosage intake in mg/kg/day for floopyrant for each week and for Weeks 1 to 4 was calculated. 🔊

4. Sacrifice and pathology

4.1 Necropsy procedure - Organ sampling

All animals were sacrificed by exsangunation under deep anesthesia (inhalation of Isoflurane) in the morning of Day 30 of the treatment phase and Day 29 of the recovery phase. Animals were diet fasted overnight prior to sactifice. A.

All animals were necropsind. The necropsy included the examination of the external surfaces, all orifices and all more organs, tissues and body cavities.

Brain and live weigher fresh at scheduled sacrifice.

Duodenum and two central sections of the liver taken from the left and medial lobes from each animal were fixed by immersion in neutral buffered 10% formalin. In addition, a piece of the median and the left lobe of the liver from each animal were collected and flash frozen in liquid nitrogen, were stored at approximately -74°C + 10°C until used for qPCR investigations. At each scheduled sacrifice, the



remaining portions of liver from each of 5 randomly selected females per group were weighed and homogenized for microsomal preparations.

4.2 Histotechnology

4.2.1 Conventional Histopathological examination

Duodenum and the two central sections of the liver were embedded in paraffi@wax.

Histological sections, stained with hematoxylin and eosin, were prepared for these two or animal in all groups.

4.2.2 Ki67 staining for Cell proliferation assessment

For each animal in each group a section of a formaling fixed paraffine embedded block containing 2 liver samples and one sample of duodenum was prepared. The duodenum was included to give as a positive control for staining as it has a high rate of cell proliferation. The mmuniphistochemical reaction in childed incubation with a monoclonal antibody raised against Ki67 amplification with a

detection of the complex with secondary biotinylated antibody and a streptavidin horse of the second streptavidin horse of the second streptaviding horses diamino-benzidine (DAB) and nuclear counter staining with hematoxylin

4.3 Histopathology

Histopathological examinations were performed on the liver samples from at animals in all groups at s. Balanse

4.4 Cell proliferation assessment

The immunohistochemical staining for Kip7 and determination of the abeling inderwas performed on all surviving animals showing sufficient Ki67 staining (estimated by duodenal Ki67 labeling) to assess cell cycling in the liver. The zonal labeling index, expressed as the number of Kiel-positive hepatocytes per thousand cells, were measured separately on random tields comprising of at least 1000 centrilobular and perilobular cells using an automatic mage analysis system. The mean and standard deviation were calculated for each group.

4.5. Hepatotoxicity testing O

At both scheduled sacrifice times, the remaining portions of the diver from five randomly selected females per group were weighed and nonogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) to check the hepatotoxic potential of the test substance. Phase II enzymatic activities were also determined by measuring UDP-glucuroposyltransferase (UDPGT) with bilirubin as substrate. Results were compared to those generated with well known reference compounds.

4.5.1 Total cytochrome P 450 content

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry

Total cytochrome P-430 content in Microsofial preparations was determined by spectrophotometry (Cary Win UV version 3.0 (182)) using a reduced CO differential spectrum. A single quantification was performed for each sample



4.5.2 Enzymatic activities

ble 5.5-73 Cyte	ochrome P-450s and typical	inducing agents:	
Family	Enzymatic activity	Activity	Typical inducing agents
CYP 1A1	} EROD	activation of mutagens	β -naphtoflatone
1A2		and carcinogens	
CYP 2	} PROD	detoxication of drugs and	Phenokarbital
2B1		chemicals	isomazid 🖉 🚽
2B2		Coctivation of	E S S
		V nitrosamines	
2E		k j	
CYP 3A1 & 3A2	BROD	setoxication of drugs and	Pregnenolone 16 a
		chemicals	Q [*] carbonitrile
			henobar bital
acifia arta almanta D	450 anzumatic activities		

Specific cytochrome P-450 enzymatic activities were valuated by spectro Duoringstry (SAFAS SP2000 version 6.10.7.4) using the following substrates:

- ethoxyresorufin (EROD) - benzoxyresorufin (BROD)

(1)

Ethoxyresorufin is a highly selective substrate for the isoform AA, the isoform 2B metabolizes preferentially the O-dealkylation of pentoxyresorulin, while the benzosyresorulin Odebenzylation is mainly metabolized by the isoform 3A Cytochrome P-450 dependents dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37°C 2

Phase II enzymatic zotivities were also determined by measuring UDP grucuronosyltransferase (UDPGT) with 4-nippphenol as sobstrate (method adapted from Zakim and Vessey. The enzymatic kinetic (disappearance of the colored 4-ritrophenol) was followed at 405mm during 3 min. at 30°C. Three replicates from each sample were assayed UDPGT with bihrubin as substrate was also determined using a spectrophotometry method (adapted from Heirwegh et al.) consisting in the determination of conjugated bile pigments after its conversion into azo pigment derivatives. Absorbance was measured at 530 nmK. Three replicates from each sample were assayed.

4.6 Quantitative PCK Analyses

4.6.1 Total RNA purification

Total cytoplasmic RNA was solated from the lifer of M surviving individual control and treated animals using KNeasy Midi kits (Qizgen), RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agrent Technologies).

4.6.2 Quantitative PCR

Ten my of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand, Applied Biosystems), 1/50 differed first strand cDNA, Fast Start Universal Probe Master mix (Roche) on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA.



Gene (Major function)	Abbreviation	Refset ID	Taqman assay ID ° (Applied Biosystems)
	METAB	OLISM: Phase I	
P450	POR	NM_031576.1	Rn00580820_m1
(cytochrome)oxidoreducta			
se			
Cytochrome P450 1a1	Cyplal	NM_012540.2	K Rn00487218_m1 S
Cytochrome P450 2b1	Cyp2b1	4331248*	€\$p2b1 € 5
Cytochrome P450 3a3	Сур3а3 (Сур3а23)	NM_013105,20 [×]	Rn01640761 gr
Cytochrome P450 4a1	Cyp4a1	MM 1758301	Rn00598510 m1
	МЕТАВ	OLISM: Phase IL	
Glutathione S-transferase	Gsta2	NM_017013.4~	Rn00566636_m7
A2	×		
Glutathione S-transferase	Gstm4	NMC 020540.1	Rn@78923 m1
mu3	A	$\overline{0}$ $\overline{0}$ $\overline{2}$	
UDP	Udpgtr2	₩M_1@295.1	Rn00756519_m
glucoronosyltransferase 2	Q' L	/ NM_0571050	
family, polypeptide B1	<u> </u>		
Epoxide hydrolase 1,	Ephx1 🖉 🖉	₩ <u>01</u> 2844.2	R5 00563 3 49_m1
microsomal	N N N		
Epoxide hydrolase 2,	Eph 2 °	NM@229361	© Rn@0576023_m1
cytoplasmic			
Sulfotranferase family 1E,	Sult1el Sult1el	© 10M_012883.1	Bn00820646_g1
member 1			
		ERATION/APOPTOSIS	
Epithelial cell adhesion	Takestd1 4	₩ <u>1386</u> 41.1	Rn01473202_m1
molecule Q [×]			
Growth arrest and NA- C	Gadd45b 🗸 🔨	, . NM 001008\$21.	Rn01452530_g1
damage-inducible 45 beta			4) ^y
Retinoblastoma 1	$Rop1 O^{\vee} \& O^{\vee}$	X 344334.3	Rn01753308 m1

Beta-microglobulin (B2m; Refset ID: MM_012512.t. Taquan assay ID: Rn00560865_m1) was selected as reference gene for the quantitative calculations of transcripts in the liver. The relative quantity (RQ) value of each test transcript was calculated using the following formula:

$$\Delta\Delta Ct = (Cttest - CtB2m) + (Ct$$

where Ct is the threshold cycle a which PCR amplification started to be significantly different from the background signal. As a Ct of \geq 35 indicates that a gene is poorly expressed in the tissue investigated, any subsequent RO that generated from such a Ct are considered as non-relevant due to an increased risk of contamination.

- II. Results and discussion
- A. Clinical signs and mortality
- 1. Clinical signs of toxicity

Treatment poase:

<u>Flue Four</u> Four females in three different treatment groups had ocular discharge from Week 3 of treatment One of the four females also had red conjunctiva in the same eye as the discharge.

<u>Phenobarbital</u>: All females displayed reduced motor activity starting from Week 2 of treatment. In addition, three females had ocular discharge and/or lacrimation in either one eye or both eyes starting



from Week 2 of treatment. Other clinical signs included increased salivation for two females during the final week of treatment and soiling around the mouth or the head.

Recovery phase:

The few clinical signs (described below) recorded during the recovery phase were considered for to be treatment-related.

Fluopyram: Two females at 1500 ppm exhibited soiled fur around the head region during week 20 if the recovery phase.

<u>Phenobarbital</u>: One female exhibited soiled fur around the nose during Week 4 of the recovery phase and one female had ocular discharge from one eye during Week 2 of the recovery phase.

2. Mortality

There was no mortality in any group during the course of the st

B. Body weight and body weight gain

Treatment phase:

Fluopyram: There were no relevant treatment relate Changes in my of the body weight parameters compared to the controls during the treatment phase

<u>Phenobarbital</u>: There were no relevant changes in any of the body werght parameters compared to the controls during the treatment phase.

Recovery phase:

Fluopyram: There were not elevant charges in any of the body weight parameters during the recovery phase compared to the controls

<u>Phenobarbital:</u> Between Days 1 and 8 of the recovery phase, there was a mean absolute body weight loss compared to the control group)-2g compared to $\pm 8g$ in the control group; -125%, p ≤ 0.01). Between Days 8 and 15 of the recovery phase there was a reduced mean absolute body weight gain compared to the control group (5c compared to 10g b) the control group -50%, not statistically significant). Thereafter, the mean absolute body weight gains were comparable to the controls.

C. Food and water consumption

Treatment phase:

<u>Fluopyram</u>: Food consumption was statistically significantly reduced at 1500 ppm during week 3 ($p \le 0.05$) and week 4 ($p \le 0.01$) of treatment. All other changes were considered non-relevant.

<u>Phenobarbital</u> Food consumption was increased by up to 12% compared to the controls for all periods during the treatment phase. The increase was statistically significant for the first (p \leq 0.05), second (p \leq 0.01) and fourth (p \leq 0.05), seek of treatment.

Recovery phase: 🦿

<u>Fluopyram</u>: A marginal, though statistically significant reduction ($p \le 0.05$) in food consumption was recorded during the second and third week of the recovery phase.

<u>Phenobarbita</u>: A significant reduction ($p \le 0.01$) in food consumption was recorded for the first week of the recovery phase, which could be attributed to two females with an apparent reduced food intake. Thereafter, food intake was comparable to the controls.



Table 5.5-75Mean achieved intake of fluopyram						
Dose group (ppm)	Mean achieved dietary intake of fluopyram of (mg/kg/day) for weeks 1-4					
30		2.2	<u> </u>			
75		5.6 0				
150		11				
600	A	44.5	\$ `}}` @			
1500 11.4 D. Sacrifice and pathology I. Terminal body weight and organ weight Treatment phase: Image: Comparison of the second s						
1. Terminal body weight and organ weight						
Treatment phase:		Â, Â				
Fluopyram: There was no change in mean terminal the controls. At 600 and 1500 ppm, mean absolute and relative when compared to controls.	l body weight in trated	mimals when	r eompared to			
At 600 and 1500 ppm, mean absolute and relative when compared to controls.	liver weight were stati	stically signif	icanily higher			
At 150 ppm, mean liver to body weight ratio and significantly higher when compared to controls.	mean liver to brain we	eight Patio we	te statistically			
These changes were considered to be treatment relation		°, °, «				
Table 5.5-76Mean absolute and relative liver fluopyram	weight changes following	28 days treat	nent with			
Mean liver weight # SD at scheduled 3 day sa		ompared to co	ntrols)			
Dose level (ppm)	🎐 🎢 Fluopyram 🌾		1 500			
	75 150	🦘 600	1500			
Abcolute liver weight (a) 4×14 (92 $\times 10^{-2}$	(AB) 16 9 6 6 48 0 77 8	». C 70 I 0 10*	7 00+0 52**			
		6.78±0.49*	7.90±0.53** (+29%)			
		(+10%)	(+29%)			
Liver to bod weigh 238 ± 0.16 $2.4220.11$ $(+2\%)$						
Liver to bod weight 238 ± 0.16 2.42 ± 0.11	(*6%) (*8%) (* 2.49±613 2.55±0.11**	(+10%) 2.69±0.15**	(+29%) 3.16±0.14** (+33%) 424.55			
Liver to bod weight 238±0.16 2.42±0.11 (+2%) Liver to brain weight 319.44 \$19.40 Tatio (%) ±18.44	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	$\begin{array}{r} (+10\%) \\ \hline 2.69\pm 0.15^{**} \\ (+13\%) \\ \hline 368.06 \\ \pm 28.60^{**} \end{array}$	(+29%) 3.16±0.14** (+33%) 424.55 ±30.85**			
Liver to bod weight 238±0.16 2.42±0.11 2 ratio (%) 238±0.16 2.42±0.11 2 (+2%) 238±0.16 2.42±0.11 2 (+2%) 2 Liver to brain weight 319.44 319.40 ±0.96 ±18.44 (0%) 2	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	(+10%) 2.69±0.15** (+13%) 368.06	(+29%) 3.16±0.14** (+33%) 424.55			
Liver to bod weight 238 ± 0.16 2.42 ± 0.11 ratio (%) $(+2\%)$ Liver to brain weight 319.44 ratio (%) ± 3.96 ± 18.44 (0%) ± 10.6 ± 10.40	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	$\begin{array}{r} (+10\%) \\ \hline 2.69\pm 0.15^{**} \\ (+13\%) \\ \hline 368.06 \\ \pm 28.60^{**} \\ (+15\%) \end{array}$	$\begin{array}{r} (+29\%) \\\hline 3.16\pm 0.14** \\ (+33\%) \\\hline 424.55 \\ \pm 30.85** \\ (+33\%) \\\hline \end{array}$			
Liver to bod weight 238 ± 0.16 2.42 ± 0.11 ratio (%) $(+2\%)$ Liver to brain weight 319.44 ratio (%) ± 32.96 ± 18.44 (0%) \star n<0.05: \star * n<0.01c	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	$\begin{array}{r} (+10\%) \\ \hline 2.69\pm 0.15^{**} \\ (+13\%) \\ \hline 368.06 \\ \pm 28.60^{**} \\ (+15\%) \end{array}$	$\begin{array}{r} (+29\%) \\\hline 3.16\pm 0.14** \\ (+33\%) \\\hline 424.55 \\ \pm 30.85** \\ (+33\%) \\\hline \end{array}$			
Liver to bod weight 238 ± 0.16 2.42 ± 0.11 ratio (%) $(+2\%)$ Liver to brain weight 319.44 ratio (%) ±0.96 ±18.44 (0%) * p<0.05: ** p<0.010	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	$\begin{array}{r} (+10\%) \\ \hline 2.69\pm 0.15^{**} \\ (+13\%) \\ \hline 368.06 \\ \pm 28.60^{**} \\ (+15\%) \end{array}$	$\begin{array}{r} (+29\%) \\\hline 3.16\pm 0.14** \\ (+33\%) \\\hline 424.55 \\ \pm 30.85** \\ (+33\%) \\\hline \end{array}$			
Liver to bod weight 238 ± 0.16 2.42 ± 0.11 ratio (%) $(+2\%)$ Liver to brain weight 319.44 ratio (%) ±0.96 ±18.44 (0%) ±0.96 ±18.44 (0%)	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	$\begin{array}{r} (+10\%) \\ \hline 2.69\pm 0.15^{**} \\ (+13\%) \\ \hline 368.06 \\ \pm 28.60^{**} \\ (+15\%) \end{array}$	$\begin{array}{r} (+29\%) \\\hline 3.16\pm 0.14** \\ (+33\%) \\\hline 424.55 \\ \pm 30.85** \\ (+33\%) \\\hline \end{array}$			
Liver to bod weight 238±0.16 2.42±0.11 2 ratio (%) 238±0.16 2.42±0.11 2 (+2%) 238±0.16 2.42±0.11 2 (+2%) 2 Liver to brain weight 319.44 319.40 ±0.96 ±18.44 (0%) 2	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	$\begin{array}{r} (+10\%) \\ \hline 2.69\pm 0.15^{**} \\ (+13\%) \\ \hline 368.06 \\ \pm 28.60^{**} \\ (+15\%) \end{array}$	$\begin{array}{r} (+29\%) \\\hline 3.16\pm 0.14** \\ (+33\%) \\\hline 424.55 \\ \pm 30.85** \\ (+33\%) \\\hline \end{array}$			

Table 5.5-75	Mean achieved intake of fluopyram	



Table 5.5-77Mean absolute and relative liver weight changes following 28 days treatment with
phenobarbital

Mean liver weight ±SD at scheduled sacrifice (% change when compared to controls)							
Dose-level	P	'henobarbital 📎 🧖					
	0 ppm	so mg/kg					
Absolute liver weight (g)	6.14 ± 0.53	$7.48 \pm 1.01^{**} (+22\%)$					
Liver to body weight ratio (%)	2.382±0.160	Q936 ± 0.352 (+23)					
Liver to brain weight ratio (%)	319.44 ± 31.96	$412,05 \pm 55(53**(+29\%))$					
**: p≤0.01							

Recovery phase:

Fluopyram: There was no change in mean-terminar body weight in treated animals when compared to the controls.

The few organ weight changes were considered to be incidental

Phenobarbital: There was no change in mean terminal body weight increated animals when compared to the controls.

The few organ weight changes were considered to be incidental

2. Gross pathology

Treatment phase:

Fluopyram: Enlarged fiver was observed starting from 75 ppm and this observation could be correlated with microscopic findings starting from 600 ppm.

Other changes were considered as incidental and not treatment-related (no correlation with microscopic findings).

	Macroscopic changes	, Q* ,		
Table 5.5-78	Macroscopic changes	in the liver follows	ng 28 davš treatme	nt with fluopyram

Aneidence of maeroscopic changes in the liver, scheduled sacrifice							
Enopyram							
Dose-level (ppm)		90	15	150	600	1500	
Enlarged		\$0/150 [°]	3/15	5/15	4/15	14/15	

<u>Phenobacoital</u>: Enlarged livers were noted in 10/15 animals treated with phenobarbital.

C

Other changes were considered as incidental and not treatment-related.

Recovery phase; [\]

Fluopyram: All changes were considered as incidental and not treatment-related.

Phenobarbhal: Alt changes were considered as incidental and not treatment-related.

3. Microscopic pathology

Treatment phase:

<u>Fluopyran</u>: Higher incidences of hepatocellular hypertrophy compared to the controls were noted at 600 and 1500 ppm and were considered to be treatment-related.



Phenobarbital: Higher incidences of hepatocellular hypertrophy and increased number of mitoses were noted and were considered to be treatment-related.

Microscopic changes in the liver following 28 days treatment with fluopyram of Table 5.5-79 phenobarbital

Process and the second s								
Incidence and severity of microscopic changes in the liver, all animals of the Terminal sacrifice								
Dose level of Fluopyram (ppm)	0	30	75	150	600	1500	P.B. 80 mug/kg)	
Number of examined animals	15	15	15	15	£0 [™] 15	15	N 150 0	
Hepato	ocellular	hypertrop	hy: entr	ilobular ᢧ	panlobula	r		
Minimal	0	0	0°0	0		Q" 7,0"	Ø 6 Ø	
Slight	0	0 🌾	[♥] 0,~°	, P		Č,	50	
Moderate	0	0 0	,¢ [×]			81 L	<u>A</u> 1 °	
Total	0	Â,		$v 0 \sqrt{2}$	4 6 A	_{≥ 14} 0 [×]		
	Ineceased number of mitoses							
Present	0	l 0%	N.		, e		p q	
Total		B	~~~0 ~~	y ev	são s			
Dogovory phaso:	Q'	is i		\$			°∼y	

Recovery phase:

Fluopyram: All changes were considered as incidental and not treatmentorelated

<u>Phenobarbital</u>: All changes were considered as incidental and not treatment if 4. Cell proliferation

Treatment phase: 🍙

Fluopyram: Centribobula and global (referred to as "Potal" in tables below) proliferation indexes were statistically significantly higher from 150 ppp when compared to controls. Periportal proliferation index was also significantly higher at 1500 ppm when compared to control Centrilobular proliferation was statistically@significantly_increased at 75 ppm. P

Phenobarbital: Centril bular and global proliferation indexes were statistically significantly higher when compared to controls A \bigcirc

0 Ô Mean cell province and index following 28 days treatment with fluopyram or Table 5.5-80 , Ô phenobarbital . O <u></u> n Ø

	0 0			
Compound	Dose		Mean ± SD ^A	
~~~ ~	Ô Ô	S W X% Change	compared to control mea	n values)
J. J		~ Centrillobulac	Periportal	Total
Control		$403 \pm 301$	$8.37 \pm 4.75$	$6.65 \pm 3.19$
Fluopyram	30 ppm	$4.23 \pm 2.42$ (-14%)	7.62 ± 3.66 (-9%)	5.93 ± 2.82 (-11%)
L. L	₹\$ ppm	$\hat{7}.\hat{23} \pm 3.55^{*}$ (+47%)	8.51 ± 3.86 (+2%)	7.87 ± 2.65 (+18%)
	150 ppm	10.16 386** (+106%)	12.51 ± 3.97* (+49%)	11.33 ± 3.30** (+70%)
Û Ô	600 ppm 🔬	10.14 ± 5.27** (+106%)	12.10 ± 8.36 (+45%)	11.12 ± 6.50** (+67%)
	500 ppm	15.54 ± 7.33** (+215%)	22.80 ± 10.49** (+172%)	$19.17 \pm 7.20 **$ (+188%)
Rhenobarbital	80 mg/kg	21.46 ± 17.90** (+335%)	10.28 ± 7.24 (+23%)	$15.87 \pm 11.74 **$ (+139%)

A: n = 15 females/group for cell proliferation determinations; *:  $p \le 0.05$ ; **:  $p \le 0.01$ . Differences in data between table above and in Appendix K are due to rounding-up.



#### **Recovery phase:**

Fluopyram: Centrilobular and global proliferation indexes were statistically significantly higher when compared to the control group.

<u>Phenobarbital</u>: Centrilobular, periportal and global proliferation indexes were significantly higher when compared to the control group.

Table 5.5-81	Mean cell proliferation index f	following Recovery Phase
1	internet of promotion index	strees of the set of t

Group		Mean ± SD ^A	U. 70, 70, 3
	Centrilobular	ر ٌ Periportal	
Control	$4.59 \pm 2.44$	8.25 ± 4.00 .	6.42 ± 3.20
1500 ppm	8.30 ± 3.75** (+81%) a	11.12 ± 6.87 (+35%)	[©] 9.71 [©] 4.77*⁄g+51%€
PB (80 mg/kg)	6.91 ± 3.44* (+51%)	$16.92 \pm 9.89^{**} (+105\%)$	11 02 ± 6.47** (+86%)

A: n = 15 females/group for cell proliferation determinations with the exception of the coupoil group where n=14 instead of 15; *:  $p \le 0.05$ ; **:  $p \le 0.01$ . Differences in data between table above and in Appendix K are due to rounding up.

### 5. Hepatotoxicity testing

### Treatment phase:

<u>Fluopyram</u>: A marginal, though nor statistically significant increase in total P50 content was recorded at 1500 ppm. No statistically significant changes in enzyme activity were observed at 30 ppm and 75 ppm. Statistically significant increases in UDPGT-bilirubin (p<001) were observed from 150 ppm. EROD activity was also statistically significantly increased from this dose level, however the increases (up to 96% at 1500 ppm) were constdered marginal in comparison with the positive control, betanaphthoflavone, which induced EROD by 800% compared to controls. BROD (p<0.01), PROD (p<0.05) and UDPGT-4-nitrophenol (p<0.01) were statistically significantly increased from 600 ppm.

Table 5.5-82	Mean extochrome P-450 content and enzymatic activities in the liver following 28 days treatment with fluopyram or phenobarbital	
1 able 5.5-62	astean eyrochronne r - 450 content and enzy maric activities on the liver following 20	
	~days freatment with fluonyram or when other bita $\mathbb{Z}_{1}^{*}$	
	( and a construction of the construction of th	

		O Mea	n Føld Change		Controls	
Fluopyram dose	Total P450 Content	EROD	BROD	© PROD	UDPGT-4- nitrophenol	UDPGT- bilirubin ^A
30 ppm	×9.94 ×	1.13	\$.24 ×	°≈9.92	0.82	1.09
75 ppm	\$ 0.97	\$ 1.08	1.44	1.27	0.95	1.21
150 ppm		1.34**	^{لا} 2-89 (	[©] 1.53	1.08	1.57**
600 ppm_@		° <u>,</u> ,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	, 9 <u>1</u> 2** 0	1.87*	1.83**	2.14**
1500 ppm	1.42	<u></u> 1.96 <b>*</b> ♥	39.09	4.76**	3.21**	2.76**
Phenobabital 80 mg/kg	1,59**	1.07	× 87,4**	8.44*	1.81**	1.38*

a: n=4 instead of 5 for the control group due to on Coutlier animal. *: p≤0.05; **: p≤0.01

### Recovery phase:

<u>Fluopyram</u>: EKOD ( $p \le 0.05$ ), BROD ( $p \le 0.03$ ), PROD ( $p \le 0.01$ ) and UDPGT-bilirubin ( $p \le 0.05$ ) activity were statistically significantly increased following 1 month on control diet; however the increases were much lows than those recorded immediately following treatment.

C

<u>Phenoharbital</u> PROP and COPGT-bilirubin activity were statistically significantly increased ( $p \le 0.01$ ) following 1 month recovery. The increase in PROD activity was much lower than that recorded immediately after treatment; however UDPGT-bilirubin activity was marginally increased compared to immediately following treatment (1.56-fold following recovery compared to 1.38-fold following treatment). EROD was also significantly increased ( $p \le 0.01$ ) following the recovery phase despite there being no activity immediately following treatment. The increase (27%) was considered marginal in



comparison with the positive control, beta-naphthoflavone, which induced EROD by 800% compared to controls.

Table 5.5-83	Mean cytochrome P-450 content and enzymatic a	ctivities in the liver following	Ś
	Recovery Phase		5

Recovery	Thase				Q°	N A
		Me	an fold chang	ge relative to c	mirols	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Dose	Total P450 Content	EROD	BROD	PROP	UDPGT-4- nitrophetrol	UBPGT-
Fluopyram 1500 ppm	1.03	1.21*	J.47*	1.64**	1.91	1.29* 0
Phenobarbital 80 mg/kg/day	1.01	1.28**	0.85 °	Ő ^{1.} 93** 。	0.98	£\$6**_@
*: p≤0.05; **: p≤0.01			6 Y		Q OY	

### 6. Gene Transcript Analyses

### **Treatment phase:**

<u>Fluopyram</u>: At 30 ppm, a clear and statistically significant  $(\vec{p} \le 0.01)$  dose-related increase in the expression of Cyp3a3 was recorded (+81% at 30 ppm/p to +4943% at 1500 ppm)

From 150 ppm, clear dose-related and statistically Significant (ps0.01) increases were seconded for the expression of the Phase I genes Cyp2b1 (+990% at 150 ppm up to +154275% at 1500 ppm) and Cyp1a1 (+711% at 150 ppm up to +35372% at 1500 ppm) In addition, a statistically significant ( $p \le 0.01$ ) increase was recorded for the expression of genes coding for the Phase II enzyones Gstm4 (+136% at 150 ppm up to 1289% at 150% ppm) and Uppgtr2 (+64% at 150% ppm up to +304% at 1500 ppm).

From 600 ppm, genes coding for additional Phase II opzymes were statistically significantly increased. Specifically, Gsta2 expression was increased by 53% (p $\leq$ 005) at this dose and phx1 expression was increased by +116% (p=0.010). A marginal, though statistically Gignificant (+75%; p=0.01), increased expression of the marker for cell proliferation (Gadd 45b) wagalso observed.

Finally, the expression of Ephy2 demonstrated a weak but dose related decrease (-3%, -10%, -13%, 27% at 30, 75, 950 and 600 opm respectively), which was statistically significant at 1500 ppm (-43%; p≤0.01). ñ

There were no clear effects on the expression of POR, 604a1 Facstd1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which

weakly expressed in the liver as evidenced by the Variable and algh Ct (cycle threshold) values, which was greater than opequal to the out-off limit of 35 for the majority of samples (59/90 control and fluopyram treated liver samples)



ble 5.5-84	Mean gen	e transcript an	alyses followi	ng 28 days treat	ment with fluop	yram
Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (28 day treatment) (% change compared to control mean values)					
	Control	30 ppm	75 ppm	150 ppm	600 <u>pp</u> m	15000pm
		MF	ETABOLISM:	Phase I	<u>S</u>	4 2
POR	$0.78 \pm 0.34$	$0.70 \pm 0.29$ (-10%)	$0.67 \pm 0.29$ (-14%)	$0.77 \pm 0.27$ (NC)	$0.81 \pm 0.24$	$0.89 \pm 0.30$ (+14%)
Cyplal	1.06 ± 0.70	1.87 ± 1.25 (+76%)	2.43 ± 1.48 (+129%)	8.60 ± 7.02** (+711%)	107.0 ± 28.03** (+999%)	\$76.0 ± \$90.43*\$ \$243537\$%) \$
Cyp2b1	1.26 ± 1.15	3.38 ± 7.17 (+168%)	$2.09 \pm 1.91$ (+66%)	13.74 10:57** (±090%),	° 267 69 ± 4 193.99** ° (+21145%)	1945/13± 1618.28* (154275%)
Сур3а3	1.66 ± 0.65	3.01 ± 1.34** (+81%) &	06.19 ± 2.66 (+27,2%)	× 8.72 + 2.690+ (+425%) ~	28.37¥ 7.22** (1909%)	$ \begin{array}{c} 83 72 \pm \\ 26 02^{**} \\ 4943 \\ \end{array} $
Cyp4a1	$0.78 \pm 0.30$	$0.65 \pm 0.25$ (-17%)	$0.69 \pm 0.20$		$0.63 \pm 0.08$ (-	⁴ 0.55 ± 0.08 (- 30%)
		S ME	<b>FABOLISM:</b>	Phase 🏗 💍		L.
Gsta2	3.62 ± 2.63	3.76±2.19	3 49 ± 2.30 (-5%)	3.28 ≠ 2.37 - 5 9% Q	\$94 ± 254* (+530%)	³ √10.85 ± ³ √6.21** (+200%)
Gstm4	0.56 ± 0.39	0.60±0.365	0.92 ± 0.62	1.32 ± 0.48** 0.48** (+136%)	3,42/± 2.82**	7.78 ± 6.64** (+1289%)
Udpgtr2		1.08 ≠ 0.40 @ \$20%}	1.04 0.58 (+16%)	1.48± 0 9.51** (+64%)	2.29 ± 0.92** 0(+154%)	3.64 ± 1.51** (+304%)
Ephx1	1.06 0.22	) 1.34 ⊕0.60 ‰ (∉26%) ₄	0 1.18 (★1%) \$	) 1.49 30.35 (+41%)	2.29 ± 0.58** (+116%)	4.49 ± 2.04** (+324%)
Ephx2	1.73 ± 0.64	67±074	$1.95 \pm 0.74$	1 = 0.66 (-	1.27 ± 0.54 (- 27%)	0.98 ± 0.29** (-43%)
Sult1e1	ND 2	∕ <u>́</u> ∭ND_Ô	Y NO &	, AND	ND	ND
			DLIFERATIO	N/APOPTOSIS		
Tacstd1	1.09 9.31	Ĵ.30 €9.38 (+19%) ∕	₹1.16 + 9.23 (+6%)	1.35 ± 0.25 (+24%)	$1.23 \pm 0.28$ (+13%)	$\begin{array}{c} 1.35 \pm 0.32 \\ (+24\%) \end{array}$
Gadd455	0.79 ± 0.23	$0.95 \pm 0.44$	$0 @ 6 \pm 0.20$	0.75 ± 0.20 (- 5%)	1.38 ± 0.49** (+75%)	1.75 ± 0.89** (+122%)
Rb1	1.15 0.26	1.14¥0.19 NC) Q	1.10 <b>0.19</b>	$1.21 \pm 0.18$ (+5%)	$1.16 \pm 0.19$ (NC)	$1.16 \pm 0.23$ (NC)

Table 5.5-84Mean gene transcript analyses following 28 days treatment with fluopyram

NC: no change; *:  $p \le 0.05$ ; **:  $p \le 0.01$  DID: considered as not detected due to the variable and high Ct values, which were greater than or equal to the cut-off light of 35 for the rajority of samples (59/90 control and fluopyram treated liver samples). In the prove table the presented so changes do not always calculate exactly from the presented mean data. This is due to rounding up differences

<u>Phenobartital</u>: The gene expression data indicated that treatment with phenobarbital leads to doserelated increased expression of Phase I and Phase II enzymes. In particular, Cyp2b1, Cyp3a3, both isoforms of glutathione (Cistm4 and Cista2), Ephx1 and Udpgt were all statistically significantly increased. In contrast there was a statistically significant ( $p \le 0.01$ ) down-regulation of Cyp4a1 and Ephx2 expression.

The marker for cell proliferation (Gadd45b) was also statistically significantly increased.



There were no clear effects on the expression of POR, Cyp1a1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (8/15 phenobarbital treated liver samples).

	Mean Relative Quant	ity ± standard deviation of gene transcripts (28 day treatment)
Gene transcripts	(% change of	nonnafrad ta contrating maan valuas 🖕 👋 🖉 👘 🗍
	Control	Phenoparbital (80 mg/kg/day)
	METABOLIS	W Phase I
POR	0.78 ± 0.34	$\sim 0.00 \pm 0.2$ $\sim 0.00$ $\sim 0.00$
Cyplal	1.06 ± 0.70	$0^{\circ}$ $0^{\circ$
Cyp2b1	1.26 ± 1.15 0 (	$2930 \pm 5 \pm 2678.47 \pm (+232475\%)$
Cyp3a3	1.66 ± 0.65	$ \begin{array}{c} \textcircled{0} \\ \end{array}{0} \\ \textcircled{0} \\ \textcircled{0} \\ \textcircled{0} \\ \textcircled{0} \\ \textcircled{0} \\ \end{array}{0} \\ \textcircled{0} \\ \end{array}{0} \\ \textcircled{0} \\ \end{array}{0} \\ \end{array}{0} \\ \begin{array}{0} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\$
Cyp4a1	$0.78 \pm 0.30$	3 $3$ $3$ $3$ $3$ $3$ $3$ $3$ $3$ $3$
	METABOLIS	
Gsta2	MET&BOLIS 3,62 ± 2.60	<b>VI:</b> Phäse II $(-5\%)$ (+5%) (+5%) (+5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%) (-5%
Gstm4	0.56 ±0.39 @	2373%)
Udpgtr2	0 90 7 0 490	3.92@ 2.66** (+336%)
Ephx1		0° 3.6° ± 1.7°** (+246%)
Ephx2	1.73 ± 0.64	0.95 ± 0.27** 0.45%)
Sult1e1		ND
Ĩ	CEEL PROLIFERAT	ION/AP@PTOSIS
Tacstd1	0 1.09 ± 0.31	<u> </u>
Gadd450	$0.79 \pm 0.230^{\circ}$	1.33 [±] 0.59** (+68%)
Rb1 ô	$1.15 \pm 0.26$	$23 \pm 0.23 (+11\%)$

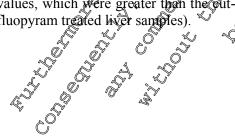
rubie ele de fifeun gene transcript analyses fonoving 20 augs treatment with presson brai	Table 5.5-85 Mean gene transcript analyses following 28 days treatment with pl	henobarbital
-------------------------------------------------------------------------------------------	--------------------------------------------------------------------------------	--------------

NC: no change  $*: p \le 0.05$ ;  $*: p \le 0.01$ ; D: considered as not detected due to the variable and high Ct values, which were greater than of equal to the co-off linear of 35 for the majority of samples (19/30 for controls and phenobarbital). In the above table the presented the presented the presented to be always calculate exactly from the presented mean data. This is due to rounding-up differences.

### Recovery phase

<u>Fluopyram</u>: The expression of genes coding for the Phase I enzymes Cyp1a1 (+83%), Cyp2b1 (+70%) and Cyp3a3 (+157%;  $p \le 0.01$ ) and the Phase I enzyme Gstm4 (+73%;  $p \le 0.05$ ) were still increased compared to the controls. Gadd45b (#42%;  $p \le 0.04$ ) and Rb1 (+13%;  $p \le 0.05$ ) were also statistically significantly increased compared to the controls. In all cases however; the % changes were much lower than those recorded primediately following treatment.

Sult le1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which were greater than the ut-off limit of 35 for the majority of samples (25/30 control and fluopyram treated liver samples).





Gene transcripts	(Rec	tandard deviation of gene transcripts
	Control	1500 ppm
	<b>METABOLISM: Ph</b>	ase I
POR	$0.54 \pm 0.24$	0.62±0.18 (+15%)
Cyplal	0.72 ± 0.23	$32 \pm 1.11 (+8\%)$
Cyp2b1	0.30 ± 0.24	0.51 ± 0.36 @70%)
Cyp3a3	2.67 ± 1.61	6.86 ± 3.75 (+157%)
Cyp4a1	0.68 ± 0.29	$\frac{1}{1000} = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0.00 = 0$
	METABOLISM: Pha	ise AF in a straight
Gsta2	$1.55 \pm 1.37$	$1.67 \pm 1.42 (+8\%)$
Gstm4	$0.74 \pm 0.48$	$1.28 \pm 0.95* (+3\%)$
Udpgtr2	0.83 40.33	$\begin{array}{c} 0 \\ \hline 0.28 \pm 0.95 * (+5\%) \\ \hline 0.65 \\ \hline 0.25 \\ \hline 0.$
Ephx1	$0 \times 0 \pm 0 \times $	0  0  0  0  0  0  0  0  0  0
Ephx2	0.85±0,26	$\sqrt[9]{0}$ $\sqrt[9]{11 \pm 0}$ $\sqrt[9]{9} \times (\frac{1}{10})$
Sult1e1		ST O UNDER ST
	ELL PROLIGERATION/A	COPTOSIS O &
Tacstd1	£ € 1.21 ± 9.29 £ 0	(2.27 (+8))
Gadd45b	y _ 1.3♥±0.4₫ y	1.96 0.57 (+42%)
Rb1 🔬	$\bigcirc$	⁶ (1.15±Q)7*(+13%)

NC: no change; *:  $p \le 0.05$  *:  $p \le 0.01$ ; ND, considered as not detected due to the variable and high Ct values, which were greater than or equal to the cut-off limit 0.35 for the majority of samples (0.3730 for controls and fluopyram). In the above table the presented % changes to not always calculate exectly from the presented mean date. This is due to rounding-up differences.

differences. <u>Phenobarbital</u>. The expression of genes coding for the Phase I enzymes Cyp2b1 (+107%; p $\leq$ 0.01), Cyp3a3 (+149%; p $\leq$ 0.01) and Cyp4a1 (+46%;  $\geq$ 0.01) and the Phase II enzyme Gstm4 (+181%; p $\leq$ 0.01) were still increased compared to the controls. Ephx(+21%; p $\leq$ 0.05) was also increased following the recovery phase. However, with the exception of Cyp4a1 and Ephx1, which were not increased immediately following treatment, the % changes were much lower than those recorded immediately following treatment.

following treatment. Sult1e1 was only wearly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (24/30 control and phenobarbital treater liver samples).

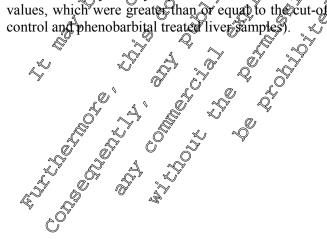




 Table 5.5-87:
 Mean gene transcript analyses following Recovery Phase in phenobarbital treated group

group		
Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (Recovery Phase) (% change compared to control mean values)	
	Control Dhonohouhita/2000 mg/lig/day)	· · · ·
	METABOLISM: Phase I 🗸 🔿 🔊	Ņ,
POR	ControlPhenobarbitarioso ing/kg/day)METABOLISM: Phase I $0.54 \pm 0.24$	
Cyp1a1	$0.72 \pm 0.23$ $0.87 \pm 0.45$ $1\%$	, Ô
Cyp2b1	$0.30 \pm 0.24$ $0.62 \neq 0.46 $ $(+10\%)$	Î Î Î
Cyp3a3	$2.67 \pm 1.61$ $3^{\circ}$ $3^{\circ}$ $7.46 \pm 5.62^{**}$ (+ $0.9\%$ ) $3^{\circ}$ $3^{\circ}$	Ş
Cyp4a1	$0.68 \pm 0.29$ $0^{\circ}$ $0^{\circ}$ $0.99 \pm 0.29^{\circ}$	_ 0
	MEAABQLASM: Phase II Q O' O'	
Gsta2	$1.55 \pm 1.37$	, y
Gstm4	$0.74 \textcircled{0.48} \end{array}{0.48} \textcircled{0.48} \textcircled{0.48} \textcircled{0.48} \end{array}{0.48} \rule{0.48} \textcircled{0.48} \end{array}{0.48} \rule{0.48} \end{array}{0.48} \rule{0.48} \rule{0.48}$	
Udpgtr2	$0.89 \pm 0.367 - 77 - 77 - 969 \pm 0.367 - 1728 - 77$	
Ephx1	$0.90 \pm 0.19$ (2) (2) (2) (2) (2) (2) (2) (2) (2) (2)	
Ephx2	$0.85 \pm 0.26$ $1.03 \pm 0.40 + 21\%$	
Sult1e1	W WD D L A A NO	
,	CELL PROLIFERATION/APOPTOSIS	
Tacstd1	$1.20 \pm 0.29$ $1.20 \pm 0.26$ NC)	
Gadd45b	$5 = 538 \pm 0.42$ $5 = 0.41 (+14\%)$	
Rb1	$\frac{1.02 \pm 0.140}{1.102 \pm 0.140} = \frac{1.16 \pm 0.22}{1.16 \pm 0.22} (+14\%)$	

NC: no change;  $p \le 0.05$  *:  $p \le 0.01$ ; ND: considered as not detected due to the variable and high Ct values, which were greater than opequal to the cut-off limit @ 35 for the majority of comples (24/30 for controls and phenobarbital). In the above table the presented % changes to not all ways calculate exactly from the presented mean data. This is due to rounding-up differences.

### E. Deficiencies

No specific deficiencies were noted in the study.

## III. Conclusions

Overall, treatment with fluopyraph for a fleast 28 days induced clear and statistically significant changes in the lixer (cell proliferation hypertrophy and enzyme activity as well as associated changes in gene expression). These changes were dose-related beginning from 75 ppm. The dose of 30 ppm was considered as a no observed adverse effect level (NOAEL; based on the increased gene expression of cyp3a3, with no other correlated findings, at this dose level). The hepatic changes appeared to be reversible as evidenced by the reduced hepatic responses recorded in females previously treated with 1500 ppm fluopyraph following the recovery period.

any following the



### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on the effects and target organs of fluopyram in the rat

Overall, treatment with fluopyram for at least 28 days induced clear and statistically significant changes in the liver (cell proliferation, hypertrophy and enzyme activity as well as associated changes in gene expression). These changes were dose-related beginning from 75 ppm. The dose of 30 ppm was considered as a no observed adverse effect level (NOAEL; based on the increased gene expression of cyp3a3, with no other correlated findings, at this dose level). The depatic changes appeared to be reversible as evidenced by the reduced hepatic responses recorded in females previously treated with 1500 ppm fluopyram following the recovery period.

Data Point:	KCA J.J/0/
Report Author:	
Report Year:	
Report Title:	Fluopyram: Encyme and DNA synthesis induction in cultured human hepatocytes
Report No:	CXR1241 $(1)$ $(1)$ $(1)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$ $(2)$
Document No:	M-4501560Y-1
Guideline(s) followed in	No specific guidefine
study:	
Deviations from current	Current guideline: none
test guideline:	
Previous evaluation:	Not premously evaluated a set of the set of
2	
GLP/Officially	Not previously ovaluated Yes conducted under GLP Officially recognised testing facilities
GLP/Officially recognised testing	
Acceptability/Reliability; (	Yes Y Y Y Y Y

### **Executive Summary**

• Female buman hepatocytes were cultured in the presence of the test item fluopyram, and phenobarbital (PB) or epidermal growth factor (EGF) as two positive control compounds.

• A decrease in ATP concentration commencing at  $100 \mu$ M with a slight decrease followed by a marked decrease at 300  $\mu$ M fluopyram was observed. This was indicative of cytotoxicity. Fluopyram was not cytotoxic at  $300 \mu$ M.

• Culturing primary female human hepatocytes with fluopyram had no effect on replicative DNA synthesis Likewise no effect was observed following exposure of hepatocytes to phenobarbital.

• Culturing primary bumar hepatocytes with fluopyram resulted in an increase in pentoxyresorufin-O-depentylation (PROD) activity of up to a maximum of 1.9–fold at 100  $\mu$ M. This indicates that fluopyram is an inducer of CYP2B.

• Culturing pointary human Repatocytes with fluopyram resulted in an increase in benzyloxyresorufin-O-debenzylation (BROD) activity, up to a maximum 2–fold at 300  $\mu$ M. This indicates that fluopyram is an inducer of CYP2B/CYP5A.

• Culturing primary adman depatocytes with fluopyram resulted in an increase in benzyloxyquinoline-O-debenzylation (BQ) activity up to a maximum of 1.8–fold at 10  $\mu$ M. This indicates that fluopyram is an induce of CYP3A at low concentrations.

• In conclusion, these data suggest that fluopyram is a weak activator of CAR and PXR, with no compound-stimulated DNA synthesis in human hepatocytes



#### I. Materials and methods

A.	Materials	Q° S
1.	Test material:	Fluopyram (AE C656948) Beige powder EDFL013235 98.7% 658066-35-4 Stable in 0.1% (v/v) a µL Dimethol sulfoxide (DMSQ) mL
	Description	Beige powder
	Lot / Batch #:	EDFL013235
	Purity:	98.7%
	CAS#	658066-35-4
	Stability of test compound:	medium, prepared daily
	Vehicle and / or positive ntrol:	Phenobarbital sodium salt (PB), positive control
	Lot / Batch #:	P-5178 $\checkmark$
	Supplier:	Sigma Cliemicals
	CÂŜ #	
	Stability of test compound:	Stable in 0.1% (v/x) $\mu$ L Dimethyl sulfoxide (DMSO) mL medium, prepared daily $\sigma$
3.	Vehicle and / or positive	E9644 Sigma Chemicals Stable in 02% (vA), 1 µC Dimethyl suffoxide (DMSO)/mL
co	ntrol:	Agide and growth races (ECA), positive cantion
	Lot / Batch #:	E 9644 7 5 5 5 5 5
	Supplier:	Signa Chemicals of L of C
	Stability of test compound:	
4.	Test system:	
	Species: 🌱 🔬	Primary female frumar frepatocytes, plateable cryopreserved
	Viability:	fin excess of 70% (one donor was used)
	Source:	fn excess of 70% (one donor was used) Invittegen, 7 Kingstand Grange, Warrington, Cheshire, UK
B.	Study designed	
	$\sim$ $\sim$ $\sim$ .	$5^{\circ}$ 07 December 2012 $-$ 20 December 2012
	Study outline and treatment?	
Thi	s study investigated the potentia	for fluopyram to induce the characteristic effects of CAR/PXR
acti	vation in cultured human hepet	ocytes Stimulation of CYP2B activity, CYP3A activity and cell
pro	liferation (measured as the chan	ge in replicative DNA synthesis [S-phase of the cell cycle]) were
dete	ermined in primary of tures of isc	lated female human hepatocytes. Phenobarbital (PB) and epidermal
		Pas positive control reference items for induction of CYP2B/3A
acti	vities (phenobarbital outy) and c	ell proliferation.
	A. X	

Table S. 20				
Group #	Treatment of		CYP Enzyme Activity	ATP
1	Control	$\hat{Q}$ $\checkmark$	$\checkmark$	$\checkmark$
2	PB LOAM *	$\checkmark$	$\checkmark$	$\checkmark$
3	Ϋ́ Ϋ́ PB 900μMs	$\checkmark$	$\checkmark$	$\checkmark$
4	PB 1000 M	$\checkmark$	$\checkmark$	$\checkmark$
	² βuopyram 1μM	$\checkmark$	$\checkmark$	$\checkmark$
<i>4</i> €6 ∠	Fluopyram 3µM	$\checkmark$	$\checkmark$	$\checkmark$
70	Fluopyram 10µM	$\checkmark$	$\checkmark$	$\checkmark$
8	Fluopyram 30µM	$\checkmark$	$\checkmark$	$\checkmark$

# Table 5.5.88 Study design



Group #	Treatment	BrdU for Sphase	CYP Enzyme Activity	ATP °
9	Fluopyram 100µM	$\checkmark$	$\checkmark$	
10	Fluopyram 300µM	$\checkmark$	✓ 🔉	√ 5 ⁷ 0 ⁷
11	EGF (+ve control BrdU)	$\checkmark$	- 5	

### 3. Statistics

Aken v a g Statistical comparisons between treated hepatocytes and their control group were undertaken

for all numerical data sets using a 2-tailed Student's t-test.

### C. Methods

### **1. Hepatocyte Culture**

Primary monolayer cultures of hepatocytes were prepared in collager coated 25 cm2 flastes, 96- and 6well plastic tissue culture plates, using Leibowitz HCL15 (Mitchell A.M. et al 1984) Arch Toxicol. 55, 239-246) as the medium. In all 96-well plate cultures the outside wells were not used, but filled with sterile phosphate buffered saline.

Hepatocytes were resuscitated in Revorteserved Hepatocyte Recovery Medium (CHRM®) then cultured in Cryopreserved Hepatocyte Photing Wedium (CHRM) for approximately 6 1 to allow adherence. Following a visual check for adhesion, the medium was then changed to Leibowitz HCL15 (Laboratory Method Sheet (LNOS) Tie-002) medium and the hepatocytes were exposed to PB at 3 concentrations (10, 100 and 1000  $\mu$ M), to floopyram at 6 concentrations (1, 3, 10, 30, 400 and 300  $\mu$ M, determined in the preliminary dose ranging study and to EGF (25 ng/mL). A rehicle control (0.1% v/v DMSO) was included. The media, with test of reference items, was replepished daily for a further 3 days. There were 3 replicates for each goncentration in 25 cm2 flacks for enzymetactivity measurements. 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (S-phase) analysis and 6 replicates for each concentration 96 well plates for cell toxicity (ATP) measurements.

The test and reference items were formulated in DMSO, and administered such that the final DMSO concentration is all contures @as 0.1% (v/x) 1 µL DMS@mL medium,

### 2. Assays

After 96 hours in culture, heppiocytes were fixed, of harvested by scraping them into SET buffer (0.25 M sucrose, 5 mM DTA 20 mM Tos-HQI pH74), someating the mixture and storing it at approximately -700°C until analysis. Protein was determined by the method of Lowry et al (1951) J. Biol. Chem. 193, 265-273, (LNS Spec-001)

### 2.1 Adenosin@ 5'-Triphosphate (ATP)

Hepatocyte toxicity was assessed following 96 hours of culture as indicated by ATP depletion. Cellular ATP was determined by luminometry according to LMS FLUOR003, using an assay kit supplied by Promega (CellTitre, Glo luminescept cell adability assay).

Results are expressed as appercentage of the maximum amount of ATP released (i.e. the value of control cells).

## 2.2 Replicative DNA Synthesis (S-Phase)

The number of hepatocytes undergoing replicative DNA synthesis (S-phase) in any given cell population can be determined by the incorporation of BrdU followed by immunostaining. S-phase was determined immunocytochemically following the incorporation of BrdU into hepatocyte nuclei over the last 3 days of culture. Innunostaining was performed after fixation at 96 h. Data are expressed as a labelling index (% of total hepatocytes that have incorporated BrdU).

EGF (25 ng/mL, n=5) was included as a positive control for induction of replicative DNA synthesis.



C

### 2.3 Pentoxyresorufin-O-depentylation (PROD)

The activity of CYP2B in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al* (1985) Biochem. Pharacol. 34, 18, 337-3345, according to LMS Fluor-002.

### 2.4 Benzyloxyresorufin-O-debenzylation (BROD)

The activity of CYP2B/3A in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin, as described by Burke *et al* (1985), according to LMS Fluor-002.

### 2.5 Benzyloxyquinoline-O-debenzylation (BQ)

The activity of CYP3A in cultured hepatocytes was determined spectroflyprometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline as described by GEVTEST HTS technical bulletin, according to LMS Fluor-005.

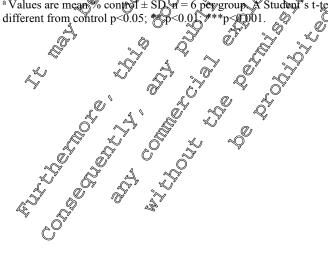
### II. Results and discussion

### 1. Adenosine 5'-Triphosphate (ATP)

Fluopyram (1 - 30  $\mu$ M) had little, or no effect on cellular AFP concentrations after 96 bours. However, at a concentration of 100  $\mu$ M a slight decrease in in ATP concentration was observed, whilst at 300  $\mu$ M a marked decrease in ATP concentration was observed, indicating the start of cytotoxicity at 100  $\mu$ M, which was clearly evident at 300  $\mu$ M.

Table 5.5-89	Adenosine 5'-Tr	iphosplate	o 4		)
	Treatment			ATP & Control	)
	Vehicle Control			\$ \$100.0 ± 8.7 ^a	
	@B 10,µM 🔮		S a	$O_{110.0} = 5.2*$	
	ΥPB_100 μM2γ			$103.4 \pm 7.1$	
	^С РВ∂ 000 µ́М́			$\frac{2}{\sqrt{108.4 \pm 7.1}}$	
Ő	Flaopyrand µM			©108.9 ± 1.6*	
	Fluopyram 3 µM	4 A Y	10 ¹ W		
- A	Fluons am 10 M	£ . 2		$111.5 \pm 11.5$	
* %	Fluopyram 30 µM			$126.3 \pm 8.6$ ***	
	Endopyram 100 1001		O 🏷	$91.9\pm6.9$	
<i>@</i>	Fluopyram 300 µM		\$ <u>\$</u>	$29.1 \pm 1.4$ ***	

^a Values are mean % control  $\pm$  SD H = 6 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; *p<0.01 ***p<0.01.





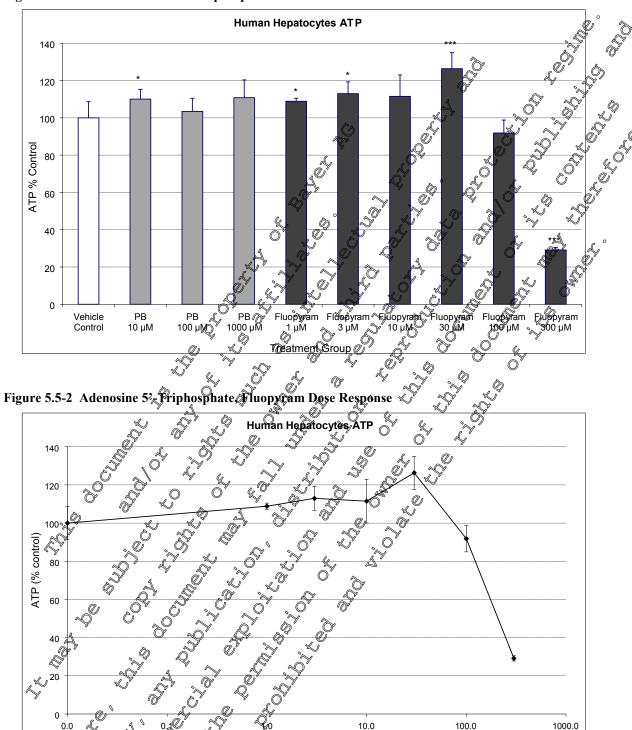


Figure 5.5-1 Adenosine 5'-Triphosphate



0.0

Treatment with 25ng/mL EOF resulted in a statistically significant increase in replicative DNA synthesis to 15-fold control values. This indicates that the hepatocytes could proliferate following exposure to proliferative stimuli, and therefore demonstrating their suitability for use in investigations involving assessing induction of proliferation.

Fl@pyram Concentration (µM)

10.0

100.0

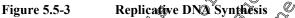
1000.0

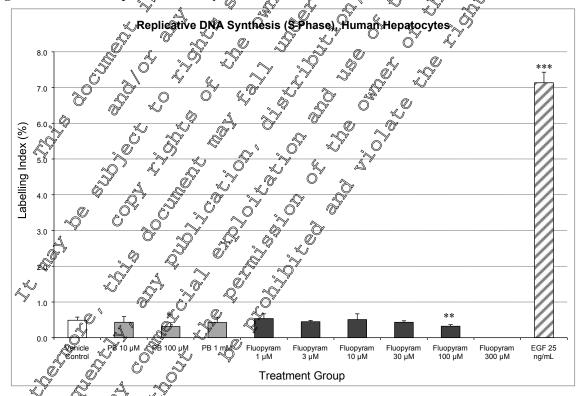


No biologically significant changes in replicative DNA synthesis were observed following treatment with either PB ( $10 - 1000 \mu$ M) or fluopyram ( $1 - 30 \mu$ M). However, at a concentration of  $100 \mu$ M fluopyram, there was a decrease in hepatocyte confluency, with fewer adherent hepatocytes present, possibly indicating the beginning of dose-dependent toxicity. Analysis of the cells exposed to 300  $\mu$ M fluopyram was precluded due to extensive cytotoxicity.

Table 5.5-90	<b>Replicative D</b>	NA Synthesis	
Trea	itment		Labelling Index (%)
Vehicle	e Control		$0 = 0.09 (100 @ \pm 18.5)^{a}$
PB 1	l0 μM		$0.43 \pm 0.16$ (88) 4 ± 32.4) $0.43 \pm 0.16$ (88) 4 ± 32.4)
PB 1	00 μM		$0.31 \pm 0.11$ (63.8 ± 22.2) $0$
PB 10	000 μM	ĺ	$0.43 \pm 0.15$ (87.4 $230.30$
Fluopyr	am 1 μM		$0.54 \pm 0.13 (109.9 \pm 27.2)$
Fluopyr	am 3 μM	×	$0.45 \neq 0.03(91.7 \pm 9.1)$
Fluopyra	am 10 µM		
Fluopyra	am 30 µM		
Fluopyra	m 100 μM		$0.32 \pm 0.04^{**}$ $(66.1 \pm 9.1)^{b}$
Fluopyra	m 300 μM	Q° 4 [°]	
	25ng/mL		$7.13 \pm 0.30$ * (1461.4 ± 60.7)
a Values are Mean =	± SD. Values in pare	nthesis are mean % cont	ntrol SD n z per group. b Decrease in hepatoevie confluency.

^a Values are Mean  $\pm$  SD. Values in parenthesis are mean % control SD n  $\neq$  per goip. ^b Decrease in hepatoevic confluency, fewer adherent hepatocytes present. ^cApalysis unable to to performed due to excessive cytooxicity. OStudent's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001





# 3. Pentoxyresorutin-O-Depentylation (PROD)

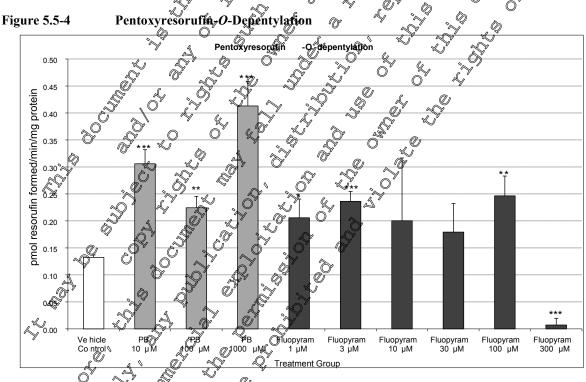
Culturing primary human hepatocytes for 96 hours in the presence of phenobarbital resulted in increases in PROD activity to a maximum 3.1-fold at 1000  $\mu$ M. This is consistent with historical data.



Addition of fluopyram to the hepatocytes resulted in increases in PROD activity to a maximum 1.9 fold of control value at 100  $\mu$ M. Treatment with 300  $\mu$ M fluopyram reduced PROD activity to 5% of the control value, reflecting the cytotoxicity shown by the ATP and S-phase results (Table 4 and Figure 4). Clear dose-dependent induction by fluopyram was not seen, suggesting that maximal effects were seen at the low concentrations of fluopyram.

Table 5.91Pentoxyr	esorufin-O-Depentylation	
Treatment	PROD (pmol resoruf	in formed/min/mg protein)
Vehicle Control	$0.32 \pm 0.0$	$005 (100.0 \pm 3.5)^{a}$
PB 10 μM	$0$ $\bigcirc 06 \pm 0.026$	5***Q231.1 ± 19.8%
PB 100 μM	$0.224 \pm 0.02$	$10^{10}$ (169.6 ± 16.0)
PB 1000 µM	$0.413 \pm 0.042$	$5^{***}(\mathcal{D}^{2.0 \pm 34.2})$
Fluopyram 1 µM	<u>کې 206 ± 3.02</u>	35*(U55.6,=26.2) 35* 3
Fluopyram 3 µM	O [*] \$.236 0.018	87 ** (17896 ± 1399) 2 ~ ~ ~ ~ ~
Fluopyram 10 µM		46 (151.4 ± 827)
Fluopyram 30 µM	0.179 ≢0.0	153 (435.5 \$ 90.0) × 5
Fluopyram 100 µM	<i>∞ 4</i> × 0.246 <i>⇒</i> 0.03	7 (186: ± 27.8) ~ O
Fluopyram 300 µM	$\frac{1}{\sqrt{2}} \frac{1}{\sqrt{2}} \frac{1}{\sqrt{2}$	12***(5.4±9(4)
Values are Mean + SD Valu	ues in parenthesis are»mean ‰ control ±	SDOn = 3 over grown. A Student's t-test

^a Values are Mean  $\pm$  SD. Values in parenthesis are mean % control  $\pm$  SDOn = 3 per group. A Student's t-test (2sided) was performed on the results; *statisticall@different from control % <0.05 ** p <0.01; *** p <0.001.



## 4. Benzyloxyresorufin-Q Debenzylation (BROD)

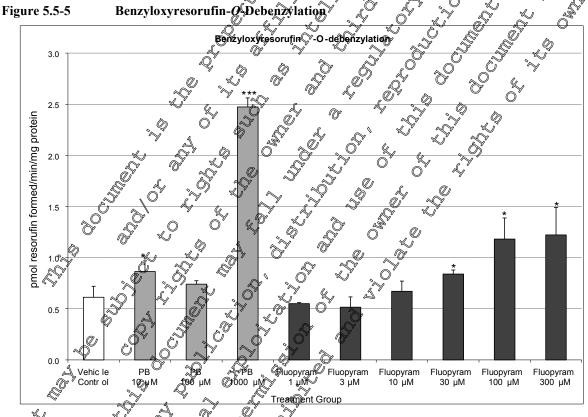
Culturing primary human heperocytes, for 96 hours, with phenobarbital resulted in increases in BROD activity up to maximum 4 fold increase at 1000  $\mu$ M. This is consistent with historical data.

Culturing topatocytes with fluopyram resulted in a dose-dependent increase in BROD activity, up to a maximum increase of 2-fold at 300  $\mu$ M. This level of induction could be affected by the cytotoxicity shown by the ATP and S-phase and PROD results.



Treatment	BROD (pmol resorufin formed/min/mg protein) 🔬
Vehicle Control	$0.612 \pm 0.107 (100.0 \pm 17.5)^{a}$
PB 10 μM	$0.865 \pm 0.105^{*} (141.2 \pm 17.5)$
PB 100 μM	$0.741 \pm 0.033 (121.0 \pm 55)$
PB 1000 μM	$2.473 \pm 0.089^{***}$ (403.8 ± 14.5)
Fluopyram 1 µM	$0.550 \pm 0.011 (89.9 \pm 1.8)$
Fluopyram 3 µM	$\frac{0.515 \pm 0.102 (842) \pm 16.6)}{0.671 \pm 0.100 (309 5 \pm )164 \sqrt{2}} \xrightarrow{7} \sqrt{2}$
Fluopyram 10 µM	0.671 ± 0.100 (019.5± )16.4 2 2 2 2
Fluopyram 30 µM	$\sqrt{0.840 \pm 0.046}$ (137.1 ± 6.5) (137.1 ± 6.5)
Fluopyram 100 µM	$0^{1}$ 1.182 ± 0.206* (1950 ± 355) $0^{1}$ $0^{1}$
Fluopyram 300 µM	$1.222 \pm (269*(199.5 \pm 013.9))$

^a Values are Mean  $\pm$  SD. Values in parenthesis are mean to control  $\pm$  SD. n = 5 per group. A Student's t-test (2sided) was performed on the results; *statistically different from control p<0.03; ** p<0.01; *** p<0.01.



# 5. Benzyloxyquinoline-QDebenzylation (BQ)

Culturing primary human hepatocytes, for 56 hours, with phenobarbital resulted in a dosedependent increase in BG activity of up to 5.2 fold at 1000  $\mu$ M. This is consistent with historical data.

Exposure of hepatocytes to fluopyram resulted in an increase in benzyloxyquinoline-Odebenzylation (BQ) activity up to a maximum of 1.8-fold at 10  $\mu$ M. This indicates that fluopyram is an inducer of CYP3A at low concentrations. Higher concentrations led to decreases in activity, with cytotoxicity combinencing at 100  $\mu$ M and clearly evident at 300  $\mu$ M.

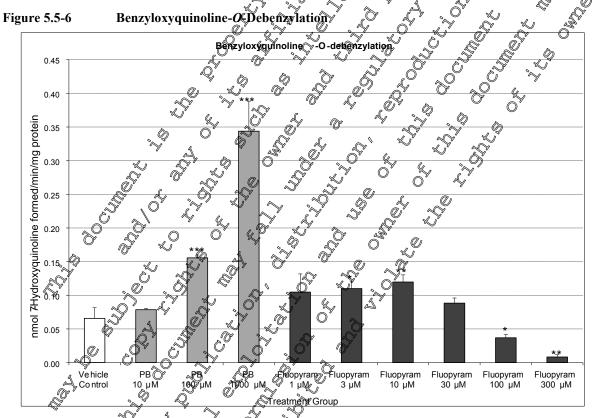




Treatment	BQ (nmol 7-Hydroxyquinoline formed/min/mg protein)
Vehicle Control	$0.066 \pm 0.016 (100.0 \pm 24.2)^{a}$
PB 10 μM	$0.079 \pm 0.002 (119.6 \pm 2.5)$
PB 100 µM	$0.156 \pm 0.004^{***} (236.8 \pm 5)$
PB 1000 μM	$0.344 \pm 0.045^{***} (523.3 \pm 67.9)$
Fluopyram 1 µM	$0.105 \pm 0.027 (159.441.1)$
Fluopyram 3 µM	$0.140 \pm 0.011* (160.5 \pm 16.9)$
Fluopyram 10 µM	$0.120 \pm 0.011 ** 0.82.2 \pm 16.1)$
Fluopyram 30 µM	$0.088 \pm 0.008$ (134.4 $\pm 11.6$ )
Fluopyram 100 µM	$0.037 \pm 0.005*(56.2 \pm 7.2)^{\circ}$
Fluopyram 300 µM	0.008 ± 0.003 ** 12.7 ± 0.3)

Table 5.5-93 Benzyloxyquinoline-O-Debenzylation

^a Values are Mean  $\pm$  SD. Values in parenthesis are mean % control  $\pm$  SD = 3 per group. % Student's t-test (2sided) was performed on the results; *statistically different from control pc0.05; *4-0.01



### A. Deficiencies

A. Deficiencies of the second in the second 

#### Conclusions III.

Fluopyram and phenobabital appeared to exhibit similar properties in human hepatocytes, inducing cytochromes P450 via the programe x receptor (PXR) and the constitutive androstane receptor (CAR). The increases on productivity seen after exposure to even low concentrations of fluopyram indicate that fluopyram is an inducer of CYP2B. The induction of BROD (at higher concentrations) and BQ (at lower concentrations) activity suggests that fluopyram is also a CYP3A inducer. Ĉ



Human CAR/PXR did not mediate compound-stimulated dna synthesis in human hepatocytes. Neither phenobarbital nor fluopyram stimulated replicative dna synthesis (s-phase) in the hepatocytes, whilst the positive control EGF elicited a 15-fold increase in S-phase.

In conclusion, these data suggest that fluopyram is a weak activator of CAR and PXR, with r compound-stimulated dna synthesis in human hepatocytes.

### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanist information on the effects and target organs of fluopycam in the rat

Fluopyram and phenobarbital appeared to exhibit similar properties in human hepatocytes, inducing cytochromes P450 via the pregnane x receptor (PAR) and the constitutive and ostane ecceptor (CAR). The increases in PROD activity seen after exposure to even low concentrations of Duopyram indicate that fluopyram is an inducer of CYP2b. The induction of BROD (at higher concentrations) and BQ (at lower concentrations) activity suggests that fluopyram is also a CYP3a inducer. Human CAR/PXR did not mediate compound stimulated DNA synthesis in human hepatocytes. Neither phenobarbital nor fluopyram stimulated replicative DNA synthesis (s-phase) in the hepatocytes, whilst the positive control egt chicited a 15 fold increase in S-phase. In conclusion, these data suggest that fluopyram is a weak activator of CAR and PXR with no compound-stimulated DNA synthesis in human hepatocytes.

Data Point:	46CA 5008 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
Report Author:	
Report Year:	
Report Title:	Fluopyram: Enzone and DNA synthesis induction in cultured rat hepatocytes
Report No:	CXR1242 X A A A
Document No:	$\frac{\text{MCXRQ242}}{\text{M-4450157},01-1} \sim 7 \sim 7 \sim 7 \sim 7$
Guideline(s) for wed	No specific guideline
study:	No specific guideline
Deviations from current 🔬	Current guidettige: none Q
test guideline:	
Previous evaluation:	Not previously evaluated
GLP/Officially	Wes, conducted under GLP/Officially recognised testing facilities
recognised testing	
GLP/Officially recognised testing facilities:	S & S S
Acceptability keliability:	Ves.
Y .	

## Executive Summary @

• Female rat hepatoc des were cultured in the presence of the test item fluopyram, and phenobarbital or EGF as two positive compolicoppounds.

• A decrease in ATP concentration at 300  $\mu$ M fluopyram was observed. This was indicative of cytotoxicity Duopyram was not cytotoxic at 100  $\mu$ M.

• Culturing primary female rat bepatocytes with fluopyram stimulated replicative DNA synthesis in a dose-dependent manner. At  $90 \mu$ M, S-phase was increased by approximately 3.9–fold. Similar, but smaller, increases were observed following exposure of hepatocytes to phenobarbital (2.9–fold at 100  $\mu$ M).

• Culturing primary rat hepatocytes with fluopyram resulted in an increase in pentoxyresorufin-O-depentivation (PROD) activity of up to a maximum of 2.8–fold at 30  $\mu$ M. This indicates that fluopyram is an inducer of CYP2B.



• Culturing primary rat hepatocytes with fluopyram resulted in an increase in benzyloxyresorufin-Odebenzylation (BROD) activity, up to a maximum of 4.2-fold at 30 µM. This indicates that fluopyram is an inducer of CYP2B/CYP3A.

• Culturing primary rat hepatocytes with fluopyram resulted in a concentration-dependent increase in benzyloxyquinoline-O-debenzylation (BQ) activity up to a maximum of 18.0 fold at 100 ft. This indicates that fluoruram is an inducer of CVP2A indicates that fluopyram is an inducer of CYP3A.

- In conclusion, these data suggest that fluopyram is an activator of both CAR and PXR
- I. Materials and methods A. Materials 1. Test material: Fluopyram (ADE C656948 Beige powder Description Lot / Batch #: EDFL013235 **Purity:** 98.7% CAS# 658066-35-4 thyl softoxide (DMSO) Stable in 0,1% (v/ I KF **Stability of test compound:** medium, prepared daily 2. Vehicle and / or positive Phenobarbital sodium salt (PB control: *S* Lot / Batch #: ธ์178 Supplier: Sigma Chemicals CAS# ∞50-06-6 Stable in MM% (x/v), 1 µ Dimethyl suffoxide (DMSO)/mL Stability of test compound: medium prepared daib 3. Vehicle and / or positiv Epidermal growth factor (EGF), positive control control: Lot / Batch E-9644 0 Supplier Sigma Chemicals Stability of test compounds Amedium, prepared daily Bul Donethyl sulfoxide (DMSO)/mL 4. Test animals: Species: hale only) Reat -Plan Wistar (out-bred HsdHanTM: WIST) Strain: Acclimation period weeks approximately at start of treatment 150 to 200 g at aprival Harlan WK Ltd, Shaw's Farm, Blackthorn, Oxon, UK At least 5 days RMUpelleted diet (Special Diet Services Ltd., Stepfield, Witham Essex, UK), ad libitum Pap water, ad libitum **∦**Water: Anipals were housed in groups on saw-dust in solid-Housing: _Ø bottom, polypropylene cages. Environmental conditions: Temperature ~©19-23℃ 40-70% Humidity 14-15 air changes per hour Air changes: Photoperiod 12 hours light, 12 hours dark Test system: Species: Primary monolayer cultures of hepatocytes Viability: In excess of 80% (hepatocytes from two independent perfusions were pooled)



- B. Study design
- **1. Experimental period:** 
  - 07 December 2012 20 December 2012

### 2. Study outline and treatment

This study investigated the potential of fluopyram to induce the characteristic effects of CAR/PXR activation in cultured rat hepatocytes. Stimulation of CYP2B activity, @P3A activity and cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cellevcless were) determined in primary cultures of isolated female rat hepatocytes. Phenobarbital (PB) and epidermal growth factor (EGF) were included as positive control reference items for induction of CYP2BOA activities (phenobarbital only) and cell proliferation.

Table 5.5-94	Study design	
Group #	Treatment	BrdU for S-phase CYP Enzyme
1	Control	
2	PB 10µM	
3	PB 100µM	
4	РВ 1000µМ _Õ [🌾]	
5	Fluopyram 1µMQ	
6	Fluopyram 3 @M	
7	Fluopyram	
8	Fluopyragn 30µNO	
9	Fluopyram 100µM	
10	Flugpyram 200 µM	
11	EGIS (+ve control Bridu)	
3 Statistics		

Statistical comparisons between treated hepatocytes and their control group were undertaken for all numerical data sets using a 2-taked Stydent's Otest.

### 1. Hepatocyte Isolation

Rats were terminally maestbetised, using euthater, and hepatocytes isolated by in situ perfusion according to Mitchell A.M et al 1984, Arch Toxicol. 55: 239-246. Viabilities of the hepatocyte preparations, determined by trypen blue exclusion, must be in excess of 80%. Hepatocytes from two independent perfusions were pooled.

### 2. Hepatocyte Culture

Primary monolayer cultures of hepatocytes were prepared in 25 cm2 flasks, 96- and 6-well plastic tissue culture plates, using Leibowitz HCL (9 (Mitchell A.M. et al (1984) Arch. Toxicol. 55, 239-246) as the medium. In all 96-well plate cultures the outside wells were not used, but filled with sterile phosphate buffered salare.

Hepatocoffes were cultured in Deibowitz CL15 (Laboratory Method Sheet (LMS) TIC001) medium for approximatel with to allow adhesion. Following a visual check for adhesion, the medium was changed and the hepatocytes exposed to phenobarbital sodium salt at 3 concentrations (10, 100 and 1000 µM), fluopyran at 6 concentrations (1, 3, 10, 30, 100 and 300 µM, determined in the preliminary dose-ranging study) and to EGF (25 ng/mL). A vehicle control (0.1% v/v DMSO) was included. The media, with test items, was replenished daily for a further 3 days. For each test and reference item, there were 3 replicates for each concentration in 25 cm2 flasks for enzyme activity measurements, 5 replicates for each



concentration in 6-well plates for replicative DNA synthesis (S-phase) analysis and 6 replicates for each concentration in 96-well plates for cell toxicity (ATP) measurements. The test and reference items were formulated in DMSO, and administered such that the final DMSO concentration in all cultures was 0.1%, 1 µL DMSO/mL medium.

### 3. Assays

After 96 hours in culture hepatocytes were fixed, or harvested by scraping them into SET buffer 10.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4), sonicating the mixture and storing it at -706c until analysis. Protein was determined by the method of Low(e *et al* (1951) J. Biol. Chem. **193**, 265-275 (MET-SPEC001).

### 4. Adenosine 5'-Triphosphate (ATP)

Hepatocyte toxicity was assessed following 96 hours of culture as indicated by RTP depletion. The ATP released from viable somatic cells was determined by luminometry according to LMS FLUQR003, using an assay kit supplied by Promega (CellTure-Glof luminoscent cell viability assay).

Results are expressed as a percentage of the maximum amount of ATP released (i.e. the value of control cells).

### 5. Replicative DNA Synthesis (S-Phase)

The number of hepatocytes undergoing replicative DNA synthesis (S-phase) in any given cell population can be determined by the incorporation of BrdU followed by immunostaining. S-phase will be determined immunocytochemically following the incorporation of BrdU into hepatocyte nuclei over the last 3 days of culture. Immunostaining was performed after fixation at 96 h. Data will be expressed as a labelling index (% of total bepatocytes that have incorporated BrdU).

EGF (25 ng/mL, n=5) was included as a positive control for induction of replicative DNA synthesis.

## 6. Pentoxyresorufin-Ø-depentylation (PBOD)

The activity of CVP2B in oultured hepatocytes was determined spectrofluor metrically by the formation of resorufin from peneosyresorufin, as described by Burke *et al* (1985) Biochem. Pharmacol. **34**, 18. 3337-3345, according to LMS Fluor 002.

 $\bigcirc$ 

 $\bigcap$ 

### 7. Benzylogyresorufin-Q-debenzylation (BROD)

The activity of CYP2B/3A b cultured hepatocytes was determined spectrofluorometrically by the formation of resorution front benzylex yresorufine as described by Burke *et al* (1985), according to LMS Fluor-002.

## 8. Benzyloxyquinoline-O-depenzylation (BQ)

The activity of CYP3A in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from berzyloxyquinoline, as described by GENTEST HTS technical bulletin, according to LMS Fluor-005.

### AII. Results and discussion

### A. Adenosine 5% Triphosphate (ATP)

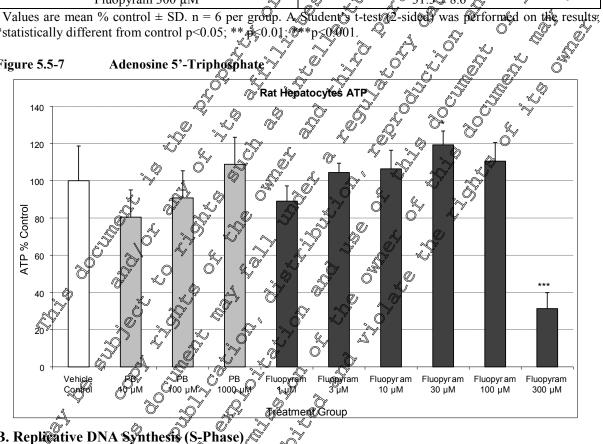
Fluopyram (10,100 µM) has little, or no, effect on cellular ATP concentrations after 96 hours. However, at a concentration of 300 µM a marked decrease in ATP concentration was observed, demonstrating cytotoxically.



Table 5.5-75. Adenosine 5 - Triphospha	ate
Treatment	ATP (% Control)
Vehicle Control	$100.0 \pm 18.7^{a}$
PB 10 μM	$80.5 \pm 14.6$
PB 100 µM	90.8±44.6
PB 1000 μM	$108.9 \pm 14.5$
Fluopyram 1 µM	
Fluopyram 3 µM	$ \begin{array}{c}                                     $
Fluopyram 10 μM	
Fluopyram 30 μM	
Fluopyram 100 µM	Q Q 10.5 ± 10.0 Q Q
Fluopyram 300 µM	المربح المربح المربح المربح

#### Table 5 5-95. Adenosine 5'-Trinhosnhate

^a Values are mean % control  $\pm$  SD. n = 6 per group. A Student's t-test/2-sides *statistically different from control p<0.05; **  $\phi \leq 0.01$ ;



### Figure 5.5-7

## B. Replicative DNA Synthesis

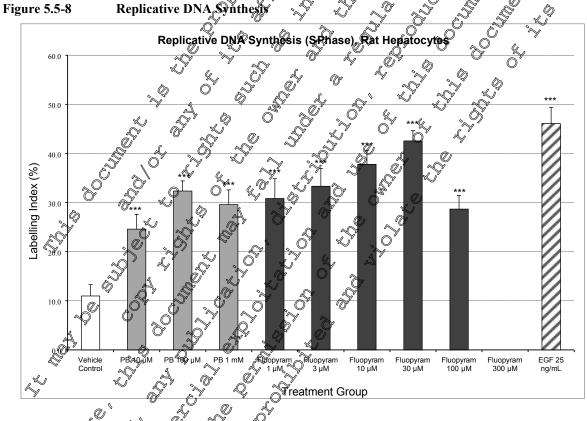
Culturing primary rat hepatocytes for 96 hours with phenobarbital resulted in a dose dependent increase in replicative DNA synthesis up to a maximum of 2.9-fold. Similarly, a dose-dependent increase of Sphase was observed, following treatment with fluopyram, with maximal stimulation at 30-fold). Exposure of Depatosytes to higher concentrations resulted in less M fluopyram (3.9 stimulation of Sphase. The second positive control, epidermal growth factor (EGF) increased replicative DNA synthesis by 4.2-fold, which is within the historical data range.



Table 5.5-96	<b>Replicative DNA</b>	Svnthesis
	itepheatite Divis	Synchesis

Treatment	Labelling Index (%)
Vehicle Control	$10.97 \pm 2.32 (100.0 \pm 21.1)^{a}$
PB 10 μM	$24.59 \pm 3.00^{***} (224.3 \pm 27.4)$
PB 100 μM	32.34 ± 2.07*** (294.9 ± 18,8)
ΡΒ 1000 μΜ	29.57 ± 2.99*** (269.7 ± 27.3)
Fluopyram 1 µM	$30.82 \pm 4.04^{***} (281 \text{ J} = 36.8)$
Fluopyram 3 µM	33,3 ± 3.66*** (30) 8 ± 33.4)
Fluopyram 10 µM	37.79 ± 2.386*** (344.6 ± 26.0) (2 3 2 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3
Fluopyram 30 µM	@2.56 ± 2.09*** (388.1 ± 19.10
Fluopyram 100 μM	$28.67 \pm 2.80^{***}$ (26924 ± 255)
Fluopyram 300 µM	by by by the by the the
EGF 25ng/mL	€6.07 € 3.29*** (420 ) = 30.05

^a Values are Mean  $\pm$  SD. Values in parenthesis are mean  $\frac{4}{3}$  control  $\pm$  SD h = 5 per group. ^b Apalysis upable to be performed due to excessive cytotoxicity. A Student's trest (2 sided) was performed on the results; statistically different from control p<0.05; ** p<0.01; ***p<0.001.



## C. Pentoxyresorutin O-Depenty Artion (PROD)

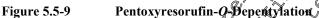
Culturing primary rat hepatocytes for 96 hours in the presence of phenobarbital resulted in a dosedependent increase in PROD activity to a maximum 4.6-fold at 100  $\mu$ M. This is consistent with historical data. Addition of floopyram to the hepatocytes resulted in a dose-dependent increase in PROD activity to a maximum 2.8-fold of control value at 30  $\mu$ M. The degree of induction decreased as the concentration of fluopyram was increased to 100 and 300  $\mu$ M.

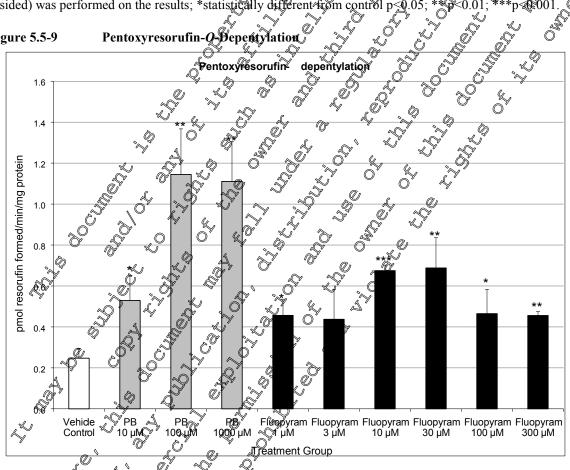


Treatment	PROD (pmol resorufin formed/min/mg protein)
Vehicle Control	$0.247 \pm 0.046 (100.0 \pm 18.7)^{a}$
PB 10 μM	0.529 ± 0.123* (213.9 ± 49.7)
PB 100 µM	1.145 ± 0.222** (462.9 ± 89.9)
PB 1000 µM	$1.110 \pm 0.196^{**} (448.9 \pm 79.1)$
Fluopyram 1 µM	$0.456 \pm 0.079*(1845 \pm 32.0)$
Fluopyram 3 µM	$0.437 \pm 0.146 (176.7 \pm 59.1)$
Fluopyram 10 µM	0 675 ± 0.027** (272.9 ± 11.0) Q
Fluopyram 30 µM	$0.688 \pm 0.148 \times (2782 \pm 555)$
Fluopyram 100 µM	$0.465 \pm 0.19*(187.9 \pm 48.2)$
Fluopyram 300 µM	9.456 ± 0.019 ± (184 4 ± 7.8)

Table 5.5-97 Pentoxyresorufin-O-Depentylation

^a Values are Mean ± SD. Values in parenthesis are meter % control ± SD. n 3 per group & Student's t-test (2sided) was performed on the results; *statistically different from control p≤@ 001. © 0.05





# D. Benzylox vesorufin-O-Debenzylation (BROD)

Culturing primar rat hepatocytes, for 96 hours, with phenobarbital resulted in a dosedependent increase in BROD activity up to a maximum 5.8-fold increase at 100 µM. This is consistent with historical data. Culturing hepatocyces with fluopyram resulted in a dosedependent increase in BROD activity, up to a maximum increase of 4.2-fold at 30 µM.

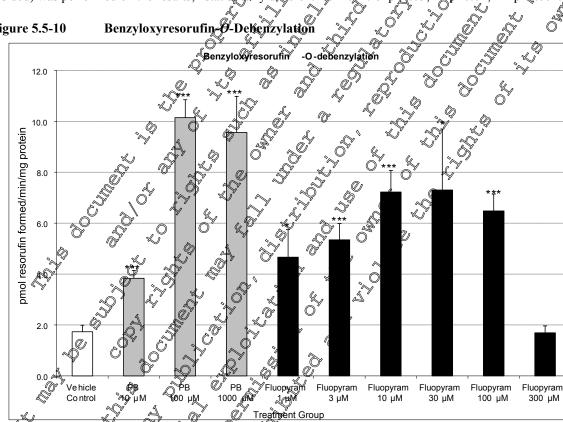
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1 able 5.5-98Benzyloxyresorul	in-O-Debenzylation
Treatment	BROD (pmol resorufin formed/min/mg protein) 🖉 🔭
Vehicle Control	$1.734 \pm 0.255 (100.0 \pm 14.7)^{a}$
PB 10 μM	3.835 ± 0.309*** (221.1 ± 17%)
ΡΒ 100 μΜ	10.142 ± 0.704*** (584.8 ± 0.6)
PB 1000 μM	$9.560 \pm 1.419^{***} (551.3 \pm 81.8)$
Fluopyram 1 µM	$4.664 \pm 1.087*(2687 \pm 62.7)$
Fluopyram 3 μM	5.349 ± 0.639*** (308.1 ± 36.8)
Fluopyram 10 µM	7223 ± 0.844*****(416.5 ± 48.7)
Fluopyram 30 µM	$7.299 \pm 2.398 (4200) \pm 1383 $
Fluopyram 100 μM	$6.480 \pm 0.662 *** (373.7 \pm 38.1)$
Fluopyram 300 µM	(1.693) = 0.217 (97.7 ≠ 15.7)

Table 5 5-98 **Renzyloxyresorufin-***O***-Debenzylation** 

^a Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n 🖲 X Student's t-test per group, p**≪@**:001. (2sided) was performed on the results; *statistically different/from control





## E. Benzyloxyquinoline-O-Depenzylation (BQ)

Culturing prinary rat hepatocytes for 96 hours, with phenobarbital resulted in a dose-dependent increase in BQ activity of p to 12-fold 1000 µM. This is consistent with historical data. Exposure of hepatocytes to the popyrand resulted in a large, dose dependent increase in BQ activity of up to 18-fold at

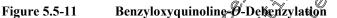


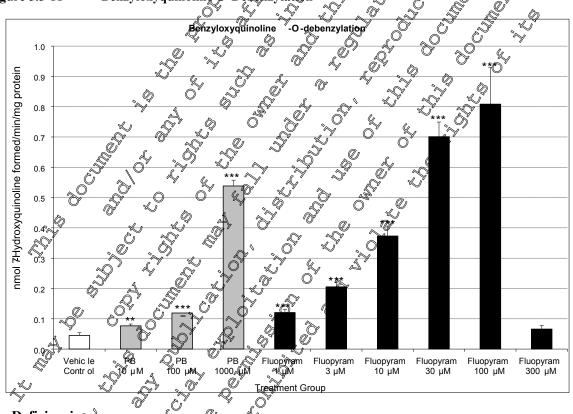


Treatment	BQ (nmol 7-Hydroxyquinoline formed/min/mg protein) $\mathscr{Q}$
Vehicle Control	$0.045 \pm 0.009 \ (100.0 \pm 20.5)^{a}$
PB 10 μM	0.077 ± 0.006** (170.8 ± 13 ^(h) )
PB 100 μM	0.119 ± 0.004*** (265.5 ± 4.4)
PB 1000 µM	0.538 ± 0.019*** (1198 ± 43.2)
Fluopyram 1 µM	0.12 + 0.011*** (268 7 ± 24.3)
Fluopyram 3 µM	$0.206 \pm 0.015^{***}$ ( $57.9 \pm 34.1$ )
Fluopyram 10 µM	∯\$73 ± 0.033*** (831.1 ± 73.8) Q
Fluopyram 30 µM	0,701 ± 0.048* (1563.5 ± 167.8)
Fluopyram 100 µM	$0.808 \pm 0.12$ *** (1800.9 ± 269.2)
Fluopyram 300 µM	0.066 0.011 (147.2 25.5)

Table 5.5-99 Benzyloxyquinoline-O-Debenzylation

^a Values are Mean ± SD. Values in parenthesis are mean % control SD for 3 percence. A Student's t-test (2sided) was performed on the results; *statistically different from control p < 0.001. <0.03





#### B. Deficiencies

were noted in the study. No specific deficiencies C

Conclusions III.

The clear increases in productivity seen after exposure to fluopyram indicate that fluopyram is an inducer of XP2b The induction of BROD and, particularly, BQ activity suggests that fluopyram is also a CYP3a inducer.

In contain with phenobarbital, fluopyram stimulated replicative DNA synthesis (S-phase) in the hepatocytes.



In conclusion, these data suggest that fluopyram is an activator of both CAR and PXR.

#### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on the effects and target organs of fluopyram in the rat.

The clear increases in prod activity seen after exposure to fluopyram indicate that fluopyram is and inducer of CYP2B. The induction of BROD and, particularly, BQ activity suggests that fluopyram is also a CYP3A inducer.

In common with phenobarbital, fluopyram stimulated replicative DNA synthesis (S-phase) on the hepatocytes.

In conclusion, these data suggest that fluopyram is an activator of both CAR and BAR.

#### Supporting data regarding male mouse thyroid tumors

In the mouse carcinogenicity study (199568-01-1), a higher incidence of thyroid folligular cell tumors was observed in male mice exposed to AE C656948 at 750 ppm (105 mg/kg bw/d) for 78 months compared to the corresponding controls. There were 7/50 chimals affected in the high dose group compared to 1/50 in the control group. This finding was associated with a statistically significantly higher incidence of the pre-reoplastic lestor follicular cell hyperplasia? Retrospective examination of the thyroid gland of males sacrified after 1 year of treatment reveated that 2/10 animals had thyroid follicular cell hyperplasia?

Thyroid effects were seen in association with marked liver effects at this dose level including centrilobular to paralobular hepatocellular hypertrophy, hepatocellular cholestasis, single cell degeneration/necrosis, interstinal mixed cell infiltrates eostnophilic inclusion bodies and multinucleated hepatocytes. AE/C656948 did not show any genotoxic patiential (see section 5.4).

These data alone support the hypothesis of a fion-genotoxic threshold mechanism for the thyroid follicular cell adenoma formation.

In addition, AE C656998 being a moderate phenoloarbital like cytochrome P-450 inducer in the rat (see section 5.3.1 and 5.5 (mechanistic studies), one can hypothesize that an indirect mechanism of action for thyroid follicular cell adenoma, secondary to a liver enzyme induction in the mouse since the liver is heavily involved in the thyroid homone metabolism through different enzymatic activities (Kelly, 2000⁵). In order to test this hypothesis, a series of studies were conducted including:

(1) an *in vitro* study on potential interaction of AE CC56948 on thyroid peroxidase-catalysed reactions i.e. direct effect on thyroid hopmone synthesis,

(2) ancin vivo study to investigate the effects of AE C656948 on liver enzymes and on pituitary/thyroid hormone balance in the marker mouse.

(3) two *in vire* studies to nvestigate the effects of AE C656948 on the pharmacokinetics of intravenously administered 251-thyroxine in the male mouse.

(4) an *in two* study to investigate the effects of AE C656948 on the expression of a selection of genes involved in the clearance of thyroid hormones and the detoxification of xenobiotics in the liver using qPCR analysis.

For the *invevo* studies a similar investigation was performed using phenobarbital as a "positive control" reference compound for comparison. The same dose levels of AE C656948 and phenobarbital were used for each of the three investigations. A dose level of 2000 ppm AE C656948 was selected after evaluation of the results of a 28-day toxicity study in the mouse with this substance (see section 5.3.1), where 5000



ppm clearly exceeded the maximum tolerated dose and a 90-day toxicity study in the mouse (see section 5.3.2), where at the highest dose level tested, 1000 ppm, slight toxicity was observed. Therefore, the dose level of 2000 ppm was selected as an appropriate high dose level to be used in this study. Although, 2000 ppm was higher than the high dose used in the mouse carcinogenicity study (750 ppm), this dose level was selected to allow a clearer evaluation of the effects of AE C656948 without causing pressive toxicity. A dose level of 80 mg/kg/day phenobarbital was selected based on previous studies conducted with phenobarbital and was expected to provoke a positive response.

These studies are summarized hereafter.

The data generated indicate that

• AE C656048 did not affect the thyroid hormone synthesis at the hyroid peroxidase level supporting the absence of a direct effect on hormone synthesis

• AE C656948 induced a similar hepatic enzymatic profile in male mice as phenobarbital i.e. induction of cytochrome P-450 content and specific induction of BRQD and PROD activities.

• AE C656948 is able to modify the normal thyroid formofie level in male mice, in particular by causing a decrease in T4 level associated with an increased level of T5H after only 30 as of treatment by also consistently after 2 weeks of treatment  $2^{\circ}$ 

• AE C656948 induced a more rapid learance of 14 from the blood that in the corresponding controls over the 24 hour period following 251-thyroxine administration.

Overall, the data on AE C656948, support the hypothesis of a non-genotoxic indirect threshold mechanism for the formation of thyroid follicular cell adenomas in male mice. In particular, it was shown that when administered to male mice, AE C656948 was able to induce the metabolism capability of the liver and to cause a decrease in the T4 level which was associated with an increase in TSH levels. This increased level of TSH will stimulate the thyroid gland to produce thyroid hormones in order to restore their normal physiological tevels and this constant stimulation at mid-or long-term is known to induce follicular cell hypetrophy hyperplasia (observed in the mouse oncogenicity study already after 12 months in a few animals and clearly after 18 months in a large tumber of animals). In addition, the increased rate of clearance of T4 from the blood observed in AE C656948 treated animals was similar to that seen with the reference compound phenobarbital, which is known to induce microsomal  $\beta$ -D-glucuronyl transferases, which statalys the metabolism of the rotative to its glucuronide conjugate in the rodent (Bastomsky, 1973⁶). In the qPCR analysis asay, the gene transcrips in the liver that were upregulated following freatment with AE C656948 and phenobarbital, i.e., the sulfotransferases and UDP glucoronosyltransferases, are known to encode for enzymes that inactivate T3 and T4 via glucoronide and sulfate derivatives.

In order to show a dose response for the key events leading to Car/Pxr-induced thyroid follicular cell tumors observed in male once to lowing life-tune exposure to AE C656948 at the high dose level of 750 ppm a series of 28 day feeding addies in the male mouse have been conducted using a range of dose levels which included dose levels used in the mouse carcinogenicity study, plus intermediate dose levels. The following parameters were essessed, specific cytochrome P-450 enzyme activities which acted as markers for activation of the Car/Pxr paclear receptors and UDPGT species isoenzyme profiles, plasma levels of T4 and TSH, the gene expression of *Tsh* in the pituitary gland and thyroid follicular cell proliferation. Reversibility of changes seen following 28 days of treatment with AE C656948 was also assessed, phenobarbital was included as a "positive control" reference compound. A dose concordance was established for each key event and changes observed after up to 28 days of treatment with fluopyrap were found to be reversible following cessation of treatment for 28 days.

In addition, pivotal mechanistic study conducted to demonstrate Car/Pxr receptor activation as the first key event was a 28-day mouse study using both the wild-type (WT) C57BL/6J mouse and a genetically modified mouse that does not have functional Car or Pxr receptors (Pxr-Car KO), this study (<u>M-449890-01-1</u>) is summarized hereafter. Mice were exposed to AE C656948 at the tumorigenic dose (750 ppm) in the mouse cancer bioassay and above (1500 ppm), equivalent in terms of mg/kg/d to the rat



tumorigenic dose level. In this study, a significant induction of liver enzymes, liver enlargement and hepatocellular hypertrophy was seen in the WT mouse, but was not observed in the Pxr-Car KO mouse.

Other plausible modes of action (MoAs) for thyroid tumor formation have effectively been excluded.

The induction of chemical-specific thyroid tumors in rodents may be caused by the following MoAs (Capen, C.C. (1997). Mechanistic data and risk assessment of selected toxic and points of the thyroid gland. Toxicol. Pathol. 25(1):39-48: KIIA 5.5.4 /37;Capen, C. C.;1997; <u>M-435031-01-1</u> and Hurley *et al.*, 1998) Mode of carcinogenic action of pesticides inducing thyroid follocular cell tumors in odents Environ. Health Perspect. 106(8):437-45, KIIA 5.5.4 /38;Horley, P. M.; Hill, R. N.; Whung, R. J.;1998; <u>M-086436-01-1</u>.

I. DNA reactivity

II. Inhibition of the active transport of inorganic is dide into the follicular cell (redide pump)

III. Inhibition of thyroid peroxidase that converts inorganic fodide into organic fodide and couples iodinated tyrosyl moieties into thyroid hormone

IV. Damage to follicular cells

V. Inhibition of thyroid hormone release into the blood

VI. Inhibition of the conversion of Toto Taby 5' monode odinase at xatious sites in the body

VII. Enhancement of the metabolism and excretion of thyroid formore by the live Aargely through the action of UGT

Concerning MoA I; *DNA reactivity*, this mechanism can be ruled our based on *in-vitro* and *in-vivo* genotoxicity studies which show that fluopyram is not a genotoxic chemieal. MoA II - V are the result of direct thyroid gland effects, whereas VI and VII are peripheral to the thyroid gland. A direct MoA is unlikely based on the pivotal Pxr-Car-KO study which provides compelling evidence to demonstrate that fluopyram is a Car/Pxr inducer and is not a direct thyroid toxicant. More specifically, concerning MoA III; *Inhibition of the oid peroxidase*, mechanistic studies using hog thyroid microsomes showed fluopyram did not affect thyroid gland in the mouse and tal have never shown overt cytotoxicity, only hyperplasia/adenoma in the mouse following chemic treatment and hypertrophy and hyperplasia in the rat. As for the indirect MoA VI: *Inhibition of the conversion of T4 to T3 by 5'-monodeiodinase at various sites in the body*, is unlikely, as serum levels of T9 were not changed in either the mouse or rat exposed to fluopyram. This indicates that the indirect MoA VII; *Enhancement of the metabolism and excretion of thyroid hormone by the liver*, *largely through induction of UGT enzymes*, is the most likely mechanism for thyroid function and is strongly supported by the mechanistic data.

Sex- and species differences

Although the basic functions of the pypothalamic pituitary-thyroid axis are similar in animals and humans, long-term alteration of the pituitary-thyroid axis by chemicals in laboratory rodents (rats and mice) are more predisposed to give a higher incidence of hyperplasia and follicular cell tumors in those animals than in humans. Bats and mice are particularly sensitive to the decreased availability of T4 and T3 and respond with hypertrophy and hyperplasia of follicular cells and in long-term studies there is an increased incidence of thyroid tumors (Capen, 1992⁷). By contrast, humans are much less sensitive to elevated TSE levels or alteration of the myroid function which are usually not associated with increase in thyroid cancer incidences (Hill *et al.* 1998⁸). For instance, there is no evidence that chemicals such as phenobarbital which affect thyroid function through a peripheral mechanism involving thyroid hormone metabolism, are associated with thyroid neoplasia in humans (McClain *et al.*, 1989⁹; Curran and De Groot, 1999¹⁰). The only verified cause of thyroid cancer in humans is exposure to ionizing radiation. The reasons for the interspecies difference in sensitivity are not completely known although one factor that probably plays a key role is the protein carriers of thyroid hormones. Both humans and rodents have nonspecific low affinity protein carrier of thyroid hormones (e.g. albumin). However, humans, other



primates and dogs also have a high affinity binding protein (i.e. thyroxine-binding globulin) which binds preferably with T4 and which is missing in rodents and lower vertebrates. Hence, in rats, T4 is primarilybound to albumin and to a lesser extent to pre- and post-albumin. In mice, T4 is also begind preferably to albumin and postalbumin, whereas in humans T4 is bound to thyroxine binding grobulin (van Raaij, 2002¹¹). Consequently, the proportion of unbounded T4 (therefore more susceptible to metabolism and excretion) is greater in rodents than in humans (Capen, 1992). In rodents, males have higher serum TSH levels than females and are usually more sensitive to goitrogenic stimulation and thyroid carcinogenesis (Chen, 1984¹²). In humans, there is no sex difference in hormone levels athough females develop thyroid tumors more frequently than males (Boring *at die*, 1994¹³).

An *in vitro* CYP and UGT induction study in human and Wistar rat hepatocytes with AE C C656948 (2020), M-759019-01-1, KCA &5/22), was conducted to address the T-nodality as part of the endocrine disruption assessment, which also provides additional evidence for the species differences between rat and humans for the the tumor assessment. This study demonstrated AE C656948 was a strong CYP3A at  $\geq$ 10 µM and to a lesser extent & CYP1A2 inducer in Wistar rat hepatocytes. At  $\geq$ 10 µM AE C656948 was a CYP1A2, CYP2B6, CYP3A and UCT1A1 inducer in human hepatocytes, but does not increase UGT-T4 activity in human hepatocytes.

Overall, the mechanistic studies together with the standard repeat dose studies, dearly demonstrate the mode of action for the thyroid turbors in the mouse following a life time exposure to fluopyram is mediated through and secondary to liver enzymes induction *via* activation of the CarPxr nuclear receptors. The rodent specificit of liver mediated through folloular cell tumors well recognized and accepted as being non-relevant to humans.

Further detailed argumentation regarding mode of action and relevance to humans of the mouse thyroid tumors following exposure to fluopyram (AE 4656948) are presented in the following Expert Summary documents:

Position Paper

Expert Sumpnary Report

Fluopyran: Mode of Action and Paiman Relevance Framework Analysis for Fluopyram-Induced Rodent Liver and Thyroid Tumors MIIA Sec 3 702; 2013; M-454439-02-1

5 Kelly G. (2000) Periphera netabolism of the roid hormones a review. Alternative Medecine Review, Vol. 5 N°4, 306-333. KIIA 5.5.4 /10; Kelly, G 200835-01-1

6 Bastomsky C.H. (1973) The biliary excretion of thy xine and its glucoronic acid conjugate in normal and Gunn rats. Endocrinology, 92, 35-40. KUA 5.5 418;Bagomsky S. H.;1953;<u>M-308397-01-1</u>

8 Hill R.N., Crisp T.M., Hurley P.S., Resenthal S.L. and Singh D.V. (1998) Risk assessment of thyroid follicular cell tumors. Environmental Heath Perspectives 06 (8) 447-450 KIIA 5.5.4 /12; Hill, R. N.; Crisp, T. M.; Hurley, P. M.; Rosenthal, S. L.; Singh, D. V. 0998; M= 300844 01-1

9 McClain R.M., Levin A.A., Osh R. and Downing J.C. (1989) The effect of phenobarbital on the metabolism and excretion of thyroxin on rats. Joxicology and Applied Pharmacology, 99, 216-228. KIIA 5.5.4 /13; McClain, R. M.; Levin, A. A.; Posch, R.; Downing, J. C. 1989; <u>M-103769 01-1</u>

10 Curran P. Cand Degroot 12. (1991) The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid glass. Endocrane Reviews, 12-22), 135-150. KIIA 5.5.4 /14; Curran, P. G.; DeGroot, L. J.; 1991; <u>M-066260-01-1</u>

11 Van Ranj M.T.M. (2002) Follicular cell thyroid tumors in rodents. In "Factsheets for the (eco)toxicological risk assessment strategy of the national institute for public health and the environment (RIVM), RIVM report 601516009, pp 27-42, Part II, editors Luttik, R.; Pelgrom, S. M. G. J. KIIA 5.5.4 /15; Luttik, R.; Pelgrom, S. M. G. J.; 2002; <u>M-300839-01-1</u>



12 Chen H.J. (1984) Age and sex differences in serum and pituitary thyrotropin concentrations in the rat : influence by pituitary adenoma. Experimental Gerontology, 19, 1-6. KIIA 5.5.4 /16; Chen, H. J.; 1983; M-300821-01-1

13 Boring C.C. (1994) Cancer statistics 1994. A Cancer Journal for Clinicians, 44, 7-26 KIIA 5.5.4/17; Boring, C. C.; Sachres, T. S.; Tong, T.; Montgomery, S.; 2008; <u>M-300849-01-1</u>

Data Point:	KCA 5.5/09
Report Author:	
Report Year:	
Report Title:	AE C656948 (Fluopyram) In vitre studies on the potential interactions with thyroid peroxidase-catalyzed reactions
Report No:	AT04481
Document No:	$\underline{M-299276-01-1}$
Guideline(s) followed in	No specific guideline
study:	
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011) Yes, conducted under GLP/Officially recognised testing factities
GLP/Officially	Yes, conducted under GLP/Officially recognised testing facilities of O
recognised testing facilities:	
Acceptability/Reliability:	Yes $\sim$ $o$ $o$ $\sim$ $\sim$ $\sim$ $\sim$ $\sim$ $\sim$
Frequitive Summary	

#### **Executive Summary**

To investigate a potential effect of AE @656948 on thyroid hormone synthesis at the level of thyroid peroxidase (TPO), interactions of this compound with TPO-catalyzed reactions were studied *in vitro* using solubilized hog thyroid microsomes as an enzyme source. Amitrole and Ethylenethiourea (ETU) served as positive control substances.

Amitrole, a potent inhibitor of thygoid peroxidase, strongly inhibited TPO-catalyzed oxidation of guaiacol and formation of jotine. About 50% inhibition was observed in the presence of 1  $\mu$ M Amitrole for guaiacol oxidation and in the presence of 0.1  $\mu$ M Amitrole for isdine formation. Ethylenethiourea, which is not a TPO inhibitor, but a trap of the iodinating intermediate generated by TPO from iodide, temporarily suppressed iodine formation.

In contrast, AE C656948 drd not affect PPO-zeralyzed guaiacol oxidation up to 300  $\mu$ M, the highest concentration tested. Similarly, TPO-zeralyzed iodine formation was not affected by 300  $\mu$ M AE C656948.

These findings strongly strengest that AE 0656948 does not affect thyroid hormone synthesis at the level of TPO.

I. 🌋 Materials and method	
A. Materials	
1. Test material: 🐴 🔪 🧹	AE C636948
1. Test material:	Light beige powder
Lot /Batch &	Mix-Batch:08528/0002
Pursity:	94.7%
CAS # Or Ar Ar	658066-35-4
Stability of test compound:	Stable for a period covering the study duration
2. Vehicle and / or positive control:	Dimethylsulfoxide



Amitrole (3-amino-1,2,4-triazole) from Sigma (Lot number 083K0649)

ETU (ethylenethiourea, 2-imidazolidinethione) from Riedel-

Haën (Lot number 3223X)

**4.. Biological raw material:** Hog thyroid glands from domestic pigs were obtained from Bayer CropScience, Monheim. They were trimmed free of excess fat and connective tissue. They were stored at -80°C until use.

#### B. Study design

1. Experimental period:

3. Positive control substances:

The study was conducted on October 4, 2007

2. Microsome preparation: Interactions of AE C656948 with TPO-catalyzed reactions were studied *in vitro* using solubilized hog thyroid microsomes as an enzyme source. Amitrole and Ethylenethiourea (ETU) served as positive control substances. Solubilized hog thyroid microsomes were prepared according to a standard procedure as described in Neary et al. 1984

#### 3. Statistics:

No statistical evaluation was performed

#### B. Methods

#### 1. Determination of TPO-catalyzed geniacol axidation

Guaiacol oxidation was used as a measure for peroxidative activity incubations were carried out at room temperature in 0.1 M potassium prosphate buffer, pH 74 in a total volume of 1.0 mL. Guaiacol (125  $\mu$ L of 40 mM solution in water, final concentration 5 mM), TPO (approximately 0.1  $\Delta$ E/min, corresponds to 3.5  $\mu$ L of microsomal preparation) and test compound were prencubated for 1 minute, then the reaction was initiated by addition of hydrogen peroxide (20  $\mu$ L of 12.5 mM solution in water, final concentration 250  $\mu$ M). Test compounds were added in 20  $\mu$ L DMSO, likewise control incubations lacking test compounds contained the same amount of solvera.

The following that concentrations were used

The initial linear increase (XE/min) of the absorption at 470 mm was used to calculate the peroxidase activity.

## 2. Determination of PO-catalyzed iodine formation @

Incubations were carried out as described above however, guaiacol was replaced by potassium iodide (100  $\mu$ L of 100 mM solution in water final concentration 10 mM).

The following final concentrations were used:

AE C656948: 3.0 🕉 – ἔρο μΜ

The initial linear increase ( $\Delta E$  min) of the absorption at 350 nm was used to calculate the enzymatic activity  $\Delta = \Delta E$ 

## ²II. Results and discussion

The results of the TPO-catalyzed oxidation of guaiacol are summarized in Table 5.5.4-52. Amitrole, the positive control, at a concentration of 1  $\mu$ M inhibited the initial rate of thyroid peroxidase (TPO)-



catalyzed oxidation of the model substrate guaiacol by more than 50%. AE C656948, at concentrations up to 300  $\mu$ M did not affect this reaction.

Table 5.5-100	Effect of AE C656948 on TPO-ca		
Compound	Concentration (µM)	$\Delta \mathbf{E}/\mathbf{min.} \pm \mathbf{SD}$	🔗 🦷 % of control 🔨
Vehicle	-	0.121 ± 0.006	
AE C656948	3	$0.122 \pm 0.002$	200.8 ~
	30	©0.123 ± 0.005	
	300	$0.124 \pm 0.00^{\circ}$	× 100.5 × 0
Amitrole	1	0.054 ± 0003 °	44.6 Č C

The results of the TPO-catalyzed iodine formation are summarized in Table 5.5.4-53. Up to 300  $\mu$ W AE C656948 did not affect TPO-catalyzed iodine formation. Nother the initial rate of the reaction was affected, or a temporary suppression of iodine formation was observed. In contrast, Ethylenethiourea (ETU), a trap of the iodinating intermediate, temporarily suppressed iodine formation, whils Amitrole at a concentration of 0.1  $\mu$ M inhibited the initial rate of this reaction by 50%.

		20		$\sim$		v	C.
Table 5.5-101	Effect of AE	C 6560 18	on TOO	of tolyz	iboilli	Morm	ation
1 abic 3.3-101	Ellect of AL	CUSWHO	<b>UI V</b> I <b>U</b>	-s, avai y L	cu avun		aupun

a

Compound	Concentration ( $\mu M_D$ $\Delta E/m n. \pm SD$ $D$ of control
Vehicle	$0^{259} \pm 0^{12}$ $0^{259} \pm 0^{12}$ $0^{210}$
AE C656948	
	$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$
Amitrole	₩ 10.1 ₩ 1.131 ±0.013 × 50.6

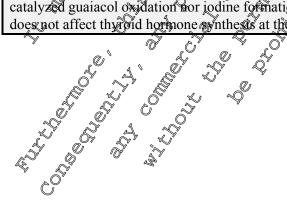
#### III. Conclusion

AE C656948, at concentrations up to 300 µV (the highest concentration tested) neither affected TPO catalyzed guaracol oxidation nor iceline formation. These findings strongly suggest that AE C656948 does not affect thyfoid hormone synthesis at the level of TPO.

#### Assessment and conclusion by applicant

Study meets the cupent guidance and the requirements in 283/2013 as it provides mechanistic information on the potential interactions of fluppyram with thyroid peroxidase-catalyzed reactions. AE C656948, at concentrations up to 300  $\mu$ M (the highest concentration tested) neither affected TPO-catalyzed guaiacol oxidation for iodine formation. These findings strongly suggest that AE C656948 does not affect thyroid hormone synthesis at the level of TPO.

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Data Point:	KCA 5.5/10
Report Author:	
Report Year:	2008
Report Title:	AE C656948 Mechanistic 14-day toxicity study in the mouse by dietary
	administration (hepatotoxicity and thyroid hormone investigations)
Report No:	SA 07215
Document No:	<u>M-299522-01-1</u>
Guideline(s) followed in	No specific guideline
study:	
Deviations from current	Current guideline: none
test guideline:	
Previous evaluation:	Yes, evaluated and accepted in the DAR (200).
GLP/Officially	Yes, conducted under Gor Officially recognise testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes A A A A A A A A A A A A A A A A A A A
E	
<b>Executive Summary</b>	

#### **Executive Summary**

AE C656948 was administered continuously via the diet to 2 groups of 15 male C57BL/6J mice for 3 or 14 days at a concentration of 2000 ppm, equating approximately to 308 and 314 mg/kg body weight/day, respectively. Two signilarly constituted groups of 15 mates received unreated diet and acted as a control groups. Clinical signs were recorded daily, body weight and food consumption were measured weekly. A detailed physical examination was performed weekly. On study Day 4 and study Day 15, before necropsy; blood samples were taken for hormone level measurements (T3, T4 and TSH). The liver and thyroid gland were weighed and sampled for rocroscopic examination. In addition, hepatic cytochrome P-450 isoenzymes and UDPGT activities were also measured using 4-nitrophenol as substrate.

After 3 days of exposure at 2000 ppm, mean 73 level was not changed when compared to the untreated control group whereas mean T4 level was decreased (-30%) while at the same time, mean TSH level was increased (+18%) when compared to controls. Arean absolute and relative liver weights were increased by approximately 60% when compared to control animals. At macroscopic observation, enlarged fiver was found in albhe treated animals of microscopic examination, diffuse centrilobular to panlobular hepatoce Dular hypertrophy and an increased number of mitoses were observed in all examined treated animals. In addition, hepatocellula single cell necrosis was observed in 1/5 treated animals. No significant microscopic mange was observed in the thyroid gland.

Total cytoch ome P 450 content, was markedly increased (+116%) by the treatment. EROD activities were marginally increased (+230%), whereas ROD and BROD activities were markedly increased (respectively +2890% and +87) 7%) when compared to controls. No significant changes were observed in UDPOT.

After 14 days of exposure at 2000 ppm over all the results were very similar to those obtained after 3 days of exposure, Mean T3 level way not changed whereas mean T4 level was still decreased (-27%) and mean TSH evel was still increased (+7%), when compared to controls. Mean absolute and relative liver weights were increased by approximately 60% when compared to the control animals. At macroscopic observation, enlarged liver was found in 13/15 treated animals, compared to no incidences in the controls, Furthermore, Cark liver was observed in 14/15 treated animals compared to only 1 in the control animals. An microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy was seen in all examined treated animals. In addition, hepatocellular single cell necrosis was observed in 4/5 treated animals. No significant microscopic change was observed in the thyroid gland. 🗘



Total cytochrome P-450 content was moderately increased (+71%) by the treatment. EROD activities were marginally increased (+165%) whereas PROD and BROD activities were markedly increased (respectively +2163% and +9061%) when compared to controls. No relevant changes were observed in UDPGT activities.

In conclusion, this study demonstrates that AE C656948 dietary administration at a monimal concentration of 2000 ppm (equivalent to between 308 and 314 mg/kg bw/da/) in the C57BL/6J mouse for 3 and 14 days induced a phenobarbital-like P-450 hepatic enzymatic activity profile and that the ability to modify the normal thyroid hormone balance (in particular to cause a sustainable decrease by T4 level and increase in TSH level), which in turn can indirectly induce thyroid changes.

I. Materials and methods A. Materials 1. Test material: AE C656948 covering the study duration Light bege powder Description Lot / Batch #: Mix-Batch:08528/094 94.7% **Purity:** CAS# 658066,35-4 Stable in rodent diet **Stability of test compound:** 2. Vehicle and / or positive control: 3. Test animals: Mouse Male only **Species:** Strain: C57BL/6L® 8 weeks pproximately at start of treatment Age: Weight at dosing थ्री.2 to 25.1 g⊂ Source: Acclimation period 5 to 6 days Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Diet: Orge, France), ad bibitum Ľ Municipal tap water, addibitum Water Animals were caged individually in suspended stainless steel Housing: vire mesh cages. A Environmental conditions Temperature: -70% **Humidity** 10-05 air changes per hour Air changes: **Photoperiod:** Afternating 12-bour light and dark cycles (7 am- 7 pm) B. Study design Sosing period for the day exposure groups: 01 October – 04 October 2007. 1. In life dates: Dosing period for the 14-day exposure groups: 18 September – 02 October

# 2. Animal assignment and treatment

The dose level was set after valuation of the results of a subacute toxicity study in the mouse with this substance (see section 5.3.1). In this study, 5000 ppm clearly exceeded the maximum tolerated dose as alkinales, and most females had to be sacrificed for humane reasons before the end of the study. Therefore, the dose level of 2000 ppm was selected as an appropriate high dose level to be used in this study. Although, 2000 ppm was higher than the high dose used in the mouse carcinogenicity study (750 ppm), this dose level was selected to allow a clearer evaluation of the effects of AE C656948 without



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causing excessive toxicity. Males only were used in this study as an increased incidence of thyroid follicular cell adenoma was observed in this sex only.

Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Faropéennes 358, 38 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Table 5.5-102	Study design	Ó		
Test group	Treatment	Dose level in ppm (mg/kg bw/day)	Duration of treament	Animalsassigned
1	Control	0 4	days °	
2	AE C656948	2000 (208)	∽y 3 da&s ~~	
3	Control	&9 &°	ے الالمays م	\$ ~15 V
4	AE C656948	2000 (314)	4 days	

The stability of the test substance in the diot at 20 and 10000 ppm has been demonstrated in a previous study after a freezing period of 95 days followed by storage periods at room temperature of 10 days or 105 days. Before the start of the study homogeneity and concentration were checked on the study mix at 2000 ppm. The mean value obtained from the homogeneity check was taken as measured concentration. The study mix was found to be acceptable for use on the study as the homogeneity and concentration checks were within the range of 102 to 103% of the homoments.

#### 3. Diet and water

Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France) and filtered and softened tap water from the municipal water supply in individual bottles were available ad libitum, except prior to savifice when animals were diet fasted overnight. Routine analyses of food and water indicated that there was no contamination which could have compromised the study.

#### 4. Statistics $\gg^{\circ}$

• - Body weight change parameters,

- Terrefinal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

The F test was performed to compare the homogeneity of group variances.

If the F test was not significant  $0^{>0.050}$  mean of the exposed group was compared to the mean of the control group using the test (2 sided)

If the F test was significant ( $p \le 0.05$ ), mean of the exposed group was compared to the mean of the control group using the modified test (Q-side Q).

- - Body weigh and average food consumption/day parameters
  - Total cytochrome P450 content
  - Hormorial parameters

Mean and standard deviation were calculated for each group and per time period for average food construction day parameters.

The F test was performed to compare the homogeneity of group variances.

If the F test was not significant (p>0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).



If the F test was significant (p < 0.05), data were transformed using the log transformation. If the F test on log transformed data was not significant (p>0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.

If the F test was significant ( $p \le 0.05$ ) even after log transformation, the mean of the exposed group compared to the mean of the control group using the modified t-test (2-sided).

If one or more group variance(s) equal 0, means were compared using non-parametric procedu

• - Enzymatic activities and cell proliferation parameter

Mean of the exposed group will be compared to the mean of the composition group using the Whitney test (2-sided). O

Group means will be compared at the 5% and 1% levels of significance

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Ephanced Statistics) except for liver enzyme parameters and cell proliferation parameters which were analyzed using SAS programs version 8.2.

#### C. Methods

#### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all and mals and detailed physical examinations were performed pleast weekly during the treatmen operiod. The nature onset, severity, reversibility, and duration of any clinical spens were recorded.

#### 2. Body weight

Each animal was weighed at least once during the acclimatization phase on study Day 1 and then weekly during the treatment period. Additionally, diet fasted anituals were weighed before scheduled necropsy.

#### 3. Food consumption

Ì The weight of Good supplied and of that remaining at the end of the food consumption period was recorded weekly for all unimals during the reatment period to calculate the mean daily food consumption. Any food spillage was noted.

Ø

#### 4. Hormonal investigation

#### **Blood sampling**

On study Day 4 (Groups 1 and 2) and on study Day 15 (Groups 3 and 4), blood samples were taken from all animals by pencture of the setro-or Pital venous plexus. Animals were anesthetized by inhalation of Isoflurane (Baxter, Magrepas, France prior, & bleeping. Blood was collected on lithium heparin for hormone level evaluation.

## Hormone measurements

Plasma was prepared from each blood sample and kept frozen at approximately -80°C until the determination of TSH, T3 and T4 hormone vevels with specific radio-immunoassay kits (supplied by Amersham for TSH and by DIASQRIN for T3 and T4).

Ophthalmoscopic examination was not conducted in this study.

## 5. Sacrifice and pathology

## Necropsy procedure - Organ sampling

On study Day 4 and on study day 15, all animals from groups 1 and 2 and from groups 3 and 4, respectively were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane (Baxter, Maurepas, France). All animals were necropsied. The necropsy included the examination of the



external surfaces, all orifices and all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled except upon decision of the Study Pathologist. Brain and liver were weighed fresh.

A piece of median and left lobe liver of 5 animals as well as the thyroid gland (with parathyroid glands) of all the animals were sampled and fixed in 10% neutral buffered formalin for histological examination. The remaining portions of livers from those 5 animals and the entire liver of the other 10 animals of each group were used for microsome preparation.

#### **Histotechnology - Histopathology**

Liver portions and thyroid glands sampled for microscopy were embedded in paraffit sections, stained with hematoxylin and eosin, were prepared and examined

#### 6. Hepatotoxicity testing:

At scheduled sacrifice dates, in each group the givers of 3 animals were pooled (each time 2 entire livers and the remaining portion of 1 liver used for histological examination) and pomogonized for microsomal preparations. In total, 5 microsomal samples, per group were prepared. Total cytochrome P-450 content and specific cytochrome P-450 isozyme profile (including EROD, BROD and PROD activities) were measured to check the hepatotoxic potential of the est substance In addition. Phase II enzymatic activities were also determined by measuring UDPs neuroposyltransferase (UPPGT), with 4-nitrophenol as substrate.

#### II. **Results and discussion**

#### A. Observations

#### 1. Mortality

There was no mortality in any group shroughout the

#### 2. Clinical signs

There were no treatment-related clinical signs,

## B. Body weight and body weight gain &

There was no effect on body weights of body weight gains throughout the study. Ś S.

## C. Food consumption

There was a slight reduction in food consumption at the beginning of treatment with AE C656948

(-12.5% at the end of the 3-day exposure period and 5.1% after 1 week in the 14-day exposure period). Thereafter, food consumption was comparable to controls

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#### D. Hormone analysis

The results of TSH, T3 and T4 levels are summarized in Table 5.5.4-55.

The results are very comparable Between the time-points in both cases, while T3 levels remained unaffected, a significant decrease in T4 levels (-30 and -27%, respectively) together with a significant increase in TSH levels (+18 and + 18, respectively) was seen in treated groups compared to control groups. These results are consistent with the known feedback regulation mechanism of thyroid hormones where when ovroid hormones levels are low, the pituitary gland will produce TSH to stimulate the thread gland in order to restore the normal level of thyroid hormones.



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Table 5.5-103	Mean levels of thyroid/pituitary hormone after 3 and 14 days of exposure to AE
	C656948 (mean±SD)

Group	3-day	exposure	14-day o	exposure
	Control	2000 ppm (308 mg/kg bw/d)	Control	2000 prom (314 mg4,g bw/d9)
T3 (nmol/L)	$1.62 \pm 0.15$	1.64 ± 0.25 (+1%)	$1.45 \pm 0.18^{\circ}$	1.52 ± 0.38 (+5%)
T4 (nmol/L)	$43.7 \pm 8.1$	30.7** ± 6.0 (-30%)	38.1 ± 9.1	27.7* ± 8.7° - 27%
TSH (ng/mL)	$3.81 \pm 0.23$	4.48** ± 0.3 (+18%)	3.81 0.28	4,09*±0,44 (+7%)
': p≤0.05; **: p≤0.01		, O		

#### E. Sacrifice and pathology

As seen in other studies in the mouse (see section 5.2), liver weights were markedly increased in the treated group compared to control groups after only days of exposure and after 14 days of exposure (approximately +60% in all cases). This was associated with charged and/or dark over seen at the macroscopic examination and with hepatocellular hyperrophy in all examined animals and single cell necrosis on many occasions especially after [4/day of exposure. An increased number of mitoses was also observed in all animals after 3 days of exposure

Table 5.5-104 Patho	logical liver effects afte	\$3 and \$4 days of	f exposure_toAE	C656948 (mean±SD)
---------------------	----------------------------	---------------------	-----------------	-------------------

			sy in lo	
Group 🕺	🎽 🥎 3-day	/ exposure 😽 🧳	14-da	y exposure
, and a second s	Control	200% ppm 308 mg/kg bw/d)	Control	2000 ppm (314 mg/kg bw/d)
Liver weights Absolute weight	1.22 ±0.120	(+59%)	1.25±0.10	1.99±0.22** (+59%)
Body weight ratio (?	\$.40±0.57	S 8.71 → 0.54**	3.23±0.33	8.42±0.94** (+61%)
Brain weight rate (%)	∀ 279¢99±29.24 © &	448.34±34.51** (+@%)	28649±21.42	462.30±52.04** (+61%)
Histopathological findings	Q A A		L.	
Centrilobûtar to panlobular hepatose fular hypertrophy	×		0/5	5/5
Increase mitosis	0/50	x x 5/5 Å	0/5	0/5
Single cell necrosis	O QIS	° 1/5°∂r	0/5	4/5
*: p≤0.05; **: p≤0.01 Q		\$ \$		

No relevant changes were observed in the thyroid gland,

## Hepatotoxicity testing

The results of hepatotoxicity testing are described in Table 5.5.4-57.

At both time points, treament with AE C656748 induced a clear increase in total cytochrome P-450 content and a marked increase in BR@D and PROD activities compared to controls. EROD activity was only slightly increased whereas 4-mitrophenol UDPGT activity was unaffected. These results are very comparable with those obtained with phenobarbital with the same study design (see following study). They are also quite similar to the changes observed in the rat with the exception of the 4nitrophenol UDPGT activity which was also induced in the rat but was not affected in the mouse.



Table 5.5-105	Cytochrome P-450 content and enzymatic activities in the liver after 3 and 14 days of
	exposure to AE C656948 (mean±SD)

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Group	3-da	y exposure	14-d:	ay exposure
	Control	2000 ppm (308 mg/kg bw/d)	Control	2000 ppm (314 mg/kg bw/d\$)
P-450 (nmol/mg Prot.)	$1.08 \pm 0.16$	2.33 ± 0.19 (+116%) **	1.26 ± 0.49	2.15 ± 0.06 (+71%) *
EROD (pmol/min/ mg Prot.)	90.25 ± 11.11	$302.52 \pm 4949$ (+235%) ***	99.05 ± 8.98	Ž62.24 €72.87
PROD (pmol/min/ mg Prot.)	4.93 ± 0.83	143.42 ± 57.05 (+2,590%) **	4.19±0.49	94.80 ± 44977
BROD (pmol/min/ mg Prot.)	$12.99 \pm 2.34$	1143.28 ± 262.93 (+8719%) ***	12.83 ± 2.11	1175,30/± 163,99 (+9061%) **
UDPGT (nmol/min/ mg Prot.)	16.04 ± 1.42	15.36 ≇ 0.53 ( <del>X</del> .C.)	0.90 17.09 0.90	1432 ± 0.65 (-16%) °
f: p≤0.05; **: p≤0.01	Ø			
F. Deficiencies	Ĵ.			
No specific deficiencies w	ere noted in this	study 🏷 💦		
III. Conclusions				
Chasa data indianta that Al		a hill the in water CE	$\mathcal{D}_{\mathbf{I}}$	~

#### F. Deficiencies

#### III. Conclusions

These data indicate that AE C656948 has the ability in male C57BL/61 mice to

- modify the thyroid hormone balance by chusing decrease in T4 levels and concomitant increase in TSH, Ô

- induce liver denges including in particular an induction of phenobarbital-like hepatic enzymes including totaDcytochrome P450, PROD, BROD activities. ġ,¥

When compared to the results obtained will phenobarbital using the same study design, these results also indicate a good similarity of effects, between AE CS56948 and phenobarbital.

#### Assessment and conclusion by applicant: A

Study meets the current guidence and the requirements in 283/2013 as it provides mechanistic information on the effects and targe organs of fluopyram in the mouse.

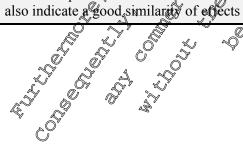
A

These data indicated hat AF C656948 has the ability in male C57BL/6J mice to:

- modify the thyroid hormone balance by causing a decrease in T4 levels and a concomitant increase in TSH, 🦃

- induce liver changes including in particulation induction of phenobarbital-like hepatic enzymes Including total cytochrome P450, BROD BROD activities.

When compared to the results obtained with phenobarbital using the same study design, these results also indicate a good similarity of effects between AE C656948 and phenobarbital





Data Point:	KCA 5.5/11
Report Author:	
Report Year:	
Report Title:	Phenobarbital mechanistic 14-day toxicity study in the mouse by oral gavage
	(hepatotoxicity and thyroid hormone investigations)
Report No:	SA 07326
Document No:	<u>M-299521-01-1</u>
Guideline(s) followed in	No specific guideline
study:	
Deviations from current	Current guideline: none
test guideline:	
Previous evaluation:	Yes, evaluated and accepted in the DAR (200).
	A Q & Y Q
GLP/Officially	Yes, conducted under Go Officially recognise testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes A A A A A A A A A A A A A A A A A A A
	Yes to
E-roomting Summary	
<b>Executive Summary</b>	

#### **Executive Summary**

Phenobarbital, a reference hepatotoxic compound (batch number: 06160228: white powder@99.6% of purity), was administered daily by gavage to 2 groups of 15 female 7BL/9 mice for 3 or 14 days at a dose level of 80 mg/kg/dag, Two similarly constituted groups of 5 females received 0.5% methycellulose for 3 or 14 days and acted as a control groups. Clarical signs were recorded daily, body weight and food consumption were measured weekly. Addetailed physical examination was performed weekly. Before necropsy, blood samples were taken for thyroid and pituitary gland hormone analyses (T3, T4 and TSH). At final sachifice times, fiver and brain were weighed and byer and thyroid gland sampled for the assessment of morphological changes. In addition, pepatic cytochrome P-450 and UDPGT isoenzyme activities were assessed.

After 3 days of prosure, mean T3 and T4 levels were decreased (respectively -10% and -27%) and mean TSH lever was not affected when compared to controls. A necropsy mean absolute and relative liver weights was increased by between 4% to 11% compared to the control group. This was associated with macroscopic findings including enlarged and/or dark lifer in several animals and with microscopic observations including diffuse centrilobular to pantobular hepatocellular hypertrophy in most animals.

Total cytochrome Red 30 content was markedly increased (+146%) by the treatment. EROD activity was marginally increased (±297%) whereas PROD and BROD activities were markedly increased (respectively +1381 and +4930%) when compared to compose. No significant change was observed in UDPGT activity when measured with 4-nitrophenol as substrate.

After 14 days of exposure at terminal sacrifice, the mean T3 level was not changed whereas the mean T4 level was statistically significantly decreased (-19%) and the mean TSH level was statistically significantly increased (+9%) when compared to controls. Effects in the liver were very comparable after J4 days of exposure than after 3 days of exposure. At necropsy mean absolute and relative liver weights were increased by between 21% to 23% compared to the control group. At macroscopic observation, enlarged liver was found in most treated animals (12/15) and dark liver was observed in 4/15 treated minals. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy were seen in all treated animal.

Total corochroppe P-450 content was slightly increased (+36%) by the treatment. EROD activity was slightly increased (#375%) whereas PROD and BROD activities were markedly increased (+1345% and 2844%, respectively) when compared to controls. No significant change was observed in UDPGT activitie Owhen measured with 4-nitrophenol as substrate.

In conclusion, this study demonstrated that phenobarbital administration at a dose level of 80 mg/kg body weight/day in the C57BL/6J mouse for 3 and 14 days induced significant liver changes including



increases in hepatic total cytochrome P-450, PROD and BROD activities. In addition, treatment with phenobarbital was associated with a modification of the normal thyroid hormone balance (in particular by a decrease in T4 and/or T3 after 3 and/or 14 days of exposure together with a concomitant increase in TSH level after 14 days.

#### I. Materials and methods

	5	~ "0
	I. Materials and methods	Phenobarbital White crystalline powder 06100228 99.6% 50-06-6 Stable for a period covering the study doration Methylcellulose 400 Mouse - Atale only 77BL/6J 8 weeks approximately at start of treatment 19.9 to 24.2 g Charles Kriver Laboratories, St German-sur P Arbresle, France of days
A.	Materials	
1.	Test material:	Phenobarbital
	Description	White crystalline powder
	Lot / Batch #:	
	Purity:	99.6% A Q & C O
	CAS #	50-06-6 $\mathcal{O}^{\mathcal{V}}$ $\mathcal{O}^{\mathcal{V}}$ $\mathcal{O}^{\mathcal{V}}$ $\mathcal{O}^{\mathcal{V}}$ $\mathcal{O}^{\mathcal{V}}$
	Stability of test compound:	Stable for a period covering the study doration
2.	Vehicle and / or positive	MathyleQuilec 200 2 2 2 2 2
co	ntrol:	
3.	Test animals:	
	Species:	Morse - Male only L of A R
	Strain:	
	Age:	8 weeks approximately at start of treatment
	Weight at dosing:	19.9 to 24,2 g & & o o o o v
	Source:	Charles River Laboratories, Sc German-sur PArbresle, France
	Acclimation period:	6 [°] daysov O [°] Q [°] Q [°] O [°]
	à ô	Certified rodent powdered and irradiated diet &04C-10 P1 from
	Diet:	S. OF.E. Scientific Animal Food and Engineering, Epinay-sur-
	Water:	Orge, France, ad libiom
	Water:	Municipal tap water, ad libuum
	Housing:	Animals were caged individually in suspended stainless steel
	Water: Housing: Environmental conditions: Temperature: Humidaty: Air, changes:	wire mester cages, S S
	Environmental conditions:	Do allo to to to to
	Temperature: S	20-24°C & & & @ 40470% & & & &
	Humidaty: Air changes:	40-0% & Composition of the second sec
	Air changes: C	Approximately 10-15 ar changes per hour
	Temperature: Humidaty: Air changes: Photoperiod:	Alternating 12-hour light and dark cycles (/ am- / pm)
B.	Study design 3	Approximately 10-15 or changes per hour Alternating 12-hour light and dark cycles (7 am- 7 pm)
	In life dates: Dostag period for	the 3-day exposure proups: 27 November – 30 November 2007.

Dosing period for the 14-day exposure groups: 20 November - 04 December 2007

#### 2. Anima assignment and treatment

Phenobarbital was administered once daily for 3 or 14 days by oral gavage to groups of 15 males at the dose of 80 mg/kg/day in \$5% agreous Solution of methycellulose 400. The dose level was selected on the basis of previous studies conducted with phenobarbital. Negative control groups with the same number of animals received the vehicle only (0.5% aqueous solution of methycellulose 400). Animal housing and ausbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised (1985) and "Le Guide Qu Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, Nº 86/609/CEE du 24 Novembre 1986".



#### Table 5.5-106: Study design

Test group	Treatment	Dose level in ppm (mg/kg bw/day)	Duration of treament	Animals assigned
1	Control	0	3 days 💊	15 5
2	Phenobarbital	80	3 days	15
3	Control	0	14 days	A5 N
4	Phenobarbital	80	14 days	~15, Q

Before the start of the study homogeneity and concentration were checked on the test formulation of 8 g/L. The mean value obtained from the homogeneity check was taken as measured concentration. The  $\bigcirc$  results of homogeneity and concentration of phenobarbital in the test formulation were found to be acceptable, being within a range of between 97% and 99% of the nominal concentration.

#### 3. Diet and water

Certified rodent powdered and irradiated diet A046 1-10 from SA.F.F. Scientific Animal Pood and Engineering, Augy, France) and filtered and softened tap water from the municipal water supply in individual bottles were available ad libitum, except prior to sacrifice when animals were diet fasted overnight. Routine analyses of food and water indicated that there was no contamination which could have compromised the study

#### 4. Statistics

- - Body weight change parameters
  - Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

The F test was performed to compare the homogeneity of group variances.

If the F test was not significant (p>0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-side).

If the F test was significant ( $p \leq 0.05$ ), the an of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

- - Body weight and average food consumption/day parameters
- Total cytochrome P450 content
- Hormonal parameters

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.

The F test was performed to compare the formogeneity of group variances.

If the F test was not significant p > 0.05, mean of the exposed group was compared to the mean of the control group using the t-test p > side d.

If the F test was significant ( $p \le 0.05$ ), data were transformed using the log transformation. If the F test on log transformed data was not significant (p > 0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.

If the t test was significant  $(\dot{p} \le 0.05)$  even after log transformation, the mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

If one  $\mathfrak{m}_{l}^{(n)}$  for group variance(s) equal 0, means were compared using non-parametric procedures.

• - Enzymatic activities and cell proliferation parameter



Mean of the exposed group will be compared to the mean of the control group using the exact Mann-Whitney test (2-sided).

Group means will be compared at the 5% and 1% levels of significance.

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Spatistics) except for liver enzyme parameters and cell proliferation parameters which were analyzed using SAS programs version 8.2.

#### C. Methods

#### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily of weekends of public holidays). Observed clinical signs were recorded at teast once daily for all animals and detailed physical examinations were performed at least weekly during the treatment perfod. The nature, onset, severity, reversibility, and duration of any clinical signs were resorded.

#### 2. Body weight

Each animal was weighed at least once during the acclimatization phase on study Day 1 and then weekly during the treatment period. Additionally, dier fasted, animals were weighed before scheduled nestropsy.

#### 3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. Any good spillage was noted.

#### 4. Hormonal investigation

#### **Blood sampling**

On study Day 4 (Groups 1 and 2) and on study Day 15 (Groups 3 and 4), blood samples were taken from all animals by puncture of the retro-orbital venous plexus. Animals were anesthetized by inhalation of Isoflurane (Baxter, Manrepas, France) prior to bleeding. Blood was collected on lithium heparin for hormone level evaluation.

#### Hormone measurements

Plasma was prepared from each blood sample and kept frozen at approximately -80°C until the determination of TSH0T3 and T4 hormone level with specific adio-immunoassay kits (supplied by Amersham for TSH and by DIASORIN for T3 and T4%

#### 5. Sacrifice and pathology

## Necropsy procedure Organ sampling

On study Day 4 and on study Day 15 all argmals from groups 1 and 2 and from groups 3 and 4, respectively were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane (Baxter, Maurepas, France) All argmals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled except upor decision of the Study Pathologist. Brain and liver were weighed fresh.

A piece of median and left lobe liver of Sanimals as well as the thyroid gland (with parathyroid glands) of all the animals were sampled and fixed in 10% neutral buffered formalin for histological examination. The remaining portions of livers were used for microsome preparation.

## Historechnology - Historethology

Liver portions and thyroid glands sampled for microscopy were embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared and examined



measuring

#### 6. Hepatotoxicity testing:

At scheduled sacrifice dates, in each group the livers of 3 animals were pooled (each time 2 entire livers and the remaining portion of 1 liver used for histological examination) and homogenized for microsomal preparations. In total, 5 microsomal samples per group were prepared. Total cytochrome P-450/content and specific cytochrome P-450 isozyme profile (including EROD, BROD and PROD activities) were measured to check the hepatotoxic potential of the test substance.

In addition, Phase II enzymatic activities were also determined by

glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate.
II. Results and discussion
A. Observations
1. Mortality
There was no mortality in any groups throughout the study.
2. Clinical signs
There were no treatment-related clinical signs.
B. Body weight and body weight gain
The first days of exposure were associated with lower body weight gains in treated groups compared to controls. For the 3-day exposure group, an overall mean body weight loss of 0.6 g was observed at the end of treatment compared to controls. For the 3-day exposure group, an overall mean body weight loss of 0.6 g was observed at the end of treatment compared to controls. For the 3-day exposure group, an overall mean body weight loss of 0.6 g was observed at the end of treatment compared to controls. For the 3-day exposure group, an overall mean body weight loss of 0.6 g was observed at the end of treatment compared to controls. end of treatment compared to gain of 0.3 g, in the control group. In the 14-day exposite group, there was a mean body weight loss of 0.3 g (p $\leq 0.005$ ) in the treated group compared with a gain of 0.4 g in the control group on study Day although overall mean bodyweight was not affected at the end of treatment period.

## C. Food consumption

the beginning of treatment but not during the second There was a slight reduction in food consumption at week of treatment.

## D. Hormone analysis

The results of TSH, T3 and T4 evels are summarized in Table 5.5 4-59

After 3 day of exposing, statistically significantly lower levels of both T3 and T4 were observed in the treated group compared to control while TSH Evel remained unaffected.

After 14 days of exposine T4 level was still significantly lower in the treated group than in the control with a concomptant significant higher lexe of TSH. An increased level of TSH is expected when T4 level is low as TSH will stimulate thyroid to preduce J4.

Table 5.5 107	Mean levels of thyroid/pinitary formone after 3 and 14 days of exposure to
×.	phenobarbital (prean±50)

$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Group	3-day exposure		14-day exposure	
T3 ( $\mu\mu\nu$ )/L) $\bigcirc$ 1.72 ± 9.25     1.54* ± 0.17 (- 10%)     1.61 ± 0.20     1.57 ± 0.19 (-29)		Control	^{Q^y} 2 [™] Phenobarbital	3 Control	4 Phenobarbital
	T3 (nmol/L)	℃1.72 ±39.25	,	$1.61 \pm 0.20$	1.57 ± 0.19 (-2%)
		36.7±6.2	26.8** ± 3.5 (-27%)	$32.4 \pm 6.5$	26.1* ± 6.7 (-19%)
	TSH (mg/mL)	4.44 ± 0.27	4.41 ± 0.54 (-1%)	$4.47\pm0.37$	4.89* ± 0.58 (+9%)



,Ø

#### E. Sacrifice and pathology

Higher liver weights were observed in animals treated with phenobarbital compared to controls after both 3 and 14 days of exposure. After 14-day of treatment, absolute and relative liver weight were higher (+21 to 23%) whereas after 3 day of treatment, only liver to bodyweight ratio was significantly higher (+11%) compared to control. This was associated in several occasions with enlarged and/or dark liver observed at the macroscopic examination and with hepatocellular hypertrophy at the microscopic examination.

Table 5.5-106 Tathological	inver effects after	5 and 15 days of ex		
	3-day e	xposure	_O [♥] 14-d≵	exposure
Group	1	<u> </u>		<u> </u>
	Control	Phenobarbital	Control	<b>Phenobarbit</b>
Liver weights Absolute weight (g)	1.30±0.12 🕵	1.36+0.09 (5%)	± 1.31∉0.16	1.60**±0.16 (+22%)
Body weight ratio (%)	5.70±0,333	6.32** <u>0.21</u>	5.38±0.50	6.65***0.47 ° (************************************
Brain weight ratio (%)	307.79-34.56	320.20±22.02	©301.33£34.16	365 46**±45.51
Histopathological findings			2 . S	
Centrilobular to panlobular	°, 0°, °,	D 1/5 8		5/5
hepatocellular hypertrophy	O S O	9 ^{4/5}	\$* <b>`6</b> 7 _~ 0	\$ 313
Increased mitosis	× ×0/5 ~	3/3	گي 0/5 _ U	© [♥] 0/5
*: p≤0.05; **: p≤0.01		NO N		Ò
Hepatotoxicity testing 📎			~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	) [°]

#### Table 5.5-108 Pathological liver effects after 3 and 14 mays of exposure to phenobarbital

# The results are described in Fable 5.3.4-61. At both time points treatment with phenobarbital induced clear increase in total cytochrome P-450 content and a marked increase in BROD and PROD activities compared to controls. EROD activity was only slightly facreased whereas 4-nitrophenol UDPGT activity was unaffected. Those results are in line the changes observed in the rat with the exception of the 4-nitrophenol UDPGT activity which was also induced in the rat.

Ô	
Table 5.5-199	Cytochrom P-450 withent and enzymatic activities in the liver after 3 and 14 days of
K.Y	exposure to phenobarbital (mean ESD) 2
· //	

	Aday exposure	14-d	ay exposure
Group	Phenobartal	3 Control	4 Phenobarbital
$\begin{array}{c} P-450 \\ (nmet/mg Prot.) \end{array} \qquad \begin{array}{c} 0.99 \pm 0.0 \\ 0.99$	2531**±@.20 (+	$0.98 \pm 0.12$	1.33* ± 0.29 (+ 36%)
(pmol/min/ mg Prof.)	37 €190,65** ± 97.94 (+ 297%)	$35.34 \pm 10.48$	167.88**±96.47 (+ 375%)
PROD (pmol/min/ mg Prot.)	89.99** ± 29.89 (+ 1381%)	$4.98 \pm 0.59$	71.97** ± 21.55 (+ 1345%)
$\begin{array}{c} \text{BROD} \\ \text{(pmol/max} \text{ mg.Prot.}) \end{array} \qquad 17.33 \pm 1.5 \\ \end{array}$	871.66** ± 148.84 (+ 4930%)	$18.82 \pm 3.30$	554.00** ± 119.35 (+ 2844%)
(nms/min/mg Prot)	$17.23 \pm 0.82$ N.S.	15.18 ± 1.28	$12.96 \pm 2.14$ N.S.
*: p≤0,65; **: p€0,01 , 5 , 4 , 4			

## F. Deficiencies

No specific deficiencies were noted in this study



#### III. Conclusions

These data indicate that phenobarbital has the ability in male C57BL/6J mice to:

- modify the thyroid hormone balance by causing a decrease in T4 and/or T3 levels and a conceptitant increase in TSH,

- induce liver changes including in particular induction of several hepatic enzymes as total cytochrome

P-450, PROD, BROD activities.

#### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283(2013 as it provides mechanisti information on the effects and target organs of phenobarbital in the mouse.

These data indicate that phenobarbital has the abolity in male C57BL/bJ mice to: - modify the thyroid hormone balance by causing a deprease of T4 and/or T5 levels and a concomitant increase in TSH,

- induce liver changes including in particular induction of several heratic enzymes as total cytochrome P-450, PROD, BROD activities.

	KCA 5.612
Data Point:	KCA 5.012
Report Author:	
Report Year:	
Report Title:	AE C656948 Mechanistic 3-day toxicity study in the mate mouse
	Capharmacokinetic investigations of the clearance of intravenously administered
	$\frac{1}{2^{125}}$ I-thyroxine) $\frac{1}{2^{5}}$
Report No:	SA08159 9 0 0 0 0 0 0 0 0
Document No:	<u>M²30830-01-1Ø 5 5 0 4</u>
Guideline(s) followed in	No specific guideline
study:	
Deviations from Current	
test guideline.	
Previous evaluation:	Yes, evaluated and accepted in the DAR (2017).
<u> </u>	
GLP/Officially	No not conducted under OLP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability	Current gindeline @rone Yes, evaluated and accepted in the DAR (2011). No hot conducted under OLP/Officially pecognised testing facilities Yes

## Executive Summary

AE C656948, a fungicide (barch number: Mix-batch: 08528/0002: light beige powder, 94.7% purity) was administered continuously via the dia at a dose level of 2000 ppm to a group of 5 male C57BL/6J mice for 3 days, a similarly constituted group of 5 males received untreated diet and acted as a control group. In addition, a further group (# 5 males received 80 mg/kg/day Phenobarbital (batch number: 06100228: white powder, 94.6% purity) by oral gavage for a 3 day period. Phenobarbital acted as a reference compound known to induce an increase in T4 clearance in the mouse through induction of T4 glucoronidation. On Study Dav 4 each animal received by intravenous injection via the tail 250 µl of diluted. T-Theroxine, solution. Approximately 3 hours post-administration with ¹²⁵I-Thyroxine, each animal received 0.1 mg of NaI in 250 µl of 0.9% sterile saline by intraperitoneal injection. A blood sample was collected from the retro-orbital venous plexus of each animal after 1 hour 20 minutes, 2, 4, 6 and 24 flours post ¹²⁵I-Thyroxine administration. The level of ¹²⁵I radioactivity was indicative of the rate of Thyroxine (T4) clearance from the blood. Animals were checked daily for mortality and clinical



signs. Body weights were recorded on Study Days 1 and 4. Due to technical difficulties encountered with the intravenous injection of ¹²⁵I-Thyroxine, Thyroxine (T4) clearance data for 9 animals only (5 control animals, 1 AE C656948 treated animal and 3 Phenobarbital treated animals) was obtained from this first group of animals (subgroup 1). Consequently, 5 additional animals were incorporated onto the study (subgroup 2). One animal acted as a control and received untreated diet, whilst the remaining 4 animals were treated with 2000 ppm AE C656948. The results obtained in both subgroups of animals were combined.

There were no mortalities or clinical signs during the course of the study. There was no statistically significant effect on body weight.

The results show that following an intravenous of ¹²⁵I. Thyroxine the radioactivity level in the blood of the AE C656948 treated animals was lower than that in the blood of the corresponding control animals? This decrease in the level of radioactivity in the blood of AE C656948 treated animals was observed at all time points examined and reflects a more rapid clearance of Thyroxine on these animals over a 24 hour period following ¹²⁵I administration, compared with the controls.

A similar response to that observed with AE C656948 treated annuals was seen in annuals treated with the reference compound Phenobarbital.

In conclusion, the results indicate that the clearance of Thyroxine from the blood of animals treated with 2000 ppm AE C656948 was increased when compared to control animals over a 24 hour period following ¹²⁵I administration. In addition, the response observed with AE C656948 treated animals was similar to that noted in animals treated with 80 mg/kg day Phenobarbital.

#### I. Materials and methods

A. Materials

1	Test and the test of the second secon	AE C656948 Light beige powder Mix-Batch:08528/0002
1.	Test material:	
	Description	Light beige powder
	Lot / Batch # O	Mix-Batch:08528/0002
	Purity:	
	CAS # 🏹 炎 🗸	658066-35-4 0
	Stability of test compound:	Stable in rodent det for a period covering the study duration
		Vehicle for AF C656948: rodent diet
2.	Vehicle and / or bositix	Positive control: Phenobarbital
co	Vehičle and / or positive ntrol: Positive control: Description: Lot / Batch :	Vehele for Phenobarbital. methylcellulose 400
	E A L	
	Positive control: 2 5	Phenobarbital S
	Description:	White crystalline powder
	Lot / Batch :	06190228
	Purito: 6	99 ⁸ 6% 2 4
	CAS:	ر50-06 کې مې ^۲
	Stability of test compound; "	Stable in redent diet for a period covering the study duration
	Test animals; $\hat{\mathcal{O}}^{Y}$	
	Species:	Mouso ² Male only
	Strain: 0 x x	C57BL/6J
	Age:	8 weeks approximately at start of treatment
	Age: Weight at dosing:	19.8 to 25.4 g
	Source: & A S	
	Acclimation period:	6 days (subgroup 1 animals), 23 days (subgroup 2 animals)
//		
~	D. C	Certified rodent powdered and irradiated diet A04C-10 P1 from
	Diet	S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-
		Orge, France), ad libitum
	Water:	Municipal tap water, ad libitum



Housing:	Animals were caged individually in wire mesh cages.	suspended stainless steel
<b>Environmental cond</b>	e	
<b>Temperature:</b>	20-24°C	
Humidity:	40-70%	$\sim$ $\sim$
Air changes:	Approximately 10-15 air changes pe	er hours 4
Photoperiod:	Alternating 12-hour light and dark c	cycles (7 am- 7 pm)
B. Study design	Č	
1. In life dates: Start o	f treatment to final sacrifice for the subgroup	p 1 animals, 23 August 27

August 2008

Start of treatment to final sacrifice for the subgroup animals: 05 September September 2008

#### 2. Animal assignment and treatment

The dose level for AE C656948 was set after evaluation of the results of a 28-day toxicity study (see section 5.3.1) and a 90-day toxicity study (see section 5.3.2) in the mouse with this substance. In the 28day study, 5000 ppm clearly exceeded the maximum Colerated dos as alkmales and most females had to be sacrificed for humane reasons before the end of the study. In the 90-day study slight toxicity was seen at the highest dose tested, 1060 ppm. Therefore, the dose level of 2006 ppm was selected as an appropriate high dose level to be used in this study. Although 2000 ppm was higher than the high dose used in the mouse carcinogenicity study (750 ppm), this dose level was selected to allow a clearer evaluation of the effects of AE C656948 without causing excessive toxicity. Males only were used in this study as an increased incidence of theroid follicular cell adenoma was observed in this sex only. In addition, 2000 ppm AE Co56948 was the doscrevel selected on other in-who mechanistic studies in the mouse. Phenobarbital was administered once daily for 3 days by oral gayage to a group of 5 males at a dose level of 80 mg/kg/day in 0.5% aqueous solution of methycellulos 400. The dose level was selected on the basis of previous studies, conducted with phenobarbital and was expected to provoke a positive response. A negative control group received untreated diet afone during the study period. 1 Col

Animal housing and husban fry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and Le Quide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CPE du 24 Novembre 9986".

Test group	O Treatment O Dose level	Duration of treatment	Animals assigned
*	Contropatiet	3 days	5
2*	AE C656948 2006 ppm	3 days	5
لا € 3*	Phenobarbinal Ø 80 mg/kg/day	3 days	5
4**	Controcdiet 0	3 days	1
5**	A SAE 656948 2 2000 ppm	3 days	4

#### Table 5.5-110 Study design

* subgroup 1 animals * subgroup 2 animals

The stability of AC C656948 in the diet at 20 and 10000 ppm has been demonstrated in a previous study after a fivezing period of 95 days followed by storage periods at room temperature of 10 days or 105 days. Before the start of the study homogeneity and concentration were checked on the study mix at 2000 ppm. The mean value obtained from the homogeneity check was taken as measured concentration. The study mix was found to be acceptable for use on the study as the homogeneity and concentration checks were within the range of 91 to 93% of the nominal concentration. The stability of Phenobarbital in aqueous methylcellulose at 8 g/l has been demonstrated in a previous study after a storage period of



29 days. Before the start of the study homogeneity and concentration were checked on the study formulation at 8 g/l. The mean value obtained from the homogeneity check was taken as measured concentration. The study formulation was found to be acceptable for use on the study as the homogeneity and concentration checks were 100% of the nominal concentration.

#### 3. Diet and water

Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France) and filtered and softened tap water from the municipal water supply in individual bottles were available *ad libitum* until Day 4. On transfer to the radioactive suite on Day 4, animals were given certified rodent pelleted and irradiated diet A04CP1-10 from S.A.F.F. Scientific Animal Food and Engineering, Augy, France). The water was supplied via water bottles. Animals were not diet fasted overnight prior to blood sampling and final sacrifice Routine analyses of food and water indicated that there was no contamination which could have compromised the study.

#### 4. Statistics

Body weight

Mean and standard deviation were calculated for each group.

The F test was performed to compare the homogeneity of group variance

If the F test was not significant (p $\neq$ 9.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ( $p \le 0.05$ ), data were transformed using the log transformation. If the F test on log transformed data was not significant (p > 0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data

If the F test was significant (p\$0.05) even after logoransformation, the mean of the exposed group was compared to the mean of the control group using the modified t-test (2) sided).

If one or more group variance(s) equal 9, means were compared using non-parametric Mann-Whitney test (2-sided).

Group means will be compared at the 5% and 1% levels of significance.

Statistical analyses were carried out using Path/Tox System V4.22 (Module Enhanced Statistics).

## C. Methods

#### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed elinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

## 2. Body weight

Each animal was weighed at least once during the acclimatization phase, then on Study Days 1 and 4.

#### 3. Food consumption

Food consumption was not recorded.

## 4. Intravenous injection of ^{CA}I-Thyroxine

¹²⁵I-Thyroxine solution (+I-T4, Nem Life Sciences, France) was diluted with 0.9 % sterile saline solution to provide a solution containing 4 µCi/ml. The certificate of analysis of the stock solution of ¹²⁵I-Thyroxine is presented in Attachment 2 in the study report. Each animal received on Day 4 by intravenous injection via the tail 250 µl of the diluted ¹²⁵I-Thyroxine solution corresponding to 1 µCi of ¹²⁵I-Thyroxine per animal. Approximately 3 hours after the intravenous injection of ¹²⁵I-Thyroxine, each



animal received 0.1 mg of NaI in 250 ul of 0.9 % sterile saline by intraperitoneal injection. Dilution of the ¹²⁵I-Thyroxine solution and preparation of the NaI solution was carried out on the day of administration.

For the animals belonging to subgroup 2, to take into account the decay of the specific activity of the ¹²⁵I-Thyroxine, a new dilution of the ¹²⁵I-Thyroxine stock solution was performed. This diluted solution was performed by mixing the same volumes of stock solution of ¹²⁵I-Thyroxine and 0.9 % sterile same as the ones used for the main experiment (subgroup 1 animals).

#### 5. Blood sampling:

Animals were not diet fasted prior to blood collection.

A whole blood sample was collected from the retro-orbital vencos plexus of each surviving animation Whole blood samples were placed into tubes and the radioactivity overe measured. Prior to blood sampling animals were anesthetized with Isoflurane (Baxter, Maurepas, France). Samples were taken at 1 hour 20 min, 2, 4, 6, and 24 hours after the dosing with the ¹²⁵I-Thyroxine.

#### 6. Measurement of radioactivity:

Two aliquots of 50 µl of each whole-blood sample were dispensed directly into tubes for radioactivity measurement. The radioativity (125I) was measured at the same time in all the blood samples generated both in subgroup 1 and subgroup 2 animals

Radioativity (125I) were measured using & Cobragampa scinthation counter (Packard

Radioactivity measurement was expressed as follows

Whole blood radioactive content.

2 Were combined and an overall mean and Data from animals belonging to both subgroups 1@and standard deviation derived for each treatment group

#### II. Results and discussion

## A. Observation

## 1. Mortality

groups (proughou There was no mortality in an

## 2. Clinical signs

There were no clinical signs observed.

# B. Body weight and body weight gain

After 3-days of exposure, no statistically significant effect on mean body weight was recorded either in the AE C656948 or Phenobarbital treated animals when compared to the controls.

#### T4 clearance С.

During the course of the study, some technical difficulties affected the process of intravenous injection of the initial batch of animals allocated to the study. Consequently, Thyroxine (T4) blood level data was obtained from 9 animals only (5 control animals, 1 AE C656948 treated animal and 3 Phenobarbital treated animals). Consequently, 5 additional animals were incorporated onto the study which consisted of 1 control animal and AE Co56948 treated animals. T4 blood level data was obtained from these 5 animals The data presented in the table below represent the combined mean and standard deviation derived from both subgroups of animals for each treatment level.



Table 5.5-111	Whole blood radioactivity after a single administration of 125 I Thyroxine Mean $\pm$
	Standard deviation (cpm)

Time point	Control	AE C656948 (2000	Phenobarbital
		ppm)	──── mg/kg/day
Number of animals	6	5	
Time point 1h20min	$11434\pm1624$	4767 ±1953	5775 2615
Time point 2h	$11025 \pm 1415$	4686 ±1999	5905±20952 ×
Time point 4h	9811±1756	4984 ±1491	5051±995 0
Time point 6h	$8692 \pm 1397$	4566 ± 12632	6021 £1046 \$ 0 [°]
Time point 24h	$2686 \pm 454$	1955 <b>J</b> 199 <u>.</u>	2309 [±] 446 [°]

The results show after an intravenous injection of ⁴²⁵I-thyroxine a decrease of the radioactivity in the blood of the AE C656948 treated animals when compared to controkanimals. This decrease in the level of radioactivity in the blood of AE C656948 Geated mimals was observed at alloime points examined and reflects a more rapid clearance of thyroxine in these mimal over a 24 hour period following administration, compared with the controls.

Similarly, the decrease of the radioachvity was also observed in animals, treated with phenobarbital a Overall these results indicate that the thyroxine clearance was increased in the AE 6656948 treated animals when compared to control animals. D. Deficiencies No specific deficiencies, were noted in this study III. Conclusions reference compound known to induce an increase in thyrexine clearance in the prouse dirough induction

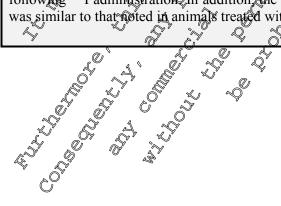
In conclusion, the results indicate that the clearance of thyrosine from the bood of animals treated with 2000 ppm AE C 656 248 was increased when compared to control animals over a 24 hour period following ¹²⁵I administration. In addition, the response Observed with & C656948 treated animals was similar to that noted in animals treated with 80mg/kg/day phenobar ital.

#### Assessment and conclusion by applicant: L

Study meets the current guidence and the requirements in 283/2013 as it provides mechanistic information on AE C 56948 0

X.

In conclusion, the results Indicate that the clearance of thyroxine from the blood of animals treated with 2000 ppm AE C656948 was increased when compared to control animals over a 24 hour period following ¹²⁵I administration In addition the response observed with AE C656948 treated animals was similar to that wited in animals treated with 80 mg/kg/day phenobarbital.





Data Point:	KCA 5.5/13
Report Author:	
Report Year:	
Report Title:	AE C656948 Mechanistic 3-day toxicity study in the male mouse (qPCR
	investigations of gene transcripts in the liver)
Report No:	SA 08151
Document No:	<u>M-308073-01-1</u>
Guideline(s) followed in	No specific guideline
study:	
Deviations from current	Current guideline: none
test guideline:	
Previous evaluation:	Yes, evaluated and accepted in the DAR (20U).
GLP/Officially	No, not conducted under ALP/Officially recognized testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes A A A A A A A

#### Executive Summary

AE C656948, a fungicide (batch number: Mix-batch: 08528/0002: light beige powder, 94,7% purity) and Phenobarbital (batch number: 06100228: white powder, 99.6% purity) were deministered daily to 2 groups of 10 male C57BL/61 mixe for 3 days at dose levels of 2000 ppon and 80 mg/kg/day, respectively. One similarly constituted group of 10 males received control diet for 3 days and acted as a control group. Clinical signs were recorded daily and body weight was measured of Study Days 1 and 4. At final sacrifice, liver was weighed and sampled for gene expression analyses by quantitative Polymerase Chain Reaction (cPCR).

There were no mortalities or body weight effects during the course of the study. Animals treated with Phenobarbital showed reduced motor activity throughout the treatment period. At necropsy, mean absolute and relative liver weights were increased by 60% to 61% for AE C656948 treated animals and by 17% to 19% for Phenobarbital treated animals, when compared to the control animals.

Quantitative PCR analyses of transcripts of genes known to be implicated in the hepatic inactivation of thyroid hormones revealed in AE 656948 treated animals on up-regulation of sulfotransferase transcripts (from +92% to +463% p $\leq$ 0.01% and ODP glucoronosyltransferase transcripts (from +173% to +273%, p $\leq$ 0.01% similarly, an up-regulation of sulfotransferase transcripts Sult 1a1 and Sultn (+62% and +96%, respectively; p $\leq$ 0.01% and DP glucoronosyltransferase transcripts (from +82% to +119%, p $\leq$ 0.01) was observed on Phenobarbhal treated animals.

In conclusion, this study demonstrates that AE C656948 at 2000 ppm and Phenobarbital at 80 mg/kg body weight/day administered to the 057BL/6J mouse for 3 days both induced an up-regulation of the sulfotransferase and 0DP glucoronosyltransferase gene transcripts in the liver. These transcripts are known to encode enzymes that inactivate 13 and 14 via glucoronide and sulfate derivatives.

I. Materials and method	San de
A. Material	
1. Test materfal: Description	AE C656948 Light beige powder
Description V V Dot / Batch #:0	Mix-Batch:08528/0002
Purity:	94.7%
CAS#	658066-35-4
Stability of test compound:	Stable in rodent diet for a period covering the study duration



2 Waltala and / an a sitira	Vehicle for AE C656948: rodent diet
2. Vehicle and / or positive	Positive control: Phenobarbital
control:	Vehicle for Phenobarbital: methylcellulose 400
<b>Positive control:</b>	Phenobarbital
Description:	White crystalline powder
Lot / Batch :	06100228
Purity:	99.6%
CAS:	50-06-6
Stability of test compound:	Stable for a period covering the study duration. $\sqrt{2}$
3. Test animals:	
Species:	Mouse - Male only $\mathcal{A}^{\circ}$ $\mathcal{A}^{\circ}$ $\mathcal{A}^{\circ}$ $\mathcal{A}^{\circ}$
Strain:	C57BL/6J $\mathcal{A}^{\vee}$ $\mathcal{A}^{\vee}$ $\mathcal{A}^{\vee}$ $\mathcal{A}^{\vee}$ $\mathcal{A}^{\vee}$ $\mathcal{A}^{\vee}$ $\mathcal{A}^{\vee}$ $\mathcal{A}^{\vee}$
Age:	8 weeks approximately at start of meatment
Weight at dosing:	19.9 to 23.4 g $\sim$
Source:	Vehicle for AE C656948: rodent diet Positive control: Phenobarbital Vehicle for Phenobarbital: methylcellulose 400 Phenobarbital White crystalline powder 06100228 99.6% 50-06-6 Stable for a period covering the study duration.
Acclimation period:	6 days
	Certified rodent powdered and irradiated diet A04C-10P1 from
Diet:	S. G.F.E. Scientific Animal Food and Engineering, Epinay Sur-
	Orge, France) and libitum Municipal tap water and libitum Animals were caged individually in suspended stainless steel whe mesh cages
Water:	Municipal tap/water/ad libutum 2 2 2
Housing:	Anymals were caged individually in suspended stainless steel
	where mesh cages and a cage of the cage of
Environmental conditions:	
	$\frac{1}{4000\%} \int_{0}^{1} \int_$
Humidity:	Approximately 10-15 air changes per hours
Air changes:	Approximately 10-15 arr changes per nouro
Photoperiod:	Approximatels 10-15 air changes per hours Alternating 12-hour light and dark cycles (7 am- 7 pm)
Photoperiod: 5 7 B. Study design 5 5 1. In life dates 5 5 Start of tre	
	atment of final sacrifice: 08 fully – 14 July 2008 ent
The dose level for AE 6656948 wa	set after evaluation of the feaults of a 28-day toxicity study (see

The dose level for AE C656948 was set after evaluation of the results of a 28-day toxicity study (see section 5.3.1) and a 90 day toxicity study (see section 5.3.2) in the mouse with this substance. In the 28day study, 5000 ppm clearly exceeded the maximum toterated dose as all males and most females had to be sacrificed for humane reasons before the end of the study. In the 90-day study slight toxicity was seen at the highest dose tested, 1000 ppm Therefore, a dose level of 2000 ppm was selected as an appropriate high dose level to be used in this study. Although, 2000 ppm was higher than the high dose used in the mouse carcinogenicity study (750 ppm) this dose level was selected to allow a clearer evaluation of the effects of AF C656948 without causing excessive toxicity. Males only were used in this study as an increased incidence of thyroid forthcular cell adenoma was observed in this sex only. In addition, 2000 ppm AE C656948 was the dose devel selected on other

*in-vivo* mechanistic studies in the more. Phenobarbital was administered once daily for 3 days by oral gavage to a group of 3 males of a dose level of 80 mg/kg/day in 0.5% aqueous solution of methycellulose 400. The dose level was selected on the basis of previous studies conducted with phenobarbital and was expected to provoke a positive response. A negative control group received untreated diet alone during the study period.

Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".



Test group	Treatment	Dose level	Duration of treament	Animals assigned
1	Control diet	0	3 days	r 100° s
2	AE C656948	2000 ppm	3 days	10 . 9
3	Phenobarbital	80 mg/kg/day	3 days	

The stability of AE C656948 in the diet at 20 and 10000 ppin has been demonstrated in a previous study after a freezing period of 95 days followed by storage periods at room temperature of 10 pays of 105 days. Before the start of the concentration were checked on the study mix at 2000 ppm. The study mix was found to be acceptable for use on the study as the concentration check was 99% of the Hominal concentration. The stability of Phenobarbital in aqueous methylcollulose at 8 g/has been demonstrated in a previous study after a storage period of 29 days. Before the start of the study concentration was checked on the study formulation at 8 g/l. The study formulation was found to be acceptable for use on the study cormutation was found to be acceptable for use on the study of 29 days. Before the start of the study concentration was checked on the study formulation at 8 g/l. The study formulation was found to be acceptable for use on the study as the concentration check was 100% of the normal concentration.

#### 3. Diet and water

Certified rodent powdered and irradiated diet A04CP1-10 from SA.F.E. (Scientific Animal Food and Engineering, Augy, France) and filtered and softened tap water from the municipal water supply in individual bottles were available *da libitum*. Animals were not diet facted overnight prior final sacrifice. Routine analyses of food and water indicated that there was no contamination which could have compromised the study.

#### 4. Statistics

- Body weight parameters
- Terminal body weight, absolute and relative organ weights parameters
- Organ weight parameter
- qPCR data

Mean and standard deviation were calculated for each group

• Terminal body weight absorbe and relative organ weight parameters.

The F test was performed to compare the nonogeneity of group variances.

If the F test was not significant p>0.05, mean of the exposed group was compared to the mean of the control group using the t-test 2-sided).

If the F test was significant ( $p \le 0.05$ ), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

• Body weight

The P test was performed to compare the homogeneity of group variances.

If the F test was not significant ( $p \ge 0.05$ ), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ( $p \le 0.05$ ), data were transformed using the log transformation. If the F test on log transformed data was not significant (p > 0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.

If the F test was significant ( $p \le 0.05$ ) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).



If one or more group variance(s) equaled 0, means were compared using the non parametric Mann-Whitney test (2-sided).  $^{\circ}$ 

#### • qPCR data

The objective of the statistical analysis was to compare mean quantities of 9 gene transcripts calculated in the AE C656948 treated group to the mean quantities of the 9 gene transcripts calculated in the control group. Mean quantities of the 9 gene transcripts calculated in the Phenobarbital treated group were also compared to the mean quantities calculated in the control group.

Because of a lack of normalization and a high variability between calculated values of gene transcript quantities, non-parametric procedures were selected. The mean quantity of each gene transcript of calculated in the AE C656948 treated group was compared to the mean quantity calculated in the Control group using the exact Mann-Whitney test (2-sided). An exact non-parametric test was preferred because of the low sample sizes (10 animals per group).

Group means were compared at the 5% and 1% levels of significance

Statistical analyses were carried out using Path/Fox Sestem 14.2.2. (Module Enhanced Statistics) except for qPCR data which were analyzed using SAS programs.

#### C. Methods

#### 1. Observations

The animals were observed twice daily for morbundity and portality (once daily on weekends or public holidays). Observed clinical spins were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

#### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, then on Study Days 1 and 4.

#### 3. Food consumption

Food consumption was not recorded?

#### 4. Necropsy procedure and tissue contection

On Study Day 4 all animals were sacrificed. All Cacrifices were performed by exsanguination under deep anesthesia (inhalation of Isoflurane) Baxter, Maurepas France). Animals were not diet fasted overnight prior to sacrifice. All mimals were recropsed. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abaormalities were recorded but not sampled except upon decision of the Study Pathologist.

The liver was weighed fresh at scheduled sacrifice.

A piece of median and left lobe liver of all the animals was collected and stored frozen at approximately -70°C for quantitative Polymerase Chaine Reaction analysis. The remaining portions of livers were discarded.

## 5. qPCR analysis:

#### Total RNA purification

Total cytoplasmic RNA was solated from the liver of individual control and treated animals using RNease Midi kits (Diagen) RNA quality controls were performed based on the ribosomal RNA electrophoretic propres using a Bioanalyser (Agilent Technologies).

## Quantitative PCR

Ten µg of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand,



Applied Biosystems), 1/50 diluted first strand cDNA, AmpliTaq Gold® PCR Master Mix on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA.

Table 5.5-115: List of Taqinan ass			
Gene family	Isoform	Refset ID	🖉 Taqman assay ID
		4	(Applied Brosystems)
Cytochrome P450	Cyplal	M_009992.3	Mm004872182m1
Cytochrome P450	Cyp2b9	MM_0100002	Mp10065790_mk
Cytochrome P450	Cyp3a11	M_007818.3	Atm007& 567 mi
Sulfotransferase	Sulta1	NM_133690.1 @	Mm06467072_m1
Sulfotransferase	Sulta2	NM_0092864	Mm02394381_g4
Sulfotransferase	Sultn	NM 016711.2	@m00502030_m1
UDP glucuronosyltransferase	Ugtla1	M_200645.2	Mm@6033375_m1 🗸 °
UDP glucuronosyltransferase	Ugt2b1	NM 352811.1	Mm00514184_m()
UDP glucuronosyltransferase	Øgt2ba	NAT 009467.1 X	Mm01623253
Beta-2 microglobulin	^{O™} B2m [™] →	M_009735.3.5	Mm09437762_m1
Beta-2 microglobulin		r	<u>Mm@#437762_ml</u>

#### Table 5.5-113: List of Taqman assays used

Beta-2 microglobulin (B2m) was selected as reference gene for the quantitative calculations of transcripts. The relative quantity (RQ) value of each test transcriptowas calculated using the following formula:

$$\Delta\Delta Ct = (Cttest - CtB2_m)_{streamed} - (Cttest - CtB2_m)_{control}$$

 $RQ = 2^{-\Delta\Delta Ct}$ 

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signals

Each RQ value obtained for a given gene was normalized by divering by the RQ value obtained for the control animal ST1102625

## II. NResults and discussion

#### A. Observations

#### 1. Mortality

There was no mortality in any groups throughout the study.

## 2. Clinical signs

Reduced motor activity was recorded throughout the treatment period in the Phenobarbital treated animals. No other clinical signs were observed during the course of study in any animals.

## B. Body weight and body weight gain

After 3-days of exposure, no effect on mean body weight was recorded either in the AE C656948 or Phenobarbita Dreated animals when compared to the controls.

## C. Sacrifice and qPCR analysis

Mean liver and mean liver weight to body weight ratio were statistically significantly higher both in AE C656948 and phenobarbital treated animals when compared to control animals.



T 11 5 5 114	
Table 5.5-114	Liver weight changes (% change when compared to controls)

	5 5 K	с <b>т</b>	
Sex		Males	<u> </u>
Test substance	Control Diet	AE C656948 (2000 ppm)	Phenobarbital (8) mg/kg/day
Mean absolute liver weight (g)	1.20±0.10	1.93**±0.12 (+61%)	0 1.40**±0 5 (+1753)
Mean liver to body weight ratio (%)	5.34±0.36	8.55%±0.40 (+60%)	6.34**#0.290 19%) 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
** p≤0.01			

#### **QPCR** analysis

Table 5.5-115Mean Relative Quantity ± standard deviation of gene transcripts % change<br/>compared to control mean values

		$\checkmark$
Gene transcripts	Control X AE C656948 (2000 Phenobarbital (80	r
	( V ppm) O y Smg/kg/day)	
Cyplal	$1.29 \pm 0.50$ 4.81** ± 19 (+2,2%) 20 ± 0.24 NS (-7%)	
Cyp2b9	$14.71 \pm 0.17 \qquad 48.61^* \pm 9.521 \qquad 0.11 \pm 0.19.88 (+43\%)$	)
Cyp3a11	$1.5\% \pm 0.5\% = 43.5\% \pm 34/38 (+2083\%) = 7.\%^{**} \pm 34/11 (+413\%)$	
Sult1a1	$1.19 \pm 9.39$ $2.29 \pm 1.20 (\pm 92\%)$ $1.920 \pm 0.46 (\pm 62\%)$	
Sult2a2	$3 \times 0.54 \pm 0.22$ $3 \times 2.90$ $\pm 2.08$ (+463%) $3 \times 0.65 \pm 0.35$ NS (+22%)	
Sultn	$\begin{array}{c c} & & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & & \\ & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & &$	
Ugt1a1		
Ugt1a1 Ugt2b1		
Ugt2b5	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	
NS · Not statistically signal	Frant 2 4 9 6 0 0	

NS : Not statistically significant

* : Statisticall alifferent from the control group 450.05

** : Statistically different from the control group ( $p \le 0.01$ )

Cyp1a, cyp2b and cyp3a gene transcript isoforms were up-regulated in the liver of AE C656948 treated animals when compared with controls. In the phenobabital treated animals the induction of cyp3a was statistically significantly up-regulated (+413%,  $p \le 0.01$ ). The cyp2b transcript was only slightly up-regulated (+43%) and the effect was not statistically significant.

The transcripts of isoforms of suffictant ferase and udp glucoronosyltransferase known to inactivate T3 and T4 via glucoronide and suffate derivatives were statistically significantly up-regulated in the liver of AE C056948 treated animals. In the liver of animals treated with phenobarbital, except for sult2a2, the same sult and ugt transcripts were statistically significantly up-regulated as with AE C656948 treated animals

#### D. Deficiencies

No specific deficiencies were noted in this study

# III. Conclusions

In conclusion, this study demonstrates that AE C656948 at 2000 ppm and phenobarbital at 80 mg/kg body weight/day administered to the C57BL/6J mouse for 3 days both induced an up-regulation of the sulfotrare ferase and udp glucoronosyltransferase gene transcripts in the liver. These transcripts are known to encode enzymes that inactivate T3 and T4 via glucoronide and sulfate derivatives.



#### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on AE C656948.

In conclusion, this study demonstrates that AE C656948 at 2000 ppm and plenobarbital at 80 mg/kg body weight/day administered to the C57BL/6J mouse for 3 days both induced an up-regulation of the sulfotransferase and udp glucoronosyltransferase gene transcripts in the liver. These transcripts are known to encode enzymes that inactivate T3 and T4 via glucoronide and sulfate derivatives.

Data Point:       KCA 5.5/14         Report Author:       2009         Report Year:       2009         Report Title:       AE C656948 Definitive Mechanistric 4-daytoxicity study iff the male monse (pharmacokinetic investigations of the clearance of intravenously administered)         1251-thyroxine)       Report No:         SA 08288       A 8288         Document No:       M-328662 44-1         Guideline(s) followed in study:       No specific guideline         Deviations from current test guideline:       Current guideline: none         GLP/Officially       Yes, evaluated and accepted in the DAR (2011)         GLP/Officially       Yes, conducted under GLP officially recognised testing facilities         Acceptability/Reliability:       Yes		
Data Point:       KCA 5.5/14         Report Author:       Image: Constraint of the constraint of th		
Report Year:       2009         Report Title:       AE C656948 Definitive Mechanistic 4-daydoxicity study iff the male more (pharmacokinetic investigations of the clearance of intracenously administered)         Report No:       SA 08288         Document No:       M-32866249-1         Guideline(s) followed in study:       No specific guidefine         Deviations from current test guideline:       Current guidefine: none         Previous evaluation:       Yes, evaluated and accepted in file DAR (2011)         GLP/Officially recognised testing facilities       Yes, conducted under GLR Officially recognised testing facilities		
Report Year:       2009         Report Title:       AE C656948 Definitive Mechanistic 4-daydoxicity study iff the male more (pharmacokinetic fuvestigations of the clearance of intravenously administered)         Report No:       SA 08288         Document No:       M-32866201-1         Guideline(s) followed in study:       No specific guideline         Deviations from current test guideline:       Current guideline: none         Previous evaluation:       Yes, evaluated and accepted in file DAR (2011)         GLP/Officially recognised testing facilities       Yes, conducted under GLP/Officially recognised testing facilities	Data Point:	KCA 5.5/14 $\mathcal{O}^{*}$ $\mathcal{O}^{*}$ $\mathcal{O}^{*}$ $\mathcal{O}^{*}$ $\mathcal{O}^{*}$ $\mathcal{O}^{*}$
(pharmacokinetic investigations of the clearance of intravenously administered)         1251-thyroxine)         Report No:       SA 08288         Document No:       M-32866209-1         Guideline(s) followed in study:       No specific guideline         Deviations from current test guideline:       Vest evaluated and accepted in the DAR (2011)         Previous evaluation:       Yes, evaluated and accepted in the DAR (2011)         GLP/Officially recognised testing facilities       Vest ponducted under GLP/OfficialDy recognised testing facilities	Report Author:	
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Report No:       SA 08288         Document No:       M-32866251/-1         Guideline(s) followed in study:       No specific guideline         Deviations from current test guideline:       Current guideline: none         Previous evaluation:       Yes, evaluated and accepted in file DAR (2011)         GLP/Officially recognised testing facilities:       Yes conducted under GLP/Officially recognised testing facilities		(pharmacokinetic investigations of the clearance of intravenously administered $\mathbb{Q}^{\mathbb{Z}}$
Document No:       M-328662 N-1         Guideline(s) followed in study:       No specific guideline         Deviations from current test guideline:       Current guideline: none         Previous evaluation:       Yes, evaluated and accepted in the DAR (2011)         GLP/Officially recognised testing facilities:       Yes conducted under GLP/Officially recognised testing facilities		
Document No:       M-328662-14         Guideline(s) followed in study:       No specific guideline         Deviations from current test guideline:       Current guideline: none         Previous evaluation:       Yes, evaluated and accepted in the DAR (2011)         GLP/Officially recognised testing facilities:       Yes conducted under GLR officially recognised testing facilities	Report No:	SA 08288 0 4 4 4 5 4 5 5 5
Deviations from current test guideline:       Current guideline: none         Previous evaluation:       Yes, evaluated and accepted in the DAR (2011)         GLP/Officially recognised testing facilities:       Yes conducted under GLP/OfficialDy recognised testing facilities	Document No:	<u>M-328662</u>
Deviations from current test guideline:       Current guideline: none         Previous evaluation:       Yes, evaluated and accepted in the DAR (2011)         GLP/Officially recognised testing facilities:       Yes conducted under GLP/OfficialDy recognised testing facilities	Guideline(s) followed in	No specific guidefine
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GLP/Officially recognised testing facilities:		
GLP/Officially     Yes conducted under GLP/Officially recognised testing facilities       facilities:     Acceptability/Reliability:	Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
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	Acceptability/Reliability:	Yes y y y y

# Executive Summary

AE C656948, a fungicite (batch number: Mix-batch: 08528/0002/ light beige powder, 94.7% purity) was administered continuous via the diet at a dose level of 2000 ppm to a group of 8 male C57BL/6J mice for 4 days, a similarly constituted group of 8 makes received untreated diet and acted as a control group. In addition a further group of 8 males received 80 mg/kg/day Phenobarbital (batch number: 06100228: white poweer, 99,5% purfy) by oral gavage for a 4 day period. Phenobarbital acted as a reference compound known to induce an increase in T4 clearance in the mouse through induction of T4 glucoronidation and sulfoontion On Study Day 3 each animal received by intravenous injection via the tail 250 of diluted 25I-Thyloxin Solution in 0.9% sterile saline. Approximately 3 hours postadministration with ¹²/₂/₂. Thyroxine, each animal received 0.1 mg of NaI in 250  $\mu$ l of 0.9% sterile saline by intraperitoneal injection. A blood sample was collected from the retro-orbital venous plexus of each animal after 40 minutes 1.5, & and 24 hours post 125I-Thyroxine administration. The level of 125I radioactivity in Each sample was measured using a Cobra gamma scintillation counter. The rate of ¹²⁵I radioactivity was inducative of the rate of Thyroxine (T4) clearance from the blood. Animals were checked darly for mortality and clinical gigns. Body weights were recorded on Study Days 1 and 5.

There were no stricel signs during the course of the study. One animal was sacrificed after intravenous injection of the radio abeled Thyroxine due to a technical problem during the injection procedure. There was to statistically significant effect on body weight.

The results show that following an intravenous of ¹²⁵I-Thyroxine the radioactivity level in the blood of the AE C656948 treated animals was lower than that in the blood of the corresponding control animals. This decrease in the level of radioactivity in the blood of AE C656948 treated animals was observed at



all time points examined and reflects a more rapid clearance of Thyroxine in these animals over a 24 hour period following ¹²⁵I administration, compared with the controls.

A similar response to that observed with AE C656948 treated animals was seen in animals treated with the reference compound Phenobarbital.

In conclusion, the results indicate that the clearance of Thyroxine from the blood of animals treated with 2000 ppm AE C656948 was increased when compared to control animals over a 24 hour period following ¹²⁵I administration. In addition, the response observed with AE £656948 treated animals was similar to that noted in animals treated with 80 mg/kg/day Bhenobarbitak 

#### I. Materials and methods

A.	Materials	<ul> <li>AE C656948</li> <li>Light beige powder</li> <li>Mix-Batch:08528/0002</li> <li>94.7%</li> <li>6580667354</li> <li>St compound:</li> <li>Stable in rodent diet for a period covering the study duration</li> </ul>
		: AE C656948 2° 5° 5° 5° 5° 5° 5°
1.	Test material	: AE C656948 & S & J & S & S
	Description	Light berge poweder to the the second s
	Lot / Batch #:	Light beige poweder of the second sec
	Purity:	94.7%
	CAS #	658066735-4 0 × 0 × 0 ×
	Stability of te	Mix-Batch:08528/0002 94.7% 658066;35-4 st compound: Stable to rode of diet for a period covering the study duration or positive Positive control: Phenokarbital Vehicle for Phenokarbital Vehicle for Phenokarbital Official
2.	Vehicle and /	or positive Positive centrol: Phenokarbital
	ntrol:	A Postrive control Phenomatolital
	Positive contr	ale Menobarbital
	Description:	ol: Phenovarbital White crystalline powder
	Lot / Batch :	$\circ$ $\circ$ Whate crystalline powder $\sim$ $\sim$ $\sim$ $\sim$
	Purity:	$\mathcal{A}_{\mathcal{A}} = \begin{pmatrix} 0 & 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 &$
	CAS :	$5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5^{-1}$ $5$
	Stability of te	White crystalline powder 06 f00228 99.6% 50-06-6 50-06-6 Stable for a period covering the study duration Mouse Mate only
3.	Test animals.	
	Species:	Mouse Materonly a
	Strain:	$\tilde{O}$ $\tilde{C}$ $C57BL/64$ $\tilde{S}$ $\tilde{O}$ $\tilde{S}$
	Age: 🔊	& Sweeks approximatel Lat start of treatment
	Weight at dos	$\sin g_{\mu}^{(1)}$ $\delta^{(2)}$ $20.3 \text{ to } 23.5 \text{ g}^{(2)}$ $\delta^{(2)}$ $\delta^{(2)}$
	Source:	
	Acclimation ₁	priod: Afteast 9 days O
	Q	Sertified rodent powdered and irradiated diet A04C-10 P1 from
	Diet:	S.A.F.C. (Scientific Animal Food and Engineering, Epinay-sur-
	4	Orge, France), addibitum
	Water:	Municipal tap water, ad libitum
	Housing:	Animals were reaged individually in suspended stainless steel
	×.	wiremesh cages.
	*Environment	S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur- Orge, France), <i>addibitum</i> Municipal tap water, <i>ad libitum</i> Animals were caged individually in suspended stainless steel wire mesh cages. al conditions: 90-24°C 40-70% Approximately 10-15 air changes per hour Alternating 12-hour light and dark cycles (7 am- 7 pm)
	I emperature	
	Air chorecos	Approximately 10-15 air changes per hour
	All Changes.	Alternating 12-hour light and dark cycles (7 am- 7 pm)
р.	Stay ucash	A XY
1. 1	a life dates:	Start of treatment to final sacrifice for the subgroup 1 animals: 14 November – 19
- La	a life dates:	November 2008
	Ĉ	
		Start of treatment to final sacrifice for the subgroup 2 animals: 16 November $-21$ November 2008
		November 2006



#### 2. Animal assignment and treatment

The dose level for AE C656948 was set after evaluation of the results of a 28-day toxicity study (see section 5.3.1) and a 90-day toxicity study (see section 5.3.2) in the mouse with this substance. In the 28day study, 5000 ppm clearly exceeded the maximum tolerated dose as all males and most females had to be sacrificed for humane reasons before the end of the study. In the 90-day study slight to study was seen at the highest dose tested, 1000 ppm. Therefore, the dose level of 2000 ppm was selected as an appropriate high dose level to be used in this study. Although, 2000 ppm was higher than the high dose used in the mouse carcinogenicity study (750 ppm), this dose level was selected to allow a clearer evaluation of the effects of AE C656948 without causing excessive to within Males only were used in this study as an increased incidence of thyroid follicular cell adenoma was observed in this sex only. In O addition, 2000 ppm AE C656948 was the dose level selected on other in-vivo mechanistic studies in the mouse. Phenobarbital was administered once daily for 4 days by oral gavage to a group of 8 males at a dose level of 80 mg/kg/day in 0.5% aqueous solution of methycellulose 400. The dose level was selected on the basis of previous studies conducted with pheneparbital and was expected to provoke a positive response. A negative control group received untreated diet alone during the study period.

Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Hearth Service, National Justitute of Hearth, NHA publication N°86-23, revised 1985) and "Le Guide the Journal Officiel des Communautés Européennes L958, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

1 able 5.5-116	Study design			¥ &
Test group	Treatment 🔬	Dose Level	Duration of 🖉	Animals assigned
	ÇQ Ö ^v		treatment	L.
1	Control diet		5 [€] 4 days [€] 8	8
2	AGE C656948	2000 ppm	O 4 days	8
3	henobarbital	%80 mg/kg/day	C 4 days	8

The stability of AE C656948 in the deet at 20 and 10000 ppm has been demonstrated in a previous study after a freezing period of 95 days followed by storage periods at room temperature of 10 days or 105 days. Before the start of the study homogeneity and concentration were checked on the study mix at 2000 ppm The mean value obtained from the homogeneity check was taken as measured concentration. The study mix was found to be acceptable for use on the study as the homogeneity and concentration checks were within the range of 92 to 98% of the nominal concentration. The stability of Phenobarbital in aqueous methyleellulose at 8 2 has been demonstrated in a previous study after a storage period of 29 days. Before the start of the study homogeneity and concentration were checked on the study formulation at 8 g/l. The mean value obtained from the homogeneity check was taken as measured concentration. The study formulation was found to be acceptable for use on the study as the homogeneity and conceptration checks were 99% of the nominal concentration.

# 3. Diet and water

Certified rodent powder and iradiated diet 04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Acey, France) and filtered and softened tap water from the municipal water supply in individual bottles were available addibitum until Day 5. On transfer to the radioactive suite on Day 5, animals were given certified rodent perfeted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animal Food and Engineering Augy, France). The water was supplied via water bottles. Animals were not dief fasted overnight prior to blood sampling and final sacrifice. Routine analyses of food and water indicated that there was no contamination which could have compromised the study.

# 4. Statistics

# Body weight

Mean and standard deviation were calculated for each group.

A



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The F test was performed to compare the homogeneity of group variances.

If the F test was not significant (p>0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ( $p \le 0.05$ ), data were transformed using the log transformation. If the F test on log transformed data was not significant (p>0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data,

If the F test was significant (p≤0.05) even after log transformation, the mean of the exposed group compared to the mean of the control group using the modified t-test (2-gided).

If one or more group variance(s) equal 0, means were compared using non-parametric Mann test (2-sided).

Group means will be compared at the 5% and 1% evels of significance.

Statistical analyses were carried out using Path Tox System 34.2.2 (Module Enhanced Statis 0 V

### Radioactivity measurements in the blood

ere conducted on the averaged For each animal, 2 replicated values were measured. Statistical analysis values calculated per animal from the preplicate values.

### Comparison between the subgroup 1 and subgroup 2

In a first step, mean of the subgroup 1 was compared to the mean of the subgroup per time point and for each group by the use of the t-test when the subgroup variances were homogeneous or by the use of the modified t-test when the subgroup variances were not homogeneous. Test sperformed were 2-sided.

Results indicated that for each group, the mean of the subgroup 1 was not statistically different from the mean of the subgroup 2 per time point? Consequently animals of the 2 subgroups were pooled for further analysis.

# Comparison between the confrol and the AF C656948 treated group

Statistical comparison were performed performed point and on proved data of subgroups 1 and 2.

Group variances were compared by the use of the F test (2-sided).

When the F test was not significant (p>0.05), means were compared using the t-test (1-sided).

When the F test was significant (p\$0.05) means were compared using the modified t-test (1-sided).

### Comparison between the control and the Phenobarbital greated group

Statistical comparisons were performed performed performed point and on pooled data of subgroups 1 and 2.

Group variances were compared by the use of the F test (2-sided).

When the F test was not significant (p>0.05) means were compared using the t-test (1-sided).

When the F test was significant (p20.05), means were compared using the modified t-test (1-sided). Group means were compared at the 5% and 4% levels of significance.

casied out using SAS programs (Version 8.2). Statistical and yses were

#### Methods С.

# 1. Observations

The mimals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.



### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, then on Study Days 1 and 3?

#### 3. Food consumption

Food consumption was not recorded.

### 4. Intravenous injection of ¹²⁵I-Thyroxine

¹²⁵I-Thyroxine solution (¹²⁵I-T4, Nem Life Sciences, France) was diluted with 0.9 % sterile saline solution to provide a solution containing 4  $\mu$ Ci/ml. The certificate of malysis of the stock solution of ¹²⁵I-Thyroxine is presented in Attachment 2 in the study report. Each animal received on Day 5 by intravenous injection via the tail 250  $\mu$ l of the diluted 25I-Thyroxine solution corresponding to 1  $\mu$ C of ¹²⁵I-Thyroxine per animal. Approximately 3 hours after the intravenous injection of ¹²⁵I-Thyroxine, each animal received 0.1 mg of NaI in 250  $\mu$ l of 0.9 % sterile saline by intraperitoneal mjection. Dilution of the ¹²⁵I-Thyroxine solution and preparation of the ^{NaI} solution was carried out on the day of administration.

## 5. Blood sampling:

Animals were not diet fasted prior to blood conection

A whole blood sample was collected from the retro-orbital ventus plexus of each surviving animal. Whole blood samples were placed into tubes and the radioactivity were measured. Prior to blood sampling animals were anesthetized with Isoflarane (Baxter Maurebas, France). Samples were taken at 40 minutes, 1.5, 4 and 24 hours after the dosing with the ¹²4-Thy axine.

## 6. Measurement of radioactivity

Two aliquots of 50 µl of each whole-blood sample, were dispensed directly into tubes for radioactivity measurement. The radioativity (¹²⁵) was measured at the same tone in all the brood samples generated both in subgroup 1 and subgroup Sanimals.

Radioativity (125) were measured using a Cobra gamma scipfillation counter (Packard).

Radioactivity measurement was expressed as follows:

Whole blood radioactive content

Data from animals belonging to both subgroups and were combined and an overall mean and standard deviation derived for each treatment group.

# II. Results and discussion

### A. Observations

### 1. Mortality

There was no mortality in any group during the 4 day treatment period. On Day 5, one animal from the Phenobarbital treated group was sacrificed due to a technical problem with the intravenous injection of the ¹⁵I-Thyroxine solution.

### 2. Clinical signs

There were the clinical signs observed.

# B. Body weight and body weight gain

After é days prexposire, no statistically significant effect on mean body weight was recorded either in the AE C656948 of Phenobarbital treated animals when compared to the controls.



#### C. T4 clearance

The data presented in the table below represent the combined mean and standard deviation derived from both subgroups of animals for each treatment level at each time point. It can be estimated; knowing that the circulating blood volume in a mouse is approximately 80 ml/kg, that at time point 0, the anyount of radioactivity in 50 µl of blood of all the animals was approximately 40000 cpms

Table 5.5-117	Whole blood radioactivity after	a single administr	ation of ²⁵ I-Th	yroxine	⁄Iean∳⊕″	
	Standard deviation (cpm)	- &.	A A	\$ \$	°~°	ŝ

Timepoint	Control	XE C656948 (2000	Phenobar Stal (80
_		, ppm) ^{O♥}	w mg/kg/day) v v
Number of animals	8	<b>8</b> Q ~ °	
Time point 40 min	19726 ± 1468	6163** ± 2025	^Q 1 <b>0</b> ¹ 1 ¹ ¹ ¹ ¹ ¹ ¹ ¹ ¹
Time point 1h 30 min	16930 ± 1004	° 63 <b>85</b> ** ±≪1982 √	0592** ± 1245
Time point 4h	$13781 \pm 1099^{\circ}$	6111**#1304	9312**±1330 °
Time point 24h	3889 # 561	2562 ** ± 482	2653**£547 Ø
** p≤0.01			

The results show after an intravenous injection of ¹²⁵I-Thyroxine there was estatistically significantly lower level of radioactivity present in the blood of animals treated with AF 2656948, at all time points, when compared with the controls. This decrease is considered a reflect a more rapid clearance of Thyroxine in AE C656948 treated animals over a 24 hour period, when comparec with the controls.

Similarly, a decrease of the radioactivity level was also observed in animal streated with Phenobarbital, a reference compound known to induce an increase in Thyroxine clearance on the mouse through induction of Thyroxine glucebonidation. O  $\bigcirc$ 

Overall these results indicate that the Dryroxine cleanance was increased in the AE C656948 treated

### D. Deficiencies

No specific deficiencies were noted in this study

# onclusions

In conclusion, the results indicate that the clearance of hyroxine from the blood of animals treated with 2000 ppm AE Co56948 was increased when compared to control animals over a 24 hour period following ¹²⁵I administration. In addition, the response observed with AE C656948 treated animals was similar to that noted in an invals treated with 80 mg/kg/day phenobarbital.

### Assessment and conclusion by applicant:

Å

Study meets the current guidance and the Pequirements in 283/2013 as it provides mechanistic information of AE C656948

In conclusion, the results indicate that the clearance of thyroxine from the blood of animals treated with 2000 ppm SE C659948 was increased when compared to control animals over a 24 hour period following ¹²⁵ Administration In addition, the response observed with AE C656948 treated animals was similar that noted in animals treated with 80 mg/kg/day phenobarbital. n



Data Point:	KCA 5.5/15
Report Author:	
Report Year:	2011
Report Title:	Fluopyram: Mechanistic 3-day toxicity study in the mouse by oral gavage
	(thyroid hormone investigations)
Report No:	SA 10241
Document No:	<u>M-408352-01-1</u>
Guideline(s) followed in	No specific guideline
study:	
Deviations from current	Current guideline: none
test guideline:	
Previous evaluation:	Not previously evaluated
GLP/Officially	No, not conducted under GEP/Officially recognised testing/facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes of the second secon

### **Executive Summary**

Fluopyram, a fungicide of the pyramide family (batch number: Mix-batch: 08528/0002: Light beige solid, 94.7% purity), was administered continuously by oral gavage to 2 groups of 15 male C57BL/6J mice for 3 days at 100 and 300 mg/kg body weight/day. Phenobarbitat batch number: 06160228: White powder, 99.6% purity) was administered continuously by oral gavage to one group of 15 male C57BL/6J mice for 3 days at 80 mg/kg body weight/day. A similarly constituted group of 15 males received the vehicle alone (0.5% methylcellulose) and acted as a control group. Chincal signs were recorded daily and body weight was measured on Day 1 and Day 3. A detailed physical examination was performed once during the treatment period. On study Day 4, before necropsy, blood samples were taken for hormone levels measurement (T4 and TSH). The call bladder was taken and the bile was collected for T4 level measurement. The pituitary gland was sampled for qPCR analysis of the Tsh transcript.

There were no nortalities and no treatment-related findings in term of chinical signs and body weight parameters during the cours of the study and the study of t

In the Fluopyram treated animals, after 1 days of exposure, at 100 and 300 mg/kg/day, mean T4 levels in the plasma were decreased (26% and -34%, p<0(01) whits mean TSH levels in the plasma were not changed when compared to controls. An increase in the levels of Tsh transcript (+21% n.s.; +49%, p<0.01) were observed in the piturtary gland.

In the Phenobarbital treated animals after 3 days exposure at 80 mg/kg/day, mean T4 levels in the plasma were decrease (-38%, p<0.01), whilst mean TSIP levels in the plasma were not changed when compared to the controls on increase in the levels of Fsh transcript (+46%, p<0.01) was observed in the pituitary gland.

In conclusion, this study demonstrates that Fh@pyram administration by oral gavage at nominal concentrations of 100 and 300 mg/kg/day, in the C57BL/6J mouse for 3 days, induced a decrease in plasma T4 levels associated with an increase in the levels of Tsh transcript (beta subunit) in the pituitary gland, whereas no increase of plasma TSH by vels were detected.

aterials and methods I.

A. Materials 1. Test material; **Description** Lot ØBatch #: **Purity:** CAS#

Fluopyram AE C656948 Beige powder Mix-Batch:08528/0002 94.7% 658066-35-4



Stable in 0.5% aqueous solution of methylcellulose for a period **Stability of test compound:** covering the study duration Vehicle for AE C656948: methylcellulose 2. Vehicle and / or positive Positive control: Phenobarbital control: Vehicle for Phenobarbital: methylcellulose methylcethulose for a period & Phenobarbital **Positive control:** White crystalline powder **Description:** Lot / Batch : 06100228 99.6% **Purity:** 50-06-6 CAS: Stable in 0.5% agreeous solution of **Stability of test compound:** covering the study duration  $\sqrt{2}$ 3. Test animals: **Species:** Mouse - Male only Strain: C57BL/6 Age: 8 weeks approximately treatment Weight at dosing: 21.1 to 25.7 g Source: **Acclimation period:** Qlays, 🖗 Ś Õ Certified rodent powdered and instituted thet AOAC-1991 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Diet: Otge, France), an libitan 0 Water: Municipal tap water, ad libitum Animals were caged individually in suspended stainless steel **Housing:** wife mesh cages Environmental conditions: **Temperature: Humidity:** 40©7760% 5 ar changes per hour Air changes Approximately hour light and dark cycles (7 am- 7 pm) Photoperiod lternating B. Study design Dosing period: 27 September 1. In life dates: eptember 2010 2. Animal assignment and treatment The dose levels for Fluopyram were strafter evaluation of the results from previous studies conducted with Fluopyram and following discussions with the US (FA) and Canadian (PMRA) authorities. The dose level of 00 mg/kg/day is equivalent to the top dose level (750 ppm) used in the mouse cancer bioassay in which thyroid tumes were observed in the males (SA 05094) and the dose level of 300 mg/kg/dawis equivalent to the dose level used in previous mouse mechanistic studies conducted with Fluopyram (SA 07215/SA 08151 and SA 08288) Owhere thyroid hormone changes were detected.

The dose level of 80 mg/kg/day for Phenobarbital is the dose level used in previous mouse mechanistic studies conducted with Phenobarbital where thyroid changes were detected (SA 07326, SA 08151 and SA 08288).

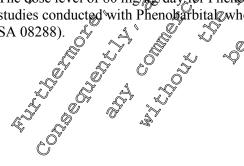




Table 5.5-118	Study design
---------------	--------------

Group	Test substance	Dose level (mg/kg/day)	Number of animals per group	Anin ident	
		Males		Subgroup	Subgroup 2
1	Control	0	15	UT1M4180 to 4186	× 4194
2		100	15 🐨	UT2)44195 to 4201	UT2014202 6 0
3	Fluopyram	300	A45	101 3M4240 to 4216	UT3MQ217 to C
4	Phenobarbital	80		UTAM422500 4230	UT4M4232 to 4239

#### 3. Dosing preparation and analysis

Fluopyram and Phenobarbital were suspended in 0.5% aqueous solution of methodicellulose to provide the required concentration. There was one preparation of the test formulation for the entire study. When not in use the formulations were stoted at approximately  $\frac{4}{2}$  °C.

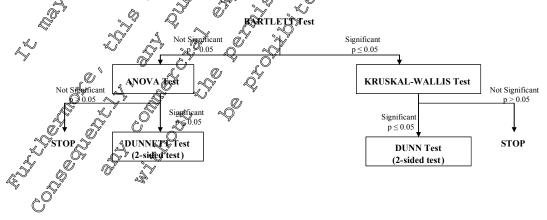
Ras been demonstrated in aprevious study (SA 04060), The stability of Fluopyram at 10 g/l and 30 g/l which covers the period of storage and usage for the current study Ô Ò

which covers the period of storage and usage for the current study.
The stability of Phenobarbital at 8 g/l has been demonstrated in a previous study (SA 03310), which covers the period of storage and usage for the current study.
4. Statistics
Variables analyzed
Body weight parameters in the plasma
Hormonal parameters in the bile
Statistical methods
Mean and standard deviation were calculated for each group.

Mean and standard deviation were calculated for each group

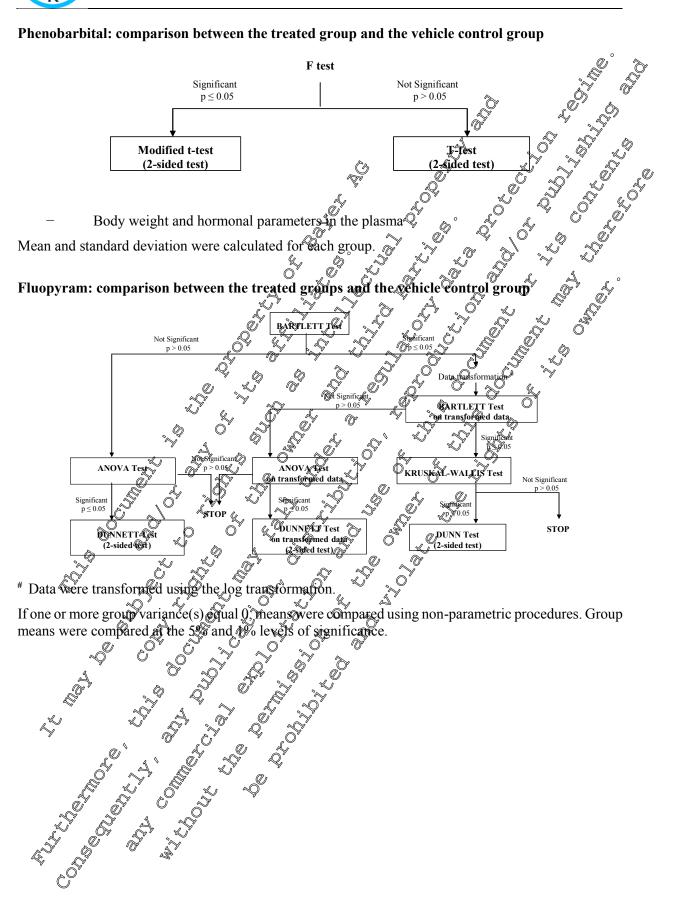
Body weight change parameters

ameters, of of of the vehicle control group Fluopyram: comparison betw





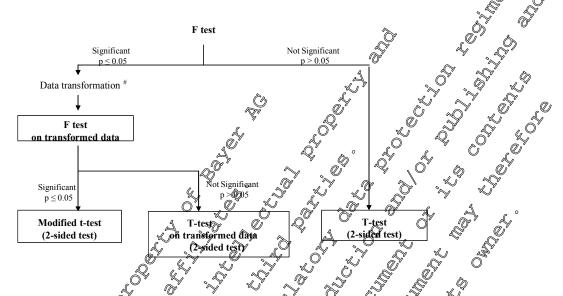
#### Phenobarbital: comparison between the treated group and the vehicle control group





#### Phenobarbital: comparison between the treated group and the vehicle control group

Mean and standard deviation will be calculated for each group



[#] Data were transformed using the Qog transformation . F

Hormonal parameters in the bile

T-tests were used for the comparison of T4bile content on treated groups compared to control group. Group means were compared at the 5% and 1% levels of significance.

Statistical analyses were carried our using Path Fox System \$4.2.2 (Module Enhanced Statistics) except the hormona@neasurements in the bile performed using Microsoft Excel.

### C. Methods

# 1. Observations

The animal were observed twice daily for more undity and mortality (once daily on weekends or public holidays Observed clipical signs were recorded at feast once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any plinical signs were recorded.

### 2. Body weight

All animals were weighed a least once during the acclimatization phase. Each animal was weighed on the first day of test substance administration, then on Day 3.

Ô

# 3. Hormonal investigation

# **Blood** sampling

On the day of saprifice, between 8:30 a.m. and 11 a.m., blood samples were taken from all animals

from the abdominal aorta, the blood sampling for each animal was performed precisely 24h after the last dose. Animals were mesthetized by inhalation of Isoflurane (Baxter, Maurepas, France) prior to bleeding Blood was collected on lithium heparin. The resulting plasma samples were stored frozen at approximately \$80°C before shipment in dry ice to the subcontracted laboratory for hormone level evaluation.

# Hormone measurements

Plasma was prepared from each blood sample and kept frozen at approximately -80°C until the determination of TSH and T4 hormone levels with specific radio-immunoassay kits (supplied by



Amersham for TSH and by DIASORIN for T4). This measurement was subcontracted at "Laboratoire d'endocrinologie clinique et toxicologique – 1, Avenue Bourgelat, Marcy l'Etoile" under the of the Principal Investigator A.S. Le Hen.

#### 4. Sacrifice and pathology

#### Necropsy procedure

On Study day 4, all designated animals from all groups were sacrificed. All sacrifices were performed by exsanguination under deep anesthesia (inhalation of Isoflurane, Baxter, Maurepas, France). Animals were not diet fasted overnight prior to sacrifice. All animals were necepsied The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled except upondecision of the Study Patrologist.

#### **Tissue collection**

A piece of the median and left liver lobes of § animals from each group as well as the thyroid gland (with parathyroid glands) and the pituitary gland of all the animals were sampled. The gall bladtler from each animal was collected and pooled in each subgroup by 3 or 4 in eppendorf tubes. The tubes were centrifuged (10000 rpm for 10 minutes) and the supernatants consisting of falle, were collected for T4 level measurements.

#### 5. :QPCR analysis

### **Total RNA purification**

Total cytoplasmic RNA was isolated from the pituitary gland of adividual control and treated animals using RNeasy Mini kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agrient Technologies).

### Quantitative PCR

Ten µg of total RNA was used for Reverse transcription (RT) asing a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in suplicate using Taqman assays (Assay on demand, Applied Biosystems), 50 diluted first strand cDNA, AmpliTag Gold® PCR Master Mix on an ABI prism 7900 H@ machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA. Actin was selected as reference gene for the manitative calculations of transcripts. The relative quantity (RQ) value of beta subunit. Bh transcript (Tsh be was calculated using the following

formula:

# $\Delta\Delta Ct = (Cttest - CtAction treated (Cttest - CtAction) control$

### $RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. Each RQ value obtained for ogiven gene was normalized by dividing by the RQ value obtained for the control animal UTFM4180.

- II. Results and discussion
- A. Observations
- 1. Mortahty

There was no mortality during the course of the study

# 2. Clinical signs of

There we no treatment-related clinical signs throughout the duration of the study.



#### B. Body weight

There was no treatment-related effect on body weight parameters.

#### C. Hormone analysis

The magnitude of the changes in the plasma hormone values relative to the controls and catistical significance between treated and control values were as follows:

T4

A statistically significant dose-related decrease in mean T4 levels (26% and -37% p2%01) (37% p3% observed in the plasma of Fluopyram treated animals. A statistically significant decreases in mean T4 related animats.

#### TSH

No statistically significant change in mean TSH levels was observ

# Table 5.5-119 Mean levels of hormones T4 and TSH hormone after exposure to Flue pyrant or phenobarbital

Group	Mean plasma hormone values ± standard deviation (% Change 5 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
	Compared to control mean values S & Q
	Control 🗸 Fluopyran 🦉 Fluopyran 🧐 Phenobarbital
	100 mg/kg/day 300 mg/kg/day
T4	$24.2 \pm 9.7$ $254^{**} \pm 6.1 (-26\%)$ $22.6^{**} \pm 4.6 (-34\%)$ $27.3^{**} \pm 3.1 (-38\%)$
(nmol/l)	
TSH	
(ng/ml)	
**. Tl	a manufal single for the top of the second for the

**: The group mean is significantly different from the control at  $p \le 0.01^{\circ}$ 

In the bile, for the prean data, the magnitude of the changes relative to the controls and statistical significance between treated and control values were as follows:

T4

No statistically significant change in mean T4 levels was observed in the bile of fluopyram treated animals.

An increase of T4 levels in the bile of phenobarbital treated animals was anticipated as a consequence of an increase of clearance of T4 Paradoxically a statistically significant decrease in mean T4 levels (-19%,  $p \le 0.04$ ) was observed in the bile of phenobarbital treated animals. This difference was most likely due to some technical reasons and was constalered not to be a treatment related effect.

# Table 5.5-120 Mean field of A 4 in the bile after exposure to Fluopyram or phenobarbital

Group	Mean T4 values	s in the bits ± standard of mean	leviation (% change con 1 values)	npared to control
Â			Males	
	Control	2 Fluopyram 100 mg/kg/day	3 Fluopyram 300 mg/kg/day	4 Phenobarbital 80 mg/kg/day
T4 0	79.2 ± 7.9	$79.0 \pm 6.1$	83.9 ± 8.5	64.5** ± 4.4 (-19%)

**: The group mean is significantly different from the control at  $p \le 0.01$ 



#### **D. QPCR** analysis

A statistical significant increase of the level of accumulation of Tsh transcript (+49%, p<0.01) was observed in the pituitary gland of Fluopyram treated animals. A statistical significant increase of the level of accumulation of Tsh transcript (+46%, p<0.01) was observed in the pituitary grand of Phenobarbital treated animals.

Table 5.5-121	Tsh b transcript levels after exposure to fluopyram or phenobarbital	4
1 abic 5.5-121	Is b transcript levels after exposure to huopyram or phenobaronal	-

Gene transcript	Mea		andard deviation of gene tr ed to control mean values)	
	Control	2 Fluopyram 100 mg/kg/day 🛛 🔍	Fluopyrach 300 mg/kg/day	Phenobarbital 80 mg/kg/day
Tsh b [#]	$0.994 \pm 0.124$	1.138 ± 0.230 (+21%)	1.40 ^{4**} ±0.253 (*49%) ×	
# Beta subunit tsh	n **: p≤0.01	2	<u>r v Q , v</u>	

 E. Deficiencies
 No specific deficiencies were noted in this study
 III. Conclusions
 In the fluopyram treated animals, after 3 days of exposure at 100 and 300 mg/kg day, mean T4 levels in the plasma were decreased (-26%) and -30%, p<0.01) whilst mean tst levels in the plasma were not changed when compared to controls. An increase in the levels of TSH transcript (+21% n.s.; +49%, p<0.01) were observed in the pituitary gland.

In the phenobarbital treated mimals after 3 day exposure at 80 mg/kg/day mean T4 levels in the plasma were decreased (-38%, p<01), whilst mean TSH levels in the plasma were not changed when compared to the controls An increase in the levels of 1sh transcript (+46%, p<0.01) was observed in the pituitary gland.

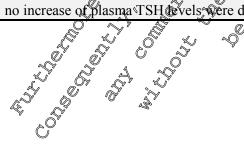
In conclusion, this study demonstrates that floopyram administration by oral gavage at nominal concentrations of 100 and 300 mg/kg/day, in the C57BL/64 mouse for 3 days, induced a decrease in plasma Te levels associated with an increase in the fevels of tsh transcript (beta subunit) in the pituitary gland, whereas no inecease of plasma TSHOevels were detected

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# Assessment and conclusion by applicant

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on AE C656948 in the mouse. ×

This study demonstrates that flugpyram administration by oral gavage at nominal concentrations of 100 and 300 mg/kg/day on the C57BL05J mouse for 3 days, induced a decrease in plasma T4 levels associated with an increase in the levels of tsh transcript (beta subunit) in the pituitary gland, whereas no increase of plasma TSH devels were detected.





Data Point:	KCA 5.5/16
Report Author:	
Report Year:	2012
Report Title:	Fluopyram: Mechanistic 3-day toxicity study in the mouse by oral gavage 🚿 🖉
	(thyroid hormone investigations)
Report No:	SA 10430
Document No:	<u>M-426994-01-1</u>
Guideline(s) followed in	No specific guideline
study:	
Deviations from current	Current guideline: none
test guideline:	
Previous evaluation:	Not previously evaluated of A S
GLP/Officially	No, not conducted under GEP/Officially recognised testing facilities
recognised testing	No, not conducted under GEP/Officially recognised testing/facilities
facilities:	
Acceptability/Reliability:	Yes O Q X Y Q A

### **Executive Summary**

The objective of this study was to investigate the mode of action by which thropyrain gives rise to thyroid tumors in the male mouse following chronic exposure. The effects of different dose levels of fluopyram (including the dose level which induced throad throad tumors) on the plasma levels of thyroxine (T4) and thyroid stimulating hormone (TSH) were determined following faily ball gavage for 3 days. Four time points were evaluated 2h, 8h, 14 cand 48h after the last gavage administration.

Fluopyram, a fungicide of the pyramide family (batch number: Mix-batch, 08528,0002: Light beige solid, 94.7% purity), was administered daily by oral gavage for 3 days to groups of 15 male C57BL/6J mice, at dose levels of 400 of 300 mg/kg body weight/day of fluopyram. T4 and TSH hormone levels were examined at four time points th, 8h 44h and 48h after the last gavage administration. One group from each dose level was sacrificed at each time point. Four similarly constituted groups of 15 males received the vehicle alone (0.5% methylcelfulose) and acted as controls. Clinical signs were recorded daily and body weight was measured on Day 1. A detailed physical examination was performed once during the treatment period. On the day of sacrifice, before necropsy, blood samples were taken for hormone tevel measurements (T4 and fSH).

There were no mortalities and no treatment related findings in term of clinical signs during the course of the study.

In the fluopyram freated animals, after 3 days of exposure, at 100 or 300 mg/kg/day, where animals were sacrificed 2h &h, 140 and 48h after the flast dose, mean T4 levels in the plasma were statistically significantly ( $p\leq0.01$ ) decreased by between -1&% and 41%. The magnitude of the response occurred in a dose-related manner at all time-points apart from the 2h time point. Mean TSH levels in the plasma were not changed when compared to controls at any of the time points investigated.

In conclusion, this study demonstrates that fluoryram administration to C57BL/6J male mice by oral gavage for three days at concentrations similate or above the top dose level administered in the mouse cancer bioassay induced a statisticatly significant decrease in plasma T4 levels at all the time points examined. However, no charge in plasma TSH level was detected in this short term assay.

I. Materials and methods

Materia

1. Test material: Description Lot / Batch #: Purity:

Fluopyram (AE C656948) Beige powder Mix-Batch:08528/0002 94.7%



CAS #	658066-35-4
	Stable in 0.5% aqueous solution of methylcellulose for a period.
Stability of test compound:	covering the study duration
2. Vehicle and / or positive	covering the study duration 0.5% aqueous solution of methylcellulose (vehicle)
control:	0.5% aqueous solution of methylcellulose (vehicle) Mouse - Male only C57BL/6J 8 weeks approximately at start of treatment 19.2 to 25.2 g
3. Test animals:	
Species:	Mouse - Male only
Strain:	C57BL/6J
Age:	8 weeks approximately at start of treatment $\sqrt{2}$
Weight at dosing:	19.2 to 25.2 g
Source:	
Acclimation period:	
	Certified rodent powdered and irradiated Bet AOAC-10P1 from
Diet:	S.A.F.E. (Scientific Animal Food and Engineering, Epinay, Sur-
	Orge, France), al libitium
Water:	Municipal tap water ad libitit of the second
Housing:	Animals were caged individually in suspended stainless steel with mesh cages
e	with mesh cages in the second se
Environmental conditions:	
Temperature:	20-248° ''' ''' ''''''''''''''''''''''''''''
Humidity:	40-70% is in the second sec
Air changes:	Approximately 10-15 ar charges perhour &
Photoperiod:	Alternating 12-hour light and dark@ycles (7 am- $\mathcal{O}$ pm)
B. Study design 🔬 Ö	Animals were caged individually in suspended stainless steel 20-24 40-70% Approximately 40-15 ar changes per hour Alternating 12-hour light and darkaycles (7 am- 0 pm) rigd: 15 fanuary 20 January 2011
1. In life dates: Dosite pe	rigd: 15 Anuary 20 January 2011
2. Animal assignment and treated	

The dose levels for fluoporam were set after evaluation of the results from previous studies conducted with fluopyram and following discussions with the US (EPA) and Canadian (PMRA) authorities. The dose level of 000 mg/kg/day is equivalent/to the top dose level (750cppm) used in the mouse cancer bioassay in which thyroid tumors were observed in the males ( $M_{-2}$  5688-01-1) and the dose level of 300 mg/kg/day is equivalent to the dose level (2000 ppm) used in previous mouse mechanistic studies conducted with fluop ram ( $M_{-2}$  9522-06-1,  $M_{-2}$  968073-01-1 and  $M_{-3}$  28662-01-1), where thyroid hormone changes were detected.

Table 5.5-1	122 Study design	ĭ _s o			
Group	A Test substance	Time point F	Bose level mg/kg/day)	Number of animals per group	Animal identity
		′~~	, Males		
Ay I	Control	_~2h ≪		15	UT1M5032 to 5046
2		2h@	100	15	UT2M5047 to 5061
3	fluopyram	2h@ 2r	300	15	UT3M5062 to 5076
4		🔊 8h 🐔	0	15	UT4M5077 to 5091
5		للا 8h	100	15	UT5M5092 to 5106
6		8h	300	15	UT6M5107 to 5121
L. P	Control 2	14h	0	15	UT7M5122 to 5136
8	Elucation	14h	100	15	UT8M5137 to 5151
9	Fluopyram	14h	300	15	UT9M5152 to 5166



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Group	Test substance	Time point	Dose level (mg/kg/day)	Number of animals per group	Animal identity
			Males		
10	Control	48h	0	15	UTXM5 67 to 5481
11		48h	100	15	UTOM518270
12	Fluopyram	48h	300		UTDM9197 to 211

### 3. Dosing preparation and analysis

Fluopyram was suspended in 0.5% aqueous solution of methylcelfulose to provide the required concentrations. There was one preparation of the test formulations for the entire study. When not in use Š the formulations were stored at approximatel  $\mathbb{Q}^{+}4^{\circ}\mathbb{C}^{\mathbb{Q}}$ Ø

The stability of Fluopyram at 10 g/l and 30 g/l has been demonstrated in a previous study (\$A 04(60), which covers the period of storage and trage for the current study. , ...uy (SA 04) which covers the period of storage and usage for the current study

#### 4. Statistics

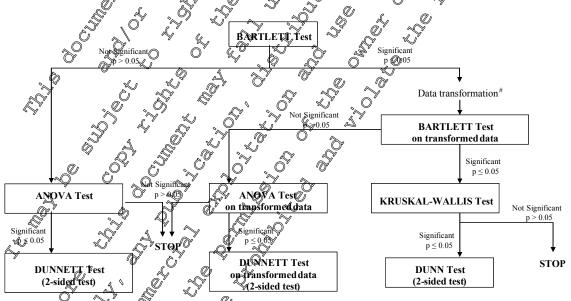
Variables analyzed

- Hormonal parameters in the plasma

Statistical methods

O Mean and standard deviation were Galculated for each group.

Fluopyram: comparison beforeen the treated groups and the ehicle control group



Data were transformed using the bg transformation. #

If one or more group variance equal 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance.

Statistical malyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).



#### C. Methods

#### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animats and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset severity, reversibility, and duration of any clinical signs were recorded.

#### 2. Body weight

All animals were weighed at least once during the acclimatization phase Each animal was weighed only a on the first day of test substance administration.

#### 3. Hormonal investigation

#### **Blood sampling**

On the day of sacrifice, between 8:30 a.m. and 1 a.m., blood samples were taken from all animals from the abdominal aorta. The blood sampling for each animal was performed precisely 2h/8h, 14r and 48h after the last dose. Animals were anestherized by inhalation of Isofterane (Baxter, Maurepas, France) prior to bleeding. Blood was collected on lithium herarin. The reculting plasma samples were stored frozen at approximately -74°C +10°C before shipment in dry ise to the subcontract of laboratory for hormone level evaluation.

#### Hormone measurements

Plasma was prepared from each blood sample and kept frozen at approximately -74°C+10°C until the determination of TSH and T4 hormone level with specific radio immunoassa kits (supplied by Amersham for TSH and by DIASORIN for T4). This measurement was subcontracted at "Laboratoire d'endocrinologie clinique et toxicol gique 01, Acenue Bourgetat, Marcy 12Foile" under the of the Principal Investigator A.S. Le Henc

# II. Results and discussion

A. Observations

1. Mortality

There was no mortality during the course of the study

# 2. Clinical signs ๙

There were no treatment-related clinical signs throughout the duration of the study.

# B. Hormone analysis

The magnitude of the changes in the plasma horm the values relative to the controls and statistical significance between treated and control values were as follows:

T4 ____

A statistically significant dose telated decrease in mean T4 levels (between -18% and -41%;  $p \le 0.01$ ) was observed in the plasma of fluory ram treated animals at all time points investigated apart from the 2h time point where the decrease was not dose-related.

### TSH

No statistically significant change in mean TSH levels was observed at any time point.



Time Point	Hormone	Control	Fluopyram (100 mg/kg/day)	Fluopyram (300 0° mg/kg/day)
			Males	
	T4	31.5 ±	22.8**± 6.1 (-28%)	240°*± 6.3 (-24%)
2h	(nmol/l)	9.1		
	TSH (ng/ml)	$2.7\pm0.5$	2.6 ± 0.5 (-1%)	$(3.7 \pm 0.5)$ ( $(4.1\%)$ ) $(4.1\%)$
	T4	38.2 ±	$28.8^{**} \pm 5.4 (-25\%)$	22.4** <b>4</b> .1 (- <b>4</b> )%)
8h	(nmol/l)	7.0	a di sa	
	TSH (ng/ml)	$2.8 \pm 0.6$	3.0 ± 0.577%)	3Q ⁴ ±0.70 ⁴ 13%
	T4	25.5 ±	20.9*** 4.5 (28%)	18.6*** 4.3 (-27%)
14h	(nmol/l)	5.3		
	TSH (ng/ml)	$3.1 \pm 0.4$		$3.1 \pm 0.5 (+2\%)$
	T4	34.5 ±	25 4** ± 6 (-26%)	C 24 * ± 4 (-30%)
48h	(nmol/l)	8.5		C 24 C* ± 4 C (-30%)
	TSH (ng/ml)	3.1 ± 0.6	3 2 + 9 5 (+29)	

Table 5.5-123:	Mean levels of hormones T4 and TSH hormone after exposure to fluopyram
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The group mean is significantly different from the pontrol at p 

**: the group mean is significantly different from the control at p<0.0

#### C. Deficiencies

no specific deficiencies were noted in this study 5

#### III. Conclusions

In the fluopyrate treated animals, after 3 days of exposure, at 100 or 300 mg/kg/day, where animals were sacrificed 2h, 8h, 14h and 48h after the last dose, mean T4O evels on the plasma were statistically significantl% (p≤0.01) decreased by between -08% and -41%. The magnitude of the response occurred in a dosected at all time-points apart from the 26 time point. Mean TSH levels in the plasma were not changed when compared to controls at any of the time points investigated.

In conclusion, this study, demonstrates, that fly opyrar administration to C57BL/6J male mice by oral gavage for three days acconceptrations similar to ocabove the top dose level administered in the mouse cancer bioassay induced a statistically significant decrease in plasma T4 levels at all the time points examined. However, no change in plasma TSH devel was detected in this short term assay.

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# Assessment and conclusion by applicant:

Study meets the current guidance and the Pequirements in 283/2013 as it provides mechanistic information on fluopyram in the mouse.

This study demonstrates that fluopyram administration to C57BL/6J male mice by oral gavage for three days at concentrations subilar to or above the top dose level administered in the mouse cancer bioassay induced asstatistically significant decrease in plasma T4 levels at all the time points examined. However, no change in plasma TSH level was detected in this short term assay.



Data Point:	KCA 5.5/17
Report Author:	
Report Year:	2012, Amended: 22-03-2013
Report Title:	Fluopyram: Mechanistic 28-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations)
Report No:	SA 11105
Document No:	<u>M-428031-02-1</u>
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evalauated
GLP/Officially	Yes, conducted under Gor Officially recognise desting facilities
recognised testing facilities:	
Acceptability/Reliability:	Yes a the open of the

#### **Executive Summary**

The objective of the present study was to investigate the mode of action by which fluopyrate gives rise to thyroid tumors in the male mouse following chronic exposure. The effects of different doses of fluopyram on the plasma levels of Thyroxine (T4) and Thyroid Stanulating Homone (FSH), the gene expression of Tsh in the privitary gland and the hepatic UDP-gluctronosyltransferases (UDPGT) activity were determined following continuous dretary administration for a teast 2% days. In addition, the reversibility of any effects observed at the highest dose level was assessed following a recovery period of one month on untreated control diet. Phenobarbital was administered by oral gavage and was used as a positive control for liver and dryroid effects. The reversibility of any effects induced by phenobarbital was also assessed following a recovery period of one month on untreated control diet.

Fluopyram, a fungicide of the pyramide family (batch number: Mrx-batch: 08528/0002: Beige powder , 94.7% purity, was administered to male C57BG/6J mice continuously for at least 28 days in the diet at dose levels of 0, 30, 75, 150, 600 and 750 ppm. These doses equated to 0, 5, 13, 25,102 and 128 mg/kg/day respectively. A group of mimals dosed with 80 mg/kg/day phenobarbital (batch number: 090500%: White crystalline powder, 100% purey) by gavage acted as a positive control for the parameters investigated in this present study. Each group consisted of 15 male mice with the exception of the control group, the 750 put fluoryram group and the phenobarbital group, where 15 additional males were fed untreated control dier for a further one month to assess the reversibility of changes induced during the 28 day treatment period. Clinical observations were performed daily, body weight and food intake were measured weekly & detailed physical examination was performed once during the acclimatization phase and at teast weekly during the dosing period. Animals were sacrificed either following at least 28 days of treatment or at the end of the recovery phase. All animals were subjected to a neeropsy and the liver was weighed of both sacrifice times, blood samples were taken for hormone analysis (T4 and TSH) and the liver and the pituitary gland from each animal were collected. The pituitary gland, was flash frozen in light ninogen and stored at approximately -74°C + 10°C until used for Tsh transcorpt analyses by quantitative Polymerase Chain Reaction (qPCR). At both sacrifice times, 5 pools of Flivers per group were hopogenized for microsomal preparations in order to determine UDPGT pecific enzyme activities using either bilirubin or T4 as substrate.

# Dosing phases

# Fluepyram,

There were no mortalities or treatment-related clinical signs. There were no changes in body weight parameters or food consumption during the course of the study.



Fluopyram had no effect on terminal body weight but induced treatment-related changes in the liver and thyroid hormonal parameters investigated as follows:

At 30 ppm, a statistically significant decrease (-28%; p≤0.01) in mean T4 levels was recorded at terminal № sacrifice.

At 75 ppm, a statistically significant decrease (-31%; p≤0.01) in mean T4 levels was recorded at terminal sacrifice. At necropsy, a slight but statistically significant increase in mean liver weight relative to body weight (+6%; p<0.05) was recorded.

At 150 ppm, a statistically significant decrease (-25% 20.01) in mean T4 levels was recorded at terminal sacrifice. At necropsy, statistically significant increases in absolute mean liver weight and mean liver weight relative to body weight (+11%; p≤0.01; , p%; p≤0.01 respectively) were recorded, pepatic UDPGT enzymatic activities were marginally, though not statistically significantly, increased (+32%) for bilirubin and +52% for T4 substrates), compared to the controls.

At 600 ppm, a statistically significant decrease (-37%; p≤0.01) in mean T4 levels was recorded at terminal sacrifice. At necropsy, statistically significant increases in absolute mean liver weight and mean liver weight relative to body weight (+27%, p≤0.01 for both parameters) were recorded. Statistically significant increases in hepatic UDPGT enzymatic activities were observed + 39% p < 0.05 for bibrubin and +83%; p≤0.01 for T4 substrates compared to controls. In addition, anite reases in the level of accumulation of Tsh transcript in the pituitary gland was accorded (+43%; p<005).

At 750 ppm, a statistically significant/decrease (-38%; p\$0.01) in mean T4 levels was recorded atterminal sacrifice. At necrossy, statistically significant increases in absolute mean liver weight and mean liver weight relative to body weight (+36%; p≤0.01; +33%; p≤0.01 respectively) were recorded. A statistically significant increase in the hepatic UDPGT-bitrubin enzymatic activity (+48%; p≤0.01) together with a marginal increase of the UDP/9T-T4@nzymatic (+33%) activity were observed. In addition, an increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded (+54%; p≤0.01).

#### Phenobarbital:

One animal treated with phenobarbital was killed for humane reasons and two others were found dead during the first week of treatment. The premature deaths of these animals may be due to the use of a concentrated phenobarbital formulation (16 gr at a dosing volume of 5 ml /kg body weight) being administered during the first week of reatment. All males dosed with phenobarbital displayed reduced motor activity. Body weight and body weight gain were reduced throughout treatment, resulting in a statistically significant 5% ( $p \le p(01)$ ) reduction in body weight and a statistically significant 42% (p≤0.01) reduction in comulative body weight gain by Day 29. A statistically significant decrease (-23%; p≤0.01) in mean 14 levels was recorded at terminal carrifice. At necropsy, statistically significant increases in absolute mean liver weight of mean liver weight relative to body weight (+10%; p≤0.01; +16%; p≤0.01 respectively) were recorded. Nonstatistically significant increases in the hepatic UDPGTbilirubin enzymatic (+42%) and UDPGTT4 enzymatic (+32%) activities were observed. In addition, an increase in the level of acoumulation of Ish transcript in the pituitary gland was recorded (+53%; p≤0∕0⁄1).

**Recovery phase: Fluopyram:** In the maps initially treated with 750 ppm fluopyram, a slight increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded (+12%;  $p \le 0.05$ ) however, the observed increase was much lower than the one observed immediately following treatment with 750 ppm (+54%; p $\leq$ 0.01). All other parameters were comparable with the control group.



#### Phenobarbital:

In the males initially treated with 80 mg/kg/day phenobarbital, body weight was 5% lower than the control group at the start of the recovery phase, the effect being statistically significant ( p < 0.05 By cthe end of the recovery phase, body weight was marginally reduced by 3% the effect was not statisfically significant. All other parameters were comparable with the control group.

In conclusion, this study demonstrates that fluopyram administration at the tumorigenic dose level of 750 ppm for at least 28 days in the C57BL/6J mouse, induced a decrease in plasma T4 levels correlated with an increase in the levels of Tsh transcript (beta subunit) in the pituitary gland, together with an increase in the UDPGT-bilirubin and UDPGT-T4 enzymatic activities on addition, absolute and relative liver weights were significantly increased. These effects were not detected after a four-week recovery phase except for a marginal increase in Tsh transcript level. All these effects occurred in a dose related manner, apart from the decrease in plasma T4 for which there was no dose-related concordance in terms of the magnitude of the response observed. At the low dose of 0 ppm, the only finding was a decrease in plasma T4 levels, the relevance of which is doubtful based on the Jack of clear dose response Materials and methods concordance.

#### I.

#### A. Materials

	Test material:	
1.	Test material:	Fluopyram (AE (\$56945)
	Description	Beige powder of a start of the
	Lot / Batch #:	Fluopyrand (AE (\$56948) Beige powder & Mix-Batch:08528/0002
	Purity: & O	94.7% O [*] ^{*O*} [*] [*] [*]
	CAS#	658066-39-4
	Stability of test compound: 2. Vehicle and for positive control:	Stable in rodeor diet for a period covering the study duration
	2. Vehicle and for positive	Vehi@e for AE C656948: rodent@iet
	control:	Phenobarbital (positive control), vehice methylcellulose
	Positive control:	Phenobarbital X X X
	Description:	White erystalline powder
	Lot / Batch : 🐨 🐇 🔊	Lot No. 09050075
	Purity; 🔬 🔬	
	CASE 6	Vehicle for YE C656948: rodent diet Phenobarbital (positive ontrol), vehicle methylcellulose Phenobarbital White crystalline powder Lot No. 09050075 100% Stable in 0,5% aqueous solution of methylcellulose for a period covering the study duration Mouse - Male only C57BL/6J 8 weeks approximately at start of treatment 18.1 to 24.7 g
	Stability of test Commond	Stable in 0,5% aqueous solution of methylcellulose for a period covering the study duration Mouse - Male only C57BL/6J 8 weeks approximately at start of treatment 18.1 to 24.7 g
	Stability of testscompanies	covering the study duration
3.	Test animals	a the the the the test of test
	Species: Q Q X	Mouse- Male only O
	Strain:	C57BL/6J6
	Age: A	8 Weeks approximately at start of treatment
	Weight at dosing:	C57BL/6J 8 Weeks approximately at start of treatment 18.1 to 24.7 g
	Source:	
	Acclimation period	13 Pp 15 days
		Certific rodent powdered and irradiated diet A04C-10 P1 from
	Diet: 🔏 🔬 💭	S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-
		Orge, France), ad libitum
	Water of of s	Municipal tap water, ad libitum
	Housing:	Animals were caged individually in suspended stainless steel
		wire mesh cages.
L	Weight at dosing:	
	Temperature:	20-24°C
	Humidity:	40-70%
	Air changes:	Approximately 10-15 air changes per hour



#### **Photoperiod:**

Alternating 12-hour light and dark cycles (7 am- 7 pm)

#### B. Study design

**1. In life dates:** Dosing period: 24 May – 23 June 2011

Recovery period: 22 June – 25 July 2011

#### 2. Animal assignment and treatment

Seven groups of male mice were dosed for at least 28 days with either control diet or the appropriate compound by the appropriate route of administration.

Six groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the remaining five groups received fluopyram atome appropriate dietary concentration (30, 75, 150, 600 and 750 ppm) at a constant level. The dose levels for fluopyramwere set after evaluation of the results from previous studies and following discussions with the US (EPA) and Canadian (PMRA) authorities. The top dose level of 50 ppm used in the present study represents the top dose level used in the mouse cancer bioassay in which thyroft tumors were observed in the males (SA 05094).

The seventh group was dosed by oral gavage with 80 mg/k@day phenobarbital suspended in 0.5% aqueous methylcellulose 400 using a dosing volume of 5 ml/kg bodyweight the first week of treatment and then using a dosing volume of 10 ml/kg bodyweight for the remaining treatment period. The volume administered to each mouse was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose was used in previous mouse mechanistic studies conducted with phenobarbital, where thyroid changes were detected (SA 07326, SA 08151, SA 08288).

Each group consisted of 15 male mice with the exception of the control group, the 750 ppm fluopyram group and the phenobarbital group where 15 additional males were feet control or test diet (750 ppm fluopyram) or were grally dosed (80 mg/kg day phenobarbital) for at least 28 days and were then allowed one month (at least 28 days) of recovery during which they were maintained on untreated control diet.

All animals were sacrificed in the morning after the last day of treatment or after the last day of the recovery phase.

¥				
Group	Test substance	Dose level	Normber of animals pergroup	Animal identity
		ົ. ບິ <u>ດັ</u> . Mo	iles 8	
1	Control 2	N N G		T1M1885 to 1914
2		(5 mg/kg/day)	15	T2M1915 to 1929
By By		0 75 9pm (13 mg/kg/d0)	15	T3M1930 to 1944
4	Fluopýrám	(25 mg/kg/day)	15	T4M1945 to 1959
5		600 ppm (102 mg/kg/day)	15	T5M1960 to 1974
		750 ppm (128 mg/kg/day)	15 + 15*	T6M1975 to 2004
7	Phenobarbital	80 mg/kg/day	15 + 15*	T7M2005 to 2034

Table 5.5 724 Study design

Recovery phase



### 3. Diet preparation and analysis

Fluopyram was incorporated into the diet to provide the required dietary concentrations.

The test formulations were stored at room temperature and issued to the animal unit in polyether formulation for each concentration used in the study and any unused diets were discarded at the end of each administration period.

The homogeneity of fluopyram in diet was verified at the lowest and highest concentrations. The mean values obtained from the homogeneity check were taken as measured concentrations. For the remaining concentrations, the dietary levels were verified for each concentration at the time of preparation. The homogeneity and concentration data for fluopyram in rodent diet and has within a range of 89 and 98% of the nominal concentration. Therefore all values were within the in-house targe range of 85-105% of the nominal concentration. The stability of fluopyram at 20 and 10000 ppm in the diet was demonstrated in an earlier study (M-085510-01-1), which covered the period of storage and usage for the present study.

Phenobarbital formulations were prepared twice during the study by suspending the test substance (w/w) in a 0.5% aqueous solution of methylcellulose 400. The method of proparation was documented in the study file. This suspension was stored in air-tight bottles at +5,  $C \pm 3$ , C when not in use. The suspension was mixed continuously before and during dosing using an electromagnetic stirrer. Any unused formulation was discarded at the end of the administration period.

The homogeneity of the first phenobarbital formulations at 16 and 8 gr were verified. The sampling was done at two levels at the surface and at the bottom. The mean varies obtained from the homogeneity checks were used as measured concentrations. For the second formulation, the sampling was done at the surface and the mean value obtained from the sampling was used as measured concentration. The homogeneity and concentration data for phenobarbital as an aqueous caspension in 0.5% methylcellulose 400 were between 93 and 10% of the nominal concentration. Therefore all values were within the in-house target range of 90-110% of the nominal concentration. The stability of phenobarbital at 8 g/l has been demonstrated in a previous study (\$\$03316), which covers the period of storage and usage for the current study.

### 4. Statistics 🏷

Variables analyzed

Body weight parameter

- Body weight gain/day parameters calculated according to time intervals
- Average food consumption/day parameters executated according to time intervals
- Alormonal parameters
- Terminal body weight, absolute and relative organ weights parameters Liver
- enzyme activities
- qPCR@etermination

Statistical methods

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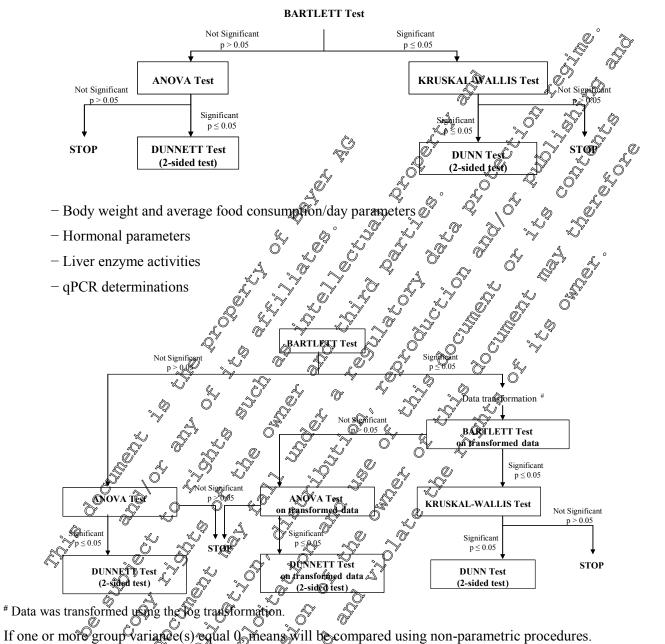
Mean and standard deviation were calculated for each group.

Fluonyram: comparison between the treated groups and the vehicle control group

- Body weight change parameters,

Freminal body weight, absolute and relative organ weight parameters,





Group means were compared at the 5% and 1% levels of significance.

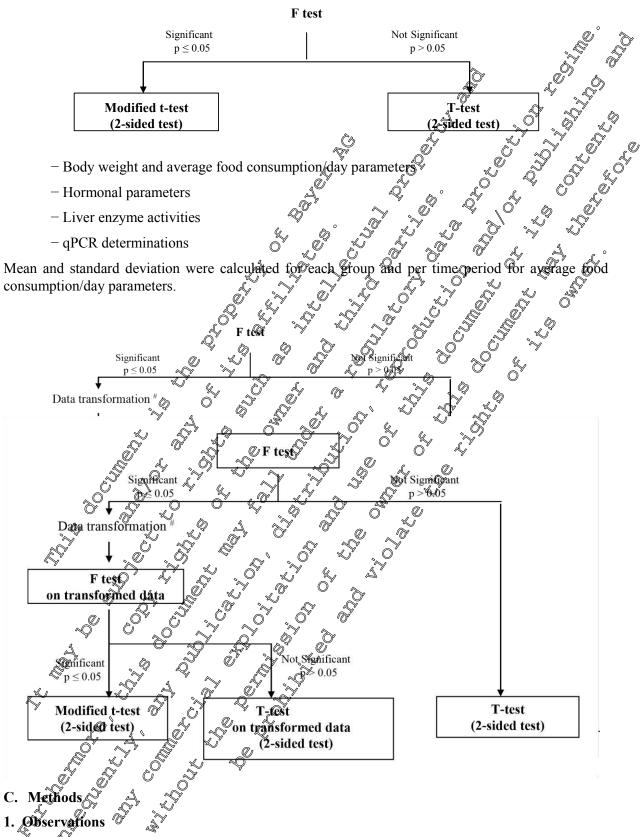
Phenobarbital: comparison between the treated group and the vehicle control group

- Body weight change parameters,

- Termisal body weight, absolute and relative organ weight parameters,

Mean and standard deviation was calculated for each group and per time period for body weight change parameters





The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical



examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

#### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, on study Day 1 and there weekly during the treatment period and recovery phase. Additionally, animals were weighed before scheduled necropsy (terminal body weight).

#### 3. Food consumption and compound intake

The weight of food supplied and of that remaining at Wie end of the food consumption period y recorded weekly for all animals during the treatment period to calculate the mean daily food consumption. Any food spillage was noted. From these records, the weekly mean achieved dosage intake in mg/kg/day for fluopyram for weeks 1 to 4 was calculated using the following formula:

Dose level (ppm) ×  $Gr^{\circ}oup$  mean food cosmumption (g/day)

Test substance intake =  $\frac{1}{Group mean body}$ the food consumption period g)at the end b

#### 4. Hormonal investigation

#### **Blood sampling**

On the day of sacrifice, between 8:30a.m. and 11 a.m., blood samples were taken from all animals

from the abdominal aorta. Animals were anesthetized by inhalation of Ischurane (Baxter, Maurepas, France) prior to bleeding. Blood was collected on linhium heparing the resulting plasma samples were frozen at approximately -74°C + 10°C before shipment in dry ice to the subcontracted laboratory for hormone measurement.

#### **Hormone measurements**

Plasma was prepared from each blood sample and kept to zen af approximately  $-74^{\circ}C + 10^{\circ}C$  until the determination of TSH and T4 hormony levels with specific radio-immunoassay kits (supplied by Amersham for TSH and by DIASORIN for TA). This measurement was subcontracted at "BioVetim, Vet Agro Sup, Campu Vétérinaire de Lyon VAile Niveau 3, Avenue Bourgelat, 69280 Marcy l'Etoile, France "

### 5. :Sacrifice and pathology

Necropsy procedure Organ sampling

All designated animals from all groups, were sacrificed on Days 29 and 30 following treatment or on Day 29 following the accovery phase. All sacrifices were performed by exsanguination under deep anesthesia (inhalation of Isoffurane, Baxter, Mancepas, France). Animals were not diet fasted overnight prior to sacrifice. All animals were newopsied The pecropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled except upon decision of the Study Pathologist.

A piece of the median and left liver lobes of 5 annuals from each group as well as the pituitary gland of all the animals were sampled.  $\int_{-\infty}^{\infty}$ 

For each group, the phuitary gland was collected and flash frozen in liquid nitrogen. These samples were stored individually at approximately -745C + 10°C until used for qPCR investigations.

# 6. Hepatotoxicity testing

At each sachfice when the livers were weighed, pooled by three in each treatment group and homogenized for microsophal preparations to determine UDPGT specific isoenzyme activities.



#### **QPCR** analysis

#### **Total RNA purification**

Total cytoplasmic RNA was isolated from the pituitary gland of individual control and treated animals using RNeasy Mini kits (Qiagen). RNA quality controls were performed based on the ribosonal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

#### Quantitative PCR

One µg of total RNA was used for Reverse transcription (CT) using a High Capacity cDNA Archive cit (Applied Biosystems). The assay was performed in duplicate using a beta subunit. Sh Tagmanassay (Assay on demand, Applied Biosystems, ref Mm03990915-g1), 9/25 diluted first strand cDNA FastStart Universal Probe Master Mix (Roche) on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA/B-microtubutin, beta Actin, Gusb and Gapdh were tested as reference genes. For the quantitative calculations of Tsh transcripts, Gusb was selected as reference gene. The relative quantity (RQ) value of beta subunit Tsh transcript (Tsh b) vas calculated using the following formula:

 $\Delta\Delta Ct = (CtTsh b - CtGusb)$  treated - (@Tsh b  $\neq$  CtGusb) control

#### $RQ = 2 - \Delta \Delta Ct$

where Ct is the threshold cycle at which CR amplification started to be significantly different from the background signal.Each RQ value obtained for a given gene was normalized by dividing by the RQ value obtained for the control animal T1XP885 for the dosing phase and T1M1900 for the recovery phase.

#### **UDPGT** activities

UDPGT activity with bilirabin as substrate was determined using a spectrophotometry method consisting in the determination of conjugated bilepionents after their conversion into azo-pigment derivatives. Absorbance was measured at 530 nm. Three replicate from each sample were assayed.

The glucuron dation of the Substrate T4 was subcontracted to CXR Biosciences Ltd, James Lindsay Place, Dundee DD1 5JJ, Szotland UK

II. Results and discussion

### A. Observations

### 1. Mortality

Fluopyram: There was no mortality in any groups throughout the study.

<u>Phenobarbital</u>: Animal T/M2020 was accriticed for furmane reasons on study day 2 and two animals (T7M2020 and T7M2023) were found dead on study day 5 and 7, respectively. The premature deaths of these animals may be due to the use of a concentrated phenobarbital formulation (16 g/l at a dosing volume of 5 ml/kg body weight) being administered during the first week of treatment. Therefore, a concentration of &g/l at a dosing volume of 10 ml/kg body weight was administered for the rest of the dosing period.

# 2. Clinical signs

Dosing phuse Q

Fluopyram: There were no treatment-related clinical signs.

Phenobarbyal: All males displayed reduced motor activity during the dosing phase.

### Recovery phase

<u>Fluopyram</u>: There were no treatment-related clinical signs.



Phenobarbital: There were no treatment-related clinical signs.

#### B. Body weight and bodyweight gain

#### Dosing phase

Fluopyram: There was no effect on body weight parameters.

Phenobarbital: Body weight and body weight gain parameters were statistically significantly reduced throughout the treatment period, resulting in a 5% ( $p \le 0.01$ ) reduction in body weight and  $a^2/2$ reduction in cumulative body weight gain by Day 29.

#### Recovery phase

Fluopyram: There was no effect on body weight parameters.

Phenobarbital: Body weight was slightly reduced at the start of the ecovery period compared to the control group, but was only marginally lower than the control group by Day 294-3%; not statistically significant).

#### C. Food consumption

#### Dosing phase

Fluopyram: Food consumption was maffected by freatment throughout the dosing phase

Phenobarbital: Food consumption was considered to becomparable to the control group throughout the dosing phase, even though consumption was marginally reduced during the first week.

#### Recovery phase

Fluopyram: Food consumption was comparable to the control group throughout the recovery O  $\bigcirc$ 

phase.

O Phenobarbital: Food consumption was considered to be comparable to the control group throughout the recovery phase, even though consumption was marginally reduced during Week 4. O CONTRACTOR

 $\bigcirc$ 

### D. Hormone analysis

The magnitude of the changes in the plasma normone values relative to the controls and statistical treated and control values were as for tows: significance between

# Dosing phase

#### Fluopyram:

#### **T4**

A statistically significant decrease in mean t4 devels ( $p \le 0.01$ ) was observed at all the dose levels in the plasma offluopyram treated mimals when compared to controls, though not in a clear dose-related manner.

No statistically significant change in mean \$H levels was observed.



Table 5.5-125	Mean levels of T4 and TSH hormones in the plasma after exposure to Fluopyram

Dose-level (ppm)			Flu	opyram	ð	
	0	30	75	150	<b>600</b>	750
T4 (nmol/l)	26.1 ±6.9	18.9** ±3.1 (-28%)	17.9** ±3.4 (-31%)	19.5** ±4.6 (-25%)	16.5** ±2.3 (-37%)	√ 1639* √2.9 √238%)℃
TSH (ng/ml)	1.4 ±0.6	2.1 ±0.9 (+50%)	1(6 40.5 7 14%)		1.0 .0.7 .0.14%	1 6 

Phenobarbital:

T4

bserved. levels A statistically significant decrease in the analysis of the statistical sector is the statistical sector in the statistical sector is the statistica n M

### TSH

No statistically significant change in mean TSH lever was observed

Table 5.5-126 Mean levels of 64 and TSH hormones in plasma after exposure to Phenobarbital
Mean plasma hormone values ± standard deviation (%
change compared to control mean values
Dose-level (nnm)
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
×YSH 2 × 1.4 × × 1.6±0.4
$\langle ng/ml \rangle = \langle 0 \rangle \langle 0 $
**: $p \le 0.01$ $(p)$ $(p')$ $(p')$ $(p')$ $(p')$ $(p')$ $(p')$ $(p')$ $(p')$
Recovery Phase: A A A A A
Fluopyram:
T4 $A$
No statistically significant charge in mean 14 levels was observed.
$TSH_{\mathcal{Y}}^{\mathcal{L}} \qquad \stackrel{\sim}{\mathcal{L}}^{\mathcal{Y}} \stackrel{\sim}{\mathcal{F}}^{\mathcal{Y}} \stackrel{\sim}{\mathcal{F}}^{\mathcal{Y}} \stackrel{\sim}{\mathcal{O}}^{\mathcal{Y}} \stackrel{\sim}{\mathcal{O}}^{\mathcal{Y}} \stackrel{\sim}{\mathcal{O}}^{\mathcal{Y}}$
No statistically significant change in grean TSH levels was observed.
T4       20.1**±2.7 (-         (nmol/l)       ±6.9         TSH       1.4         (ng/ml)       ±0.6         **: p≤0.01         Recovery Phase:         Fluopyram:         T4         No statistically significant change in mean f4 lexels was observed.         TSH         No statistically significant change in mean f5H levels was observed.



# Table 5.5-127Mean levels of T4 and T8H hormones in plasma after exposure to Fluopyram<br/>followed by recovery period

1	followed by recovery period	
Mean plasma	hormone values ± standard deviation (% o control mean values)	change compared to
	Fluopyram	
Dose-level	0	750 750 750 750 750 750 750 750
(ppm)		
T4	28.5 +5.4	
(nmol/l)	±5.4	<u>Q</u> 5% Z Z Z
TSH	1.5	1.4 0.4 (- ^Q ^Q
(ng/ml)	±0.3	
Phenobarbital:		
<u></u>		
14		
No statistically sign	ificant change in mean 14 levels was obs	erved. A Standard C
TSH	1.5 ±0.3 aificant change in mean TA levels was obs aificant change in mean TSH levels was of Mean levels of T4 and TSH hormones after recovery period Mean plasma bormone values standard of	
No statistically sign	uficant change in Grean TSH levels was of	hser@ed
No statistically sign	incant change in wheat of our revers was of	
Table 5.5-128	Mean levels of T4 and TSH hormones after	expositive to Ppenobarbital followed by
I	recovery period '>> O' 45	
	Mean plasma pormone values standard d	leviation (% change Ø
	مَنْ دَصْسَهُ compared to control mean	yalues) 29° 2
	L S / PhenoBarbital	
Dose-level (p	po s appm S S	@/ 0 4980 mg/kg
T4 🍣		28.4±7.5
		(-0.4%)
TSH		Q 1.6 ±0.5
(ngoml)		(+7%)
E. QPCR analysis	$\frac{28.959.4}{67}$	
Dosing phase		\$"
Fluopyram		Ŷ
A daga ralatadunar	in the lavel of a compulation of Tab tra	uncerint in the nituitary aland was recorded

A dose-related increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded at 600 and  $\frac{7}{2}50$  ppm (+43%; p $\leq 0.05$ ; +54%; p $\leq 0.01$ ).

Table 5.5-129	Tsip b transcript levels after exposure to Fluopyram	
~ ~		

Mean relative quantity Standa	rd deviation	FTsh b transc values)	ript (% chang	e compared to	control mean
		Fluopyram			
Dose devel to the second secon		75	150	600	750
	1.223	1.303	1.297	1.655*	1.783**
Tsh 10 1,156 ±0,334	$\pm 0.354$	±0.344	±0.451	$\pm 0.461$	±0.725
±0,334	(+6%)	(+13%)	(+12%)	(+43%)	(+54%)
*: p≤0.@1 ^{0″}		•	•		•



#### Phenobarbital

	mals (+53%; p≤0.01).
Table 5.5-130Tsh b	transcript levels after exposure to Phenobarbital
Mean relative qua	antity $\pm$ standard deviation of Tsh b transcript (% change compared $\sqrt{2}$
	to control mean values)
	Phenobarbital 2 2 2 2
	0 ppm
	1.15( 0 0 1.704** 0 0
Tsh b	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
**: p≤0.01	
Recovery Phase:	
Fluopyram:	evel of accumulation of Tsh transcript in the primitary gland stars recorded in is after the recovery phase (+12%) $p \leq 0.05$ ).
$\Lambda$ slight increase in the l	evel of accurrent attended in the night and the recorded in
fluonvram treated anima	s after the recovery phase $\ell + 12\%$ n<0.09
indopyrum treated animal	
Table 5.5-131 Tsh b	transcript levels after exposure to Fluopyran followed by Gecovery phase
Mean r	lative quantity + standard deviation of Tsh b transcript (%
Mean re	elative quantity ± standard deviation of Tsh b transcript (%
Mean r	Change compared to control mean values)
St n	Change compared to control mean values) 7 2 Pluopy am
Dose-level (ppm)	Change compared to control mean values) 7 7 Fluopy am 7 750
Dose-level (ppm)	Change compared to control mean values)
Dose-level (ppm)	Change compared to control mean values) 7 7 Fluopy am 7 750
A n	Change compared to control mean values)

No statistically significant change in the level of accumulation of Tsh transcript in the pituitary gland was recorded in phenobarbital treated animals after the recovery phase.

Table 5.5-132	Tshe transpript levels after exposure to Phenobarbital followed by recovery phase
Mean relativ	auantity ±standart deviation of Ash b transcript (% change compared to control mean

	( /s change compared to control mean
A Phenobarbital	
Dose-level (ppm)	80 mg/kg
	1.090
Tsh b	±0.139
	(+4%)

F. Post mortem examinations ~Ç Terminal body weight and organ weight

# de la companya de la

Dosing phase Fluopyram

There was no change in mean terminal body weight in treated animals when compared to the controls.



At 75 ppm, mean liver to body weight ratio was statistically significantly higher when compared to controls. From 150 ppm, mean absolute and relative liver weight were statistically significantly higher when compared to controls.

These changes were considered to be treatment-related.

Table 5.5-133	Mean liver weight changes after exposure to Fluopyram
---------------	-------------------------------------------------------

Mean liver weight ±sd at scheduled sacrifice (% change when compared to controls)							
		Fluop	yramÖ				
Dose-level (ppm)	0	30	75	A\$50	<u>600</u> 750		
Absolute liver weight (g)	1.31 ±0.08	1.37 ±0.13 (-5%)	€ 1.40 ±0.09 (+7%)	↓1.45** ↓0.43° (+ 4%)	$ \begin{array}{c} \bigcirc 1.67^{*} \\ \pm 0.42 \\ (\div 27\%) \\ \swarrow \\ (\div 27\%) \\ \checkmark \\ (\div 27\%) \\ \swarrow \\ (\div 27\%) \\ $		
Liver to body weight ratio (%)	5.338 ±0.310	5%\$70 ±0.306 _≪ ≰(+4%)⊘	\$.667* ±0.278 (+@%)	5.8 <b>25*</b> * ±02	$\begin{array}{c} 75 \\ & 9.786^{**} \\ \pm 0.468 \\ & \pm 0.341 \\ & (\pm 2)\% \end{array} $		
p≤0.05; **: p≤0.01 henobarbital:							

There was no change in mean terminal bood weight in treated animals then sompared to the controls. Mean absolute and relative liver weight were statistically significantly Digher when compared to controls Ö following phenobarbital treatment. M 81 Ø Ô

Table 5.5-134	Mean liver weight	changes after exp	osure to Phenobarbital
14010 010 10 1			osti e to i nen www.situty

Mean liver weight ±sd	at scheduled sacrifice (% change	when compare for controls)
2 S'	Phenobarbitar O	
Dose-level (ppm)	K ~ Oppm X a	80 mg/kg
		1.44**
Absolute liver weight (g)		±0.12
		<i>©</i> (+10%)
		6.190** ±0.291
Liver to body weight ratio (%)	$\sim 10.30^{\circ}$	(+16%)
**: p≤0.01		
**: $p \le 0.01$ Recovery Phase:		
**: p≤0.01 Recovery Phase:		

There was to change in mean terminal body weight, for treated animals when compared to the controls. There we no significant charge in liver weight parameters in treated animals when compared to the controls. K,

Table 5.5-135	Mean fiver weight changes after exposure to Fluopyram followed by recovery period	
---------------	-----------------------------------------------------------------------------------	--

Mean liver weight sd a	at scheduled sacrifice (% change wh	en compared to controls)
	) 🖓 Fluopyram	
Dese-lev@ (ppm)	0	750
Absolut liver Seight &	1.32 ±0.10	$1.37 \pm 0.18 (+4\%)$
Liver to body weight ratio (%)	4.984 ±0.296	5.190 ±0.500 (+4%)



#### Phenobarbital:

There was no change in mean terminal body weight in treated animals when compared to the controls.

# Table 5.5-136 Mean liver weight changes after exposure to Phenobarbital followed by recovery period

Mean liver weight ±sd at	scheduled sacrifice (% chang	e when compared	
	Phenobarbital	Q	
Dose-level (ppm)	0 ppm		80 mg/kg
Absolute liver weight (g)			1033 =0.09 (+1%) (+1%)
Liver to body weight ratio (%)	20.296 20.296		5.085 ±0.282 & \$4-2%
G. UDPGT activities			

#### Dosing phase

#### <u>Fluopyram</u>:

A dose-related increase in the UDPGT enzymatic activity using bilirthin as substrate was recorded at 600 and 750 ppm (+39%;  $p \ge 0.05$ ; 48%  $p \ge 0.04\%$ .

An increase in the UDPGT enzymatic activity using thyroxine as substrate was recorded at 600 ppm (+83%;  $p \le 0.01$ ). An increase of this activity was also detected at 150 and 750 ppm, although not statistically significant.

# Table 5.5-137 Mean enzymatic BPGT activities after exposure to Fluopyram

Mean enzymatic U	DPGT activitie	s ± stand	ard deviation	n (% change	exompared	to control me	ean values)
		<b>A</b>	Fluopyra		107		
Dose-level (ppm	e 😓 🕺	. 4	»` 30 ⁰ ".	[*] 75 [°]	150	600	750
		Å,	2.217	≥ 2.394	2.624	2.758*	2.947**
UDPGT	1.255±	0.577	≪ <b>≢</b> 0.375	±0327	±0.198	±0.324	±0.422
(Biiiiubiii) ^g			(+12€)	(*21%)	(+32%)	(+39%)	(+48%)
UDPGT		Ý 🕎	0,768	0.854	1.168	1.412**	1.026
	0.750±	0.144	<b>д</b> 0.190 🖉	±0.198	$\pm 0.360$	±0.253	±0.271
(Thyroxine)	. Ø	Ŵ,	<u>ک</u> (0%) ک	(+11%)	(+52%)	(+83%)	(+33%)
*· n<0.05 [*] *· n<0.01		ć					

Phenobarbital:

Non-statistically significant increases of the prepatic UDPGT enzymatic activities (+42% and +32%) were recorded in the phenobarbitaktreated animals.

were recorded in the phenobarbitaktreated animals.



	matic UDPGT activities = ange compared to contro	
(70 81	Phenobarbital	
Dose-level (ppm)	0 ppm	
UDPGT	1.985±0.577	2.821±0.676 (+42%)
(bilirubin)	1.903±0.377	
UDPGT (Thyroxine)	0.770±0.144	1.020±0.216(+32%)
Recovery phase	A	
Fluopyram:		
There was no significant change in	the hepatic UDP of enz	zomatic activities in the fluopyram treated
• •		
Table 5.5-139   Mean enzymatic	UDPGT activities after ex	posure to Fluop am followed by receivery
period		
Mean enzymatic UDPGT activ	vies ± standard deviation	n (% Change compared to controlimean
Q	<u>values)</u>	
Dose-level (ppm)	V OFluop am	2 ⁵⁷ 2 ⁵ 2 ⁶ 2 ⁶ 2 ⁶ 2 ⁷ 2 ⁶ 2 ⁶ 750 ⁵
UDPGT		→ → → → → → → → → → → → → → → → → → →
(bilirubin) s	1,795±0,313	
UDPGT		0.774±0.327 (-6%)
(Thyroxyre)		
Phenobarbital:		
There was no significant change in t	e hepatic UDPGT enzy	matic activities in the phenobarbital treated
mimals after the recovery phase.		
Table 5.5-140 Mean enzymatic	UDPGT activities after ex	クレージ posure to Phenobarbital followed by
recovery period		
Mean enzymentic UDPGT activ	vities ± standard deviation	• (% change compared to control mean
	vatues) 🖉	
	<u>Phenobarbital</u>	
Dose-level (ppm)		80 mg/kg
(bilirubin)	1.795±0.313	1.992±0.293 (+11%)
UDPGT (Thyroxine)	· · · · · · · · · · · · · · · · · · ·	0.808±0.096 (-2%)
H. Deficiencies A &	Ş [×] Q [×]	
No specific deficiencies were noted	in this study	

In conclusion, this study demonstrates that fluopyram administration at the tumorigenic dose level of 756 ppm for at least 28 days in the C57BL/6J mouse, induced a decrease in plasma T4 levels correlated with an increase in the levels of Tsh transcript (beta subunit) in the pituitary gland, together with an increase in the UDPGT-bilirubin and UDPGT-T4 enzymatic activities. In addition, absolute and relative



liver weights were significantly increased. These effects were not detected after a four-week recovery phase except for a marginal increase in Tsh transcript level.

All these effects occurred in a dose-related manner, apart from the decrease in plasma T4 for which there was no dose-related concordance in terms of the magnitude of the response observed. At the low dose of 30 ppm, the only finding was a decrease in plasma T4 levels, the relevance of which is doubted based on the lack of clear dose response concordance.

#### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic s information on fluopyram in the mouse.

In conclusion, this study demonstrates that fluoporam administration at the tumorigenic dose level of 750 ppm for at least 28 days in the C57Bk/6J mouse, induced a decrease in plasma T4 levels correlated with an increase in the levels of Tsh transcript (beta submit) in the pitottary gland, together with an increase in the UDPGT-bilirubin and UDPGT-14 enzymatic activities. In addition absolute and relative liver weights were significantly increased. These effects were not detected after a four-week recovery phase except for a marginal increase in Tsh transcript level.

All these effects occurred in a dose-related manner, apart from the decrease in plasma T4 for which there was no dose-related concordance in terms of the magnitude of the response observed. At the low dose of 30 ppm, the only finding was a decrease in plasma T4 levels, the relevance of which is doubtful based on the lack of clear dose response concordance

	KCA5.5/18 ~ 17 ~ 2 ~ 7
Data Point:	KCA/5.5/18
Report Authors	
Report Year:	
Report Title?	Fluopyram: 28 day toxicity study for proliferation assessment in the C57BL/6J
	mate mouse , St J O
Report No:	\$\$\$11123 \$\$
Document No:	<u>M1-428993-0184</u>
Guideline(s) followed in	No specific guideline
study:	
Deviations from current	Gurrent grideling nong
test guideline:	
Previous avaluation:	Not previously evaluated, 😒
GLP/Officially	Yes, conducted under GLA Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yest &
	Yes, U, Y

# Executive Summary

The objective of the present study was to investigate possible thyroid cell proliferation, caused by fluopram administration to C57BL/6J male mice following continuous dietary administration for at least 28 days at a dose level (750 ppm) which caused an increase in thyroid follicular cell adenomas following chronic exposure.

Fluopyram, (batch number: Mix-batch: 08528/0002: a beige powder, 94.7% w/w), a fungicide of the pyramide family, was administered daily in the diet to groups of adult male C57BL/6J mice (15



mice/dose level) for at least 28 days at a dose level of 750 ppm. This dose equated to 127 mg/kg/day. A group of animals dosed by oral gavage with 80 mg/kg/day phenobarbital (a reference compound known to induce thyroid changes in rodents, batch number: 09050075; a white crystalline powder, 100% purty) acted as a positive control for thyroid effects since it was used in a previous mouse mechanistic study where thyroid hormone level changes were detected. A control group received untreated thet. All animals were exposed to BrdU in their drinking water during the last week of the study. Animals were observed daily for clinical signs and twice daily for moribundity and mortality except on weekends when they were checked once daily. Physical examinations were performed weekly. Body weight and food consumption were recorded once weekly. All animals were sampled and fored. The thyroid way examined microscopically. Additional thyroid slides were stained for BrdU cell proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations.

#### Fluopyram at 750 ppm in the diet

There were no clinical signs and no effects of food consumption of body weight parameters for the group treated with fluopyram.

At necropsy, there was no change in mean terminal body weight when compared to control anomals. Enlarged and dark livers were found in 24/15 and 5/19 animals, respectively. No microscopic changes in the thyroid gland were noted.

Assessment of cell proliferation in the thyroid revealed a 1.7 fold increase  $fp \le 0.01$  in BrdU labeling index when compared to the controls.

Phenobarbital (reference item) at 80 mg/kg day by gavage

Clinical signs consisting of reduced motor activity were recorded for all mice dosed with phenobarbital from Study Day 2 onwards. In addition, mean body weight was reduced by between 3.5% ( $p\leq0.05$ ) and 4.8% ( $p\leq0.01$ ), throughout the study. Following an initial body weight loss between Study Days 1 and 8, overall cumulative body weight gain was reduced by 52% ( $p\leq0.01$ ) by Study Day 29, compared to the controls.

At necropsy, near terminal body weight was lower (-4.4%,  $p \le 0.01$ ) when compared to control animals. Enlarged and dark livers were found in 3/15 and 4/15 animals, respectively. No microscopic changes in the thyroid gland were noted.

Assessment of cell proliferation in the thredid repealed no change in the BrdU labeling index when compared to the controls. These results are consistent with the literature, which show that phenobarbital induces thyroid cell proliferation mice at a dose level of 1800 ppm, which corresponds approximately to 200 mg/kg/day.

In conclusion a clear increase in thyroid cell proliferation, as evidenced by a 1.7 fold increase ( $p \le 0.01$ ) in BrdU labeling index, compared to the controls, was observed following dietary administration of fluopyram at 750 ppm for at least 28 days, to the male C57BL/6J mouse.

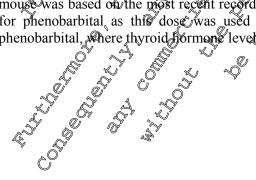
<ul> <li>I. Test material: Description</li> </ul>	
1. Test material	Flu@pyram (AE C656948)
Description of of of	Berge powder
Lot Batch#:	Mix-Batch:08528/0002
Parity: 5 5	94.7%
GAS HO O SY	658066-35-4
Stability of test compound:	Stable in rodent diet for a period covering the study duration
2. Vehicle and / or positive	Vehicle for AE C656948: rodent diet
control:	Phenobarbital (positive control),
control.	Vehicle for Phenobarbital: methylcellulose



Phenobarbital **Positive control: Description:** White crystalline powder Lot / Batch : Lot No. 09050075 100% 50-06-6 Stable in 0.5% aqueous solution of methyleellulose for a period **Purity:** CAS: **Stability of test compound:** 3. Test animals: Mouse - Male only C **Species:** C57BL/6J Strain: 8 weeks approximately at start of treatment Age: 19.6 to 24.8 g Weight at dosing: Source: **Acclimation period:** 14 days O Certified oden bowdered and irradiated die A04C-10 P1 from S.A.F.E. (Scientific Animal Good and Engineering, Epinar-sur-Diet: Orger Prances, ad Itbitum Municipal rap water, ad libitur Water: nouspended stainless steel Animals were caged individual **Housing:** wire mesh cases. Environmental conditions: 2**®**,24°C® **Temperature:** 40-70% **Humidity:** Approximately 10-05 air changes per hon Air changes: Alternating 12-bour light and thank cycles (7 aph- 7 pm) **Photoperiod: B.** Study design Rosing period 8 June 1. In life dates: 2. Animal assignment and treatment Three groups of make mice were dosed for at least 28 days with the appropriate compound by the appropriategoute of administration. Each group consisted of 15 male mice. Two groups were dosed by dictary administration. One group consisted of control animals that received untreated diet and the second group received fluopyram at 750 ppm at a constant level. The level for

fluopyram was set after evaluation of the results from a previous study conducted with fluopyram ( $\underline{M}$ -<u>408352-01-1</u>) and following discussions with the US (EPA) and Canadian (PMRA) authorities. The level of 750 ppm used in the present study represents the highest level used in the mouse cancer bioassay in which an increase in thy foid tumors was observed in the males ( $\underline{M}$ -295688-01-1).

The third proup was dosed by oral gavage with 80 mg/kg/day Phenobarbital suspended in 0.5% aqueous methylcellulose 400 using a dosing volume of 10 ml/kg bodyweight. The volume administered to each mouse was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose was used in a previous mouse mechanistic study conducted with phenobarbital, where thyroid formane level change was detected (M-299521-01-1).





Group	Test substance Dose level		Number of animals per group	Animal identity		
		Males				
1	Control	0 ppm	15	T1M2166 to 2180		
2	Fluopyram	750 ppm (127 mg/kg/day)	15	T2M218 to 2005		
3	Phenobarbital	80 mg/kg/day	15	T3M2196 to 2210 0		

#### 3. Diet preparation and analysis

Fluopyram was incorporated into the diet to provide the required dietary conceptrations.

The test formulation was stored at room temperature and issued to the animal unit in polyeth lene containers. There was one formulation used in the study and any usused thet was discarded at the end of each administration period.

The homogeneity of fluopyram in diet was verified, the mean values obtained from the homogeneity check was taken as measured concentration. For the remaining concentrations, the dietary levels were verified for each concentration at the time of preparation. The homogeneity and concentration data for fluopyram in rodent diet were between 98 and 99% of the nominal concentration. Therefore the values were within the in-house target range of 85 415% of the nominal concentration. The stability of fluopyram at 20 and 10000 ppm in the diet was demonstrated in an earlier study (<u>M-085510-01-1</u>), which covered the period of storage and usage for the present study.

Phenobarbital formulations were prepared twice during the study by suspending the test substance (w/w) in a 0.5% aqueous solution of methylcellulose 400 This dispersion was stored in air-tight bottles at  $+5^{\circ}C \pm 3^{\circ}C$  when not in use The suspension was mixed continuously before and during dosing using an electromagnetic stirrer. Any objused formulation was discarded at the end of the administration period.

The homogeneity of phenobarbital in aqueous 0.5% methylcelbilose 400 suspension was verified on first formulation to demonstrate adequate formulation preparation. The mean value obtained from the homogeneity check was taken as the measured concentration. The concentration of the second formulation was also checked. The homogeneity and concentration data for phenobarbital as an aqueous suspension in 0.5% methylcellulose 400 were all at 99% of the nominal F test concentration. Therefore all values were within the in-house target range of 90 010% of the nominal concentration. The stability of phenobarbital at 8 g/bhas been demonstrated in a previous study (M-232813-01-2), which covers the period of storage and usage for the current study.

BrdU (5-Bromo-2'-deoxyDriding), an apalogue of thymidine (batch number: HMBB6206: an off white powder, 90% purity), supplied by Sigma-Altrich, Germany, was used to evaluate cell proliferation in the study and was stored at 0 to -20 °C.

Brdty in filtered tap water from municipal water supply was prepared twice during the study at 80 mg BrdU/100 ml in drinking water. These solutions were stored in air-tight light-resistant bottles at room temperature when not in use. The unused solution was discarded at the end of the administration period.

The concentration data for BrdkL as an aqueous solution were between 96 and 105% of the nominal concentration and were therefore within the in-house target range of 90-110% of the nominal concentration

## 4. Statistics

Group poans were compared at the 5% and 1% levels of significance.



With the exception of those used for the cell proliferation data, all statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). SAS programs (version 9.2) were used for the cell proliferation data.

- Body weight change parameters,
- Terminal body weight,

Mean and standard deviation were calculated for each group and per time perio weight change parameters.

The F test was performed to compare the homogeneity of group variances

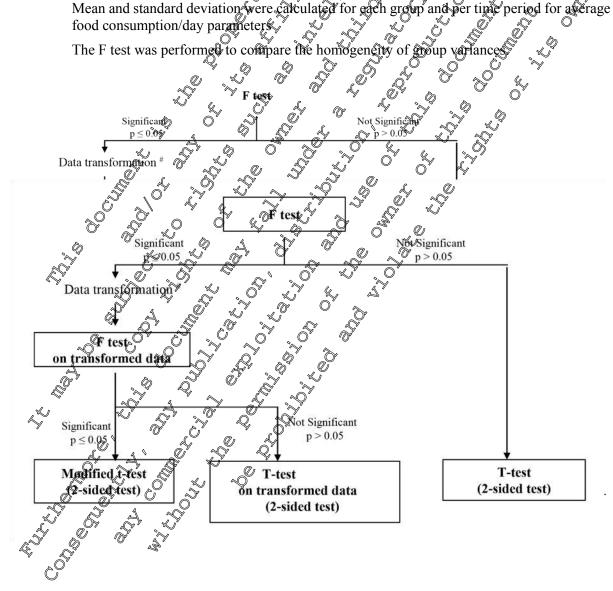
If the F test was not significant (p>0.05), mean of the exposed group was compare to mean of the control group using the t term (2 gided) , Q Ó mean of the control group using the t-test (2-sided)

If the F test was significant ( $p \le 0.05$ ), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

· Body weight and average food consumption/day garameters

time period for average Mean and standard deviation were calculated for each . R food consumption/day parameters

The F test was performed to compare the homogeneity





### C. Methods

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public, holidays). Observed clinical signs were recorded at least once daily for all animats and detailed physical examinations were performed at least weekly during the treatment period. The hature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed at least once during the acclimatization phase on study Day 4 and then weakly during the treatment period. Additionally, animals were weighed before scheduled necropsy (terminate body weight).

### 3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period to calculate the mean daily food consumption. Any food spillage was noted. The weekly mean achieved dosige intake of the tieff in mg/kg/day for each week and for Weeks to 4 was calculated for each sex using the following formula:

Test substance intake =  $\frac{Dose level (ppm) \times Group mean food cosmumption (g/day)}{Group mean bodyweight (g) at the ond of the food consumption period}$ 

### 4. Water consumption

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration (Study Day 23). Empty water bottles were weighed at least once before scheduled sacrifice (Study Day 30).

### 5. :Sacrifice and pathology

## Necropsy procedure Organ sampling

On Study Day 30 all animals from all groups were sarrificed by exsanguination under deep anesthesia by inhalation of Boflugane (Baxter, Maurepas, France).

All animals were necessoried. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Significant/nacroscopic abnormalities were recorded and sampled upon the decision of the study Pathologist.

The following organs or tissues were sampled:

- Thyroid grand (with trachea)
- Liver
- Duedenum

Macroscopic findings were sampled at the discretion of the Study Pathologist.

The thyroid gland, the duddenum and the liver from each animal in all groups were preserved in 10% neutral buffered formalin.

## Conventional Arstopathological examination

Histological samples containing 6 thyrord sections, one piece of duodenum and 2 sections of liver from all animals in all groups were embedded in paraffin wax. Histological slides, stained with hematoxylin and eosin, were prepared for these three organs from all the animals in all groups.

### Brad staining for cell proliferation measurement

For each animal in each group a section of a formalin-fixed paraffin-embedded block containing 6 thyroid samples and one sample of duodenum was prepared. The duodenum was included to serve as a positive control for staining as it has a high rate of cell proliferation. The immunohistochemical reaction



included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish complex, detection of the complex with diaminobenzidine (DAB) and nuclear counter staining with hematoxylin.

### 6. Histopathology

Histopathological examinations were performed on the thyroid samples from an animals in all groups Following the initial histopathological examination, a review of representative slides was performed a second pathologist according to standard operating procedures.

### 7. Cell proliferation assesment

The staining for BrdU and determination of the labeling index was performed on all animals showing sufficient BrdU incorporation (estimated by water consumption @duodenal BrdU labeling) @ asses cell cycling in the thyroid. The labeling index pressed as the number of Brd Opositive thyroid follicular cells per thousand, was measured separately on multiple fields comprising of at least 1000 cells using an image analysis system (slide scapper, Harnamatsu). The mean and standard deviation were calculated for each group.

#### II. **Results and discussion**

### A. Observations

### 1. Mortality

There were no mortalities during the course of this stor

### 2. Clinical signs

Ø Fluopyram: There were no reatment-related clinical signs

Phenobarbital: All mice displayed reduced motor activity form Study Day 2 on wards.

## B. Body weight and bodyweight gain ~

Fluopyram: There was weight parameters

<u>Phenobarbital</u> Mean body weight was reduced by between 3 % and 4.8% throughout the treatment period, the effect being statistically significant on Study Days 8 ( $p \le 0.05$ ), 22 ( $p \le 0.05$ ) and 29 ( $p \le 0.01$ ). This effect resulted from a mean bod@weight loss of 0.07 g (p≤0,01), compared with a 0.02 g mean weight gain in the controls, during the first week of treatment. Thereafter, mean body weight gain was slightly lower than in the controls during the last two weeks of treatment, resulting in an overall cumulative body weight gain of  $\emptyset$ .0 g, compared to 2.9 g in the controls (-52%; p $\leq$ 0.01), by Study Day 29.

## C. Food consumption

Fluopyrand Food consumption was undiffected by treatment throughout the dosing phase.

Mas unaffected by treatment throughout the dosing Phenobarbital: Food consumption

phase.

## D. Water consumption

Fluopyram; Mean consumption of water containing BrdU was decreased by 7.9% (not statistically significant, compared to the control group.

Phenotratbital Mean consumption of water containing BrdU was decreased by 7.9% (p≤0.05), compared to the control group.



### E. Post mortem examinations

### **Terminal body weight**

Fluopyram: There was no change in mean terminal body weight in treated animals when compared the controls.

Phenobarbital: Terminal body weight was 4.4% lower than the controls and was statistically si (p≤0.01).

### F. Gross pathology

Fluopyram: Enlarged and dark livers were noted in 14/15 and 5/15 treated animals, respectively animals, respectively.

Phenobarbital: Enlarged and dark livers were noted in 3/15 and 4/15

### G. Microscopic pathology

Only the thyroid glands were examined.

Fluopyram: No treatment-related microscopic change wagnoted

Phenobarbital: No treatment-related michoscopic change

### H. Cell proliferation

be approximately 1. Fold higher (p=0.01) than found Fluopyram: the mean brdu labeling index was the controls. M N

Phenobarbital: the mean brdw Jabeling index was similar to the controls

Table 5.5-142	Mean brdu labeling index (groliferation rate/100 cells)	
Group	Control 2 Chuopyram O A Phenobarbital	
Ν	\$ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~	
Mean	2 12.45 4 109 ** 12.68	
STD	6.17 6 5 8 0 3.71	
**: P≤0.01 °		

## I. Deficiencies

No specific deficiencies were noted in this

#### III. Conclusions

In conclusion a clear increase in by roid cell proliferation, as evidenced by a 1.7 fold increase ( $p \le 0.01$ ) in BrdU labeling index, compared to the controls, was observed following dietary administration of fluopyran at 750 ppm for at least 28 days, to the male C57BL/6J mouse.

## Assessment and conclusion by applicants

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on fluopyran in the mouse

In conclusion a clear increase in thyroid cell proliferation, as evidenced by a 1.7 fold increase  $(p \le 0.64)$  in BrdU abeling index, compared to the controls, was observed following dietary administration of auopytam at 750 ppm for at least 28 days, to the male C57BL/6J mouse.



Data Point:	KCA 5.5/19	
Report Author:	Blanck, O.	
Report Year:	2013, Amended: 05-14-2013	ð
Report Title:	Fluopyram: 28-day toxicity study for thyroid cell proliferation in the C57BL J	Ş
	male mouse	r
Report No:	SA 12058	
Document No:	<u>M-449821-03-1</u>	
Guideline(s) followed in	No specific guideline	2
study:		
Deviations from current	Current guideline: none	
test guideline:		Ő¥.
Previous evaluation:	Not previously evalauated $\sqrt{2}$	1
GLP/Officially	Yes, conducted under GLP officially recognised testing favilities	
recognised testing	Yes, conducted under GLP+Officially recognised testing familities	
facilities:		
Acceptability/Reliability:	Yes O Q X X M A	

### **Executive Summary**

The objective of the present study was to characterize the thyroid effects eaused by the administration of different dose levels of fluopyram to C57BL/61 male mice following continuous dietary administration for at least 28 days and to determine a threshold for thyroid follicular cell proliferation. In addition, the reversibility of any effects observed at the righ dose level was assessed following a recovery period of at least 28 days on untreated control diet

Fluopyram, (batch number: Mix-batch: 08528/0002: a beige powder, 94.7% ww purity), a fungicide of the pyridinyl-ethylbenzainide (pyramide) family, was administered daily in the diet to groups of adult male C57BL/6J mice for at least 28 days at dose levels of 0.30, 75, 150, 600, 750 and 1500 ppm. These doses equated to 0, 5, 93, 25, 99, 124 and 247 mg/kg/day, respectively, 6 ach group consisted of 15 male mice with the exception of the control group and the 500 ppm fluopyram group, where 15 additional males were fed unireated control diet for a further one month to assess the reversibility of changes induced during the 28 day treatment period/All animals were exposed to BrdU in their drinking water during the last week of the study. Animals were observed daily for clinical signs and twice daily for moribundity and mortality except or weekonds when they were checked once daily. Physical examinations were performed weekly. Body weight and food consumption were recorded once weekly. All animals were saveficed other following at least 28 days of neatment or at the end of the recovery phase. All surviving animals were subjected processor and the thyroid and the duodenum were sampled and fixed. The thyroid was examined microscopically. Additional thyroid slides were stained for BrdU cell proliferation deferminations.

### Dosing phase

No treatment-related clinical signs offects on botto weight parameters, food or water consumption were observed at any dose level bested a sign of the second secon

At necropsy, there was no charge in mean terminal body weight when compared to control animals at any dose level tested. No treatment related macroscopic and microscopic changes in the thyroid gland were noted a set of the thyroid gland

Assessment of folicular cell poliferation in the thyroid revealed a 1.21, 1.40, 1.61 and 2.33 fold increase in Brdt labeling index at 150, 600, 750 and 1500 ppm, respectively, when compared to the controls, the effect being statistically significant at the three highest dose levels.

### Recovery phase

In group initially dosed at 1500 ppm, there were no treatment-related clinical signs and no effects on body weight parameters, food or water consumption.



At necropsy, there was no change in mean terminal body weight when compared to control animals. No macroscopic changes in the thyroid gland were noted.

Assessment of cell proliferation in the thyroid revealed no change in BrdU labeling index in the 500 ppm dose group when compared to the controls.

In conclusion, a clear dose-related increase in thyroid follicular cell proliferation, as evidenced by a 1.21, 1.40, 1.61 and 2.33 fold increase in BrdU labeling index at 150, 600, 750 and 1500 ppm, respectively, compared to the controls, was observed following dietary administration of Puopytam for, at least 28 days, to the male C57BL/6J mouse. This increase in thyroid follicular cell proliferation at 1500 ppm was found to be reversible after a 28 day washout period

Å

#### I. Materials and methods

A.	Materials	Fluopyram (AE C656948) Beige bowdeo Mix-Batch 08528/0002 947% 658066 35-4 Stable in rodent diet for a period covering the study duration Vehicle for AE C656948 rodent diet No positive control Mouse - Male only C57BL/65 weeks approximately at start of treatment 19.4 (20.23.9 g
1.	Test material:	Fluopyram (AEC656948)
	Description	Beige powder Q Q Q Q Q
	Lot / Batch #:	Mix-Batch:085280002
	Purity:	940%
	CAS#	Mix-Batch 08528/0002 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7
	Stability of test compound: $\sqrt{2}$	Stable in rodent diet for a period covering the study duration
2.	Vehicle and / or positive	Veltocle for AE (\$56948) roden diet
	ontrol:	No positive comprol
3.	Test animals:	
	Species: 🏷 🔿	Mouse - Male only
		C59BL/6
	Strain:	C57BL/68 weeks approximately at start of treatment
		19.4 to 23.9 g 2 v 0 4
	Source: & A .	
	Source: Acclimation period:	CS4BL/64 & week approximately at start of greatment 19.4 to 23.9 to 10 14 days
		Certified rogent powdered and irradiated diet A04C-10 P1 from
	Diet:	S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-
		Orge, France), ad libition
	Diet: Water: Housing:	Municipal tap water, ad libioim
	Housing:	Animals were caged individually in suspended stainless steel
	Housing.	wife mesh cages
	Housing: Environmental conditions: Temperature: Humidity: Air changes: Photococical	
	Temperature: 🖉 🖉	20-249C 0° 0°
	Humidity:	40,70%
	Air changes:	Approximately 10-15 air changes per hour
	r ngao periou: 🔊 👋	
B.	Study design 🗸 🌧 💭	
1.	In life dates: Dosing pe	rigd: 09 May – 07 June 2012
		Seriod for animals allocated to the recovery phase: 07 June $-$ 04 July

## 2. Animal assignment and treatment

Severy groups' were dosed by dietary administration. One group consisted of control animals that received undreated diet and the remaining six groups received fluopyram at the appropriate dietary concentration (30, 75, 150, 600, 750 and 1500 ppm) at a constant level. The levels for fluopyram was set after evaluation of the results from previous studies conducted with fluopyram ( $\underline{M-408352-01-1}$ ) and ( $\underline{M-428303-01-1}$ ) and following discussions with the US (EPA) and Canadian (PMRA) authorities. The



level of 750 ppm used in the present study represents the highest level used in the mouse cancer bioassay in which an increase in thyroid tumors was observed in the males ( $\underline{M-295688-01-1}$ ).

A solution of BrdU at 80 mg of BrdU/100 ml of drinking water was administered to all animals dring the last week of the study.

Each group consisted of 15 male mice with the exception of the control group and the 4500 ppm fluopyram group, where 15 additional males were fed control or test diet (1500 ppm fluopyram) for at least 28 days and were then allowed one month (at least 28 days) of recovery during which they were maintained on untreated control diet.

All animals were sacrificed in the morning after the last day of treatment or after the last day of the covery phase.

able 5.5-14	3 Study de	esign	
Group	Test item	Dose level	Number of Animal identity
		, ST	Males A Or & C
1	Control	0 ppm	√
2	Fluopyram	3(Oppm (5 mg/kg/day)	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
3	Fluopyram	75 papin A13 mg/kg/day)	T3090591 % 0605
4	Fluopyram 4	(23  mg/kg/aay)	
5	Fluopyram	6000ppm (99.mg/kg/day)	<b>x</b> <b>x</b> <b>x</b> <b>x</b> <b>x</b> <b>x</b> <b>x</b> <b>x</b>
6	Fluopyram	/ \$750 ppm 124 ppg/kg/dayy	7 5 15 T6M0636 to 0650
7	Fluoppram	(12 + 42 kg day) 0 1500 pprk (247 mg/kg/day)	Y     15     Y     T6M0636 to 0650       Y     Y     T7M0651 to 0680
Recovery ph	ise		A D O

Table 5.5-143	Study design
1 abic 5.5-145	Study design

3. Diet preparation and analysis

Fluopyram was incorporated into the diet to provide the required dietary concentrations.

The test formulations were stored at foom temperature and issued to the animal unit in polyethylene containers. There was one formulation used in the study for each concentration and any unused diet was discarded at the end of each administration period.

The homogeneity of fluopyram in diet was verified at the lowest and highest concentrations, the mean values obtained from the homogeneity check were taken as measured concentrations. For the remaining concentrations, the dietary level, were verified for each concentration at the time of preparation. The homogeneity and concentration data for fluopyram in rodent diet were between 80 and 108% of the nominal concentration. All calues were within the in-house target range of 85115% of the nominal concentration, which the exception of the analysis at 30 ppm, which was at 80% of the nominal concentration. As this value was only slightly outside the target range and in isolation, it was considered to be acceptable. The stability of fluopyram at 20 and 10000 ppm in the diet was demonstrated in an earlier study (M-083 10-01 1), which covered the period of storage and usage for the present study.

BreU (5-Bromo-2'-deoxyUridine), an analogue of thymidine (batch number: HMBB6206: a white powder, 99% purity), supplied by Sigma-Aldrich, Germany, was used to evaluate cell proliferation in the study and was stored at -20 °C  $\pm$  5°C.



BrdU in filtered tap water from municipal water supply was prepared twice during the study at 80 mg BrdU/100 ml in drinking water. These solutions were stored in air-tight light-resistant on transformed data bottles at approximately 4°C when not in use. The unused solution was discarded at the end of the administration period. 0

The concentration of BrdU in tap water was verified on both formulations and was found to be The concentration of BrdU in tap water was verified on both formulations and was found to be between 106 and 107% of the nominal concentration, which were within the in-house target range of 90 10% of the nominal concentration. The stability of BrdU in aqueous solution was demonstrated in an earlier study (SA 01416), which covered the period of storage and usage for the present study.

#### 4. Statistics

Variables analyzed

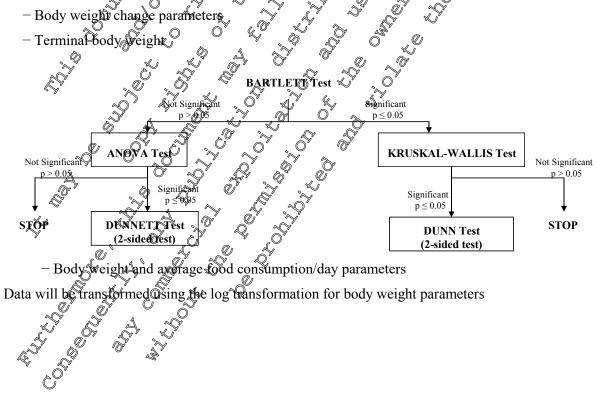
- Body weight parameters
- Body weight change parameters calculated according of time intervals _
- calculated according to time interval Average food consumption/day parameter
- Terminal body weight
  - Thyroid cell proliferation parameters , P

#### Statistical methods

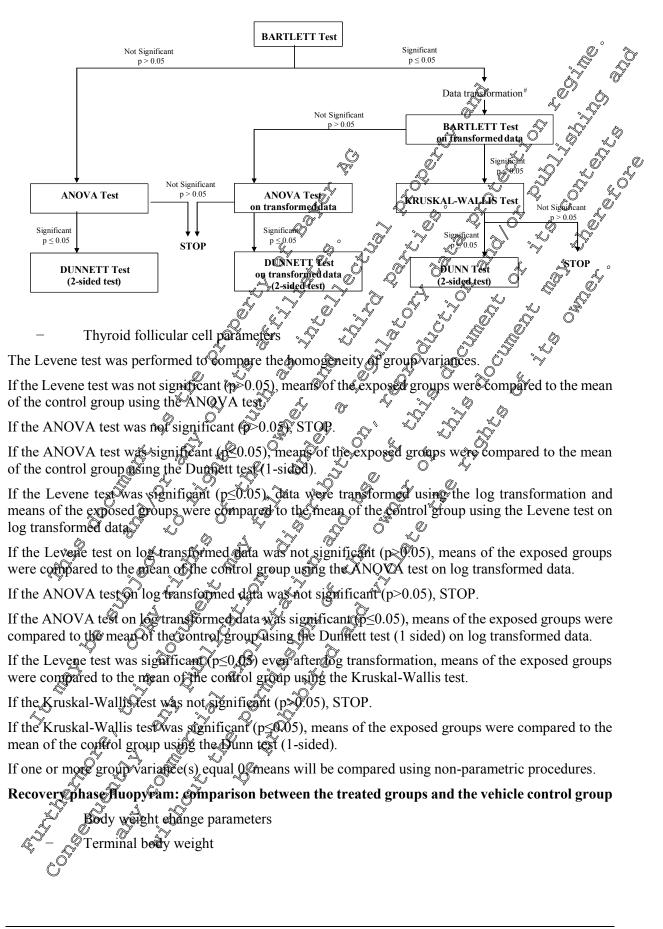
Group means were compared at the 5% and the levels of significand

With the exception of those used for the cell prohferation data, all statistical analyses were carried out using Path/Tox System V4.2.2.4 (Module Enhanced Statistics). SAS programs (Sersion 9.2) were used for the cell proliferation data **K** 0

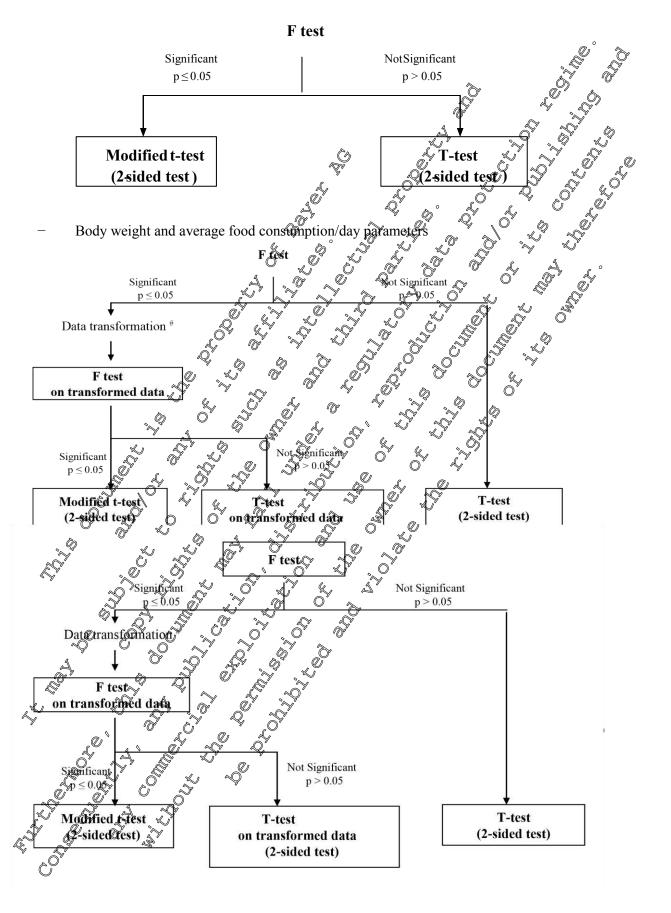
# Dosing phase fluors ram comparison between the greated groups and the vehicle control group













### C. Methods

#### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public, holidays). Observed clinical signs were recorded at least once daily for all animats and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed at least once during the acclimatization phase on study Day P and then weakly during the treatment period and recovery phase. Additionally, animals were weighed before scheduled necropsy (terminal body weight).

### 3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period to calculate the mean daily food consumption. Any food spillage was noted. The weekly mean achieved dosage intake of test item in mg/kg/day for each week and for Weeks 4 to 4 was calculated for each sex using the following formula:

Test substance intake =  $\frac{Doe elevel (ppm) \times Group mean food cosnumption (g/day)}{C}$ 

tance intake =  $\frac{1}{Group}$  free the set of the set of the ford construction period

### 4. Water consumption

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration (Study Day 23 following treatment and Study Day 22 following the recovery phase) Empty water bottles were weighed at least once before scheduled sacrifice (Study Day 30 following treatment and Study Day 29 following the recovery phase)

### 5. Sacrifice and pathology

## Necropsy proceedire - Organ sampling

On Study Day 30 and 31 following treatment of on Study Day 29 following the recovery phase, all surviving animals were sacrificed by exsanguination order deep anothesia by inhalation of Isoflurane (Virbac, Carros, France). Animals were not diet fasted prior to sacrifices.

All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body savities. Significant macroscopic abnormalities were recorded and sampled upon the decision of the Study Pathologist.

The following organs or tissues were sampled:

- Theroid gland (with trackea)
- Duodenum

Macroscopic findings were sampled at the discretion of the Study Pathologist.

The thyroid gland, the duodenum and the liver from each animal in all groups were preserved in 10% neutral buffered formalin.

## Histological preparation

Histological samples containing 6 thyroid sections and one piece of duodenum from all animals in all groups were embedded in paraffin wax. Histological slides, stained with hematoxylin and eosin, were prepared for these two organs from all the animals in all groups.



BrdU staining for cell proliferation measurement

For each animal in each group a section of a formalin-fixed paraffin-embedded block containing°6 thyroid samples and one sample of duodenum was prepared. The duodenum was included to serve as a positive control for staining as it has a high rate of cell proliferation.

The immunohistochemical reaction included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish complex, detection of the complex with diamino-benzidine (DAB) and nuclear counterstaining with hematoxylin.

### 6. Histopathology

Histopathological examinations were performed on the thyroid samples from all animals in all groups Following the initial histopathological examination, a review of representative shdes was performed a second pathologist according to standard operating procedures.

### 7. Cell proliferation assessment

The staining for BrdU and determination of the labeling index was performed on all animals showing sufficient BrdU incorporation (estimated by water consumption or duodenal BrdU labeling) to assess cell cycling in the thyroid. The labeling index, expressed as the number of BrdU-positive thyroid follicular cells per thousand, was measured separately on multiple fields comprising of at least 1000 cells using an image analysis system (lide scanner, Hamanatsu). The mean and standard deviation were calculated for each group. 

#### II. **Results and discussio**

### A. Observations

### 1. Mortality

There were no treatment-related mortalities during th e of this stady.

### 2. Clinical signs

There were no treatment-related clinical signs

Ó

### B. Body weight and bodyweight gain

ght parameter There was no treatment rela

#### Food consumption С.

Food consumption was unaffected

## D. Water consumption

Water consumption was thaffected by

#### Post mortem examinations Е.

### Terminal body weight

weight was treatment. Mean terminal body

### F. Gross pathology

No treatment-related macroscopic findings were observed.

#### Miccoscopic pathology G.

Only the thy foid glands were examined.

No treatment-related microscopic changes were noted



### H. Cell proliferation

#### Dosing phase

A 1.21, 1.40, 1.61 and 2.33 fold increase in BrdU labeling index was observed at 150, 600, 750 and 1500 ppm, respectively, when compared to the control group, the effect being statistically significant the three highest dose levels.

	4
T.LL <i>E E 144</i>	$M_{1} = D_{1} H_{1} H_{1} H_{2} H_{2} H_{2} H_{1} = (-1)^{2} H_{2} H_{$
Table 5.5-144	Mean BrdU labeling index (proliferation rate/100 cells) 🖉
1	internet and the second s

Dose group	Control	30 ppm	75 ppm	150 рран	600 ppm	750 ppm	1500 pp
Ν	14	15	15	Â.	J.		× 15 .0×
Mean	21.55	17.81	19.51	26.09	30.11**	34Q78***0	
STD	4.75	7.37	5.64 🔬	8.62°	~ 8.52 ×	Ø 7.6	10.2
**: p≤0.01 *	***: p≤0.001		Ő				, o
Recovery	<u> </u>		A	$\sim$			
No change	e in prolifera	tive index wa	as noted at 1	500 ppm whe	compared to	y controls.	
No change in proliferative index was noted at (500 ppm) when compared to controls.							

		Q 4.V		$\sim$		.0
Table 5.5-145	Mean BrdU labeli	ng index (r	oroliferat	ion ^y rate	190 ce	lký

Group	Control & Support
Ν	
Mean	\$ 0 \$17.57 \$ 11.58
STD	2 5.08 5° 0° 4 4 6078

I. Deficiencies

No specific deficiencies were poted in this stud

III. Conclusions

In conclusion, a clear dose-related increase in thyroid folligular coll proliferation, as evidenced by a 1.21, 1.40, 1.61 and 233 for increase in BrdU Tabeling index at 150, 600, 750 and 1500 ppm, respectively, compared to the controls, was observed following the tary administration of fluopyram for at least 28 days, to the male C57BL/61 mouse. This increase in thyroid follicular cell proliferation at 1500 ppm was found to be reversible after a 28 day wash our period.

## Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on fluopyrant in the mouse

In conclusion, a clear dose-related increase on thyroid follicular cell proliferation, as evidenced by a 1.21, 1.40, 1.61 and 2.33 fold increase in BrdU labeling index at 150, 600, 750 and 1500 ppm, respectively. Compared to the controls, was observed following dietary administration of fluopyram for at least 28 days, to the male C57BD/6J mouse. This increase in thyroid follicular cell proliferation at 1500 ppm was found to boreversible after a 28 day wash out period.

X i



Data Point:	KCA 5.5/20
Report Author:	
Report Year:	2013
Report Title:	Fluopyram: 28-day dietary study to determine potential role of the nuclear pregnane X receptor (Pxr) and constitutive androstane receptor (Car) on the thyroid changes following the administration of fluopyrate to male mice (C57BL/6J and Pxr KO/Car KO)
Report No:	SA 12162
Document No:	<u>M-449890-01-1</u>
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evaluated a second sec
GLP/Officially	No, not conducted under GLP/Officially recognised testing facilities
recognised testing facilities:	
Acceptability/Reliability:	Yes A A A

### **Executive Summary**

Fluopyram (batch number: Mix-bach: 08528/0002, a beige powder 34.7% Furity, a fungrcide of the pyridinyl-ethylbenzamide family, was administered continuously via the dot to goups of C57BL/6J or Pxr KO/Car KO mice (15/group/strain) for at least 28 days at concentrations of 0, 756 and 1500 ppm, corresponding to 125 and 256 mg/kg/day in C57B/L/6J male mice and to 130 and 247 mg/kg/day in Pxr KO/Car KO male mice. A similarly constituted group received untreated diet and acted as a control. All animals were exposed to BrdU in their drinking water during the last week of the study. Animals were observed at least once daily for clinical signs and wice daily for moribandity and mortality except on weekends when the were checked once daily. A detailed physical examination was performed weekly throughout the study. Body weight and food consumption were recorded once weekly. Before necropsy a blood sample was collected from the abdominal abrea of each atomal for possible further analysis. All animals were decropsed, brain and liver were weighed Liver Thyroid gland, duodenum and pituitary gland were sampled. Thyroid gland, duodenum and two central sections taken from the median and left lobes of the liver were fixed and examined microscopically Additional thyroid slides were stained for BrdU coll proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell profiferation investigations. The pituitary gland was collected and flash frozen in liquid nitrogen and stored at approx mately -74°C+ 10°C for gene transcript analyses by quantitative Polymerase Chain Reaction ( PCR) In addition, one piece of both the median and the left lobe of the liver from each animal were collected and flash frozen in liquid nitrogen and stored at approximately -74°C + 10°C for possible gene transcript analyses. The remaining portions of the liver from all animals were collected and flash frozen in liquid nitrogen prior to storage at approximately -70°C until shipped to CXR Bioscences for microsomal preparations in order to determine total cytochrome P-450 content, cytochrome P-450 isoenzyme and DPGV specific enzyme activities.

### C57BL/6J mice

No clinical signs were observed throughout the study. Body weight, food consumption and water consumptions parameters were unaffected by treatment throughout the study when compared to the controls.

C

### At 1500 ppm

At necrops, mean absolute and relative liver weights were statistically significantly higher when compared to controls. Enlarged livers were noted in 14/15 animals, compared with 0/15 animals in the control group.



 $\bigcirc$ 

Microscopic examination revealed hepatocellular hypertrophy, hepatocellular single cell necrosis, increased number of mitoses and interstitial mixed cell infiltrate.

Assessment of cell proliferation in the thyroid revealed a 2.6 fold ( $P \le 0.001$ ) higher BrdU labelling index, when compared to the controls.

Regarding the hepatotoxicity testing, total cytochrome P-450 content was increased (3.7 fold,  $P \le 0.001$ ) as were PROD (150 fold,  $P \le 0.001$ ), BQ (7.9 fold,  $P \le 0.001$ ), T4-GT (1.9 fold,  $P \le 0.001$ ) and BL2-GT (2.0 fold,  $P \le 0.001$ ) activities.

In the pituitary gland, *Tshb* gene transcripts were up-regulated (1.7 for  $P \le 0.001$ ) when compare to the controls.

#### At 750 ppm

At necropsy, mean absolute and relative liver weights were statistically significantly higher (+3.9% to +42%, P $\leq$ 0.01) when compared to controls. Enlarged by ers were noted in 7/P5 animals, compared with 0/15 animals in the control group.

Microscopic examination revealed hepatocellular hypertrophy, hepatocellular single cell necrosis and interstitial mixed cell infiltrate.

Assessment of cell proliferation in the provid revealed a 1.8 fold  $(P \le 0.00^4)$  higher Brd (labeling index, when compared to the controls.

Regarding the hepatotoxicity testing, total cytochrome P-456 content was occreased (3.6 fold, P $\leq$ 0.001) as were PROD (70 fold, P $\leq$ 0.001), BO(5.5 fold, P $\leq$ 0.001), F4-G7(1.8 fold, P $\leq$ 0.001) and BIL-GT (1.8 fold, P $\leq$ 0.001) activities.

In the pituitary gland, *Tshb* gene transcripts were up-regulated (1.6 fold,  $\mathbb{R} \ge 0.01$ ) when compared to the controls.

### Pxr KO/Car KO mue

No treatment-related clinical signs were observed throughout the study. Boty weight, food consumption and water consumption parameters were unaffected by treatment throughout the study when compared to the controls.

### At 1500 ppm

At necropsy, mean absolute and relative liver weights were statistically significantly higher when compared to controls.

Regarding the hepatotoxicity testing, PROD activity was Hightly induced (1.4 fold, P $\leq$ 0.01) whereas BQ and T4-GF activities were decreased (P.7 fold, P $\leq$ 0.001 and 1.3 fold, P $\leq$ 0.01; respectively).

In the pituitary gland, TShb gene transcripts were, Sughtly down-regulated (1.2 fold, P $\leq$ 0.05) when compared to the controls.

### At 750 ppm

At necropsy, mean absolute and relative liver weights were statistically significantly higher when compared to controls  $\sqrt{\sqrt{2}}$ 

Regarding the hepatotoxicity testing, PROD activity was slightly induced (1.4 fold, P $\leq$ 0.001) whereas BQ activity was decreased (1.5) P $\leq$ 0.001).

No were no changes in *Tshb* gene transcripts.

### Conclusion

The cleap increases in PROD activity seen in wild type C57BL/6J mice, with minimal PROD induction in Pxr KO/Car KO mice, after exposure to fluopyram, indicates that fluopyram is an inducer of Cyp2b



in wild type mice. The induction of BO activity in wild type mice and decrease in BO activity in Pxr KO/Car KO mice shows that fluopyram is also a Cyp3a inducer in wild type mice.

These data, together with the increases in the glucuronidation of bilirubin and thyroxine seen in wild type mice but not in Pxr KO/Car KO mice after exposure to fluopyram, clearly demonstrate that fluopyram is a Car and Pxr activator in mice. This is translated as in an increase of Tshb gene transcripts and in an increase of thyroid follicular cell proliferation.

#### Materials and methods I.

un	in an increase of anyrota formear	
	I. Materials and methods	
A.	Materials	
1.	Test material:	Fluopyram (AE (656948) Beige powder Mix-Batch:08528/0002 94.7% 658066-35-4 Stable in rodent diet for a period sovering the study dutation Vehicle for AE (656948 rodent diet No positive control Mouse - Male only 65/BL/60 No weeks approximately at start of greatment 22.1 to 26.9 g wildtype mice and 20.5 to 25.0 g Pxr KO/Car KO mice
	Description	Beige powder
	Lot / Batch #:	Mix-Batch:08528/0002 0 10 10 10 10 10 10 10 10 10 10 10 10 1
	Purity:	94.7%
	CAS #	658066-35-4 2 Q Q O O O A A
_	Stability of test compound:	Stable in rodent diet for a period govering the study detation
	Vehicle and / or positive	Vehrcle for AE Co56948 rodent diet
	ntrol:	
з.	Test animals:	Mouse Male only
	Strain:	C57BL/Gr & A A A A
	Age:	10 weeks approximately at start of greatment
		22.1 to 26.9 g wildtype mice and 20.5 to 25.0 g Pxr KO/Car KO
	Weight at dosing:	mige of a way of way
	Source:	
		and Taconic Parms (Germantown, New York, 12526 USA,
	Acclimation period of	respectively, for wildtype and Pxr KO/Car KO mice
	Acclimation periods	10 days > S S Contraction of the second seco
		S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-
		Orge, France), addibitum
	Water?	Municipal tap water, and libitum
	Diet: Water: Housing: Environmental conditions Temperature: Humidito: Air changes:	wite mesh cages ~ ~
	Environmental conditions Temperature:	
	Temperature:	
	Humidito O	Approximately 6-15 air changes per hour
	Air changes:	Approximately 67-15 air changes per hour
	Photoperiod:	Alternating 12-Hour light and dark cycles (7 am- 7 pm)
B.	Study design N A	
1.	In life dates: Dosing pe	riod: 24 September – 23 October 2012

## 2. Animal assignment and freatment

For both the wildt we and Pxr KO/Car CO mice, one group of control animals received untreated diet. whilst other two proups received fluopyram at the appropriate dietary concentration (750 ppm and 1500 ppm) at a constant level.

The dose levels for luopy am were set after evaluation of the results from previous studies conducted with fluotyram (M-408352-01-1, M-428303-01-1) and following discussions with the US (EPA) and Canadian (PMRA) authorities. The dose level of 750 ppm is the top dose level used in the mouse cancer bioassay in which an increase in thyroid tumors was observed in the males (M-295688-01-1). The top dose level of 750 ppm used in the mouse cancer bioassay has been shown to induce a slight but



statistically significant increase in thyroid follicular cell proliferation following 28-day treatment ( $\underline{M}$ -<u>428303-01-1</u>).

An additional dose level (1500 ppm) in the current study was investigated to better elucidate the vel of thyroid follicular cell proliferation induced after a high dose level of exposure of the test item.

A solution of BrdU at 80 mg of BrdU/100 ml of drinking water was administered to all animals during the last week of the study.

Table 5.5	-146 S	tudy desi	gn		, M				, e
		Dose		'animals oup		Animal i			
Group	Test item	level	C57BL/6J	Pxr KO/CaQ KQ	SG1	sen		CarKO K SG2	,
1	Control	0	15	10	TUM 2739 to	70M27520to Q 2759	TAM12744 to	T1M2760 to	
2		750 ppm	15		T2M2767	T2M2782 tC	T2M2775 to	T2M2789 to	
3	Fluopyram	1500 ppm	1564	\$15 °	₹3M2¶97 to 2803	∂73M2892 to 2819	2851 2851	T3M2820 to	
SG: Subgro	oup				S D	à à	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	X	

### 3. Diet preparation and analysis

Fluopyram was incorporated into the dier to provide the fequired dietary concentration. The method of preparation was documented in the study file. The test item formulation was stored at room temperature and issued to the animal unit in polyethylene containers. There was one formulation and any unused diet was discarded at the end of each administration period.

The homogeneity of fluopyram in diet at the two concentrations was verified to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as the measured concentration. The homogeneity and concentration data for fluopyram in rodent diet were between 93 and 97% of the nominal concentration. Abvalues were within the in-house target range of 85-115% of the nominal concentration. The stability of fluopyram at 20 and 10000 ppm in the diet was demonstrated in an earlier study (M-085510-01-10 which covered the period of storage and usage for the present study.

BrdU in filtered ap water from multicipal water supply was prepared during the study at 80 mg BrdU/100 ml in drinking water. This solution was stored in air-tight light-resistant bottles at approximatel 4°C when no in use. The unused solution was discarded at the end of the administration period.

The concentration of BrdU in tap water was verified and was found to be between 107% of the nominal concentration. This value was within the in-house target range of 90-110% of the nominal concentration. The stability of BrdU in aqueous solution was demonstrated in an earlier study (SA01416), which covered the period of storage and usage for the present study.

### 4. Statistics

## Variables analyzed

- Body weight parameters
- Body weight change parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Terminal body weight, absolute and relative organ weights parameters



Ľ,

- Total cytochrome P-450 content and liver enzyme activities
- Cell proliferation parameters
- Quantity of gene transcripts

#### Statistical methods

Mean and standard deviation were calculated for each group.

òpriy P-450 content and enzymatic activities were not statistically analyzed in Pristima; statistics are presented.

Group means were compared at least at the 5% levels of significance.

Copp. Statistical analyses were carried out using Pristiman ersion 6.3.2 build 19, Xybon

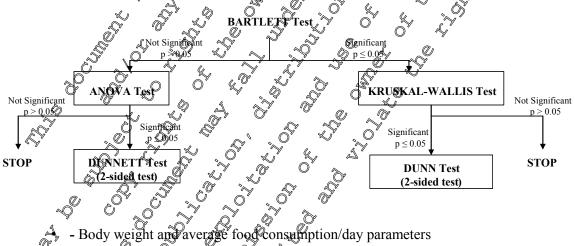
• - Total cytochrome P-450 content and liver enzyme activities

Statistical comparisons between fluopyram freated mice and their respective control groups were performed for all numerical data sets using a 2-tailed Student's t-test. 

- - Body weight change parameters
- Terminal body weight, absolute and relative organ weight parameters,

parameters for body weight change Mean and standard deviation were alculated for each gooup parameters.

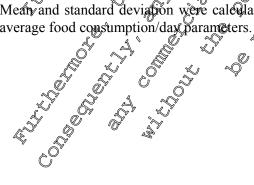
- Terminal body weig



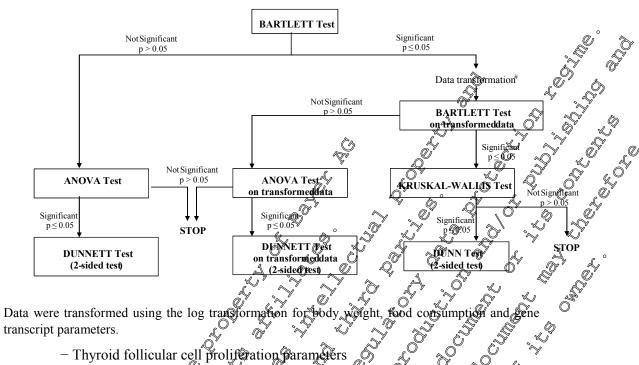
Body weight and average

Quantity of gene transcripts

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.







The Levene test was performed to compare the homogeneity of group variances

If the Levene test was not significant (p>005), preans of the exposed groups were compared to the mean of the control group using the ANOVA test.

If the ANOVA test was not significant (p>0.05), STOP

If the ANOVA test was significant ( $p \le 0.05$ ), means of the exposed groups were compared to the mean of the control group using the Dunnett test (T sided), T

If the Levene test was significant ( $p \le 0.05$ ), data were transformed using the log transformation and means of the exposed groups were compared to the mean of the control group using the Levene test on log transformed data.

If the Levene test on log transformed data was not significant (p>0.05), means of the exposed groups were compared to the mean of the control group using the ANOVA test on log transformed data.

If the ANOVAgest on or significant (p>0.05), STOP.

If the ANOVA test on logoransformed data was significant ( $p \le 0.05$ ), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1 sided) on log transformed data.

If the Levene test was significant  $(p \ge 0.05)$  even after log transformation, means of the exposed groups were compared to the mean of the control group using the Kruskal-Wallis test.

If the Kruskal-Wallis test was not significant (p>0.05), STOP.

If the Kruska Waltis test was significant  $p \le 0.05$ ), means of the exposed groups were compared to the mean of the control group using the Dumn test (1-sided).

If one primore group variance (s) equal 0, means will be compared using non-parametric procedures

## C. Srethods

## 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical



examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

#### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, on study Day 1 and there weekly during the treatment period and recovery phase. Additionally, animals were weighed before scheduled necropsy (terminal body weight).

#### 3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period recorded weekly for all animals during the treatment period to calculate the mean dail food consumption. Any food spillage was noted. The weekly mean achieved dosage intake in mg/kg/day each week and for Weeks 1 to 4 was calculated for each sex using the for Howing formula:

Dose level (ppm) ×  $G_{1}^{\circ}$  oup mean food coshumption (g/day)  $Test \ substance \ intake = \frac{1}{Group \ mean \ body weight (g) at the \ end \ of \ the food \ consumption \ period$ 

### 4. Water consumption

Drinking water bottles containing Brd Wwere weigher on the first day of BrdU raministration Study Day 23). Empty water bottles were werghed at least once before scheduled sacrifice (Study Day 30).

### **5.** Clinical Chemistry

On Study Day 30, blood samples were taken from al animals in all groups by princture of the

n

abdominal aorta. Animals were anesthetized by inhalation of IsoMuranes arros, France). Blood was collected on heparin for possible further analysis

### 6. Sacrifice and pathodogy

## Necropsy procedu Q – Organ sampling

On Study Day 30 all surviving animals were sacrificed by exsanguingtion under deep anesthesia by inhalation of Isoflurane (Virbac, Carros, France). Animals were not diet fasted prior to sacrifices.

All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Significant macroscopic abnormalities were recorded and sampled upon decision of the Study Pathologist.

At final sacrifice the

Tissue collection and histot

The following organs wer

- V• Duodenum

  - Pituitary gland

  - Thyroid@land

For each animar, the pryroid gland, two central sections taken from the median and left lobes of the liver and aportion of the duoder um were preserved 48 hours in 10% neutral buffered formalin.

Six sections of the thyroid gland, one piece of duodenum and two pieces of liver from all animals were processed and embedded in paraffin wax. Histological sections were prepared for all animals in all groups and stained with hematoxylin and eosin.



An immunohistochemical staining demonstrating the incorporation of BrdU and the determination of the labeling index was performed to assess thyroid follicular cell cycling on all study animals. A section from a formalin-fixed paraffin-embedded block containing 6 thyroid sections and one duodenum sample was prepared. The immunohistochemical reaction included incubation with a monoclonal artibody raised against BrdU, amplification with a secondary biotinylated antibody and a speptavidin-how radish peroxidase complex, detection of the complex with the chromogen diaming benzidine (DAB) and nuclear counterstaining with hematoxylin.

For each group, one piece of both the median and left lobes of the liver plus the pituitary ghand from each animal (both strains) were collected and flash frozen in liquid nitrogen. These samples were stored at approximately  $-74^{\circ}C + 10^{\circ}C$  until used for qPCR investigations.

The remaining portion of the liver from all animals were collected and flash frozen in liquid introgen prior to storage at approximately -70°C until shipped to CXR Biosciences for microscinal preparations.

### Histopathology and cell proliferation assessment

Histopathological examinations were performed on thyroid gland and liver for all antinals. Following the initial histopathological examination, a review of representative stides was performed by a second pathologist (Laëtitia ELIES) according by standard opprating procedures.

A digital image of the whole slide containing the throid gland was acquired using the Manozoomer 2.0 series (Hamamatsu). The labeling fudex, expressed as the number of BrdU positive thyrote follicular cells per thousand, was measured on multiples fields by counting in total a Peast 1900 cells. The mean and standard deviation were calculated for each group.

### Hepatotoxicity testing

At final necropsy, the remaining portions of the liver from all animals were collected and sent to CXR for liver subcellular fractionation. The liver was processed according to CXR LMS Cent-001. Microsomes were isolated and stored at approximately 40°C until required for total cytochrome P-450 content, specific concerning P-450 isoenzyme profile and aucoronidation assessments to check the hepatotoxic potential of the test item.

### Total cytochrome p-450 confent

Total cytochrome P-450 content of the liver microsomal fractions was determined according to LMS Spec-0062 (Omura and Sato, 364).

### Protein determination

The protein concentration of the liver microsomes was determined in aqueous solutions using a modification of the method of Lowfy et al. (1951) and bovine serum albumin standards, according to LMS Spec-009. Results were maintained in the study file.

### Cytochrome P-450 activity assays

Microsomal pentoxy esorufin-O-depentylation (PROD) was used as a marker for Cyp2b activity, and was measured according to LMS pluor-0002, and EQ-001 (Burke et al 1985).

Cyp3a11 activity was measured as the O-debenzylation of benzyloxyquinoline (BQ) according to LMS Fluor-0003, and EQ-001.

### Thyroxine (14) glucuron dation activity

Mouse fiver microsomes were incubated with 125I-thyroxine and the formation of T4-glucuronide was determined by HPLC with radioflow detection.

### Bilirubin grucuronidation activity

Bilirubin glucuronidation activity was assessed in mouse liver microsomes. Full details of the method used and the results were maintained in the Study File.



### Quantitative pcr analysis

### Total RNA purification

Total cytoplasmic RNA was isolated from the pituitary gland of all individual control and related animals using RNeasy Mini kits (Qiagen). RNA quality controls were performed based on the riposonal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

#### Quantitative PCR

An appropriate quantity of total RNA were used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Targaan assays (Assay on demand, Applied Biosystems), diluted first strand cDNA FastStart Universal Probe Master Mix (Roche) on an ABI prism 7900 HT machine (Applied Biosystems). β-actin was used as the reference gene for the quantitative calculations.

### Table 5.5-147 The list of Taqman assays used was as follows:

<b>1</b>	· 0		<u> </u>
Gene family	kotorni Réfs	et ID	n assay D (Applied ) Biosystems)
Thyroid Stimulating Hormone beta	TSAB AM 0		
subunit			S S S
Beta actin	Actb NM 00	0739201 0 4	1m00607939 <u>~s</u> 1
•			

Beta-actin (Actb) was selected as reference gene for the quantitative calculations of transcripts. The relative quantity (RQ) value of each test transcript was calculated using the following formula:

$$\Delta\Delta Ct = (Cttest - CtB2m)$$
, treated - (Cttest - CtB2m) control

### $RQ = 2 - \Delta \Delta Ct$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. Each RQ vitue obtained for a given gene was formalized by dividing by the RQ value obtained for the control animal T1M2737 (C57BL/6J mice) or T1M2744 (Pxr KO/Car KO mice).

### II. Results and discussion

### A. Observations

### 1. Mortality

There were no treatment related mortalities duffing the course of this study.

### 2. Clinical signs

### C57BL/6J mice:

No clinical signs were observed throughout the study

### Pxr KO/Car KO mice.

One control mouse had hair loss on both hand limbs on Study Days 29 and 30, whilst one mouse (T3M2825) from the 1500 poin treatment group was noted to have a swollen penis on Study Days 29 and 30. These clinical signs were considered to be incidental.

### B. Body weight and bodyweight gain

Body weight parameters were unaffected by treatment throughout the study.

Pxr KQ/Qar KO mice:

C57BK/6J mi€€



At 750 ppm, mean body weight gain was higher than in the controls during the first week of treatment, resulting in a cumulative body weight gain of 0.35 g, compared to a cumulative body weight loss of 0.06 g in the controls ( $p \le 0.05$ ), by Study Day 8. This difference was considered incidental in the absence of an overall effect on cumulative body weight change at 750 ppm and since body weight parameters were unaffected by treatment at the higher dose level of 1500 ppm.

### C. Food consumption

C57BL/6J mice:

Food consumption parameters were unaffected by treatment throughout the study compared to controls.

#### Pxr KO/Car KO mice:

At 750 ppm, mean food consumption was decreased by between -9% ( $\ge 0.01$ ; Study Day 19) and 4% (not statistically significant; Study Day 8) compared to the controls. We the absence of an effect on mean food consumption at the higher dose level of 0.500 ppm, the decreased food consumption noted at 750 ppm was considered to be incidental.

#### Achieved dosages

The mean achieved dietary intake of Buopyram expressed on mg/kg/day, eccived by the animals during the study was as follows:

Table 5.5-148	Mean achieved	dietary intake	of fbyopyram	Ŕ
1 4.5.10 2.10 1.10	and the second			Ô

Diet concentration     C578L/6J (mg/kg/tay)     C778L/6J (mg/kg/tay)     C778L/6J (mg/kg/tay)       (ppm)     750     750     7125     130       1500     1500     256     747     247	Mean	achieved dietary intake of fl	aopyram (Weeks 1 -	Å.
	Diet concentration		/kg@ay)	
	~~ °0	\$ ~ \$ 1255		130
	1500	256		247

### D. Water consumption

water consumption was unaffected by treatment.

### E. post mortem examinations

### Terminal body weight and organ weights

At terminal sacrifice, there were no changes in mean terminal body weights between control and wildtype (C57BL/GP) mouse groups or between control and Knock-Out (Pxr KO/Car KO) mouse groups.

In wild type animals, at 1900 and 750 ppm, mean absolute and relative liver weights were statistically significantly higher when compared to controls. These changes were dose-related and were considered to be treatment-related.

Mean over weight ±sd at scheduled sacrifice, wild-type groups					
Males					
Frippyratin dose level (ppm)	0	750	1500		
Mean absolute liver weight		1.8035**	2.115***		
(g)	1.277	$\pm 0.15861$	±0.19106		
Õ	$\pm 0.06859$	(+41%)	(+66%)		
Mean liver to body weight ratio (%)	4.9285	6.8482**	7.9883***		

### Table 5.5-149 Mean nver weight changes; Wild-Type Mice



Mean liver weight ±sd at scl (% change when	heduled sacrifice, w a compared to cont		
Sex		Males	
Fluopyram dose level (ppm)	0	750 嶡	1500
	±0.18549	±0.44559	±0.48285
		(+39%)	(+62%) ×
Mean liver to brain weight ratio (%)	200.0077	409 6459**	480.1812*** ×
	288-9877 ±17775730	<b>⇒3</b> 3.06164	±43.7024
	±1¥.73730	Q (+42%)	¢ (€66%)√ {
≤0.01; ***P≤0.001	and the second s	Å Ö	

In Knock-Out animals, at 1500 and 750 ppm, mean absolute and relative fiver weights were statistically significantly higher when compared to controls. These changes were close-related and were considered to be treatment-related. A Contraction 0

Table 5.5-150	Mean liver weight chan	igês; Knóck-	OutWice	A ST			
Table 5.5-150       Mean liver weight changes; Knock-Out Mice         Mean liver weight sid at scheduled sacrifice, knock-out groups         (% change when compared to controls)							
	Sex A	Ø Y		y Males	Š. 4		
Fluopyran	n dose level (ppm)			م 75 <b>0</b> ر	1500		
Mean absol	lute liver weight (g) 📎	1.29	45	© 1.4998** °	0~1.4307**		
			045 (1)	±0,07952 (+8%)	±0.07729 (+11%)		
Mean liver to	body weight ratio (%)	501	19 2		5.7970**		
		¢ £9.24	617	(+7%)	±0.23142 (+9%)		
Mean liver to	brain weight ratio (%)	$\sim$ $20\pi$	051	© (+7%) © 315.5933* © ±26.98886	336.8503**		
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		±5.48	8669°	±26.98886	$\pm 18.1761$ (+13%)		
*P≤0.05; **₽€0.01	;***P≤0,001 √ →		O^y ((*1570)		
F. Gross pathol							

C57BL/6J mice:

Enlarged livers were noted at 1500 and 750 ppm These changes were associated with microscopic hepatocellular hypermophy and were considered to be treatment-related.

ð Macroscopic changes in the Liver Wild-Type Mice Table 5.5-151

ficidence of macroscoffic changes in	the liver, scheduled	I sacrifice, wild	d-type groups
Sex Sex		Males	
L Huopyram dos level (ppm)	0	750	1500
Kalarged Q	0/15	7/15	14/15

Pxr KO/Car KOnnice: No macroscopic change was noted. G. Microscopic pathology

C57BI



At 1500 ppm, hepatocellular hypertrophy, hepatocellular single cell necrosis, increased number of mitoses and interstitial mixed cell infiltrate were noted. $^{\circ}$

At 750 ppm, hepatocellular hypertrophy, hepatocellular single cell necrosis and interstitial mixed cell infiltrate were noted.

ible 5.5-152 Milcros	copic changes in the Liver; wild-Type Mic
Incidenc	e of microsconic changes in the liver scheduled sacrifice wild-type grows
Sex	Males
Fluopyram dose level (ppm)	
Number of examined animals	
Не	patocellular hypertrophy: contrilogular to panlobular
Minimal	patocellular hypertrophy: centrilokular topanlohular
Slight	
Moderate	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
Total	B 4 4 4 4 5 6 7 0 Hepatocellular single cell necrepts: focal 5 5 6 7 7
	Hépatocellular single cell necrosis: focal
Minimal	
Slight	
Total 🗞	
~	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
Present	
ŠĆ	Anterstitial mixed cell mfiltrate: fogat
Minima D	9 11
Minima Slight	
v Potal 🔬	
r KOVCar KO mice	

Table 5.5-152Microscopic changes in the Liver; Wild-Type Mic

No relevant microscopic, change as noted at \$500 or \$50 ppm.

H. Cell proliferation

C57BL/6J mice:

Higher proifferative indexes were not (2.6) fold at (500 ppm and 1.8 -fold at 750 ppm) when compared to controls.

Table 5.5-153 Mean BrdU Bbeling index Groliferation rate/100 cells); Wild-Type Mice

Tryroid grand proliferation index						
Fluopyrant dose level	750	1500				
N 2 A 15	15	15				
Moon 5 14.28	26.08***	36.61***				
3.96	7.16	10.27				

***P≤0.000

Pxr KO/Car KO mice:



No change was noted between control and treated groups.

Table 5.5-154Mean BrdU la	beling index (prolife	eration rate/100 cells); Knock	-Out Mice
	Thyroid gland pro	oliferation index 🛛 🖉	
Fluopyram dose Level (ppm)	0	750	[™] 1500 👘 💭
N	15	15	15
Mean	10.05	9.91	\$27 Q 4
Std	3.88	<u>گ</u> 4.06	3.38 6

I. Hepatotoxicity testing

C57BL/6J mice:

The total cytochrome P450 content of the liver microsomal fractions from male C57BL/6L mice administered fluopyram at 1500 ppm and 750 ppm for 28 days was increased 3.7- and 3.6-fold, respectively, over the concurrent control.

Administration of fluopyram at 1500 ppm and 750 ppm for 28 days increased hepatic microsomal PROD (a marker for Cyp2b) 151- and 70-fold in male C57BL/61 mice respectively, over the consurrent control.

28 day exposure to fluopyram at 500 ppm and 750 ppm increased microsomal BQ (a marker for Cyp3a11) activity by 7.9- and 5.5-fold concurrent controls in male C57BLGI mice, respectively.

Following 28 days exposure to fluopyrom at 1500 ppm and 750 ppm, microsomal thyroxine glucuronosyl transferase (T4-GT) activity was increased by 1.9- and 1.8-fold consurrent controls in male C57BL/6J mice, respectively.

Bilirubin glucuronidation in male C57BL/69 mice administered Huopyram at 1500 ppm and 750 ppm for 28 days was increased 2.0-, and 1.8-fold, respectively, over the concurrent control.

Table 5.5-155	Res	Henatot	w" ovicity	Aestin	g; Wild-	Twne I	И	2
1 abic 5.5-155		insparoz	DAICITY	uspun	g, , , nu-	r spc 1	The contract	- K

Parameter	Control 0 0	Fluor 780	Fluopyram 1500 ppm
	2 ppm 7 & C57	BE/6J S	pp
Total P450 nmol/mg	≪0.34 ±€.22		1.25 ± 0.18 ***
protein S	@(100.0≇ 63.5)	\bigcirc^{\vee} (363.3 ± 47.2)	(367.3 ± 51.3)
PROD pmols esorutio formed/min/mg protein	(100.0 ± 10.0)	$5 14621 \pm 15.11^{****} \\ (6991.0 \pm 753.5)$	$302.14 \pm 84.76^{***} \\ (15065.3 \pm 4226.4)$
BQ printing quinolifie formed /min mg	2.77 ± 0.54 (100.0 ± 2.3)	15.20 ± 1.89*** a (549.3 68.3)	$21.94 \pm 1.83^{***}$ (792.8 ± 66.1)
T4-GT pmol T4-	0.68 ± 0.157 (100.0 ± 29.9)	$1.06 \pm 0.17^{***a}$ (183.8 ± 29.2)	$\begin{array}{c} 1.09 \pm 0.25^{***} \\ (189.5 \pm 43.6) \end{array}$
BiL-GT nmol behrubin@lucuronide formed/mm/mg protein	$ \begin{array}{c} & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & & \\ & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & \\ & & & & & \\ & & & & & & \\ & & & $	$1.30 \pm 0.22^{***a}$ (177.4 ± 30.0)	$\begin{array}{c} 1.43 \pm 0.42^{***} \\ (195.6 \pm 57.6) \end{array}$

a Values are Mean \pm SD Values in parenthesis are mean % control \pm SD. n = 15 per group; a n = 14

A Students' (seest (2-sided) was performed on the results; *statistically different from control p<0.05; **p<0.01; *** p<0.001



Pxr KO/ Car KO mice:

Fluopyram had no effect on the total microsomal P450 content in the Pxr KO/Car KO mice.

Both at 1500 and 750 ppm, Pxr KO/Car KO mice showed little induction of PROD in comparison to wild type animals, even if the effect was statistically significant (1.4 fall) Ô Ô

Administration of fluopyram at 1500 ppm and 750 ppm to male Pxr KO/Car KO mice for 28 days decreased BQ activity to 1.7-fold and 1.5-fold of concurrent control values respectively.

Administration of fluopyram to male Pxr KO/Car KO mice for 28 days had no effect on T4-GT at 750 ppm, and decreased T4-GT activity to 1.3-fold of concurrent control values at 1500 ppm.

Results of Hepatotoxicity testing; Knock-Out Mice

Parameter	Control of	Fluopyram 750 ppm	Fluopyram 1500
	ppm 🕺		ppm y
	× Pxr ŘØ/	Car 🕅 🔗 🚽 🤅	
Total P450 nmol/mg	0.2×0.09	$\mathcal{O}' \mathcal{O}' = 0 \mathcal{O}' = 0 \mathcal{O}' = \mathcal{O}' \mathcal{O}' = \mathcal{O}$	$5^{\circ} 0.2\vec{z} \neq 0.12$
protein	$(1.08.0 \pm 36.5)$	an 2 3 + 29 7) . C	(1100 + 475)
1			
PROD	Q2.27 + 0.30	>> 3.20€0.81*	∂.24 ±3√16**
pmols resorufin	- (10000 10 1000	(101.3 ± 25.6)	(142.9 ± 51.0)
formed/min/mg protein	(10000 ± 13.0)		(142.9 ± 31.0)
BQ nmols 7-OH	4. E ^Q 4.		Ô.
quinoline formed /min/mg	^{3.51} ⊅0.39 0 [°]	©2.40±0.40***	209 ± 0.35***
	(100.0°± 11.0°	$(68x3 \pm 113)$	(59.4 ± 10.1)
protein			
T4-GT pmol T4-		0.66 ± 0.170	
glucoronide	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	0.66±0.170	0.43 ± 0.15*
formed/min/mg.potein	s (100,0,°± 34,0)	(159 ± 299)	(75.3 ± 26.7)
	V & /		
BILOT	0.70 ± 0.24 a	0.66 a 0.24 ª	0.61 ± 0.28
nmol bilirubm-gluguronide	(100.0 ± 34.1)	(94.5 ± 34.4)	(86.5 ± 39.4)
	KI A O		(00.0 - 57.1)
$3 \mathbf{V}_1$ \mathbf{N}_1 \mathbf{V}_2 \mathbf{V}_1	~ 1		14

^a Values are Mean \pm SD. Values in prenthesis are mean % control \pm SD n = 15 per group; ^a n = 14 A Students' t-test (2-sided was performed on the results; *statistically different from control p<0.05; **p<0.01; *** p<0.001

J. Q-PCR analysis

C57BL/6J mie

Tshb gene transcripts were up-regulated in a dose related manner (1.7-fold, p≤0.001 and 1.6-fold, p≤0.01 and at 1500 and 750 ppm, respectively) in the pituitary gland of fluopyram treated male mice when ¢, compared with controls. Ô

Tshb gene transcript levels; Wild-Type Mice Table 5.4-157

Gene	A Mean Relative Quar	ntity ± standard deviation of gen compared to control mean valu	e transcripts (% change ues)
transcripts	Convol	Fluopyram 750 ppm	Fluopyram 1500 ppm
	^y 1,23 ± 0.289	$1.92^{**} \pm 0.306 \ (+56\%)$	2.05*** ± 0.586 (+67%)
**₱≩0.01:,¢¥*P≤0.00	01		



Pxr KO/Car KO mice:

Tshb gene transcripts were slightly down-regulated (1.2-fold, p≤0.05) at 1500 ppm and very slightly down-regulated (1.1-fold, not statistically significant) at 750 ppm in the pituitary gland of male mice treated with fluopyram when compared with controls. Due to the low amplitude of these deregulations. these changes were considered as non-biologically relevant.

Table 5.4-158 Tshb gene transcript levels; Wild-Type Mice

Gene	Mean Relative Qua	ntity ± standard@eviation of gen compared to control mean value		ts (9% change	
transcripts	Control	Fluepyram 750 ppm	° (opyratin 15005 ppm 0	
Tshb	1.25 ± 0.264	0 ¹ .14 ± 0.177	°¶∕04*∖	±0.165¢(17%)	Ũ
*D <0.05					<u></u>

*P≤0.05

K. Deficiencies No specific deficiencies were noted in this study III. Conclusions

The clear increases in PROD activity seen in wild type C57BL/6Pmice with mining PRODAnduction in Pxr KO/Car KO mice, after exposure to fluopyram, indicates that duopyram is an inducer of Cyp2b in wild type mice. The induction of BQ activity in wild type mice and detease in BQ activity in Pxr KO/Car KO mice shows that Thiopyram is also a Cyp3a inducer in wildaype mice.

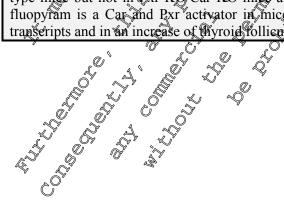
These data, together with the increases in the guicuronidation of bits ubin and thyroxine seen in wild type mice but not in Pxr KO/Car KO mice after exposure to fluopyram, clearly demonstrate that fluopyram is a Car and Pxr activator, if mice. This Otranslated as in an increase in Tshb gene transcripts and in an increase of hyroid follied ar cell proliferation

Assessment and conclusion by applicant

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on fluopycam in the mouse.

The clear increases in PROD activity een in wild type \$77BL/6J mice, with minimal PROD induction in Pxr KO/Car KO mice, after exposure to Diopyram, indicates that fluopyram is an inducer of Cyp2b in wild type mice The induction of BQ activity in wild type mice and decrease in BQ activity in Pxt/KO/Car KOmice shows that flugpyram is also a Cyp3a inducer in wild type mice.

These data, together with the increases in the fucuronidation of bilirubin and thyroxine seen in wild type mice but not in Bxr KQ/Car KO mice after exposure to fluopyram, clearly demonstrate that fluopyram is a Car and Pxr activator in mice. This is translated as in an increase in Tshb gene transcripts and in an increase of Myroid follicular cell proliferation.





Data Point:	KCA 5.5/21
Report Author:	
Report Year:	2013
Report Title:	Fluopyram: Assessment of pentoxyresorufin-o-depentylation and
	benzyloxyquinoline-o-debenzylation in 50 liver microsomal samples
Report No:	CXR1284
Document No:	<u>M-451628-01-1</u>
Guideline(s) followed in	No specific guideline
study:	
Deviations from current	Current guideline: none
test guideline:	
Previous evaluation:	Not previously evaluated
GLP/Officially	No, not conducted under GAP/Officially recognized testing facilities
recognised testing	No, not conducted under GAP/Officially recognized testing facilities
facilities:	
Acceptability/Reliability:	Yes V V V V

Executive Summary

- Microsome samples (50 samples) were received, frozen, by CXR on 03-Aug-20 M and stored at approximately -70 °C prior to analysis. Microsome preparation and measurement of total protein concentration was carried out by Bayer S.A.S (Sophia Antipolis, Grance, where in-life phase of the study was conducted under the Study number SA 11005, Documentation No. <u>M-428031-02-1</u>, Rouquie D. (2012): Fluopyram $\stackrel{\frown}{=}$ Mechanistic 28-day toxicity study in the nouse by dietary administration (hepatotoxicity and thyavid hopmone forestigations).
- The activity of Cyp2b was measured in the mouse liver microsome preparations as the rate of depentylation of pentoxyresorufin (PROD).
 28 days dietary administration of fluopyram resulted in a dose dependent increase in PROD
- 28 days dietary administration of fluopyrum resulted in a dose dependent increase in PROD activity to a maximum 47.2-fold at 750 ppm. 28 days administration of phenobarbital at 80 mg/kg/day resulted in a 3207 fold increase in PROD activity
- Following a 28 day recovery period on control diet, PROD activity in both fluopyram and phenobarbhal treated mice returned to control levels
- The activity of Cyp3a was measured in the mouse liver microsome preparations as the rate of debenaviation of benavious guinoline (BQ).
- 28 days dietary administration of fluopyran resulted in a cose-dependent increase in BQ activity to a maximum 62-fold at 750 ppm. 28 days administration of phenobarbital at 80 mg/kg/day resulted in a 3.0-fold increase in BQ activity
- Following a 28 day recovery period on control diet, BQ activity in both fluopyram and phenobarthal treated more returned to control levels.

I. Materials and methods

A.	Materials	
1.	Test material: "" . O	Eluopyram (AE C656948)
	Description Lot / Bach #:	Beige powder
	Lot / Batch #:	⁹ Mix ₂ Batch:08528/0002
	Purity & S &	94.9%
	CAS# O	658066-35-4
	CAS# Stability of test compound:	Stable in rodent diet for a period covering the study duration
2	Vehicle and / or positive	Vehicle for AE C656948: rodent diet
4		Phenobarbital (positive control)
CO	htrok	Vehicle for Phenobarbital: methylcellulose
	Positive control:	Phenobarbital
	Description:	White crystalline powder



Lot/Batch:	Lot No. 09050075
Purity:	100%
CAS:	50-06-6
Stability of test compound	Stable in 0.5% aqueous solution of methylcellulose for a period
	covering the study duration
3. Test animals:	
Species:	Mouse - Male only
Strain:	C57BL/6J (2) (2) (2) (2)
Age:	8 weeks approximately at start of treatment $\sqrt{2}$
Weight at dosing:	Stable in 0.5% aqueous solution of methylcellulose for a period of methylcellulose for a perio
Source:	
Acclimation period:	13 to 15 days \mathcal{A}^{\vee} \mathcal{Q}^{\vee} \mathcal{A}^{\vee} \mathcal{A}^{\vee} \mathcal{A}^{\vee}
*	Certified rodent powdered and irradiated Stet AQAC-10P1 from
Diet:	S.A.F.E. (Scientific Animal Food and Engineering, Epinay-Sur-
	Orge, France), and libitian
Water:	Municipal tan water and libitum
	Animals were caged individually in suspended stainless steel with mesh cages
Housing:	with mesh cages a frequency of the second seco
Environmental conditions:	
Temperature:	20-24 C V V V V V
Humidity:	40-70%
Air changes:	Approximately 10-15 ar changes perhour &
Photoperiod:	Alternating 12-hour light aperdark@ycles (7 am- \$\vec{P} pm)
• ~ &	20-24 40-70% Approximately 10-15 ar changes per hour Alternating 12-hour light and dark oycles (7 am- Φ pm)
B. Study design	
1. In life dates: Dosing per	igd: 24 September – 20 October 2012
	Alternating [2-nour light and dark coycles (7 am- ϕ pm) right 24 September – 25 October 2012 beriod 22 June – 26 July 2011
2. Microsomal analysis dates	14 March – 18 March 2013
3. Animal assignment and greating	
Seven groups of male mice were do	sed for at least 28 days with either control diet or the appropriate

compound by the appropriate oute of administration

Six groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the remaining five groups received Duopyram at the appropriate dietary concentration (30, 75, 150, 600 and 750 ppm) at a constant level. The dose levels for fluopyramwere set after evaluation of the results from previous studies and following discussions with the US (EPA) and Canadian (PMRA) authorities. The top dose level of 750 ppm used in the present studyrepresents the top dose level used in the mouse cancerboloassay in which thyroid tumors wereobserved in the males.

The seventh group was dosed by oral gavage with 80 mg/kg/day phenobarbital suspended in 0.5% aqueous methodcellulose 400 using a dosing volume of 5 ml/kg bodyweight the first week of treatment and then using a dosing volume of 0 ml/kg bodyweight for the remaining treatment period. The volume administered to each more way based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose was used in previous mouse mechanistic studies conducted with phenobarbital, where thyroid changes were detected.

Each group consisted of 15 male mice with the exception of the control group, the 750 ppm fluopyram group and the phenobarbital group, where 15 additional males were fed control or test diet (750 ppm fluopyram) or were orally dosed (80 mg/kg/day phenobarbital) for at least 28 days and were then allowed one month (at least 28 days) of recovery during which they were maintained on untreated control diet.

All animals were sacrificed in the morning after the last day of treatment or after the last day of the recovery phase.



Table 5.5-159	Study design
---------------	--------------

Group	Test substance	Dose level	Number of animals per group	Animal identity
		Male	es	
1	Control	0	15 + 15*	T1M1885 to 1914
2		30 ppm (5 mg/kg/day)	<u>کہ</u> 15	T2M1915 76 1929
3		75 ppm (13 mg/kg/day) _{(ب ب ب الم	T3M1930 to 944
4	Fluopyram	150 ppm (25 mg/kg/day)		T4M1945 to 1959
5		600 ppm (102 mg/kg/day)		T 50 1960 to 19742
6		75¢ ppm (128mg/kg*day)	€ ¹⁵ ± €5*	T6M4975 to 2004
7	Phenobarbital	mg/kg/day	15+15*	5 KM200 Lo 2034

4. Statistics

4. Statistical comparisons between treated microsomes and their control group were undertaken for all

C. Methods

1. Microsomal samples

Microsome samples were received, frozen, by CXR on 03-Aug-2011 and stored at approximately -70° C poor to analysis. Microsome preparation and measurement of total protein concentration was carfied out by Bayer S.A.S (Sophia Antipolis France, Study number SA 11105). Õ

2. Pentoxyresorufit-O-depentylation (PROD)

The activity of Cyp2b in microsomal samples was determined spectrofluorometrically by the formation of resortin from penerxyresorufin, as described by Burke et al. (1985) Biochem. Pharmacol. 34, 18. 3337-3345, according to LMS Fluor- 002°

Ľ

3. Benzyloxyquinoline-O-debenzylation (BO)

Ô The activity of Cyp3a in microsoma samples was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from bergyloxyquinoline, as described by GENTEST HTS technical bulletin, according to LMS Fluor 005.

Results and discussion Ц,

A. Pentoxyresorufin-odepentylation

The activity of cyp2b was measured in the mouse liver microsome preparations as the rate of depentylation of pentoxyresorufin (PROD).

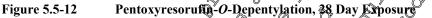
28 days detary administration of fluopyram resulted in a dose-dependent increase in PROD activity to a maximum 472-foldat 750ppm. 28 days administration of phenobarbital at 80mg/kg/day resulted in a 32.7-fold increase in prod activity.

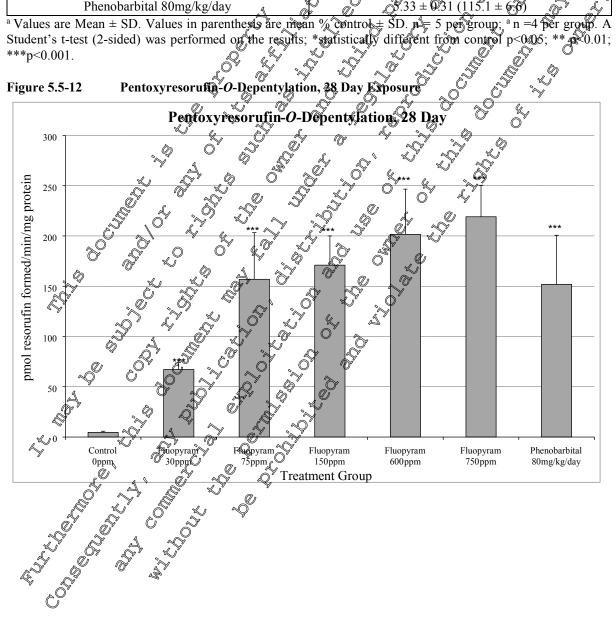
Following 28 day recovery period on control diet, PROD activity in both fluopyram and phenobarbital treated mice returned to control levels.



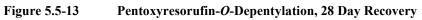
PROD (pmol resorufin formed/min/mg protein)°
$4.65 \pm 1.12 (100.0 \pm 24.2)^{a}$
67.22 ± 6.95*** (147.1 ± 149.5)
156.89 ± 46.37*** 37377.5 ± 998.3
$170.99 \pm 29.09 $ (3680.8 ± 626.2)
$201.51 \pm 45.02^{***} (4338.0 \pm 69.1)^{1}$
219.06 ± 30.31*** (4715 ± 6749)
$151.87 \pm 48.84^{***} (3269.3 \pm 1021.4)$
5.27 ± 0.75 (113.99 16.1) av
A Q.33 ± Q.31 (115.1 ± G)

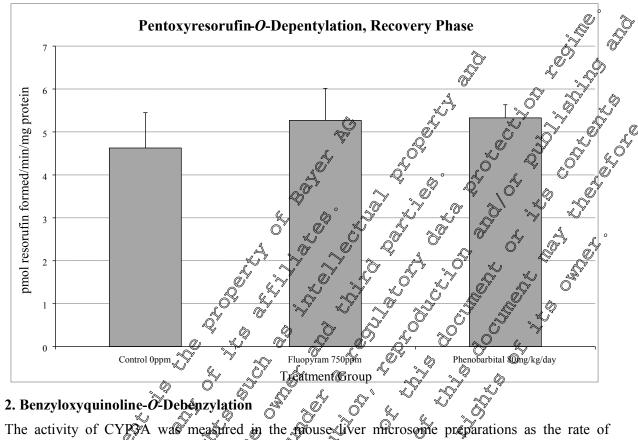
Table 5.5-160	Pentoxyresorufin-O-Depentylation
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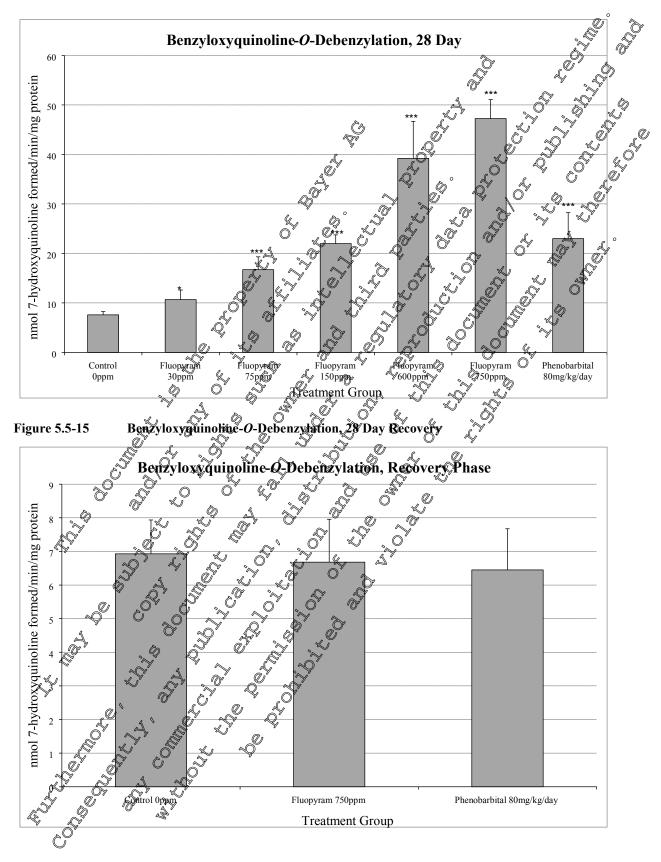
debenzylation of benzyloxyquinobne (BQ). 28 days detary administration of fluopyram resulted in a dose-dependent increase in BQ activity to a maximum 6.2 fold at 50ppp. 28 days administration of phenobarbital a080mg@rg/day resulted in a 300-fold increase in bg activity. Following a 28 day recovery period on control dies BQ sofivity in both Huopy am and phenobarbital treated mice returned to control levels. . U

Table 5.5-161 Benzyloxyquinoine-O-leebenzylation	
Treatment 28 day exposure	BQ (nməl 7-Hydroxyquinoline formed/min/mg protein)
Control 5 6	$7.59 \pm 0.67 (100.0 \pm 8.8)^{a}$
Suopyram 30ppm	
A Fluopyram 75ppm S	$10.68 \pm 1.92^{\circ} (140.7 \pm 23.3)$ $16.71 \pm 2.61^{***} (220.2 \pm 34.4)^{\circ}$
Fluopyram 150ppm	21.98 ± 1.76*** (289.7 ± 23.2)
🔬 Fluopykam 600kpm 🖉 🖉	39.20 ± 7.49*** (516.6 ± 98.6)
Fluopyram 750ppm C	$47.24 \pm 3.85^{***} (622.6 \pm 50.8)$
Phenobarbitak 80mg/1@/day	$23.00 \pm 5.24^{***} (303.1 \pm 69.1)$
Treatment, 28 day recovery	
Contra 5	$6.93 \pm 1.01 \ (100.0 \pm 14.5)$
Fabopyram 750ppp	$6.68 \pm 1.27 (96.4 \pm 18.4)^{a}$
Phenobarbinal 80mg/kg/day	6.45 ± 1.22 (93.1 ± 17.7)

^a Values are Mean \pm SD. Values in parenthesis are mean % control \pm SD. n = 5 per group; ^a n = 4 per group. A Student's 4-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.



Figure 5.5.4-14 Benzyloxyquinoline-O-Debenzylation, 28 Day Exposure





B. Deficiencies

No specific deficiencies were noted in this study

III. Conclusions

28 days dietary administration of fluopyram resulted in a dose-dependent increase in PROD activity to a maximum 47.2-fold at 750ppm. 28 days administration of phenobarbital at mmg/kg/day resulted in a 32.7 fold increase in PROD activity. Following a 28 day recovery period on control diet, PROD activity in both fluopyram and phenobarbital treated mice returned to control levels.

The activity of Cyp3a was measured in the mouse Wer microsome preparations as the rate debenzylation of benzyloxyquinoline (BQ). 28 days dietary administration of fluopyrano resulted in a dose-dependent increase in BQ activity to a maximum 6.2-fold a0750ppm. 28 days administration @ phenobarbital at 80mg/kg/day resulted in a 3.0-fold increase in BQ activity. Following 28 day recovery period on control diet, BQ activity in both fluopyram and phen@barbitat treated mice returned to control levels.

Assessment and conclusion by applicant:

OR ¢ ₽ ₽ Study meets the current guidance and the requirements in 283/2015 as it provides mechanistic information on the effects and target organs of fluopyram in the mouse.

28 days dietary administration of fluopyram resulted in a dose-dependent increase in PROD activity to a maximum 47.2-fold at 750ppm. 28 days administration of planobarbital at 80mg/0g/day resulted in a 32.7 fold increase in PROD activity. Following a 28 day recovery period on control diet, PROD activity in both fluopyram and phenobarbital treated mice returned to control levels.

The activity of Cyp3a was measured in the morse liver microsome preparations as the rate of debenzylation of benzyloxyquinctine (BQ). 28 days dietary administration of fluopyram resulted in a dose-dependent increase in BQ activity to a maximum 6.2 fold at 750ppm. 28 days administration of phenobarbital at 80mg/kg/day resulted in/a 3.0 fold increase of BQ activity. Following a 28 day recovery period on control diet, BQ activity in both fluopyram and phenobarbital treated mice

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Data Point:	KCA 5.5/22	
Report Author:		
Report Year:	2020	Ô
Report Title:	In vitro CYP and UGT induction in human and Wistar rat hepatocytes by	, The second sec
	Fluopyram	
Report No:	KLC-BA20-06	
Document No:	<u>M-759019-01-1</u>	
Guideline(s) followed in	No specific guideline)
study:		
Deviations from current	Current guideline: none	
test guideline:		Š
Previous evaluation:	No, not previously submitted A S S S	-
GLP/Officially	No, not conducted under GAP/Officially recognised testing/facilities	
recognised testing	No, not conducted under GEP/Officially recognised testing/facilities	
facilities:		
Acceptability/Reliability:	Yes O Q & A M A	

Executive Summary

Primary cultures of cryopreserved plateable male human and Wistor rat hepatocyces were used to assess the potential of Fluopyram to induce CXP and UGR expression and to picrease thyroxine (T4) glucuronidation, after 3 or 7 days of daily treatment.

Induction study was performed with 3 male human and 3 male Vistar rat hepatocyte cultures treated daily for 3 days and 7 days with Fluopyran at 10, 30, 60 and 100 μ M-of with positive control inducers beta-Naphthoflavone (BNE), rifamicin (BIF) and phenobarbital (PB) for human hepatocytes and BNF, 5-pregnen-3 β -ol-20-one 16a-carbonitrile (PCN) and PB for tat hepatocytes. RNA quantity established that Fluopyram at 10, 30 and 00 μ M were suitable concentrations for mRNA expression and activity analysis.

In Wistar rat hepatocyte reference inducers BNF (5μM), PB (1000 μVF) and PCN (6 μM) strongly induced CYP1A, CYB2B1 and CYR3A1 oppression, respectively and increased the related activities. PB and PCN also induced UGT2BP and to a lesser extent UGTA1 expression and BNF, PB and PCN increased UGT-T4 activity.

In human hepatocytes, reference inducers BNF ($\mathcal{F}\mu M$), $\mathcal{P}B$ ($\mathcal{P}00 \mu M$) and RIF (15 μM) induced CYP1A2, CYP2B6 and CYP3A4 expression, respectively, and increased the related activities. PB and RIF induced UGT A1 expression, BNF and RIF increased UGT-T4 activity.

The present results show that Fluopyram at 10 μ W, 30 μ M and 100 μ M is a strong CYP3A and to a lesser extent a CYP1A2 inducer in Wistar rat hepatocytes. Fluopyram at all tested concentrations also consistently induces UGT0B1 and increases UGT-T4 activity in Wistar rat hepatocytes. Fluopyram at 10 μ M, 20 μ M and 100 μ M is a CYP1A2, CYP2B6, CYP3A4 and UGT1A1 inducer in human hepatocytes but does not increase UGT-T4 activity in human hepatocytes.

Materials and methods
 Materials
 Materials
 Test materials
 Floopyram
 Description

Lot Batelr#:

Stability of test compound:

Floopyram Off-white powder Mix-Batch: 08528/0002 94.5% 658066-35-4 Stable for a period covering the study duration (Expiry date: 19 March 2022)

2. Control materials:



None (Culture medium was used as the negative control) **Negative:** Solvent / final **DMSO** concentration: Beta-Naphthoflavone (BNF) Phenobarbital (PB) sodium salt **Positive:**

5-pregnen-3 β -ol-20-one-16 α - carbonitrile (PCN) Rifampicin (RIF) male human and male Wistar rat hepatocytes

4. Test concentrations: Induction study-CYP/UGT 10, 30, 60 and 100 µM mRNA expression and

enzyme activity:

- B. Study design
- 01 September 2020 1. In life dates:

2. Hepatocyte information

3. Test organisms:

Cryopreserved male human and male Wistar rat hepatocytes provided by R France) and Bio-IVT were used Coll wight and hepatocytes provided by determined using France) and Bio-IVT were used. Cell yields and viabilities wøre exclusion.

3. Solubility test

Preliminary solubility tests with Fluopyram confirmed by macroscopic observation that the compound was soluble up to 100 mM in DMSQ. Flugevram was considered soluble in treatment medium, without any presence of crystals up to 100 µM (from a 100 mM stock solution).1% DMSQ final in the medium). Ň 0 L No crystals were observed up t_{0} 100 μ M. C

Stock solutions of BNF, RIP, PCN and Eluopysam were prepared in DMSO (100%) and diluted in treatment medium daily such that the final concentration of DMSO was 0.1%. The stock solution of PB was prepared in water and filtered (0.22 µm) and thereafted diluter in treatment medium containing a final concentration of PMSO of 0.1% \$

4. Hepatocyte culture and treatment

Hepatocycles from 3 batches per species were seeded in 96-well photes at a density of 0.07×10⁶ cells per well for human and as a density of 0.05 306 cells per well for rat, in 100 µL attachment medium per well.

All cultures were incubated in a humidified atmosphere of CO2/air (5%/95%), at 37°C. After a 6 h attachment period, the attachment medium was replaced by treatment medium (serum-free Hepatocyte Maintenance Media (HOM) supplemented with JPS (1% v/v), dexamethasone (100 nM) and penicillin/streptomycin/100,000 U/L (100 mg/L)) ... ×

The reference inducers were tested at concentrations known for their respective induction of CYPs and UGTs (historical data from KaLy-Cell)

Cells were exposed to either the reference compounds: 5 µM BNF, 1000 µM PB, 15 µM RIF (human hepatocytes) of 6 µMPCN that hepatocytes) or to test item Fluopyram at 10, 30, 60 or 100 µM. Solvent controls consisted in medium containing 0.1% DMSO. The volume of medium per well was kept constant (100 µL) throughout the treatment period. All incubations were performed in triplicate. The cell monolayers were overlaid with Matrigel at 0.25 mg/mL on Day 1 (24h after seeding) and on Day 4 of culture.

Æ,



C. Methods

1. Morphological observations

During the induction study, primary cultures of human and rat hepatocytes treated with Fluopyram were observed daily under the microscope, before medium replacement. In addition, solubility of Fluopyram in the treatment medium was daily checked by microscopic observation for the presence of existals.

2. Measurement of RNA content and CYP and UGT mRNA expression of hepatocyte monogayers

On Day 3 and Day 7 of treatment, total RNA was extracted using the King Fisher TM Flex Purffication System with the MagMAX mirVana Total RNA Isolation kit (Thermo Fisher Scientific USA) and stored at -80°C. RNA was quantified by fluorimetry using the Quantifluor RNA System (Promega USA).

The effect of Fluopyram at 10, 30 and 100 µM on aRNA expression was assessed in human bepatocytes for CYP1A2, CYP2B6, CYP3A4, UGT1A1, UGT1A6 and UGT2B and in Wister at hepatoc tes for CYP1A2, CYP2B1, CYP3A1, UGT1A1, UGTIA5/6 and UGT2B1, The street of BNF, PB, RIE (human hepatocytes only) and PCN (rat hepatocytes only) was assessed in parallel. After RNA extraction and quantification, cDNAs were synthesized from 0 1 ug total RNA using the isoppt Reverse Transcription Supermix for RT-qPCR from Biorad (France) at 42° (For 30 min., DNA samples were then diffited in water (1:5) and 5 μL of each sample was used for red time PCR amplification by using the SYBR Green kit from Biorad (France). The following program was used: a denaturation step at 95°C for 30 sec, and 40 cycles of PCR (denaturation, 95°C, 10 sec and annealing and extension 58°C 4 min). In all cases, the quality of the PCR product was assessed by monitoring a fission step at the end of the run. Cq (quantification cycle) values were determined using a multi-variable, non-linear regression model to individual well traces and then this model was dised to compute an optimal Cq yalue. Cq values are inversely proportional to the amount of target micleic acid in the sample (i.e. Yowest Cq level corresponds to the greatest amount of target nucleic acid in the sample). Co of housekeeping genes (in this study human and rat actin) was also used as positive control of quantitative PCR. The expression of mRNA was calculated as a ratio of Cq values of treated celloversus controls (DMSO). It is described in the equation below, where K is the officiency of the qPCR:

$$R = \frac{(Etarget)^{\Delta C}(vehele control - semple)}{(Eactin)^{\Delta C}}$$

3. P456 Enzyme Activity (OVP1A, CYP2B and CYP3A) measurement

The effect of Fluor ram at 10, 30 and 100 µM on CYP1A, CYP2B and CYP3A enzyme activities was assessed in human and Wistar rat hepatocytes on Day 3 and on Day 7 after daily treatment, by incubating monolayers of hepatocyte cultures with a cocktail of prototypical substrates, according to SOPs in place at KaLy-Celf. The effect of the reference inducers was assessed in parallel: BNF, PB and RIF in human hepatocytes and BNF, PB and PCN in Wistar tat hepatocytes. Cells were first washed with PBS for 15 min before addition of the cocktail of substrates.

4. T4-glucuronidation measurement 2

The effect of Fluopyram at 10, 30 and 100 μ M on T4-glucuronidation was assessed in human and Wistar rat hepatocytes on Day 3 and Day 7 after (aily treatment by measuring T4 glucuronide in hepatocyte cultures. The effect of BNF PB and PCN/RIF was assessed in parallel. T4 stock solution was diluted in DMEM medium supplemented with penicillin-streptomycin (100,000 U/L-100 mg/L) ("incubation medium"), to give a final concentration of 50 μ M.

5. Protein content measurement

Or Day 3 and Day 7 of treatment, after incubation with a cocktail of CYP and UGT-T4 probe substrates, cell monolayers were washed with PBS. Protein content was measured according to the Pierce Method using instructions described by the kit supplier (Sigma). Values of protein concentrations were calculated using a standard curve (bovine serum albumin, 0.1-1 mg/mL).



II. **Results and discussion**

A. CYP and UGT mRNA expression levels in wistar rat hepatocytes

1. CYP mRNA Expression levels

The reference inducers gave the expected responses with BNF strongly inducing CYP1A2 mRNA expression (mean 64.7-fold on Day 3 and mean 14.3-fold on Day 7), PB inducing CYP2B1 mRNA expression (mean 39.2-fold on Day 3 and mean 7.1-fold on Day 7) and both PB and PEN in Quicing CYP3A1 mRNA expression (mean 23.7-fold and 69.8-fold on Day 3 and mean 24.9-fold and 45.0-fold

B and n 24-9 by where marked in 24-9 by the being more marked in 26-9 by 25-4 by 27-9 by marked to be at 25-9 by the best of th Fluopyram strongly induced CYP3A1 mRNA expression at all tested concentrations, induction being maximal at the highest tested concentration of 100 MM, with the effects being more marked in Day (mean 50.0-fold) compared to Day 7 (mean 11, 2-fold). Fluepyram also induced OYP1A2 mRAA expression, induction being maximal from the lowest tested concentration of $\beta 0 \mu M_{s}$ with expression, induction being maximal from the lowest tested concentration of 40 µMs up to 1900 µMS with effects being more marked on Day 3 (mean 6.6 to 13.6 fold) compared to Day 7 (2.4 to 4.7 fold). The marginal increases in mRNA expression of CYP2B1 were millimal other compared to 7 for error compound indicating no clear induction. effects being more marked on Day 3 (mean 6,6 to 13,9-fold) compared to Day 7 (2.4- to 4.7-fold). The



Treatment	Compound	Concentration	Isoform	CYP mRNA
duration	•	(µM)		expression
Day 3	Fluopyram	10	CYP1A2	13.0±62
-		30		9.7±9.2
		100	4	6.6±1.5
	BNF	5		6(6±1.5 6(9.7±240)
	PCN	6 Č3 1000 V	<u>A</u>	0.9≠0,¥Ş
	PB	1000 🚿	COP2B1	5.1\\$3.3 ×
	Fluopyram	10 🗸	COP2B1	254±1.5~
		30		3.5±00
		100		3.3±2.5 [™]
	BNF	\$\$5 	O' 'N O	3%6≢1.2 √
	PCN	<u>لا</u> 6 0 م		0%6±0.5
	PB			39.2±40,4
	Fluopyram		CYP2BI CYP2BI	0 16.756.5
	Å	30		≪ 43.8±5.1 €
		$\begin{array}{c} 30 \\ 37 \\ 100 \\ 37 \\ 37 \\ 37 \\ 37 \\ 37 \\ 37 \\ 37 \\ $		\$0.0±21
	BNF	C C C		@*3.5±1.1
	PCN A	O X V		<u>69.8</u> <u></u> 33.0
	PB V	<i>© 6 ∼</i> <i>©</i> 1000 <i>© (</i> <i>0</i> 1000 <i>© (</i>)		© 23.∜±10.0
Day 7	Fluop fram		CYP1A2 O	&A.7±0.9
	K, K	<u>~</u> 30	~~~ o	© 3.3±0.5
				© 2.4±0.7
	°∼y [™] BNF ¢		CVP2B1	14.3±4.0
		O to C		0.3±0.2
	PB V	$ \begin{array}{c} $		2.6±0.3
	Fluopyrand «		CYP2B1	1.0±0.1
Å			Ű, S	0.8±0.4
~O~		169 5 5 6		0.8±0.4
0°	BNF O		õ jų	0.5±0.3
, Ø	PCNO A PENO A E PE O		a a a a a a a a a a a a a a a a a a a	0.7±0.5
	PCNO PB Ø Fluepyram	1000		7.1±1.5
Contraction of the second seco	Fluopyram		CYP3A1	4.5±0.5
4	PCNQ PB C Fluepyram	0 [°]		4.5±1.9
ĝ			4	11.4±5.0
~~	BOF OF		4	1.2±0.2
Ŷ	PB Y		4	45.0±25.7
A A	P PB Y			24.9±7.1

Table 5.5-162CYP mRNA expression levels in Wistar rat hepatocytes cultures treated with
Fluopyram (Mean±SD)

2. UGT mRNA expression levels

Reference inducers PB and PCN induced mRNA expression of UGT2B1 (mean 8.4-fold and 9.6-fold on Day 3 and mean 5.1-fold and 4.0-fold on Day 7, respectively). An approximately 2-fold induction of UGT1A1 was also seen with PB and PCN on Day 3 and with PB on Day 7 and of UGT1A5/6 with PB and PCN on Day 3

Fluopyran at all vested concentrations induced close to or over 2-fold UGT2B1 mRNA expression with a maximum attained from 10 μ M up to 100 μ M (mean 4.4- to 7.7-fold of control on Day 3 and mean 2.5- to 1.8-fold of control on Day 7). Fluopyram did not affect UGT1A5/6 nor UGT1A1 mRNA expression



Table 5.5-163UGT mRNA expression levels in Wistar rat hepatocytes cultures treated with
Fluopyram (Mean±SD)

Treatment	Compound	Concentration	Isoform	UGT mRN expression
duration	Compound	(μM)	150101111	expression
Day 3	Fluopyram	10	UGT1A1	1.5±00
Day 5	Tuopyrain	30	UGT1A1	1.8±0.1
		100	°	1.8±0.1
	BNF	5	A	<u>0.5±0.10</u>
	PCN			₹.9.5±0.7 ₹.2.5±0,7 49
	PB	6 1000 V	UOTIA5/6	10±0.5 0.5±0.10 2.5±0.10 0.5±0.10
	Fluopyram	10 🗸	UØ11A5/6	152±0.1 5 0°
		30		1.4±02
			UGTIA5/6	0.3 ± 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1
	BNF	~~ <u>5</u>		0x8≠0.4 ~
	PCN	× 6 %		2%4±0.6
	PB			₹ 1.7±0
	Fluopyram	A . 100 ~ (C	[™] UGT2B1 _S	<u> </u>
	4			≪ <u>7.3</u> ±3.2
		$\frac{30}{6} \times \frac{30}{100} \times \frac{30}{5} \times \frac{30}{5$		V.7±4.6
	BNF	N N N		@1.6±0.5
	PCN PB	¹ 0 6 °		9.6±5.0 8.4±5.3
D7	PB V	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		© 8.4±5.3
Day 7	Fluopytam		QUGITAM O	$0.1.3\pm0.4$
		$ \begin{array}{c} $	4 × Q	0 1.1±0.5
	BNF C			0.5 ± 0.0
		$\begin{array}{c} 5 \\ 6 \\ 6 \\ 6 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7$		2.0±1.1
	PCT 6	1000		2.0±1.1 2.1±0.2
_	Fluopyrady a	$\sim 10^{\circ}$		1.0±0.4
				0.8±0.3
L D	PGN PB Fluopyrad PBNF PCNQ	$ \begin{array}{c} $		1.0±0.1
	RNF O		Å ^v a,	0.3±0.1
	PCNØ A			1.2±0.3
	PCNQ PB C Fluepyram		N.	1.3±0.3
je St	C Flugovram		v O [™] UGT2B1	2.5±1.2
	Flugpyram	1000 00 4 00 4 0 100 0 100 0 5 0 5 0 5 0 5 0 5 0 5 0 5 0	JUGT2B1	1.9±1.4
Č		/ , ² 100 ² ~	~	1.8±0.9
Į Į Į	D BOF O	× 5× ~~		1.2±0.1
Ø	BSF 7			4.0±3.1
	PB PB			5.1±1.8
		¥ 67 0ĭ	I	

B. CYP and UGT mBNA expression levels in human hepatocytes

1. CYP mRNA Expression levels

The reference inducers give the expected responses with BNF inducing CYP1A2 mRNA expression (mean 15.9-fold on Day 3 and mean 8.4-fold on Day 7), PB and RIF both inducing CYP2B6 mRNA expression (mean 4.5-fold and 3.0 fold on Day 3 and mean 4.1-fold and 6.6-fold on Day 7, respectively) and CYP3A4 mRNA expression (mean 4.8-fold and 28.1-fold on Day 3 and mean 25.6-fold and 43.8-fold on Day 7, respectively).

Fluopyram induced CYP1A2, CYP2B6 and CYP3A4 mRNA expression on Day 3 and Day 7, with a maximum obtained from the lowest tested concentration of 10 μ M up to 100 μ M on Day 3, mean ranging between 3.5- and 6.9-fold of control CYP1A2 mRNA expression, between 3.5- and 3.7-fold of control CYP2B6 mRNA expression and between 12.5- to 14.6-fold of control CYP3A4 mRNA expression.



Table 5.5-164 CYP mRNA expression levels in human hepatocytes cultures treated with Fluopyram (Mean±SD)

Compound	Concentration	Isoform	
		150101 11	CYP mRNA
Elizanoment	<u>(µM)</u>	CYP1A2	
Fluopyram	10	CYPIA2 O	3.3±00 ×
	30	O ^x	4.7±¥.0
DUE	100	1	6@±1.0 .9.9±8.6
	5	× *	9.9±8.6
	<u> </u>	a y	<u> </u>
	1000	Q	
Fluopyram	10 5	COTP2B6	→ 95.9±8.00 → 1.4±0.3 → 1.300.3 → 3.7±1.0 → 3.5±1.6
	30		3.7±10 0
	1/00"		0 [∞] 3.5±1.6
	~~5	O N O	1√1 ≠0.2 ~~
	<u> </u>		3.7±1.0 3.5±1.6 1.4±0.2 ~~ 3.0±0.5
	<u> </u>		4.5±1.2
Fluopyram		CYP3A4	0 12.62.7
	$\sim 30 \sim$		≪ 14.6±3.8
Ŵ	100 × 100		2.5±50
BNF 🔗			€ ^v 0.5±0.2
RIF 🚿	0° 75 °	N N N	284≢4.2
PB 🔧 🧔) Ø1000 Ö		284≠4.2 24%8±2.9
Fluop fam 🔬		ÇYP1A2	2.6±0.5
	<u>م</u> لا کې کې کې		$O_{3.5\pm0.9}$
	<u>~ 0100 00</u>		\$.9±2.4
SA S DNE S			8.4±2.3
RATE O		\$ 0° ° °	1.3±0.4
, A [™] B , S [™]	a, 1000 x		1.1±0.2
Fluopyran 🗸		CYP2B6	3.1±1.7
	<u>~ 30</u>		2.1±0.9
	169		3.0±0.2
S PNF U	<u>~ 65 6°</u>	Ő. U	0.8±0.4
RIFQ	15 0		6.6±2.4
RB D	1000		4.1±1.8
🖉 Fluopyram 🏷		CYP3A4	
	<u>```30</u>	s"	5.9±6.7
	<u>⁄</u>		14.0±15.0
BASE T	N to a		0.4±0.2
,O [™] (RTF _∿)	<u>O', \$5</u>		43.8±51.8
	000 to		25.6±28.8
.0.	BNF RIF PB Fluopyram BNF RIF PB Fluopyram Fluopyram Fluopyram Fluopyram Fluopyram Fluopyram Fluopyram Fluopyram Fluopyram	30 100 BNF 5 RIF 15 PB 1000 Fluopyram 10 BNF 5 RIF 15 PB 1000 BNF 5 RIF 15 PB 1000 BNF 5 RIF 5 PB 1000 Fluopyram 20 W 30 W 30 W 30 W 30 W 30 W 30 W 1000 Fluopyram 10 W 30 W 30	30 100 BNF 5 RIF 15 PB 1000 Fluopyram 10 30 00 BNF 5 RIF 15 PB 1000 BNF 5 RIF 15 PB 1000 Fluopyram 40 CYP2B6 0 BNF 5 RIF 15 PB 1000 Fluopyram 40 CYP3A4 30 0 BNF 5 PB 21000 Fluopyram 10 CYP1A2 0 WB 4000 BNF 5 RIF 5 RIF 5 RIF 5 BNF 5 RIF 5 RIF 5 RIF 5 RIF 5

2. UGT or RNA expression levels

Reference inducers RYF and PB induced yout 2 rold UGT1A1 mRNA expression both on Day 3 and Day 7. UGT1A6 and UGT2B7 expression were not affected by reference inducers.

Fluopyram induced mean UGMAL mRNA expression both on Day 3 and Day 7 from 10 µM up to 100 r nuopyram mouced mean UGP1AL mKNAcxpression both on Day 3 and Day 7 from 10 μM up to 100 μM, between 9.9- to 3.0-fold of control, and Fluopyram did not affect UGT1A6 and UGT2B7 mRNA expression



Table 5.5-165UGT mRNA expression levels in human hepatocytes cultures treated with Fluopyram
(Mean±SD)

	Commoned	Concentration	I. former	UGT mRNA expression
Treatment duration	Compound	Concentration	Isoform	UGT mRNA expression
	Elizan	<u>(μM)</u>	UGT1A1	
Day 3	Fluopyram	10 30	UGT1A1	2.0±0/4
		30	- O ^x	2.0±9.4
	DNIE	100	1	20±0.4 9.5±0.30
	BNF	5	, see the second s	<u>•</u> <u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u></u>
	RIF	15 🖒		<u>₹ 2.0≠0,1</u>
	PB	1000 🚿	<u> </u>	$3 = \frac{9.3 \pm 0.3}{2.0 \pm 0.1}$
	Fluopyram	10 .	LUGTIA6	2.10±0.1 2.10±0.2 2.10±0.2 1.0±0.2 2.10±0.
		30		
				1.0±0.4
	BNF	~\$5	O X O	1.0≢0.2 √
	RIF			120±0.3
	PB		<u> </u>	1.1±0-3
	Fluopyram	A . 100 ~ (₩ UGT2B7	
	k	$\sqrt{30}$		≪ 0.8±0.1 √
	Q	<u>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~</u>		9.9±0.8
	BNF	× * * * * * * * * * * * * * * * * * * *		@1.1±0.2
	RIF 🔧	0 75 V		<u> </u>
	PB ؇ 🧯	$ \frac{1000}{1000} \xrightarrow{1000} 0 $	S (0) (0)	Ů 0.9±0.1
Day 7	Fluop tam		UGT1A6	2.0±0.5
		^{مر} کې چې		© 1.9±0.5
				2.0±0.5
	RIF C	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		2.0±0.3
	RAFY		& <u>,</u> , , , , 6)	2.6±0.7
	_A [™] B [™]	a, 1000 x	0' × ,	2.6±1.0
	Fluopyrand 🔌	$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	🖉 UĞT1A6	0.8±0.2
	, o ^x , A A	30 - 30		0.8±0.2
		<u> </u>		1.0±0.3
6	S BNF O	\$ 63 2		0.9±0.2
Ĉo	RIFÔ	× 05 × 15 × 1000	s s	1.1±0.3
		$ \begin{array}{c} $	\sim°	1.1±0.4
<u>É</u> S	🖉 Fluopyram 🛇		、 [©] UGT2B7	0.8±0.3
	C Fluggyram	0 ³	JUGT2B7	1.0±0.3
		/ _~ 100 ° ~_		0.9±0.2
Į Q̃	BASE OF	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1		0.9±0.4
	BASF OF BASF	1000 00 4 00 4 0 100 0 100 0 5 0 05 0 5 0 5 0 5 0 5 0 5		1.7±0.6
~\$	PB A			1.5±0.6
4				1.5-0.0

C. CYP activities and T4-glacuronidation in wistar rat hepatocytes

1. CYP activities

The reference inducers give the expected responses with BNF increasing CYP1A1/2 activity (mean 42.1-fold on Day 3 and mean 45.1-fold on Day 7), PB and PCN both increasing CYP2B1 activity (mean 3.7-fold and 2.8-fold on Day 3 and mean 6.7-fold and 4.0-fold on Day 7, respectively) and CYP3A1 activity (mean 30.2-fold and 108.1-fold on Day 3 and mean 68.0-fold and 188.8-fold on Day 7, respectively).

Fluopyram stongly increased CYP3A1 activity at all tested concentrations, increases being maximal at 30 µM on Day 3 (mean 54A-fold) and at 100 µM on Day 7 (mean 59.5-fold). Fluopyram also showed a concentration-dependent increase in CYP1A1/2 activity with a maximum at 100 µM on Day 3 (mean 39.7-fold). The marginal increases in CYP2B1 activity were minimal when compared to reference compound indicating no clear induction.



Treatment duration	Compound	Concentration (µM)	Isoform	CYP activity °
Day 3	Fluopyram	10	CYP1A1/2	3.9±1.8
		30		13.6±\$21
		100	Ş	39.7⊉3.6 . √
	BNF	5		420+168
	PCN	6	4	3+0.7
	PB	1000 🖏	, s	√ 1 5+2 X
	Fluopyram	1000	CVP2D1	0 1 $\overline{3}$
	гиоруган	30 K	C m 2D1	
		100	Å .	\sim $0^{\pm 0.1}$
	DNE	4		
	BNF		S O S	0 [×] 1.1±0.2
	PCN	¥6	O XY O	2;8±0.3
	PB	\$ 1000		3?/±2.7
	Fluopyram		CYP2A1 0	\$35.4±19,8
		A . 300		54.1 1.8 °
				∠ 43.3±6.0
	BNF 🖉	<u> </u>		J.1±0.8
	PCN O	<u>y</u> , <u>v</u> , <u>v</u>		¥08.1±41.6
	PB	ى 1000 ئ	N D D	30%2≠5.8
Day 7	Fluopyran	\$ 10 ° (CPP1ALO	© 2.6±1.4
-		30		3.8±1.7
		\$ 100		© 9.1±3.7
	& BNF	\$ 675 O		\$ 45.1±18.4
	°∼ PCN	\$ 6 % A		8.4±5.9
	V. PAST &	5 100 O	&, . Š	10.8±6.9
	S Fluopyram		O CYP2B1	3.2±0.5
(<i>a</i> , <i>c</i> , <i>g</i> , <i>b</i> , <i>k</i>	2.6±0.4
, S				1.8±0.1
Ď	BNF &			0.7±0.2
~0	PCN O	k X Or		4.0±2.2
	PBQ 1	× 1000		6.7±0.9
in the second se	Fluopyram		CYP3A1	18.4±9.5
20 ⁴	Fluoporam		A IPSAI	32.0±24.8
		4 × 8 -		59.5±43.5
Ő	BIND ST			1.0±0.0
S ().				188.8±137.0
~	C CAR S			68.0±52.3
¥ 11.40.4	in UGT-T4 activity fold) and PCP (mea		with reference indu vity over 2-fold on I	



Table 5.5-167 UGT-T4 activity in Wistar rat hepatocytes cultures treated with Fluopyram (Mean±SD)

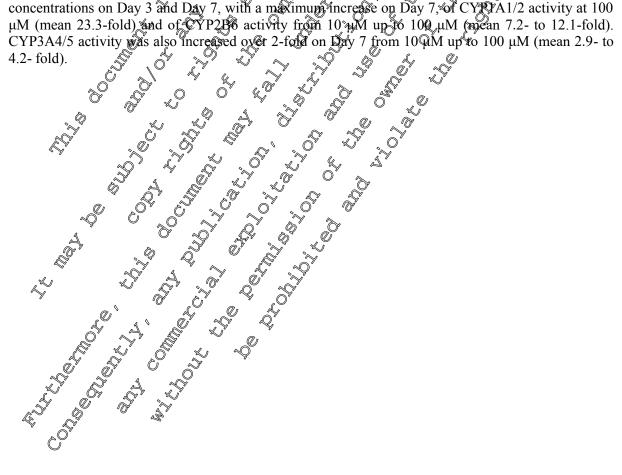
(1	(itean=5D)					
Treatment	Compound	Concentration	Isoform	UGT-T4 activity		
duration		(µM)		N O		
Day 3	Fluopyram	10	UGT-T4 🖉	1.3±000 m		
		30	UGT-T4	1.4±0.3		
		100	4	1,4±0.4		
	BNF	5	×,	\$9±0.62		
	PCN	6 🖏	<u>s</u>	√1.9≠0,3 \$		
	PB	1000 🚿				
Day 7	Fluopyram	10 🔬	JØGT-T4	$\sqrt{22\pm0.7}$		
		30		3.4±15 2		
		1/00		<u>3.8±1.1</u> 6±0≠2.9 ~		
	BNF	≪05	a in a	<u>6</u> *0≠2.9 ~		
	PCN	× 6 Q		5%9±1.6		
	PB	0 10 00 Č		2.2±0-1		
D. CYP activities and T4-glucuronidation in human hepatocytes						
	Č A					
1. CYP activities	Q,					
				- 00		

D. CYP activities and T4-glucuronidation in human hepatocytes-

1. CYP activities

The reference inducers gave the expected responses with BNK increasing CVP1A12 activity (mean 32.5-fold on Day 3 and mean 155.4 fold on Day 7), both PB and RIF increasing CYP2B6 activity (mean 5.8-fold and 2.9-fold on Day 3@and mean 2138-fold and 126-fold on Day 7) and CYP3A4/5 activity (mean 4.7-fold and 4.3-fold of Day 3 and arean 11.3-fold and 114-foldon Day 7, respectively).

Fluopyram showed an over 2-told increase in CYPIA1/2 and CYP2B6 activities at all tested concentrations on Day 3 and Day 7, with a maximum increase on Day 7, of CYPA1/2 activity at 100 µM (mean 23.3-fold) and of CYP2B6 activity from 10° µM up to 100 µM (mean 7.2- to 12.1-fold).





Treatment duration	Compound	Concentration (µM)	Isoform	CYP activity °
Day 3	Fluopyram	10	CYP1A1/2	2.6±1.5
5	1.5	30	ð	3.8±276
		100		4.9±¥.0
	BNF	5	ч <u>О</u> . д	32(5±14.8)
	RIF	15		\$\$\$9.9±0.3€
	PB	1000 🖏	L.	≪ 1.8±0.9
	Fluopyram	10 🕅	CYP2B6	3.692.1 %
	1.5	30 🗸	CXP2B6	238±2.1.5
		100		3.0±1.6
	BNF	A V		0 ⁹ 1.7 ≠ 0.2
	RIF	95		2%9≠1.1 ~℃
	PB	× 1000 %	y y y	5%8±2.8
	Fluopyram		THEYP3AJ/5	√1.7±0-4
		A . 30 . 0		0 1.7 0.1 0
				1.6±0.6
	BNF			0.8±0.6
	RIF OF	K K X	Creptato	Q 4.3±0.5
	PR 🗸	0 1000		4.₹#1.2
Day 7	Fluopyran		CAPIALO	0 8.9±5.7
,		30,5		\$1.8±8.4
		× 100		©23.3±18.9
	BNF	\$ 0 ⁵ 0		© 155.4±82.5
	N RIF	\$ 15 k		2.4±0.5
	, PB O	0 100 0	&, . Š	3.8±0.5
	S Fluopyram		O CYP2B6	11.6±11.2
		~~~ <u>~</u> 30 ~~	7/	7.2±7.3
	× `~` `0, ``	$\sim 100$		12.1±9.5
Č,	BNF &	5		2.3±0.8
8	RIF O	\$ 615 2	Å a	11.6±3.0
<i>h</i> e	PB Q	× 1000	N N	21.8±5.9
~~	Fluopsram		CYP3A4/5	27117
Ê.S			, O [#]	2.9±1.8
**		0 [°] , 100 %.	S. S	$42\pm38$
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		¥ 7 50 ×		0.9±0.3
ĊĮ [*]	BAR W	16		11.1±8.8
Ø		0,1900		11.3±7.9
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~				11.0 (1.)
14-glucuronida	ation or Si A	× Q [×] Q		
ht increases in	UGR-T4 agrivity wer	e seen on Day 7 with	reference inducers	BNF (mean 2 3-fold
RIF (mean 1 9.			Tereference madeers	Divi (incui 2.5 ioic
$\mathcal{K}$				
opyram did not	affect@GT_@4 activi	່ ັty on∰ay 3 nor on D	ay 7.	
"Oʻ		Q, ^Y		
/		¥ @1		
	affect@GT-D4 activi	<b>)</b>		
Ű ÁS	Ŭ 2 [°]			
19 D	A ~~			
S an	S ^r S			
N Q (	U .Y			

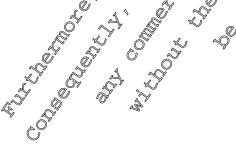




Table 5.5-169	UGT-T4 activity in human hepatocytes cultures t	reated with Fluopyram (Mean±SD)
---------------	-------------------------------------------------	---------------------------------

Treatment duration	Compound	Concentration (µM)	Isoform	UGT-T4 activity °
Day 3	Fluopyram	10	UGT-T4	0.9±0.1
		30	ð	0.9±00
		100		0.8±0.2
	BNF	5	.4	10±0.2
	RIF	15		~J.1±0,1° 4
	PB	1000 🖉	a s	≫ 1.1±0,1 \$
Day 7	Fluopyram	10 🖤	U <b>©T</b> -T4	0, 1. <b>Σ</b> \$0.4 ≪
		30 📈	O ^v	( 10,3±0.4 (
		100		1.2±0@ @
	BNF	<u></u>		<u>0</u> 2.3≱].0 0
	RIF	15	Ø X Ø	1:9#0.4
	PB	\$ 1000		1.6±0.3

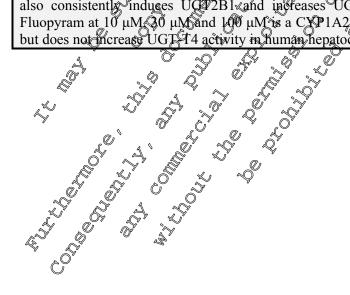
#### **E.** Deficiencies

No specific deficiencies were noted in the study III. Conclusions The present results show that Fluopyram at 10 µM, 30 µM and 100 µM is a strong CYP3A and to a lesser extent a CYP1A2 inducer in Wister rat broatenets. lesser extent a CYP1A2 inducer in Wistar rat pepatocytes. Floopyrum at all tested concentrations also consistently induces UGT2B1 and increases UGT-T4 activity in Wistar rat hepatocytes. Fluopyram at 10 μM, 30 μM and 100 μM is a CYP1AO, CYP2B6, CYP3A4 and DGT1A1 inducer but does not increase UGT-T4 activity in human heparocytes

# Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it assesses the potential of Fluopyram to Induce human and rat inducable subchrome P450 isoenzymes CYP1A, CYP2B and CYP3A as well as UDP glucuronosyltransferases UGT1A and UGT2B and to increase thyroxine (T4)-glucuronidation in the liver

The present results show that Fluopyram at 10 µM, 30 µM and 900 µM is a strong CYP3A and to a lesser extent a CXPIA2 inducer in Wistar rat Repationytes. Aluopyram at all tested concentrations also consistently induces UCP2B1 and increases UCTTA activity in Wistar rat hepatocytes. Fluopyram at 10 µM 20 µM and 100 µM is a CXP1A2 CYP2B6, CYP3A4 and UGT1A1 inducer but does not increase UGT T4 activity in human hepatocytes





#### CA 5.6 Reproductive toxicity

All studies presented in this section were conducted between 2006-2008 and complied with OECD, EU, USEPA and Japanese MAFF testing guidelines and Good Laboratory Practice (GLP).

In the rat two-generation reproduction study, The parental male systemic LOAID was 1200 ppm (82.8 mg/kg/day) based on increased clinical chemistry parameters (creatinine, total protein, albumin and urea nitrogen), increased kidney weights associated with an increased incidence of protein droplet, nephropathy and lymphocytic infiltration, and increased liver weights associated with an increased incidence of centrilobular hypertrophy. The parental female systemic LOAEL was 1200 ppm (960 mg/kg/day) based on a decline in body weight and/or body weight gain during promating decreased body weight during gestation in the P-generation, increased body weight and food consumption during gestation in the F1-generation, decreased hemoglobin and/or hematocht in the P- and/or F1-generation, increased liver weights associated with an increased incidence of centrilobular hypertrophy and minimal to slight, alveolar macrophages in the P- and/or F1 generation. Thus, the parental systemic NOAEL was 220 ppm (14.5 mg/kg/day) males, 17.2 mg/kg/day in females).

The reproductive NOAEL was 1200 ppm in both males and febrales (\$2.8 mg/kg/day in males and \$93.1 mg/kg/day females), based on no reproductive findings observed in the highest dose tested.

The offspring LOAEL was 1200 ppm (97.9 mg/kg/day). The LOAEL was based on material effects leading to secondarily-mediated effects on pup weight and pup weight gain. Also noted was a slight delay in preputial separation, (although mean body weight at attainment of social maturation was comparable to the controls 1/1 g at 1200 ppm versus 173 g in controls) and decrease in spleen and thymus weights for F2-pups (both findings considered secondary to pup weight decrease). The offspring NOAEL was 220 ppm (17,0 mg/kg/day).

In the rat developmental toxicity study, there were no maternal mortalities or treatment-related clinical signs. At the highest dose level of 0 mg@g/day mean maternal bod weight remained static between gestation days (GDS 6-8, compared to again of 6.8 g in the control group, with a reduced mean maternal body weight gain of 22% between GD 8-19 and 34% between GD 19-14, resulting in an overall reduction of 16% between GD 6-20 compared to the controls. Mean maternal food consumption was 13% to 15% lower than the controls for each interval between GD 6 and 14. Mean maternal liver weight was 40% higher than in the control group and at necropsy 4/23 females had enlarged livers. At the histopathological examination of the liver, diffuse contrilobular hepatocellular hypertrophy was observed in all females. Mean fetal body weights (combined and separate sexes) were 5% lower than the controls. There were no treatment related miding at the external fetal evaluation. At the visceral and skeletal fetal examinations there were no treatment-related malformations. The incidence of the visceral variations 'thymic cremnant present' and 'ureter convoluted and/or dilated', and skeletal variations 'at least one thoracic centrum plit/split cart@age', considered a variation as in all cases only one centrum was affected, therefore the adjacent normal vertebrae would support the spinal column. Consequently, there would be no adverse consequences on the long term functionality of the animal 2007, M-766 89-041) and 'at least one thoracic centrum dumbbell and/or bipartite/normal cartilage', was higher than in the control group

At the mid dose of 150 mg/kg day, mean maternal body weight essentially remained static between GD 6-8. Between GD 40 14, mean maternal body weight gain was 26% lower than in the controls. The overall effect between GD 6-21 was a 6% lower mean maternal body weight gain compared with the controls. Mean maternal food consumption was 10% to 18% lower than the controls for each interval between GD 6-14. Mean maternal liver weight was 15% higher than in the control group. At the histopathological cramination of the liver, diffuse centrilobular hepatocellular hypertrophy was observed on 20/23 females. No litter parameters were affected and there were no treatment-related external, visceral or skeletal findings.



At the low dose of 30 mg/kg/day, the only findings were a 31% reduction in mean maternal body weight gain between GD 6-8 and a 10% reduction in mean maternal food consumption over the corresponding and and period. This slight transient effect on body weight gain and food consumption, in the absence of other findings was considered to be a non-adverse effect.

The maternal NOAEL was 30 mg/kg/day, based on a transient reduction on maternal body weight goin and food consumption. The fetal NOEL was 150 mg/kg/day.

In the rabbit developmental toxicity study, there were no treatment-related maternal deaths or ofinical signs. At the high dose level of 75 mg/kg/day, mean body@yeight gain was reduced by between GD 1/4-18 (0.02 kg vs. 0.09 kg for controls) and between GD 8-22 (0.02 dg vs. 0.07 kg for controls), in comparison to controls, resulting in an overall body weight gain between GD 6-29 of 0.20 kg compared  $\bigcirc$  to 0.31 kg for the controls. Mean material for the controls. to 0.31 kg for the controls. Mean maternal food consumption was reduced by 22% to 34% for all intervals between GD 14-26, in comparison to compose. At necropsy, no treatment-related macroscopic findings were noted. Mean fetal body weight (combined and separate sexes) was 1/8 lower than the controls. At the external fetal examination, there were no malformations in the high dose group. The mean percentage of fetuses classified as 'runts' was 12.5% and the percentage of litters affected was 47.6%, compared with 3.0% and 23.8%, respectively, in the control group. There were no treatmentrelated visceral or skeletal findings at this dose level. Ó

There were no treatment-related maternal, here or foral findings withe mid dose of 25 the /kg/day or the low dose of 10 mg/kg/day. At C656948 were considered to be a NOEL both in the dam and in terms of fetal development in the NSW Zealand White rabbit. There were no treatment-related material, litter or total findings at the mod dose of 25 mg/kg/day or the



Type of study Doses ppm mg/kg bw/day Reference	NOAEL ppm mg/kg bw/day	LOAEL ppm mg/kg bw/day	Adverse effects / target organs
Reproductive – two ge	eneration dietar	v toxicity study	
Wistar rat 0, 40, 220, 1200 ppm	14.5♂ 17.2♀	82.8♂ 96.0♀	Parents Clinical pathology changes Parents Clinical pathology changes ↑ liver weight, protein dropter nepbropathy (♂) and centity obular perhropathy Clinical signs
<u>M-299334-01-1</u>	82.8♂ 93.1♀	>93 උ/ርሞ	Clinical signs V Body weight Reproduction Defayed social development maturation secondary to decreased body weight
	17.0 ♂/♀	97.9 31 97.9 31 97.9 31	Offspring ↓ number of implantation spes, litter size and corpora brea (secondars to severe systemic foxicity) ○
Developmental toxicity	24 s		
Doses mg/kg bw/day Reference	NOAEL mg/kg bw/day	J.OAEI mg/kg bw/day	Advorse effects / target organs
Sprague-Dawley Rat - 0, 30, 150, 450 <u>M-299438-01-2</u>	450 ∂ 450	150 3 3 3 3 3 50 2/2 3 3 50 2/2 3 3 3 50 5 1 50 5 1 50 5 1 50 5 1 50 5 1 50 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5	↓ body weight gain and food         ↓ body weight gain and food         ↓ body weight gain and food         ↓ food
New Zealand White Rabbit - $\bigcirc$ 0, 10, 25, 66 <u>M-279% 3-01-1</u>		× 4,75 5 75 ∂/2	Joint Structure       ↓ body weight gain and food consumption         Image: Structure       ↓ fetal body weight (-11%).

Table 5 6-1	Fluopyram Summar	v of reproductive and	l develonmental	toxicity studies
1 4010 0.0 1	I huppi am Summar	y of reproductive and	actopinentai	to Alcity Studies



#### CA 5.6.1 Generational studies

Data Point:	KCA 5.6.1/01
Report Author:	
Report Year:	
Report Title:	Technical Grade AE C656948: A Two Generation Reproductive Toxicity Study on
	the Wistar Rat
Report No:	
Document No:	<u>M-299334-01-1</u>
Guideline(s) followed in	OECD 416 (2001);
study:	OECD 416 (2001); US EPA Health Effects Test Guideline (OPPTS 870.3800; 1998); M A F E in Japan potification 2 Nousan NS447 (2000) guidelines
	OECD 416 (2001); US EPA Health Effects Test Guideline (OPPTS 870.3800; 1998); M.A.F.F. in Japan notification 2 Nousan N 8147 (2000) guidelines
Deviations from current	Current guideline: OECD AT6, 2001
test guideline:	
Previous evaluation:	Yes, evaluated and accepted in the DACK (2014).
GLP/Officially	Yes, conducted under GLP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes of the second secon

#### **Executive Summary**

AE C656948 was administered continuously in the feed of the Wistar Pat (30 animals/dose/sex) at nominal dietary concentrations of 0, 40, 220 and 1200 ppm. The concentration of the test substance in the feed for the females was adjusted down by 50% during the lactation period to avoid the large increase in dosage (mg/kg/day) that is otherwise associated with increased feed consumption that occurs during lactation. All test diets (including control) were available for *ad libitum* consumption; the homogeneity and stability of AE C656948 as a dietary admixture was confirmed. Body weight and food consumption determinations and detailed clinical examinations of each atimal were conducted weekly throughout the study, as well as, an evaluation of multiple reproductive parameters, clinical chemistry and hematology. Albanimals placed on study were subject to a postmeritem examination, which included (1) documenting and soving all gross lesions, (2) weighing designated organs and, (3) collecting representative tissue specimens for historations.

#### At 1200 ppm:

**P-generation Adults:** Decreased body weight and body weight gain in females during premating, as well as significant declines in body weight during gestation (Days 0-13) and on Day 0 of lactation. Clinical chemistry evaluations showed increases in creatinine, total protein and albumin in males. Females exhibited a decrease in hemoglobin and hematocrit. Increased kidney weight was observed in the males and was associated with an increased incidence of protein droplet nephropathy and lymphocytric infiltration? Increased liver weights were observed in both males and females and were associated with an increased liver weights were observed in both males and females and were associated with an increased liver weights were observed in both males and females and were associated with an increased liver weights were observed in both males and females and were associated with an increased liver weights were observed.

F1-Offspring: Pup weight gain was declined for both males and females from Days 7-14 of lactation with statistical significance observed in the males during this timeframe. A slight delay in preputial separation in the F1-males was observed, relative to control. Although statistically significant, the number of days to passing was well within this laboratory's historical control range and was considered to be secondary to the decline in male body weight gain observed during lactation. In addition, the mean body weight at attainment of sexual maturation was comparable to the controls (171 g at 1200 pm versus 173 gun controls).

**Figeneration** Adults: A slight decline in body weight gain in females was observed during the premating period. Females, during gestation, exhibited an increase in body weight gain and food consumption, relative to controls. Clinical chemistry evaluations showed increases in urea nitrogen and total protein in the males and increased cholesterol in the females. Hematology evaluations exhibited



increased white blood cell and monocyte absolute cell (Ab) counts and decreased hemoglobin in females. Increased kidney weights were observed in the males and were associated with an increased incidence of protein droplet nephropathy and lymphocytic infiltration. Increased liver weights were observed in both males and females and were associated with an increased incidence of centriboular hypertrophy in both genders and minimal to slight alveolar macrophages in the remales.

F2-Offspring: Nonstatistical declines in body weight by Day 4 for both males and females were observed with significant body weight declines observed by Day 21. Body weight gan throughout lactation for both males and females was also declined, relative to control. Decreases in spleen and thymus weights were observed in both genders and is considered to be secondary to the weight bes observed in these pups.

The data from this study demonstrate that the parental sostemic NOAR was 220 ppm (14.5 mg/kg/day in males, 1 22 mg/kg/day, in females) and that the reproductive NGAEL was 1200 ppm in both males and females (82.8 mg/kg/day in makes and 93.1 mg/kg/day females). The LOAEL for reproductive effects was >1200 ppm, the highest dose tested, because no reproductive findings were observed in the highest dose tested The offspring NOAFL was 220 ppm (17.0 mg/kg/day) based on maternal effects leading to secondarily-mediated effects on pup weight and pup weight at the LOAEL, the highest dose tested, 1200 ppm (97.8 mg/kg/day).

#### Materials and methods I.

A. Materials

Lot / Batch #: Purity: CAS # Stability of test A compound:	Beige powder 085280002 94.7% (Analyses dates of 5/4/05 and 3/30/07) 658066-35-4 Stable in the diet at concentrations of 5 ppm and 5000 ppm when stored for 7 days at room temperature followed by 28 days of storage in the freeze
^{∞2.} Venicle and / or positive control:	Acetone
3. Test animals:	
Species:	Rat



Strain: (P) 8 weeks Age: Weight at dosing: Source: **Acclimation period:** 6 days Diet: Water: Housing: **Environmental conditions: Temperature:** 18-26°C 30-70%

Wistar Han Crl: WI(HAN) (P) Males: 224.9 – 279.1 g; Females: 145.6 – 203.3 g

ð, Purina Mills Rodent Lab Chow 5002 mean ad libitum Tap water (Kansas City, MO), ad libitum ruase and as noted below for the F1 and F2 pupsion suspended stainless steel cages and deotized cage boate in the bedding trays During gestation and lactation, individual dams (and litters) were housed in polycarbonate cages with corn-cob bedding. r hour scark nals were examined

**Humidity:** Air changes: **Photoperiod:** 

#### **B.** Study design

1. In life dates:

December 2006 , ¢

At least 10 changes per hour

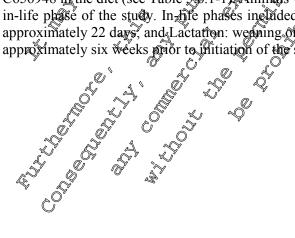
12 hours light, 12 hours dark

Ø

#### 2. Animal assignment and treatment

Following a minimum of six days of guarantine/acchimation animals were examined by a veterinarian and released for study use. The animals were randomly assigned to either a control or one of three chemically-treated groups using a weight stratification based computer program (INSTEM Computer Systems, Stone, Staffordshire, UR). Only those animals falling within 20% of the mean for all animals (per sex@were placed on study. Once animals were assigned to their dose groups, each rat on study had a microchip (Biomedic Data Systems, Thc. Scaford, DE) subcutaneously implanted on its' dorsal surface in the region between the scapulae. At a minimum, the chip was encoded with a unique number, specifying the animal's sex dose group, cage number, and study affiliation. In addition, a laminated cage card, essentially duplicating the information present on the chip, was attached to the outside of each animal's case. Pups bom alive were identified by tattoo and pups found dead were "Ŷ identified with an ordelible marling pen

Study schedule, One Andreg and twenty for ale and one mundred and twenty male rats were assigned to one of four greatment groups (20 animals/sex/group); nominal doses of 0, 40, 220 and 1200 ppm AE C656948 in the diet (see Table 59.1-1) Animals were exposed to the treated feed throughout the entire in-life phase of the study. In the phases included: Premating: 10 weeks; Mating: 14 days; Gestation: approximately 22 days, and Lactation: wearing of Day 21. F1-pups were maintained after wearing for approximately six weeks prior to initiation of the second generation.





Test group	Dose in Diet ^a		<u> </u>		
	(ppm)	P Males	P Females	F1 Males 嶡	N N
Control	0	30	30	30 8	30 5
Low (LDT)	40	30	30	30	30 2 2
Mid (MDT)	220	30	30	30	
High (HDT)	1200	30	30 30	30	^Q 30 0 ^Q 0 ^Q
^a = Diets were admini	istered from beginning	ng of the study un	til grerifice.		

Table 5.6.1-1	Animal Assignment
1 4010 0.001 1	1 minut 1 19915 minut

LDT – Low dose tested, MDT – Mid dose tested, HDT – High dose tested

Mating procedure: Males and females were exposed to the test substance for ten weeks prior to mating. Mating was accomplished by co-housing one female with one male for up to 14 consecutive days. During the mating phase, vaginal smears were taken each morning and examined for the presence of sperm and/or internal vaginal plug. Fernales found to be inseminated were placed in a polycarbonate nesting cage. The day on which insemphation was observed in the vaginal smear was designated Day 0 of gestation for that female. In order to evaluate those females, which might be inseminated without exhibiting sperm in the vaginal smear or an internal vaginal plug, all demaining females were placed in polycarbonate nesting cages, following the 14 day moting period.

#### 3. Dose selection rationale

Doses were selected based upon the prepiningly results which emetged in the ratiover the course of a pilot reproductive toxicity testing study conducted with the dest chemical at doses of 0, 30, 150, 750 and 1500 ppm AE C656948 (M-209533401-1), Milius 2008, report available in dossier. In that study, there were no compound related effects observed on body weight, food consumption or clinical observations at any dietary level tested Liver and kidney weight changes were observed in the males of the 750- and 1500-ppm dose groups Females also exhibited liver weight charges in both the 750- and 1500-ppm dose group. Changes in clipical chemistry and kematology parameters were also noted at these same dose levels in either the males or females. Based on these results, the doses selected for the twogeneration reproduction toxicity study were 0, 40, 220, and 1200 ppm AE C656948. This dose range was intended to produce evidence of toxicity at the highest distary concentration and no parental or reproductive effects at the lowest chetary concentration

#### 4. Dosage preparation and analysis

Ĩ The test substance was dissolved in acetone and then mixed with the feed. The control test diet is to be prepared in the same manner as the chemically treated test diet, excluding only the test substance. A sample of each batch of feed mixed was taken and retained in the freezer until the study was completed and the analytical data deemed satisfactory. Replacement admixtures for each treatment group were prepared weekly (or at greater intervals if within freezer stability limits) during the entire study and stored at freezer conditions until presented to the animals. Additionally, the entire batch of replacement admixture for each treatment group was used for subsequent weekly feeding if within freezer stability limits.

The concentration of the fest substance in the feed for the females was adjusted down by 50% during the lactation period (Days 0, 2) as follows, to avoid the large increase in dosage (mg/kg/day) that is otherwise associated with whereased feed consumption that occurs during lactation. Thus, during lactation days 0-21^o the dictary concentrations were reduced from 40, 220 and 1200 ppm to 20, 110 and 600 ppm respectively.

Calculation for test substance intake is: Mean analytical concentration (ppm) specific for each phase / 1000 X mean weekly food consumption (g/kg/body weight/day) for each phase. Exceptions are that



weeks 18 and 22 were analyzed but not included in substance intake calculations due to this being the developmental landmark phase in which food consumption is not measured.

The mean daily intake of the test substance (mg AE C656948/kg/day) throughout this two-generation reproduction study at nominal dietary concentrations of 0, 40, 220, or 1200 ppm, respectively, is summarized in Table 5.6.1-2.

Table 5.0.1-2 Witchi Daily Intake of the	1 est Substance			
Phase of Study	40 ppm in mg/kg/day	220 ppm in	4200 ppm in mg/kg/day*	(A)
	mg/kg/day	mg/kg/day ^a	mg/kg/day ^a	Ş
Premating (P-gen) - Male	2.7	× 15.1	\$3.1 0 ⁴	ÿ
Premating (P-gen) – Female	3.2	17 <b>6</b> 0	A 96.3	
Gestation (P-gen) – Female	∞3.0	~ *\$5.5 m	× 10.3 ×	
Lactation (P-gen) – Female		× 15.9	92.5	
Premating (F ₁ -gen) - Male	2.6	Q 13.9	0 [×] 82.4 [×]	
Premating (F1-gen) – Female	3.1	O 10.8 0	\$ 95.6	
Gestation (F1-gen) – Female	× 248 ×	14.4	\$ 95.9 O	
Lactation (F1-gen) – Female $\mathcal{L}$	× ×3.3 ×	N 48 N	\$ 1Q\$2	
Individual values were based on the marge for each	nortioular phase for a			

a Individual values were based on the means for each particular phose for each generation.

The concentration of AE C656948 in the various test diets was analytically verified for batches intended for weeks 1, 2, 3, and at monthly intervals dieteafter (Bayer CropScience LP, Environmental Research, 17745 S. Metcalf, Stilwell/KS).

Homogeneity Analysis: The mean concentrations of AE C656948 in the feed sampled from three distinct layers in the mixing bowl and containing a nominal concentration of either 5- or 5000-ppm, were determined to be 5.18 ppm (ange 500-5.39 ppm %RSD = 2.66) and 4975 ppm (range 4866-5115 ppm; %RSD = 1.99), respectively. Based on a %RSD  $\leq$  to10%, AE C656948 was judged to be homogeneously distributed in the feed over a concentration range of 5-5000 ppm.

Stability Analysis: Following 7 days of room temperature storage, the analytically-determined concentration of the AI of the test substance in the 5- and 5000-ppm admixture was determined to be 5.36 ppm (5.18 ppm or Day 6) and 5229 ppm (5,63 ppm on Day 0), respectively. Following 28 days of freezer storage, the analytically determined concentration of the AI of the test substance in the 5- and 5000-ppm admixtures was determined to be 5.02 ppm (5.18 on Day 0) and 5014 ppm (4975 on Day 0), respectively. AE C656948 mixed in ordent ration was judged to be stable at room temperature for at least seven days and following freezer storage for a minimum of 28 days, over a concentration range of 5-5000 ppm (Jensen, 2007).

Concentration Analysis Mean analytical concentrations for each dose group were 39.6, 220 and 1198 ppm, ranging from 99/100% of the corresponding nominal concentrations of 40, 220 and 1200 ppm, respectively. The concentration of the test substance in the feed for the females was reduced by 50% during lactation. Mean analytical concentrations for each dose group during lactation were 20.0, 108 and 593 ppm, ranging from 98-100% of the corresponding nominal concentrations of 20, 110 and 600 ppm, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 101% and ranged from 90-109% for rodent ration spiked with 20, 40, 220 or 1200 ppm of AE C656948 (Moore and New 2007).

#### 5. Statistics and calculation of reproductive and offspring indices

Statistical analyses: The data were analyzed using applications provided by DATATOX (Instem Computer Systems), SAS (SAS Institute, Inc.), or TASC (Toxicology Analysis Systems Customized, 1993). Parametric data (including body weight gain and food consumption) were analyzed using a univariate Analysis of Variance (ANOVA), and if significant differences were observed, a Dunnett's



Test was performed. Nonparametric data (e.g., number of estrous cycles, litter size, and number of implantation sites) was first analyzed by the Kruskal-Wallis test and then subjected to Dunn's Test if significant differences were identified. Nonparametric dichotomous data (e.g. fertility and gestation indices) were initially analyzed by the Chi-Square Test and if significance was observed between bound then by the Fisher's Exact Test with the Bonferroni adjustment. To the extent possible, the frequency of gross lesions was first examined visually, then, in the event of questionable distribution, by statistical analysis using the Chi-Square and Fisher's Exact tests. Differences between the control and test substance-treated groups were considered statistically significant when  $p \leq 0.05$  or  $p \leq 0.09$ 

#### Indices:

Reproductive indices: The following reproductive indices were calculated from breeding and pararitic records of animals in the study:

$$Mating Index (\%) = \frac{\#inseminated females^{a}}{\#of females co \pm housed} \times 100$$

$$Fertility Index (\%) = \frac{\#of pregnant females^{b}}{\#of pregnant females} \times 100$$

$$Gestation Index (\%) = \frac{\#of females with live pups}{\#of pregnant females} \times 100$$

$$Gestation Index (\%) = \frac{\#of females with live pups}{\#of pregnant females} \times 100$$

$$Fincludes pregnant females not observed spend positive or with an internet vaginal plug.$$

$$Fincludes females which did not deliver but had implantation sites.$$

$$Offspring viability indices The following viability indices were calculated from factation records of litters in the study:$$

$$Birth Index (\%) = \frac{total \#of pups Born / litter}{total \#of pups Born / litter} \times 100$$

$$Finculates (\%) = \frac{\#of live pups born / litter}{botal \#of pups / litter} \times 100$$

$$For birth Index (\%) = \frac{\#of live pups / litter on day 4 (pre - culling)}{\#of live pups / litter on day 21} \times 100$$

$$For birtholds$$

# C

#### 1. Parental animals:

#### a. Mortality and clinical observations.

Mortality checks (eageside observations) were performed twice daily (AM and PM) during the workweek and once daily on weekends and holidays. Cageside observations characterized mortality, morbidity, behavioral change, signs of difficult or prolonged delivery, and overt toxicity by viewing the animal in the cage. In the event a possible clinical sign was observed during the cageside evaluation, the animal was reproved from the cage and a detailed assessment conducted. A detailed evaluation of clinical signs included both observing the animal in the cage and removing the animal to perform a physical examplation and was conducted at least once per week throughout the entire in-life phase of the study.



#### b. Body weights and food consumption.

Body weight and food consumption was measured and fresh feed provided once per week for both males and females during the 10-week premating period. During the mating period and until sacrifice, body weight for the males and unmated females were measured once per week. Also during the mating period, fresh feed was provided for both males and unmated females once each week without preasuring food consumption. During gestation, dam body weight was measured on Days 0, 6, 13, and 20, and tresh feed was provided and food consumption measured once each week. During lactation, dam body weight was measured on Days 0, 4, 7, 14, and 21. Fresh feed was provided and food consumption measured once per week, with the exception of week one when food consumption was measured twice. Days 0-4 and 4-7).

#### c. Estrous cyclicity.

The estrous cycle (determined by examining daily vaginal smears) was characterized for all P- and F1generation females, over a three-week period prior to mating. Additionally, the estrous cycle stage was determined for all females just prior to termination.

#### d. Sperm parameters.

For all P- and F1-generation males a termination spern was collected from one testis and one epididymis for enumeration of homogenization-resistant spermatids and cauda endidymal sperm reserves, respectively. In addition an evaluation of the morphology and motility was performed on sperm sampled from the distal portion (closest to the urethra) of the vas deperent Sperm motility and counts was conducted using LVOS (Integrated Visual Operating Systems, 2005). Morphology and counts were conducted on the control and highest dose group.

#### e. Clinical Chemistry

Prior to sacrifice, 10 adult rats/sex/group were fasted overnight/with, water available prior to blood sample collection. Each adult rat was anesthetized with inhaled IsoOurane and blood samples were collected from the orbital sinus (500 µL for hematology: in tubes containing EDTA and ~1000 µL for serum chemistry using a capillary tube. Samples were transferred to the clinical pathology department as soon as possible after collection. Hematologic parameters Hematocrift (HCT) Hemoglobin (HGB) Enythrockte indices (MCV, MCH, MCHC) Total erythrocyte court (RBC Differential leokocyte count Red cell distribution width R RBC morphology Total leukocyte count (WB Demographic Distribution Width (HDW) .... include: ..... include: .... Total platelet count (PLT) (Globulin (GLOB) Alanine aminotransferase (ALT) Alkaline phosphatase (ALK) Total bilirubin (T.BIL) Cholesterol (CHOL) Urea nitrogen (BUN) Sodium (Na) Potassium (K) Chloride (Cl)



Calcium (Ca)

Phosphorus (PO₄)

Table 5.6.1-3	F ₁ / F ₂ Litter Observations

				.,			
Creatine Phosphokinase (CPK)							
Creatine Phosphokinase (CPK) 2. Litter observations: The following litter observations (X) were made: Table 5.6.1-3 $F_1/F_2$ Litter Observations							
Table 5.6.1-3         F1 / F2 Litte	er Observa	tions			<u>A 57 57 9</u>		
Observation		1	Time of 🔞 b	servation (	factation day) $\sqrt{2}$ $\sqrt{2}$		
	Day 0	Day 4 ^a	Day 4 ^b	Day 7	Day 14 Pay 21 Days (0-		
Number of live pups			1	-Q*			
Pup weight	Х	X 🖏	P .	X S			
External alterations	Х	X		X			
Number of dead pups							
Sex of each pup (M/F)	Х						
Preputial Separation	Ő		@ ⁹ Perfo	wmed post v	veaning S & S		
Vaginal Patency			Perfo	ormed post v	Caning Q		
^a Before standardization (culling)	b After stand	lard@žation (ce	ílling) 🔍 🗌	$\sim$	8.5.4		

The size of each litter was adjusted on lactation Day to yield, as bosel as possible, four males and four females per litter. If the number of male or female pupt was less than four, a partial adjustment will be made (e.g., three females and five males). No adjustments were made for atters of fewer than eight pups. Adjustments were made by fandom selection of the pups using software provided by SAS. Culled pups were sacrificed by decapitation. Grossly abnothal pups underwent a gross internal and external examination, and all colled pops were discarded. The Fir and D2-pups not colled on lactation Day 4 were maintained with the dam until wearing on lactation Day 21. On lactation Day 21, a sufficient number of F1-pups sex/ligher was maintained to produce the next generation. F1-pups not selected to become parents of the next generation and all F2-pups were sacrificed, examined macroscopically and had organs woghed. One pup/sex/litter for each generation has fissues collected and evaluated for any structural abnormalities or patheogical changes, particularly as they related to the organs of the reproductive system.

Dead pups were examined grossly for external and internal abnormalities, and a possible cause of death was determined for pups stillborp or found dead.

3. Post-mortem observations

, O ô a. Parental animals All surviving parental males were sacrificed as soon as possible after the last litters were produced. Maternal animals were sacrificed following the weaning of their respective litters (lactation Day 21). The animats were subjected to postmortem examinations as follows.

Male rats were euthanized by carbon doxide asphyxiation and a gross external examination was performed. Terminal body weights were measured and the recording of all gross pathologic alterations, weighing designated organs, and saving all gross lesions was conducted on all males. For all males at termination, perm was collected from one testes and one epididymis for enumeration of homogenization-resistant spermatids and cauda epididymal sperm reserves, respectively. In addition, the morphology and motility of sperm samples from the distal portion (closest to the urethra) of the vas deferens was aluated. Sperm motility and counts were conducted using IVOS (Integrated Visual Operating System, 2005).

Each damy both P- and F1-generations) was euthanized by carbon dioxide asphyxiation and a gross external examination was performed. Terminal body weights (fasted terminal body weights from those animals bled) were measured and the recording of all gross pathologic alterations, weighing designated



organs, and saving all gross lesions was conducted on all females. The uterus was excised and the implantation sites, if present, were counted.

Females which were sperm positive and/or had an internal vaginal plug but did not deliver-were sacrificed after gestation Day 24. Females that were never observed as being inseminated and a with an internal vaginal plug and did not deliver at least 24 days after the completion of the mating phase. were sacrificed and necropsied. A gross necropsy was performed on these animals as described above. In addition, patency of the cervical/uterine os in these females was examined via flushing of the derine horns with 10% buffered formalin. Ĉa

The following tissues were col	lected and weighed (XX). Micropathology was performed on those
tissues designated with (XXX).	lected and weighed (XX). Micropathology was performed on those
XX Brain	XXX Epididynes as A A
XXX Pituitary	XXX Coagulating Gland 🔨 🛇 🧳 🖉
XXX Liver	(k, XXX) Ovatry 🔬 🛫 🖉 😽 🕊
XXX Kidney	XXX Oviduct & S Ov A A
XXX Spleen	XXX Prostate XXX Septimal Vesicle
XX Thyroid	XXXX Seminal Vesicle x 2 x
XX Thymus	Y XXX Seminal Vesicle
XXX Adrenal	XXX Uterus S S S
XXX Cervix	XXX Semmal Vesicle

Animals found moribund while on study were sachliced and a pross pecrops performed. Animals found dead were necropsied as soon as possible. Necropsy examinations included those parameters previously described. Pups found dead or terminated in a moribund condition underwent a gross necropsy for possible defects and/or to determine the cause of death.

**b.** Offspring The F1-offspring not selected as patental unimals and all F2-offspring were sacrificed at 21 days of age. These animal overe subjected to ostmottem examinations (macroscopic and/or microscopic examination as follows

The following tissues from Q1-day wearkings were collected collected and weighed (XX), and micropathology was performed (XXX); n

XX	Brain	XXX Uterus A A	) ^y XXX	Testis
XX	Spleen	XXX Ovary	XXX	Epididymis
XX	Thymus	Vagina XXX	XXX	Prostate
Х	Gross Les	stons A XXX Cetvix	XXX	Coagulating Gland
	Q	S SXX Sviduco S	XXX	Seminal Vesicle
Anve	ross lesion	was documented and collected		

Any gross lesion was documented

#### II. Results and discussion

#### A. Mortality

There were no test substance-related mortalines observed during the course of this study at any dietary level tested in either generation.

## B. Observation

## Clinical signs

There were portest and the related clinical observations observed during the course of this study at any detary evel tested in either generation.

#### C. Body weight

a. Males



The *P*-generation males did not exhibit any test substance-related effects on body weight or body weight gain at any dietary level tested after 15 weeks of exposure. No test substance-related findings were observed on food consumption during the 10-week premating period at any dietary level tested.

The F1-generation males did not exhibit any test substance-related effects on body weight or body weight gain at any dietary level tested. No test substance-related findings were observed on food consumption during the 10-week premating period at any dietary level tested. Sporadic statistical significances on food consumption, considered not to be test substance-related, were observed to both the 220 and 1200 ppm dose groups.

#### **b.** Females (premating)

The F1-generation females of the 1200 ppm dose group exhibited declines in body weight gain (declines) 9.8%) when compared to the controls. No test substance-related findings were observed on food consumption during the 10-week premating period at any dietary level tested. A slight decline in bod consumption from Day 56 to 63 on a g/animal/day was observed in the 1200 ppm abse group and was ×, , Ø considered to be incidental. Õ Õ

The P-generation females showed slight declines in body weight in the 1200 ppm dose group with statistical significance observed on Day 63 (declined 5,5%) and body weight gain declined 20% when compared to controls. No test substance-related findings were observed on food consumption during the results (premating) are summarized in Table 5 6024. 10-week premating period at any dietary level tested. A slight decline in foot consumption from Day



	Dose Group			<u></u>
Observations/study week	Control 0	LDT	MDT	HDT 1209>ppm
P Gener	ppm ation Males	40 ppm	220 ppm	
Mean body weight (g) - Week 15 S.E.	444.5	456.2 5.99 👟	458.6 7.69	5 448C4
Mean weight gain (g) - Weeks 1-15	7.47 196.3	2109	207	39195.8
Mean food consumption (g/animal/day) - Weeks 1-10	23.3	Q23.800°	23.5 K	23.5 L
Mean food consumption (g/kg/day) - Weeks	\$68.8 \$	#0.2 L	66,4	\$ 69.6
P Generation For	males - Pre-m	atting		
Mean body weight (g) - Week 10 S.E.	244.9 0 2.8 k	287.6 0.52 ×	24 <b>¥.0</b> 2,72	234 2 2 39
Mean weight gain (g) - Weeks 1-10	72.9	y 640	66.85	58.3
Mean food consumption (g/animat/day) Weeks	\$ 17.2°	Q16.9	0 ^{6.9}	16.8
Mean food consumption (g/kg/day) Weeks	80.8	89.5	× 840	80.7
L S F1 Gener	ation Males			
Mean body weight () - Week 14 ST	462.0 ~7.09 Q	45P.9 & 21	465.6 6.78	456.5 5.74
Mean weight gan (g) Weeks 1-14	198.7	186.5	191.5	189.2
Mean food consumption (g/animal day) Weeks 7		22.7	22.8	23.8
Mean food consumption (g/k@/day) Weeks	\$64.2 Å	[≫] 64.4	62.2	66.2
F1 Generation Fe	males - Pre-n	nating		
Mean body weight (g)- Week 10 S.E.	237.5	244.1	244.5	230.6
A B B	3.51	3.97	3.63	3.14
Mean weight gain (g) - Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q	× 62.4	66.7	61.3	56.3
Mean food consumption (granimaticay) - Weeks 7 1-10	16.2	16.8	16.4	15.8
Mean food consumption (g/keday) Weeks	77.1	78.6	75.4	76.8

#### Table 5 6 1-4 Mean (S.E.) Body Weight and Food Consumption

## c. Gestation

Statistically significant body weight declines, relative to control, were observed in the P generation females of the 1200 ppm dose group from gestation Day 0-13 (mean decline of 5.5%). The lower dose groups did not exhibit any effects on body weight. There was no effect on body weight gain during gestation at any dietary level tested. There were no effects on food consumption observed at any dietary level tested.



In the 1200 ppm F1generation dose group, a significant increase in body weight gain (increased 13.2% relative to control) was observed and correlates with the increased food consumption on both, a g/animal/day and g/kg/day basis observed in this same dose group. No effect on body weight, weight gain or food consumption was observed at any other dietary level tested.

Reported body weight and selected food consumption results during gestation are summarized in Tables 5.6.1-5 and 5.6.1-6 for P generation females and F1 generation females, respectively.

Gestation	A.		ð		L.O
P Generat	tion Females - C	Gestation	×,	Q A 4	0 ^v
	A	Q Dose	Group 🖌 🏑		<i>y</i>
<b>Observations/study week</b>	Control 0		MDT	HDT	
	🤇 pprác	م ² LD ۴ ک 40 ppm	220 ppm	`∕∕1200 ppm	
Mean body weight (g) - Day 0 S.E.	24J.4 3.28	3.95 A	243.1 \$2.46	27** ° 2.50 °	
Mean body weight (g) - Day 6 S.E.	263 8 3 12	۲ 2548° ۲ ≰48 č	َمَّ 261 مَ کَلْمَ 261 مَ	248 5*** 3.51	
Mean body weight (g) - Day 13 S.R	286.0 × × × × × × × × × × × × × × × × × × ×	276.9° 4.61°	283.85 0 2.80	×272.6** ≫ 3.01	
Mean body weight (g) - Day 13 S.Q [*] Mean body weight (g) - Day 20 S.E.	340.7 K	353.6 ~6.21 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	3\$\$.7 \$4.35	² 336.1 3.83	
Mean weight gain (g) - Days 0-20 S.E.		94,4 ⁹ 4.28	100%6 320	103.4 2.90	
Mean food consumption (g/artimal/day) Days	\$20.0 °	20.1	20.0	20.0	
Mean food consumption (g/kg/day) Days	520.0 5 7 7 5 7 5 .3 6	28.0 V	75.8	79.6	
** Statistically different/prom constrol, p < 0.01.					

# Table 5.6.1-5 Mean (S.E.) Body Weight and Food Consumption for P Generation Fémales During Gestation



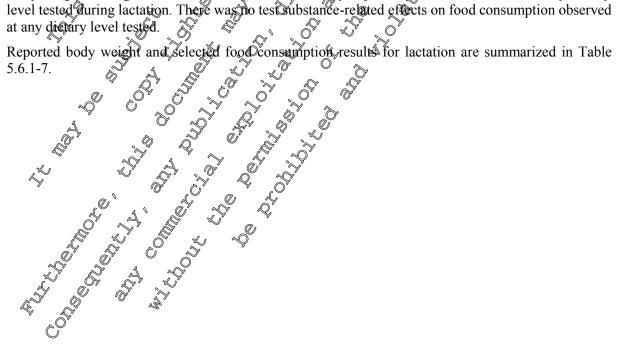
Table 5.6.1-6

Mean (S.E.) Body Weight and Food Consumption F1 Generation Females During Gestation

F1 Genera	tion Females - (	Gestation		
		Dose (	Group	
<b>Observations/study week</b>	Control 0	LDT	MDT	<b>MDŢ</b>
	ppm	40 ppm	<u>220 ppm</u>	1200 ppm
Mean body weight (g) - Day 0 S.E.	240.2	247.5	241.5	2392
	3.74	4.17	3.54	¥.51 0 ^v
Mean body weight (g) - Day 6 S.E.	253.3	261.5	2562	249 ¢
filean body weight (g) Duy 0 5.2.	3.78	4,62	3,09	<u>* 3,54 @</u> *
Mean body weight (g) - Day 13 S.E.	200.8	~279.6	¢\$73.4 ۲	<i>2</i> 69.7
Weal body weight (g) Duy 15 5.E.	3.98 .	@ 4.14 Y	J 3.89	<i>√</i> 4.06 <i>∽</i>
Mean body weight (g) - Day 20 S.E.	32903	\$ 3 <b>3</b> 873 1	325.8	332.1
Mean body weight (g) - Day 20 S.E.	<b>Å</b> 87 Ø	Q.51 O	5.75	<b>A</b> .92 <b>S</b>
Mean weight gain (g) - Days 0-20 S.E.	89.1	الم € 90.	_م گُ [*] 85.3	100.9
Wealt weight gain (g) - Days 0-20 S.E.	~ 2.8° ~	3.04 \$	y 4,466	231
Mean food consumption (g/animal/day) Days				J. 919.6
0-20	× ×0.0	× 10.4 ×	0 ^{17.2}	\$19.0
Mean food consumption (g/kg/day) Days	10° 72°8			70.4
0-20	7200		<b>0</b> 07.9	78.4
Statistically different from control, p<0.65,			~~~~ (A	·
Lactation	Å L			

In the 1200 ppm P generation dose group, a statistically significant decline in body weight, relative to control, was observed on Day 0. No other effect on body weight in this dose group during lactation was observed. Body weight was not affected by the test substance at any other dietary level tested. There was no test substance-related effects on food consumption observed at any dietary level tested.

Body weight of F1 generation animals was unaffected by exposure to the test substance at any dietary level tested during lactation. There was no test substance-related effects on food consumption observed





P Genera	tion Females - L	actation		<u> </u>
		Dose (	Group	
Observations/study week	Control 0	LDT	ŴЮТ	A TOBE
Observations/study week	ррт	40 ppm	220° ppm	1200 ppm
	267.9	264.2	270.3	€ ⁶ 255 <b>S</b> *
Mean body weight (g) - Day 0 S.E.	3.34	3.94	ປິ້ 2.79 ຼ <u>ົ</u> ້	2,65
	276.5 🗇	266.2	276.8	~267.2 [©]
Mean body weight (g) - Day 4 S.E.	3.500	4.60	2.80	3.69
Maan hade weight (g) Day 7 S E	282.7	24Q.9	° 2 <b>8</b> 4.7	2\$3.1
Mean body weight (g) - Day 7 S.E.	Q0.63	~4.31 O	~\$.00 \ O*	\$3.50 Ø
Maan hade weight (g) Day 14 S.E.	ر 299.3°	یک ² 290	299 <b>@</b> ``	289%
Mean body weight (g) - Day 14 S.E.	O 4,26	<u>4</u>	∑ 3 <del>3</del> 9% _∠	310
Mean body weight (g) - Day 21 S.E.	, <b>28</b> 7.5 C	280.4	286.7 ^O	277.9°
Weight (g) - Day 21 S.E.	3.67	<u></u> 4.06	°∼ 2.85	2.89
Mean food consumption (g/animal/day)@ays				
0-21			\$3.5 Û	44.1
Mean food consumption (g/kg/day) Days	0 ² 1610	5 1555	1526	مُحْ 161.4
0-21				101.4
🗸 🗸 🖉	tion Remales - I	Lactation 📎		
		Dose (	Scoup 🗸	-
Observations/study week	Control 0	°`_ <b>⊈∠D</b> T "ຶ	<b>NOD</b> T	HDT
	Sppm S	<b>40</b> ppm	220 ppm	1200 ppm
Mean body weight g) - Day 0 Str.	2570	266.8	<i>©</i> 260.6	258.5
	3,25 ~	5° 4037 ~~	4.56	3.95
Mean body weight for - Das A S.E.	263.0	\$274.4	271.4	263.0
Wiedli body weight () - Dago + S.L.	× 4.31	4.455	4.63	3.70
Mean body weight (g) Day 5.E.	278.3	282.6	279.9	271.3
wican body weight (gr Day Op.E. 👒	Q.14 🔍	×4.62	4.63	4.18
				291.3
	293	297.5	296.8	
	293 5 x 3,85	297.5 5.19	296.8 4.14	3.91
Mean body weight (g) - Day 14 get.		<b>5.19 290.6</b>	4.14 293.3	3.91 284.5
Mean body weight (g) - Day 14 g/E.		5.19	4.14	3.91
Mean body weight (g) - Day 14 g/E. Mean body weight (g) - Day 21 S E	3 85	290.6 4.45	4.14 293.3 3.75	3.91 284.5 4.59
Mean body weight (g) - Day 14 get.		<b>5.19 290.6</b>	4.14 293.3	3.91 284.5
Mean body weight (g) - Day 14 g/E. Mean body weight (g) - Day 21 S E. Mean food consumption (g/anipal/day) Days		290.6 4.45	4.14 293.3 3.75	3.91 284.5 4.59

# D. Compound intake

~Q Based on food consumption, body weight and dietary analyses results, the doses expressed as mean daily no test substance/kg body weight during the pre-mating period (10 weeks) are presented in Table 3.6.1-8. Calculation for test substance intake is: Table 5.6.1-8. Ĩ

Mean analytical concentration (ppm) specific for premating / 1000 X mean weekly food consumption (g/kg/body weight/day) during premating.



		Male			Female	
Generation	LDT 40 ppm	MDT 220 ppm	HDT 1200 ppm	LDT 40 ppm	MDT 220 ppm	1200 ppm
Р	2.7	15.1	83.1	3.2	10	5 96 V
F1	2.6	13.9	82.4	3.1	16.8	95.6 0 Q

#### Table 5.6.1-8Mean test substance intake during premating (mg/kg body weight/day)

#### E. Reproductive function

#### 1. Estrous cyclicity and periodicity:

There were no test substance-related effects observed on the estrous cycle number or length in either generation at any dietary level tested.

#### 2. Sperm measures:

There were no test substance-related effects observed on any sperm parameter evaluated at any detary level tested for either generation. These data are summarized in Table 5.6,1-9.

m

Table 5.0.1-5 Sperm	Silver Si		6 1. s		<u>~</u>
			Dose Grou	up (ppm) 👋	
Sperm Ana		Control	[√] √LD⊉	,_ [©] MDT	HDT
		🖉 0 ppm	40 ppm	<b>⊳ 220≰p</b> pm	1200 ppm
/	P Cent	eration Males		, Š ^v	
Same Matilit	🖉 % Motile	\$ 89.8	89.50	× 89.8	89.9
Sperm Motility	% Progressive ~	, 64Q0 (	647	Ø 64.6	63.3
	Testis	\$8.2 [~]	N/A V	N/A	34.92
Sperm Counts (Sperm/gram)	Epididymis .	¢ 240¢	°N/A€	N/A	219.7
	Normal C	198.7	NO A	N/A	197.3
Spern Morpholog	S Abnormal	°°0.9 ↔	©N/A	N/A	2.0
	Detached Head		Å″N/A	N/A	0.8
Q A		neration Male	š		
	8 % Motile O	\$ 0 ³ 87.8 ¹ 0 ³	86.4	87.0	87.2
Sperm Qotility	% Progressive	\$ 6 <b>0</b> ,4	61.1	61.8	61.9
Sporm Counts 🚬 🖏	Testers X	\$ 28.3	N/A	N/A	29.1
(sperm/gram)	Epididymic	م بم 189.4	N/A	N/A	161.9
	Normal (	197.0	N/A	N/A	195.3
Sperm Morphology (mean tanal number)	Abnormal 9	2.0	N/A	N/A	4.2
	Abnormal Q Detached bead	1.0	N/A	N/A	0.5

Table 5.6.1-9	Sperm Measur
	P

N/A - Indigues evaluation deemed unnecessary.

## F. Reproductive performance

Overall reproductive performance was not affected for any parameter (e.g., mating, fertility or gestation indices, days to insemination, gestation length, or the median number of implants) in either generation at any detary level tested.

Results for both the P- and F1-generation animals are summarized in Table 5.6.1-10.



		Dose Gr	oup (ppm)	
Observation	Control 0 ppm	LDT 40 ppm	MDT 220 ppm	HBÌP 5 ⁷ 12,00 ppn5
P Gener	ration – F1 Off	spring	^A	
Number Cohoused	30	30 ్	نگ 30 ک	
Number Mated	30 🚿	29	30	
Number of Animals Delivered	30	25	20	
Number of Animals with Implants	a de la companya de l	~25.	280 280 289	د کی 26
Mating Index	≰_ 100 <b>,⊘</b> °	2 96 F	£ 100 ¢	× 93.3
Fertility Index		<b>8</b> 6.2 C	96.7	9 2.9 × °
Gestation Index	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	∂100.Q	°,0°96.6≪	100.65
Days to Insemination Mean (S.E.Q.	3.0 (9.43)	2.4 (0.22)	2.7 (43)	2.3 (0.20)
Gestation Length (days)\Mean (K.)	21.9 (0,12)	21.8 (0.10)	20.8 (0.09)	×24.8 (0.09)
Ø "FiGene	ration - F2 Off	wring O	ð ₂ 0 «	~
Number Cohoused	× 30	~30 ~~	ر پر کاری کاری کاری کاری	30
Number Mated	S 29G	<u>~</u> 30℃	30 ⁵	30
Number of Animals Derivered	27 .~~		27	28
Number of Animals with Impoints	27,5	© 27	27	29
Mating index 4 &	<b>96</b> .7	1.00.0	100.0	100.0
Fertifity Index	\$93.1.C	© 90.0 €	90.0	96.7
Gestation Hidex		¢ 140.0	100.0	96.6
Days to Insemination Mean (S.E.)	2 (0.41)	3.9 (0.43)	2.3 (0.23)	2.8 (0.32)
Gestation Length (days) Mc (S.E.)	©21.8 (0.11)	21.7 (0.09)	21.6 (0.11)	21.5 (0.10)

#### Table 5.6.1-10Reproductive Performance

#### G. Clinical pathology (parental animals)

Ĉ

## a. Clinical Chemistry

*P***-generation**- Test substance related clinical chemistry changes were limited to creatinine, total protein, and albumin that were increased in 1200 ppm males.

**F1-generation**- Test substance related Ginical chemistry changes were limited to urea nitrogen (UN) and total protein that were increased in 1200-ppm males, and cholesterol that was increased in 1200-ppm females

#### b. Hematology

*P***-generation** Fest substance related hematology changes were limited to decreased hemoglobin and hematocrit in the 1200 ppm females.

**Ff-generation**- Test substance-related hematology changes were limited to increased white blood cell and monocyte Ab counts and decreased hemoglobin in 1200-ppm females.



#### H. Necropsy

#### 1. <u>Terminal Body Weight and Organ weights</u>:

<u>P-Generation Adults</u>: There were no test substance-related effects on adult terminal body weights for the males or females. Test substance-related organ weight changes for males and/or females were fimited to the kidneys, liver, and spleen. Kidney (right and left) weights were increased in 1200-ppm males (absolute and relative), spleen weights were decreased in 1200-ppm females (absolute), and fiver weights were increased in 1200-ppm males and females (absolute and relative). The decrease in the absolute spleen weights in the females was considered not to be an adverse effect since to corresponding micropathology or hematology findings were observed to support this inding.

<u>F1-Generation Adults</u>: There were no test substance related effects on adult terminal body weights for the males or females. Test substance-related organ weight changes for mates and of females were limited to the kidneys, liver and spleen. Kidney (right and left) weights were increased in 1200 ppm males (absolute and relative), spleen weights were decreased in 1200 ppm females (absolute and relative), and liver weights were increased in 1200-ppm males and females (absolute and relative). Mean relative spleen weight was also decreased in 220-ppm females (absolute and relative). Mean relative spleen weight was also decreased in 220-ppm females (absolute and relative), but was considered not to be test substance-related for the following reasons; (1) the mean terminal body weight for the 220-ppm dose level was numerically increased over the controls enhancing the decrease in mean relative weight, (2) the decrease was not statistically significant in absolute weights and (2) there were no statistically significant decreases in splech weights (absolute and relative) at 220-ppm in the Pgeneration females.

The decrease in absolute spleen weights (absolute and/or relative) in 1200- and 220-ppm females was considered not to be biologically of toxicologically relevant since no corresponding micropathology or hematology findings were observed to support this finding. Therefore, the absolute spleen weight decrease was considered not to be an adverse effect.

#### **Pathology**

#### 1. Macroscopic examination?

No test substance-related gross necropsy findings were observed in either the males or females at any dietary level tested.

### 2. Microscopic examination

Test substance-related micropathology findings for P-generation males and/or females that were statistically significantly different from controls included kidneys - increased incidence of protein droplet nephropathy and tymple cytic infiltration in 1200-ppm males, and liver - increased incidence of centrilobular hypertrophy in 1200-ppm males and females. There was no evidence of test substance-related changes in the kidneys and liver of the 220-ppm males and females.

#### 3. Ovarian Follicle Counts from &-Generation Females:

None of the mean prepriordial (preantral) follicular antral follicular, or corpora luteal counts for F1generation females were statistically different from controls. Ovarian follicular counts, therefore, were not affected by test substance administration.

#### I. Offspring

#### 1. Viability and clinical signs

There was no test substance related effects observed on the viability of the pups at any dietary level tested. There were to test substance-related clinical observations observed in either generation at any dietary level tested.

Mean litter size and pup viability (survival) during lactation are summarized in Table 5.6.1-11.



		Dose Group (ppm)					
Observation	Control 0 ppm	LDT 40 ppm	MDT 220 ppm	HDT 1200 ppm			
	$\mathbf{F}_1$	Generation					
Total Number of Implantation Sites (Mean)	377 (12.6)	274 (11.0)	323 (1.1)				
Total Number born	354	2357	303	2 395 J			
Number stillborn	1	Å 1	0 ×0	Q 1 5 4			
Sex Ratio Day 0 (% male)	53.2	54.0	€ 646.0 Å	لم 44.6			
Mean litter size	11.8	10.3	10.8	× 11.3 ~~			
Birth index	93.9	91.8	L 89.6 S	96 ₂ 8			
Live birth index	99.7	~ 990 Q	900.0	0 20.1 L			
Viability index	99.7°°° *	× × × × × ×	J 99.9 L	97.8			
Lactation index	98 ⁸	≪ 99.5~¥ ¥	99.6 D	\$ 99. <b>\$</b>			
	$\int_{0}^{0} \sqrt{F_2}$	Generation 🗸					
Total Number of Implantation Sites (Mean)			297011.0) 297011.0)	323 (11.1)			
Total Number born	287	L 289 L	مَحْ 289	303			
Number stillborn 👾	46			3			
Sex Ratio Day 0 (% male)	¢7.4 0	49, D ^V &	49.1 S	44.1			
Mean litter size	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	5 . J. 1	O 10.7 √	10.8			
Birth index O	مَ ^م َ ² 95% م	s, \$96.3 €	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	91.0			
Live birto index	98.7 UT	100.0	⁴ 99.7	99.2			
Viability ind 🐼 🐇	چ 99.6	\$ \$8.2 O	<u>م</u> 98.1	98.3			
Lactation index Before and ardization (c@ling), (	× 2806 O	94.9 ×	98.8	99.6			

Table 5.6.1-11	Litter parameters for F1 and F2 generatio	ns
----------------	-------------------------------------------	----

b Before Standardization (colling) c After standardization (culling) d Does not include number stillborn

# 2. Litter and Pup Weights

<u>F₁-Pups</u>: Pup body weights at birth for all three treated groups were comparable to the control group. In the 1200 ppm dose group, pup weight gain was defined for both males (statistically declined 7.8%) and females (non-statistically declined 6.2%) from Days 7-14 of lactation. No test substance-related effects were observed on body weight or body weight gain at any other dietary level tested.

<u>F₂-Pups</u>: Pup body weights at birth for all three treated groups were comparable to the control group. In the 1200 pprodose group, non-statistical declines by Day 4 (6.1% less than control) were observed with significant body weight declines observed by Day 21 (decline of 8.1%). Overall body weight gain throughout factation was declined 8.6% relative to control. There were no test substance-related effects on pup body weight observed adapt other dietary level.

Selected mean pup body weight data are presented in Tables 5.6.1-12, 5.6.1-13, 5.6.1-14 for combined sexes males and females, respectively.



F1 Generation					F ₂ Generation					
Lactation Day	Control 0	LDT 40	MDT 220	HDT 1200	Lactation Day	Control 0	LDT 40	MDT	HDT 1200>	
0 S.E	5.9 0.09	5.9 0.10	6.0 0.09	5.9 0.07	0 S.E	6.0 0.09	5.8 0.07	5.8 0.13	5.7 C0.08	Ş A
4 ^b S.E.	9.7 0.21	9.3 0.22	9.8 0.21	9.5 0.22	4 ^b S.E.	9.8 0.28	9.6 ©0.19	9.4 ×	9:2 [°]	Ś,
4° S.E.	9.7 0.21	9.3 0.22	9.8 0.20	9.5 0.22	4° S.E.	9.8 0 0.28	9.6 0,19	0.26	\$9.2 0.18	
7 S.E.	15.6 0.29	14.9 0.39	15.5 0.29	15.2 0.34	7 S.E.	15.6 0.41	0.30	15.0 9.46	↓4.5 ≪0.31 ~	Û,
14 S.E.	32.1 0.46	30.9 0.72	31.8 0.50	30.6 0 53	S.E.	31 8 Q2	90.87	0.76 ⁵	29.6 0749	Ś
21 S.E.	49.1 0.74	47.2 0.96	48.8 0.75	46.8 0.80 2	7 24 S.E. ,	\$49.2 1.090	48.D	St 10 a	45.2* 0.86	
GAIN Before stan	43.2 dardization	41.4 (culling)	42,00 Q	40/9	GAL	470:2	3 ^{42.9} 5	41 41 A	<b>39</b> .5*	
: After standa Statistically	rdization (cu	ulling)			F F		~~~ . &	8 ⁰ %	, ,	
able 5.6.1-1	13 M	ean (SE.)	M de Pu	p Weight	V(g)			L.		

Table 5.6.1-12	Mean (S.E.) Male/Female Combined Pup Weights (g)
----------------	--------------------------------------------------

Table 5.6.1-13 Mean (SE.) Mode Pup Weights (g	Mean (& E.) Mae Pup Weight (g)	Mean (SgE.) Made Pi	Table 5.6.1-13
-----------------------------------------------	--------------------------------	---------------------	----------------

				6 6		<u>_^</u>	~~~		
	<b>F</b> ₁ <b>C</b>	Generation	r s			$\int_{0}^{2^{\prime}}$ $\langle \langle F_{2} \rangle F_{2} \rangle$	Generatio	Ó	
Lactation Day	Control		₹ 220	HDT *	Lactation	Control		MDT 220	HDT 1200
0 S.E	©I 0.10 0	€ € 0.1€	6.1 0.10	0.07 ×	² 0 S C	Q.11	6.0 0.08	5.9 0.13	5.9 0.09
4 ^b S.E	9.9 0.23	9.6 0.23 Ô	×10.0 7	9.7° 0.23	S.E~S	10.00 028	9.8 0.20	9.6 0.28	9.4 0.18
4° S.E.	10.0 0.25	9.6 023	H0.1	$09.7 \ll$	4°CS.E.	▲10.0 r 0.28	9.8 0.21	9.6 0.28	9.4 0.19
7 S.E.	₩6.0 ~90.29	0¥5.4 030	0.22 15.9 0,20	0.37 ¢	0 ⁵⁷ 7 0 ⁵⁷ ⁹ SH2.	16.0 0.42	15.5 0.32	15.2 0.47	14.8 0.33
14 S.E.	32.7 0.48	32.0 20.60	∑32.3 0 0,53	3120 Ø.53	ζ≪14 ⁽¹⁴ ) ⁽¹⁴⁾ S.E.	32.3 0.69	31.4 0.88	31.1 0.79	30.1 0.51
ZY S.E.	50.2 ≪ 0.75	49.00 0.88	\$49.7 .00.82	47.6 0.83	21 S.E.	50.3 1.09	49.6 0.88	47.8 1.17	46.1** 0.81
b: Before star	dardization.	(culling)	* ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	Ŵ					

b: Before standard zation (culling) ** Statistically different from ontrol, p 50.01



F1 Generation				F ₂ Generation						
Lactation	Control	LDT	MDT	HDT	Lactation	Control	LDT	MDT	HDT	
Day	0	40	220	1200	Day	0	40	<b>220</b>	1200	~
0 S.E	5.8	5.6	5.8	5.7	0 S.E	5.8	5.6	5.6	5⁄6	-9
0.0.1	0.10	0.08	0.09	0.08	0 D.L	0.09	0.06	0.12	~0.08	
4b	9.5	9.0	9.7	9.4	4b	9.6	\$3	9.0 🗞	9:1	
S.E.	0.21	0.19	0.20	0.23	S.E	0.29	Ø.18	0.30	Ø.¥1	
4c	9.5	9.0	9.7	9.3	4c	9.6		L.	A. 1	
S.E.	0.22	0.19	0.21	0.23	<b>,⊗</b> ∕Ĕ.	0.29	0.19	_O.30 _	$0.20^{\circ}$	, O
7	15.3	14.4	15.2	14.9	0 ⁷ 7	15.4	A4.9 4	14.5	14.3	a s
S.E.	0.30	0.36	0.30	0.35	S.E.	Ø.42 ×	y 0.30	0,48	ר.31 ×	¢"
14	31.5	30.2	31.4	30.2	0 ¹ 4 ×	31.4	30.6	30.3 0 775	29.4	
S.E.	0.46	0.66	0.49	0,54	S.E.	0,05	Ø.86	0.76	0.51	Ś
21	48.0	46.0	48.2	<b>45</b> .9	× 21×	A8.2	47.80 [°]	45,6	44.6*	
S.E.	0.77	0.84	0.75	$\sqrt[3]{0.86}$	SE	× 1.120 [×]	47.80° Q77	P.09	0.84	
b: Before star	ndardization	(culling)	Ĩ,Ô ^v						, Ĉa	

Table 5.0.1-14 Mican (5.12.) Female 1 up Weights (g)	Table 5.6.1-14	Mean (S.E.) Female Pup Weights (g)
------------------------------------------------------	----------------	------------------------------------

c: After standardization (culling) * Statistically different from control, 1000 000

#### 3. Sexual maturation:

A slight delay in preputial separation in the F1-males of the 1200 ppm dose group (mean = 42.5 days) was observed, relative to control. Although statistically significant, the number of days to passing was well within this laboratory's historical control range (40.7 044.0) and is considered to be secondary to the decline in male body weight gand observed during lactation. In addition, the mean body weight at attainment of sexual maturation was comparable to the contols (171 g at 1200 ppm versus 173 g in controls). There were no andings on preputial separation in any other dietary level tested. There was no effect observed on vaginal patency at any dietary level tested. Anogenital distance was performed on lactation Day O for the F2-pups with no effects of this measurment noted.

#### Offspring postmortem results: 4.

<u>F₁-Juveniles</u>: The F₁ wearing PNIP21) of the 8000 ppm dose group (note that the dose was decreased to 4000 ppm between the pup age range of 26-38 days old) exhibited significant declines in body weight, plative to controls, pon passing criteria for balanopreputial separation and vaginal patency. Male body weights were declined 3.3% and female body weights were declined 13.6%. There was no effect on body weight at passing these developmental landmarks, compared to the control group, at any other dietary level ested

<u>F₂-Juverties</u>: There was no effect on body weight, relative to controls, upon passing criteria for balanopreputial separation and vaginal patency for the F2-juveniles (after weaning PND 21) at any dietary level tested.

#### 5. Anogenital distance (F2 pups)

As the effection body weight and attain then of puberty landmarks was affected in the F₁ generation, measurement of apogenital distance on LD/PND 0 in the F₂ generation was added to the protocol. There was no meatment-related effect on an energy distance measured at birth in either male or female  $F_2$ offspring, as shown in the table below.



	Males			a,°
Group (ppm in diet)	Number of litters		Mean (mm)	
0	26		3.5	
150	30		<b>3</b> .5	
1000	28	(	\$ 3.7	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
4000	29	4	3.5 🗬	2
	Females	×,	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	°Ó, K
0	26 🖉		2.20	
150	30 🚿	Q.	2.0 2	\$` \$
1000	28 🖧	JO A	× 2.0 Q	
4000	22		2.1	Ô, Ô
ffenring nestmostom results	Q [*]		Q, 0, 0	n Q

Table 5.6.1-15	Anogenital distance	e F2 generation at birth
1 4010 01011 10	Series and the series of the s	

#### J. Offspring postmortem results:

#### 1. Organ weights:

There were no statistically significant differences in the mean organ weights for F1 male and female pups, relative to controls. Test substance-related organ weight changes for F2 pups that were statistically significantly different from controls included spleen and thymus which were decreased in 1200-ppm males and/or combined pups (absolute); females and/or combined pups (absolute and relative).

Mean organ weights for F2-pups that were statistically significantly different from controls included brain – increased in 1200-ppm male pups (relative). This change was not considered to be test substancerelated for one or more of the following reasons: the organ weight difference was associated with the statistically significant decrease in day 21 thean body weights, the change was not dose-related, and/or the change was relatively shall.

#### 2. Pathology

Macroscopic examination All gross lesions for F1 and F2 pups were considered to be incidental and not test substance related

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Microscopic examination All micropathology lesions in the 21 day F1 and F2 pups were considered to be incidental and/or background and were not considered to be test substance-related.

# J. Deficiencies

None

#### III. Conclusions

The parental male systemic DOAEC was 200 ppm (828 mg/kg/day in males) based on increased clinical chemistry parameters (creatining total protein, albumin and urea nitrogen), increased kidney weight associated with an increased incidence of protein droplet nephropathy and lymphocytic infiltration, and increased licer weights associated with an increased incidence of centrilobular hypertrophy.

The parental female systemic LOAEL was 1200 ppm (96.0 mg/kg/day in females) based on decline in body weight and/or body weight gain during premating, decreased body weight during gestation in the P-generation, increased body weight and food consumption during gestation in the F1-generation, increased cholesterol and increased while blood cell and monocyte Ab counts in the F1-generation, decreased hemospobin and/or hematocrit in the P- and/or F1-generation, increased liver weights associated with an increased incidence of centrilobular hypertrophy and minimal to slight, alveolar macrophages in the P- and/or F1-generation.

The parental systemic NOAEL was 220 ppm (14.5 mg/kg/day in males, 17.2 mg/kg/day in females).

The reproductive NOAEL was 1200 ppm in both males and females (82.8 mg/kg/day in males and 93.1 mg/kg/day females), based on no reproductive findings observed in the highest dose tested.



The offspring LOAEL was 1200 ppm (97.9 mg/kg/day). The LOAEL was based on maternal effects leading to secondarily-mediated effects on pup weight and pup weight gain. Also noted was a slight delay in preputial separation and decrease in spleen and thymus weight for F2-pups (both findings considered secondary to pup weight decrease. Additional data to support the association between decrease in body weight and decrease in organ weight are provided in documents KIIA 5.6.1.04;

.; 2009; <u>M-345012-01-1</u> (Fluopyram - Evaluation of OFCD joint review dossier - Questions addressed from German BfR - Metabolism, toxicology, dated February 23, 2009) and XIIA 5.6.1/06; Yang, Y. G.; 2005; <u>M-329297-01-1</u> (EPA DER for Oral (diet) dovelopmental immunotoxicity study of TI 435 (clothianidin) in Crl: CD(SD) rats). The offspring MOAEL was 220 ppm (170 mg/kg/day).

#### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/201

Based on this study the parental systemic NOAEL was 220 ppm (14.5 mg/kg/day in makes, 17.9 mg/kg/day in females) based on Clinical pathology changes, increased liver weight, protein droptet nephropathy (males) and centrilobular pephropathy. The reptoductive NOAEL was 1200 ppm in both males and females (82.8 mg/kg/day in males and 93.1 mg/kg/day remales). The LOAEL for reproductive effects was >1200 ppm, the bighest dose tested, because no reproductive findings were observed in the highest dose tested. The offspring NOAEL was 220 ppm (07.0 mg/kg/day) based on maternal effects leading to secondarily-mediated effects on par weight are pup weight at the LOAEL, the highest dose tested, 1200 ppm (97.9 mg/kg/day).

CA 5.6.2 Developmental toxicity studies	LOALL, the m	ignest dose test	±u, j∠00	) hhim	77.7 mg/	Kg/uay)	· L		Ro	
			о ^х	S.			× ·	Ĵ,		
	CA 5.6.2	Developm	ntat to	Õ			0 O	Š		5

Data Point:	
Report Author Report Year:	
	2008
	AE C656948 Developmental toxic ty stud (In the rat by gavage
Report No:	SA05276 & 0 0
Document No:	Mr 299438-01-2 × × ×
Guideline(s) followed in	ØECD 914 (2001); EPA Hearth Effects Test Guideline (OPPTS 870.3700; 1998);
study: 🖉 🛁	M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
Deviations from current	current guideline DECD 14, 2018
test guidelineQ O	Deviations: an genital distance and thyroid hormones were not measured. These
	deviatons do not impact the acceptability of the study
Previous valuation:	Yes evaluated and accepted in the DAR (2011).
GLP4Officially	Thes, conducted under GEP/Officially recognised testing facilities
recognised testing	× č [×] [×] ⁰
facilities:	
Acceptability/Reliability:	

## Executive Summary

In a developmental toxicity study AE C656948 (Mix-Batch 08528/0002, 94.6% purity) was administered daily by gavage from gestation day (GD) 6 to 20 to groups of 23 pregnant Sprague-Dawley female rats per dose-group. The doses given were 0, 30, 150 and 450 mg/kg/day in suspension in aqueous solution of 0.5% methylcellulose 400.



Clinical observations were recorded daily and body weights were recorded for all females on GD 0, 6, 8, 10, 12, 14, 16, 18 and 21. Food consumption was also measured for all the females during the intervals GD 1-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18 and 18-21. At scheduled sacrifice, on GD 21, the dams were subjected to a macroscopic examination of the visceral organs, the gravid uterine weight was recorded and the dams were evaluated for number of corpora lutea, number and status of implantations (resorptions, dead and live fetuses). In addition, the liver was weighed from all pregnant females. The liver was retained from all females and was examined histopathologically at all dose levels. Live fetuses were removed from the uteri, counted, weighed, sexed and examined externally. Approximately half of the live fetuses from each litter were fixed in Bouin's solution and subsequently dissected for internal examination. The remaining half were eviscerated, fixed in absolute ethanol and statued according to a modification of the Tyl and Marr technique for skeleta examination of bone and cartilago

Up to and including the highest dose level tester of 450 mg/kg/day, there over no mortalities, no treatment-related clinical signs during the course of the study and no treatment related effects on external fetal observations. The pregnancy rate was 96% in all groups including the controls.

#### At 450 mg/kg/day

Mean maternal body weight remained static between GD 6 to 8, compared with a mean maternal body weight gain of 6.8 g over the corresponding period in the control group. Between GD 8 to 10 and 10 to 14, mean maternal body weight gain was 22% and 54% fower than in the controls, respectively. The overall effect between GD 6 to 21 was a 16% lower mean maternal body weight gain compared with the controls. Mean maternal corrected body weight change (maternal body weight change independent of the uterine weight) was less pronounced at 450 mg/kg/day (37.65) than in the control group (59.4 g). Mean maternal food consumption was 13% to 15% lower than in the control group and at autopsy, 4/23 females had enlarged livers. At the histopathological examination of the liver diffuse centrilobular hepatocellular hypertrephy was observed in all females.

At Cesarean section mean fetal body weight was 5% fower for both the combined and separate sexes compared to the controls No other littler parameters were affected and separate sexes

At the external Petal examination, no treatment-related malformations or variations were observed and the incidence of rundieuses was similar to the controls

At the visceral fetal examination, there were no treatment related malformations. The incidence of the variations 'thymic remnant present' and 'preter convoluted and or dilated' was higher at this dosage than in the controls of both the fetal and littler level.

At the skeletal examination, there were no treatment-related malformations. The incidence of the variations 'at least one thoracic centrum split split cartilage' and 'at least one thoracic centrum dumbbell and/or bipartite/normal cartilage' was higher than in controls at both the fetal and litter level. The finding 'at least one thoracic centrum split/split cartilage' was considered a variation as in all cases only one centrum was affected, therefore the adjacent normal vertebrae would support the spinal column. Consequently, there would be no adverse consequences on the long term functionality of the animal (2007, M-766189-04-1).

#### <u>At 150 mg/kg/day</u>

Mean maternal body weight essentially remained static between GD 6 to 8, compared with a mean maternal body weight gain of 6.8 g in the controls over this period. Between GD 10 to 14, mean maternal body weight gain was 26% lower than in the controls. The overall effect between GD 6 to 21 was a 6% lower mean maternal body weight gain compared with the controls. Mean maternal corrected body weight charge was ess pronounced at 150 mg/kg/day (45.8 g) than in the control group (59.4 g). Mean maternal food consumption was between 10% to 18% lower than the controls for each interval between GD 6 and 14. Mean maternal liver weight was 15% higher than in the control group. At the



histopathological examination of the liver, diffuse centrilobular hepatocellular hypertrophy was observed in 20/23 females.

At Cesarean section, no litter parameters were affected.

There were no findings considered to be treatment-related at the external, visceral or skeleral examinations.

#### At 30 mg/kg/day

Mean maternal body weight gain was 31% lower than in the control group between GD 6 to 8 thereafter, body weight gain was similar to the controls. Mean maternal food consemption was 19% lover than the controls between GD 6 to 8, but was similar thereafter. This slight transient effect on body weight gain and food consumption, in the absence of other findings was considered to be a non adverse effect.

No litter parameters were affected at this dosage

There were no treatment-related findings at the external, visceral or skeletal fetal examinations,

In conclusion, AE C656948 administered by oral gavage to the Sprague Dawley rat Quised Sprifts ant maternal toxicity in terms of lower maternal body weight gain and food consumption, higher liver weight and diffuse centrilobular hepatocellular by perfrontly a 450 and 150 mg/kg/day. Developmental foxicity was observed at the high dose level only in terms of slightly lower fetal body weight, and a slightly increased incidence of two visceral and two skeletal minor variations. In this study the maternal No Observed Adverse Effect Level (NOAE) was 30 mg/kg/dag and the fetal No Observed Effect Level (NOEL) was 150 mg/kg/day.

## Materials and methods I.

#### A. Materials

1. Test material:	SAE @ 569485 5 2 0 0 4 1
Description 🖉 🔬 💭	Beige Powder
Description 5 5 5 Lot / Batch 7: Purity: 50	Mix-Batch 08528/0002
Purity: 🖉 🖉 💧	94.680 LY & LY
CAS # 0 v v	658066-35
	Stable in Suspension in the vehicle (aqueous solution of
Stability of test compound:	Biethylcellulose 400 at 0.5% at concentrations of 0.0868 and
Standing of test compound:	250 of for pariod of 32 have under similar conditions to those
	of the current study.
2. Vehicle and or positive &	The velfiele was an aqueous solution of methylcellulose 400 at
control:	Q0.5% O O
2. Vehicle and or positive control:	
Species;	Rat S
<ol> <li>Vehicle and % or positive \$         <ul> <li>control:</li> <li>Test animals:</li> <li>Species:</li> <li>Stram:</li> </ul> </li> </ol>	Crl:CD(SD) Sprague-Dawley
Weight at dosing: Source: Acclimation period: Diet:	24Q to 30 g for the females
Source:	
Acclimation period:	S days prior to mating
	Certified rodent pelleted and irradiated diet A04C-10 from
Diet: S	S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-
	Orge, France), <i>ad libitum</i>
Water: & A X	Tap water, <i>ad libitum</i>
	Animals were caged individually in suspended stainless steel
ÆHousing:	wire mesh cages
Environmental conditions:	
Temperature:	$22 \pm 2^{\circ}C$
Humidity:	$55 \pm 15\%$
munnuny.	$55 \pm 1570$



Air changes:	Approximately 10 to 15 changes per hou
Photoperiod:	12 hours dark / 12 hours light

- B. Study design
- 04 January 2006 to 09 February 2006 1. In life dates:

#### 2. Animal assignment and treatment

Adult virgin female rats were mated on a one-to-one basis with stock product of the same strain and supplier for each group. Each morning following pairing, dats showing spermatozoa in a vaginal smear or sperm plug in situ were considered as pregnant. The day where evidence of mating was found, was designated as GD 0. The females were assigned to control and treated groups each day of pairing using a body weight dependent randomization procedure. Body weight means were checked after the mating period to ensure similar means among all groups

#### 3. Test substance dosage formulations and analysis

The appropriate amount of AE C656948 was suspended (www) in as aqueous solution of methy cellulose 400 (Fluka, Mulhouse, France) at 0.5% and stored at approximately 5°C (±5°C). Formulations were prepared twice (F1 and F2) during the study. Homogeneity of the suspensions was checked on the first formulation (F1) for the lowest and the highest concentrations (3 and 45 JL). In addition, the intermediate concentration (15 g/L) of the first formulation (El Pand all concentrations of the second formulation (F2) were checked. Homogeneity and consentration checks were between 99 and 102% of nominal values. Stability of the test substance in suspension in the vehicle at concentrations of 0.0868 and 250 g/L was determined in a previous study and was found to be stable for 33 days under similar conditions to those of the current study. ő,

Test group	Test substance	Dost levels ng/kg/day	Concentrations gL	volume ⊘ (mL/kg)	Number of animals
1				¥ 10	23
2	2. 20	30		10	23
3 🦓	AE C656948	150	@ 15 _ O	10	23
	<u> </u>	<u>450</u>	^ب √ 4 <u>5</u> 0′	10	23

Study design and animal assignment Table 5.6.2-1

Dose levels were chosen based on the findings of a range-finding study ( 2004, M-246660-01-2), where groups of spern positive SD rats were dosed at 0, 50, 200 and 500 mg/kg/day.

Clear materna toxicity was observed at 500 mg/kg/day, where mean body weight gain was reduced by 72% between GD 6-8 and by 31% between GD 10-14, when compared to controls. Maternal corrected body weight change was 19% lower than controls. Mean food consumption was reduced by 14% between GD 6-8, whilest mean liver weight was markedly increased by 43%. At 200 mg/kg/day, maternal corrected body weight mange was 19% lower than controls. Mean food consumption was reduced by 14% between GD  $6_7$ 8, whilst mean liver weight was moderately increased by 21%. No treatment-related changes were observed at 50 mg/kg/day.

Doses were deministered daily by gavage to each female from GD 6 to 20, based on the animal's most recent body weight, and at a volume of 10 mL/kg. Control animals received an equivalent volume of vehicle alone (Q3% aqueous Dethylcellulose).

### Methods

## 1. Observations

All rate were observed daily for clinical signs and twice daily for mortality (except at weekends and public holidays when checking was carried out once daily).



#### 2. Bodyweight

Body weights were measured on GD: 0, 6, 8, 10, 12, 14, 16, 18 and 21.

#### **3.** Food consumption

Food consumption was measured at the following intervals: full feeder on GDQ1, 6, 8, 10. and 18 and empty feeder weights were measured on GD: 6, 8, 10, 12, 14, 16, 18, and 21.

#### 4. Cesarean sections

On GD 21, all females were sacrificed by inhalation of carbon dioxide, for examination of utegoie content. Autopsies were performed blind with regard to the animal study identification. Bach female was first subjected to macroscopic examination of the visceral organs. The liver was weighed for pregnant females, retained from all females and subjected to a microscopic examination.

The reproductive tract was weighed (gravid uterine weight), dissected out and the following parameters recorded: number of corpora lutea, number of implantation sites, number of resorption sites (classified as early and late), number of live and dead fetuses sex and individual weights of live fetuses. Dead fetuses: were defined as fetuses showings distinct digits visible on fore and hind-paws. Knnt fetuses were defined as live fetuses weighing less than 4 g at Cesarean section of the dam. Uterine horn(s) without visible implantations were impressed in a 10% solution of ammonium suffide for visualise any sites which were not apparent. Intra merine death was classified as early resorptions when macroscopic discrimination between fetal residues and placental material was not possible and late resorption when distinct macroscopic discrimination between feral restrictes and placental material was possible. Tissues and carcasses of dams were then discarded

#### 5. Fetal examination

All data were recorded without knowledge of treatment group. Alklive fetuses were subjected to external examination and then sacrificed by subcutaneous injection (0.02 ml/fetus) of Dolethal (18.22 g/100 ml, sodium pentobarbital). Approximately half of the live fetuses, from each litter were fixed in Bouin's solution and subsequentlodissected for internal examination. The repraining half were eviscerated, fixed in absolute ethanol and stained according to a modification of the Tyl and Marr technique for skeletal examination obone and carplage.

were classified as fol Structural deviations

Malformations:

A permanent structural change that is likely to adversely affect the survival or health

Variations:

° O A change that occurs within the normal population under investigation and is unlikely to adversely affect survival or health (this might in Pude a delay in grow for morphogenesis that has otherwise followed a normal partern of development)

#### 6. Statistics

Means and standard deviations for all maternal and litter parameters were calculated for each group. Statistical analyses were performed on the following variables using TERATEST Phase 1, Version 12, TERATEST Phase 4, Version 4 or SAS programs (Version 8.2).

Maternal ordpoints: body weight change calculated according to time periods, calculated corrected body weight Grange average food consumption calculated according to time periods, liver weight.

Litter based and for al endpoints: number of corpora lutea, number of implantation sites, number of resorption gearly and late), pre- and post-implantation loss percentages, fetal sex, fetal death status, fetal body weight.



Homogeneity of variances between control and treated groups was evaluated using the Bartlett test. If not significant, means were compared using the analysis of variance (ANOVA), which was followed by the Dunnett test (2-sided) if ANOVA indicated significance.

If the Bartlett test was significant (for body weight change, corrected body weight change, number of corpora lutea, number of implantation sites or number of resorptions parameters), group means were compared using the non parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance.

If the Bartlett test was significant, a log transformation (for food consumption, liver weight or tetal body weight) or an arcsine root transformation (for pre- or post-implantation to spectate process) was performed. If the Bartlett test on transformed data was not significant, means were compared using the analysis of variance (ANOVA), which was followed by the Dunnett test (2-sided) if ANOVA indicated significance. If the Bartlett test on transformed dat@was significant, group means wer@compared using the non parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance.

If the Bartlett test was significant for fetal sex (made vs. Jemale betuses) and fetal death status (live vs. dead fetuses) endpoints, control group and each exposed group were compared using the Chi-square test (for fetal sex parameter) or the Fisher Exact test (2-sided) (for fetal death status parameter). Death status was analyzed both using the foetus as the statistical unit and using the littler as the statistical unit.

If one or more group variance(s) equaled 0, means were compared using non-parametric procedures.

The homogeneity of group variances; results of the ANOVA or the Kruskal-Wallos tests were evaluated at the 5% level of significance. Group means were compared at the 5% and 1% levels of significance.

#### Results and discussion II.

#### A. Mortality

There were no mortanties during the course

### B. Clinical observations

O There were norreatment-related clinical stans

### C. Pregnancy rate

There was no effect our pregnancy rates, which we 96% in all treated group and in the controls.

 $\bigcirc$ 

### **D.** Body weight

At 450 mg/kg/day, mean maternal body weight remained static between GD 6 to 8, compared with a mean maternal body reight gain of 5.8 gover the corresponding period in the control group, the effect being statistically significant (p\$2.01). Between GD \$6 10 and 10 to 14, mean maternal body weight gain was  $\frac{22}{2}$  (not statistically significant) and 54% (p≤0.01) lower than in the controls, respectively. At other intervals maternal weight gain was similar to the controls. The overall effect between GD 6 to 21 was a 16% lower mean thaternal body weight gain ( $p \le 0.01$ ) compared with the controls.

At 150 mg/kg/day, mean mater al body weight essentially remained static between GD 6 to 8, compared with a mean maternal body wight sain of Q8 g in the controls over this period. Between GD 10 to 14, mean mater a body weight gain was 26% lower than in the controls. The effect was statistically significant  $(p \le 0.05)$  or  $p \le 0.01$ ) at both these intervals. At other intervals maternal weight gain was similar to the controls. The overall effect between GD 6 to 21 was a 6% lower mean maternal body weight gain compared with the controls, though the effect was not statistically significant.

At/30 mg/kg/day, mean maternal body weight gain was 31% lower than in the control group between GD 6 to 8, though the effect was not statistically significant. Thereafter, body weight gain was similar to the controls.



Mean maternal corrected body weight change (maternal body weight change independent of the uterine weight) was less pronounced at 450 mg/kg/day (37.6 g) and 150 mg/kg/day (45.8 g) than in the control group (59.4 g), the effect being statistically significant ( $p\leq 0.01$ ) at 450 mg/kg/day.

At 30 mg/kg/day, mean maternal corrected body weight change was similar to the controls.

#### E. Food consumption

Mean maternal food consumption was between 13% to 15% lower than the controls at 450 mg/kg/day and between 10% to 18% lower than the controls at 150 mg/kg/day, for each interval between GD 6 and 14, the effect being statistically significant at each interval ( $p\leq0.01$  at 250 mg/kg/day and  $p\leq0.02$  or  $p\leq0.01$  at 150 mg/kg/day). Thereafter, food consumption was comparable to the controls at both dose levels. At 30 mg/kg/day, mean maternal food consumption was 10% lower than the controls between GD 6 to 8, the effect being statistically significant ( $p\geq0.01$ ), thereafter, food consumption at this dosage was similar to the controls.

#### F. Maternal liver weights, necropsies and microscopic findings

Mean maternal liver weights were 40% higher at 450 and 05% higher at 150 mg/kg/da9, compared with the controls. The effect was statistically significant ( $p \le 0.01$ ) aboth to be levels.

Mean maternal liver weights were similar to the controls at 30 m

Table 5.6.2-2	Mean liver	weight of	pregna	nt females	

		Š Š I		$\sim$
Dose group		F AU P	150 0	^{&amp;} ∕∽ 450
(ing/kg/uay) 🕓 👔				
Number	22	Ŭ & &	[₩] 22 Š [°]	21
Mean liver weight (g) 🖉		√J4.06 °	16.05**	19.47 **
± SD	°\$\$,4.09	≈ ± 1.60	± 2.11	$\pm 2.33$
(% of controls)			~(115)	(140)
** p≤0.01	V 00 V		Ŵ	

SD: Standard deviation  $\beta$ 

At autopsy of the dams enlarged liver was observed in 4/2 Gremales at 450 mg/kg/day compared with 0/23 cases in the control group

Histopathologic changes were observed in the liver at \$50 and \$50 mg/kg/day only.

Diffuse centrilobular hepatocobular hypertrophy was seen in a dose related manner at 450 and 150 mg/kg/day.

Sex Sex Sex	Ŷ ^y	Fei	males	
A Dose group A A A A A A A A A A A A A A A A A A A	0	30	150	450
Number of animal examined	23	23	23	23
Centrilobuter hepatocelluter hypertrophy, diffuse	e			
	0	1	8	0
slight A A moderate A A	0	0	10	1
moderate 27 . 27	0	0	2	21
A marked a s	0	0	0	1
Total	0	1	20	23

Table 5.6.2 Incidence of changes in the liver



#### G. Litter data

man and a start of the second **C. Litter data**At 450 mg/kg/day, mean fetal body weight was 5% (p≤0.05) lower for both the combined and separate sets of the source of the control group.
At 30 mg/kg/day, mean fetal body weight for the combined sexes and the separate sets of the source of the control group.
At 30 mg/kg/day, mean fetal body weight for the combined sexes and the separate sets of the source of the control group.
At 30 mg/kg/day, mean fetal body weight for the combined sexes and the separate sets of the source of the control group.
At 30 mg/kg/day, mean fetal body weight for the combined sexes and the separate sets of the source of the control group.
At 30 mg/kg/day, mean fetal body weight for the combined sexes and the separate sets of the source of the control group.
At 30 mg/kg/day, mean fetal body weight for the combined sexes and the separate sets of the source of the control group.
At 30 mg/kg/day, mean fetal body weight for the combined sexes and the separate sets of the source of the control group.
At 30 mg/kg/day, mean fetal body weight for the combined sexes and the separate sets of the source of the control group.
At 30 mg/kg/day, mean fetal body weight for the control group.
At 30 mg/kg/day, mean fetal body weight for the control group.
At 30 mg/kg/day, mean fetal body weight for the control group.
At 30 mg/kg/day, for the for th At 450 mg/kg/day, mean fetal body weight was 5% (p≤0.05) lower for both the combined and separate

of the boundary of the boundar



Dose group (mg/kg/day)	0	30	150	450 °
Number of litters	22	22	22 嶡	2,20
Mean litter body weight (g) for combined sexes ± SD (% of controls)	5.51 ± 0.39 (100)	$5.48 \pm 0.20$ (100)	5.39 ± 0.35 (98)	5.26% ± 0.36
Mean litter body weight (g) for males ± SD (% of controls)	5.67 ± 0.34 (100)	5 \$ ± 0.26 (99)	(98) (98)	5.37*±0.36 (95) **
Mean litter body weight (g) for females ± SD (% of controls) * p≤0.05, SD: Standard Deviation	5.40 ± 0.42 (100)	$5.38 \pm 0.19$ (100)	\$25±\$37 (97) \$0 \$0 \$0 \$0 \$0 \$0 \$0 \$0 \$0 \$0 \$0 \$0 \$0	4,5.13 * ± 0.37 495) ~ ↓ 495) ~ ↓

or late resorptions and dead freuses, were Other litter parameters including number of h unaffected by treatment.

#### H. Fetal necropsy findings

#### 1. Fetal evaluation: external obse

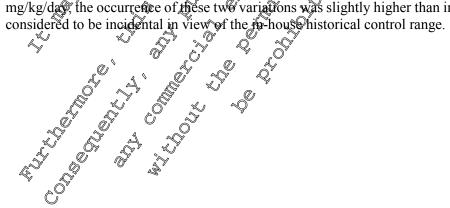
variations observed at the external fetal There were no malformations of treatment-related increased observation. Į. 1 P

#### 2. Fetal evaluation: visceral observations

There were two cases at 450 and 150 mg/kg/day and one case at 30 mg/kg/day of the malformation 'renal pelvis; dilated severe compared with no cases in the control group. However, the incidence at both the fetal and litter level was within he house variability avail three dose levels, when data from the low dose from a pecent m-house study, where there was clearly no treatment-related effect on this parameter, was taken into consideration. Therefore, at such a low incidence with no clear indication of a dose response, this finding was considered to have occurred by charge.

There were two other mattermations observed at the visceral examination, 'eventration of the diaphragm and caudate lung lobe beenton one fetus at 450 mg/kg/day, and situs inversus (total)' in one control fetus. In isolation, these findings were considered to have occurred spontaneously.

At 450 mg/kg/day, the incidence of the variations 'thymic remnant present (unilateral/bilateral)' and 'ureter (unilateral/bilateral); convoluted and 'or dilated' was higher at the fetal and/or litter level than in the control group, and was outside the in-house instorieal control range for both parameters. The higher incidence of these two variations was considered to be treatment-related at this dose level. At 150 mg/kg/da@, the occurrence of these two variations was slightly higher than in the control group, but was





Caracter			group g/day)		Historical Control	Dose group (mg/kg/day)			Historical Control	
Group	0	30	150	450	Range	0	30	150	450	Range
Observations			etal incid of fetuse		d)*	Litter in	ncidence	(% of lit	tters affe	sted)
Thymus remnant present (unilateral/ bilateral)	6/146 (3.9)	7/147 (4.6)	14/155 (9.2)	21/149 (14.5)	2/153- 11/175 (1.3-6.0)	5/22 (22.7)	5,82 (D2.7)	8/22 (36.4)*	(45.5) (45.5)	(5.3, <b>5</b> 3.3) ¢
Ureter (unilateral /bilateral) convoluted and/or dilated	46/146 (33.2)	57/147 (36.9)	72/155 (46.2)	88/149 (58,6)	€20.5- © 745.1	547/22 (77.39,	×7 717/22 (77.3)	(90,9)	20/22 (90.9)	23/24 23/24 (68.04) 95.69

* mean % of litters affected defined as: sum of % of live fetures affected per litter/no. Witters with live fetures affected per litter/no.

## I. Fetal evaluation: skeletal observations

The only malformation observed was one case of the finding two (except atlas and axis) cervical centrum (unilateral) cartilage fused, at 450 mg/kg/day. In isolation, this finding was considered to be fortuitous.

fortuitous. At 450 mg/kg/day, there was a higher incidence of the variations at least one thoracic centrum split/split cartilage' and 'at least one thoracic centrum: duribbell and/or bipartite / normal cartilage', compared with the control group. The incidence was outside the n-house historical control range at both the fetal and litter level for both findings. The increased incidence of these two variations was therefore considered to be treatment-related at this dose level. At 050 mg/kg/day, the incidence of the variation 'at least one thoracie centrum: dumbbell and/or bipartite / normal cartilage' was slightly elevated, but was considered to be incidental at this dose level, when compared with the in-house historical control range

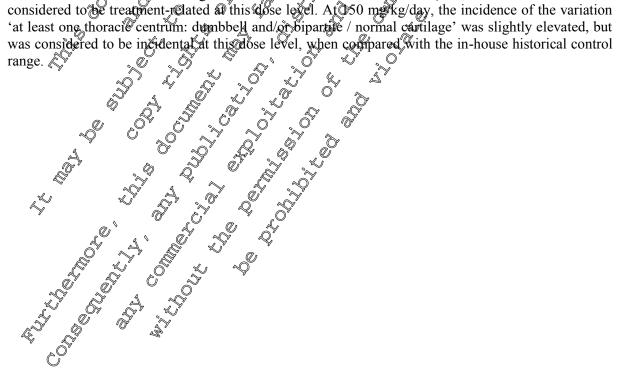




Table 5.6.2-6	Skeletal findings
---------------	-------------------

Group	Dose group (mg/kg/day)		Historical Control	Control (mg/kg/day)			Historical Control			
Group	0	30	150	450	Range	0	30	150	450	Range
Observations			ncidence es affect	e (mean % ed)*		Lit	ter incide	ence (%	of litters	affected))
At least one thoracic centrum: split/split cartilage	0/157 (0.0)	1/160 (0.6)	0/167 (0.0)	4/159 (2.6)	0/189 1/155 (0.020.6)	0/22 (0.0)	1022 (4.5)	0/22 (0.0)	24/22 (18.2Q	0/25-¥21 (0.64.8)
At least one thoracic centrum : dumbbell <i>or</i> <i>incomplete</i> <i>ossification</i> and/or bipartite/normal cartilage	3/157 (1.8)	9/160 (5.3)	12/167 (7.1)	29 159 5 (20.8)	₹ 1/144 124176 (0,₹7.2), 5 2 5 2 5 2 5 2 5 2 5 2 5 5 5 5 5 5 5 5 5 5 5 5 5	2/22# (9.1)	7,622 (3) 1.8) (3) 1.8)	9 22 (40.9)	(63.6) (63.6) (63.6) (7) (7) (7) (7) (7) (7) (7) (7) (7) (7	\$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$

* mean % of litters affected defined as sum of % of live cetuses affected or litter to of litters with ve fetuses examined or incomplete ossification : additional toon included in the in-house historical control range. With regards to 'At least one thoracic centrum: ant/split cartilage' in all cases, only one centrum was affected, therefore the adjacent normal vertebrar would support the spinal column. Consequently there would be no adverse consequences on the long-term functionality of the animal support the spinal column.

#### J. Deficiencies

The study has been carried out infline with the OEOD 414 requirements in force at the time it was conducted. No measurements of the test material in the plasmal anogenital distance or of thyroid hormones was carried out in this study, because at the time the study was conducted these requirements were not included. Study meets the current guidance and the requirements in 283/2013.

## III. Conclusions

In conclusion, AE C656948 administered by oral gavage to the Sprague-Dawley rat caused significant maternal toxicity in terms of lower maternal body weight gain and food consumption, higher liver weight and diffuse centricobular bepate cellular hypertrophy at 450 and 150 mg/kg/day. Developmental toxicity was observed at the logh dose level only in terms of slightly lower fetal body weight, and a slightly increased incidence of two visceral and two skeletar minor variations. In this study, the maternal NOAEL was 30 mg/kg/day and the fetal NOEL was 130 mg/kg/day.

## Assessment and conclusion by applicant:

The study has been carried but in the with the OECD 414 requirements in force at the time it was conducted.

AE C656948 administered by oral gavage to the Sprague-Dawley rat caused significant maternal toxicity in terms of lower maternal body weight gain and food consumption, higher liver weight and diffuse centrobulat hepatocellular hypertrophy at 450 and 150 mg/kg/day. Developmental toxicity was observed at the high dose level only in terms of slightly lower fetal body weight, and a slightly increased incidence of two visceral and two skeletal minor variations. In this study, the maternal NOACL was 30 mg/kg/day and the fetal NOEL was 150 mg/kg/day



Data Point:	KCA 5.6.2/02
Report Author:	
Report Year:	2006
Report Title:	AE C656948 - Developmental toxicity study in the rabbit by gavage
Report No:	SA 05014
Document No:	<u>M-279773-01-1</u>
Guideline(s) followed in	OECD 414 (2001); EPA Health Effects Test Guideline, (OPPTS 8700700; 1998);
study:	M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
Deviations from current	Current guideline: OECD 414, 2008
test guideline:	Deviations: None, the 2018 update affects rat-specific requirements in the TQ414;
	thus applies to rats and not tograbbits.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially	Yes, conducted under GLP/Officially recognized testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes v v v A ov v

#### **Executive Summary**

Twenty-three time-mated female New Zealand White Rabbit per group were exposed to AE C656948, a fungicide of the pyramide family, (Mix-Batch 08528/0002, a base powder, 946% purity) by gavage from Gestation Day (GD) 6 to 28 The does given were 0, 10, 25 and 75 mg/kg/day in suspension in aqueous solution of 0.5% methylcellulose 400.

Maternal body weights were recorded for all surviving females on GD 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29. Food consumption was measured for all surviving females during the intervals GD 3-4, 4-5, 5-6, 6-8, 8-10, 10-12, (12-14, )4-16, 76-18, 78-20, 20-22, 22-24, 24-26, 26-28 and 28-29. Clinical observations were recorded daily. At scheduled sacritice, on GD 29, a metroscopic examination of the visceral organs was performed, the gravid uterine weight and the number of ribs were recorded and the dams were evaluated for number of corpora lutea number and that so timplantations (resorptions, dead and live fetnses). The liver was retained from all females and was weighed for pregnant females at terminal secrifice. Live tetuses were removed from the uteri, counted, weighed and examined externally. The heads of fetuses from approximately thalf of each litter were immersed in Bouin's fluid and the internal structures examined after fixation. The bodies of all fetuses were dissected for soft tissue anomalies and sector. Fetuses were eviscerated, skinned and tixed in absolute ethanol before staining. A modification of the Staples and Schnell technique was applied and a subsequent skeletal examination was performed.

#### At 75 mg/kg/day

One mortality was noted on GD 21 following a gavage error. The macroscopic observation for this animal showed hemorrhaging in the lung and kemorrhaging and foam in the trachea. At this dosage, no treatment-related maternal clinical signs were noted. Mean body weight gain was reduced between GD 14 and 18 (0.02 kg vs. 0.09 kg for controls) and between GD 18 and 22 (0.02 kg vs. 0.07 kg for controls), in comparison to controls. Thereafter, mean body weight gain was similar to the controls, resulting in an overall body weight gain between GD 6 and 29 of 0.20 kg compared to 0.31 kg for the controls. Maternal corrected body weight change was more pronounced at 75 mg/kg/day (-0.25 kg), compared with the controls (-0.47 kg). Mean maternal food consumption was reduced by between 22 to 34% for all intervals between GD 14 to 26, in comparison to controls. At autopsy, no treatment-related materoscopic findings were noted.

At Cesarean section, mean fetal body weight for the combined sexes and for the individual sexes were 11% lower than the controls. No other litter parameters were affected.



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At the external fetal examination, the mean percentage of fetuses classified as 'runts' was 12.5% and the percentage of litters affected was 47.6%, compared with 3.0% and 23.8%, respectively, in the control group. There were no treatment-related visceral or skeletal findings at this dose level.

#### At 25 mg/kg/day

One female was killed for humane reasons on GD 23, following a slight loss in body weight and a reduction in food consumption between GD 20 and 22. Clinical signs in this female consisted of librited use of the right hindlimb on GD 22 and 23. The macroscopic observation showed a severe fracture of the right hindleg, in association with massive subcutaneous hemorrhaging and a distal epiphysa femoral disjunction. Consequently, this premature sacrifice was considered to be due to accidental frauma. At this dosage, no treatment-related maternal findings weigh noted.

There were no treatment-related effects on litter parameters.

There were no treatment-related external, visceral or skeletal fordings at this lose bevel.

#### At 10 mg/kg/day

No treatment-related maternal findings were noted.

There were no treatment-related effects on littler parameters

There were no treatment-related external, visceral, or skeletal fetal findings at this dose levels

In conclusion, a dose level of 75 mg/kg/day of AE C606948 administered to the pregnant remale New Zealand White rabbit caused maternal toxicity, as epidenced by reduced mean body weight gains and food consumption. Fetal toxicity at this dose level consisted of a reduced body weight in both sexes.

A dose level of 25 mg/kg/day of AP C656948 was considered to be a No Observed Effect Level (NOEL) both in the dam and in terms of tetal development in the New Zealand White rappit.

### I. Materials and methods

A. Materials

1. Test material: 🖉 🖉 🤇	OAE (\$65694) 20 2 0
Description 0	Beige Powrer of States
Description Lot / Batch #: Pustry: CAS #	OAE (\$656948)     O     Image: Second
Purity:	$94.6\%$ , $0^{9}$ $0^{9}$ , $0^{7}$
$CAS # \sqrt{2}$	658066-35+4 & S
	Stable in Suspension in the vehicle (aqueous solution of
Stability of test compound:	methyle ellulose 400 at 0.5%) at concentrations of 0.0868 and
	250 gPL for a period of 33 days under similar conditions to those
	of the current study.
2. Vehicle and / or positive	The vehicle was an aqueous solution of methylcellulose 400 at
	0.5%
3. Fest animals: Species: Strain: Age: Weight at dosing:	
Species:	Rábbit Ö
Strain:	Sew Zealand White Crl:KBL (NZW)
Age: of A	18 weeks approximately on arrival
Weight at dosing:	3 9 - 3.90 kg females
	Say - 5.90 kg temates
Weight at dosing:	
Acclimation period:	At least 5 days prior to dosing
BLAL U ST S	110 C-10 pelleted animal diet from S.A.F.E. (Scientific Animal
Diet: J O j	Food and Engineering, Augy, France) ad libitum
A YA	Filtered and softened tap water from the municipal water
Water:	
-	supply, <i>ad libitum</i> .



Housing:	Animals were caged individually in stainless s	teel wire mesh
	cages	~ °
Environmental conditions:		
Temperature:	$19 \pm 2^{\circ}C$	
Humidity:	55 ± 15%	
Air changes:	Approximately 10 to 15 changes per hour	
Photoperiod:	12 hours dark / 12 hours light	
Study design	Co L	

1. In life dates:

B.

18 October 2005 - 06 December 2005

#### 2. Animal assignment and treatment

Time-mated female New Zealand White Crl:KBL (NZW) rabbits were used in this sody. Stock makes from the same strain were used by the supplier to naturally mate nulliparous females. The day of insemination was designated as GD 0. The animals were approximately 18 weeks of age on arrival and were received on GD 1 or 2. On each day of mating, the females were advocated to control and treated groups using a computerized randomization procedure. Body weight means were checked to ensure similar means among all groups. The doses were administered daily by gavage at a volume of 4 mL/kg to each female from GD 6 to GD 28 inclusive based on the animal's most recent body weight. Control animals received an equivalent volume of vehicle alone (methylcellulase).

Table 5.6.2-7	Study	design	and	animat assig	nment	<ul> <li>.</li> </ul>
10010 01012	~~~~~					

Test group	Test substance	omg/kgjday	Concentrations	(mLØg)	Number of animals
1	\$ \$ \$			<u> </u>	23
2		& 107 ×	2.50 °	Ž 4	23
3	AGE C656948		~ 6.5 , O	4	23
4 .		$\mathbb{R}^{n/3}$	@8.75_ @	4	23
á Ca			NY ON	•	•

Dose levels were based on the results obtained in a range finding study in pregnant rabbits (KIIA 5.6.11 2004; M-122897-01-1, XE C656948 Range-finding study for developmental toxicity /02; in the rabbit by gavage DOC 56, SA 04051), where groups of 8 time-mated female NZW rabbits received 0, 50/150, 00 and 750 mg/kg/0 y from GD 6 to 28. The highest two dose levels caused marked maternal toxicity, sceeding a maximum tolerated dose, with several dams aborting and marked effects on body weight and food consumption parameters At 150 and 50 mg/kg/day, there were no maternal deaths and no dams aborted. At \$50 mg/kg/day, mean maternal body weight change was adversely affected throughout the treatment period. There was a body weight loss of 0.02 kg between GD 8 and 10 and a reduced weight gain for the subsequent intervals, in comparison with the controls. Overall, there was a mean maternal body weight gain of 0.15 kg between GD 6 and 29, compared to a mean body weight gain of 0.43 kg in the control group. Mean maternal food consumption was reduced throughout the treatment period by between 13 to 40%. At 50 mg/kg/day, overall mean maternal body weight gain between GPG and 29 was 0.35 kg compared to . 0.43 kg in the controls. Mean maternal food consumption was reduced by between 11 to 23% from GD 10 to 22.



#### 3. Test substance dosage formulations and analysis

The appropriate amount of test substance was periodically (six formulations for each dose fevel) suspended (w/v) in an aqueous solution of methylcellulose 400 (Fluka, Mulhouse, France) at 0.5% and stored at approximately 5°C ( $\pm$  3°C). Homogeneity of the suspensions was checked during the tast formulation for the lowest and highest concentrations. All concentrations were checked for all formulations. Homogeneity and concentration checks were between 97 and 106% of nominal values and were therefore inside the in-house target range of 90 to 110% of nominal concentration. Stability of the compound in suspension in the vehicle was determined before the start of the study at 0.0868 and 250 g/L in 0.5% aqueous methylcellulose in a previous study and was found to be stable for 53 days index similar conditions to those of the current study.

#### C. Methods

#### 1. Observations

Clinical signs

All rabbits were observed daily for clinical signs (except once daily on weekends and public holidays).

Mortality

All rabbits were observed twice dady for mortality (except once daily of weekends and public holidays).

Body weight

Body weights were measured on GD: 3, 6 3, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29.

Food consumption:

Full feeder weights were measured on GD: 3, 4, 5, 8, 10, 12, 16, 16, 48, 20, 22, 24, 26 and 28.

Empty feeder weights were measured on GD: 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 29.

#### 2. Cesarean sections

Animals killed in extremis by intravenous injection of Dolethan (Sanofi, Libourne, France) or found dead, were autopsied. A macroscopic examination of the viscoral organs was performed and the number of ribs counted. The number and type of implantations and corpora lutea were noted when present. In the case of no visible uterine implants, but with corpora lutea, interine horn(s) were immersed in a 20% solution of ammonum sublide according to the Salewski method (1964). The liver was taken and preserved in 10% neutral buffered formalin. Tissues and cateasses were then discarded.

On GD 29, surviving female were killed by intravenous injection of Dolethal® for examination of their uterine content. Each female was first subjected to macroscopic examination of the visceral organs and the number of ribs was recorded in the case of no visible uterine implants, but with corpora lutea, uterine horn(s) were immersed in a 20% solution of ammonium sulfide according to the Salewski method. The liver was weighed for pregnant females and was taken from all females and preserved in 10% neutral buffered formalin.

The reproductive tract was weighed (gravid define weight), dissected out and the following parameters recorded: number of corpora lutea, number of implantation sites, number of resorption sites (classified as early and fate), number of live and dead fetuses, individual weights of live fetuses. Dead fetuses: were defined a dead conceptuses showings distinct digits visible on fore and hind-paws. All the live fetuses were and subjected to an external examination. Resorption death was classified as early resorptions when macroscopic discrimination between fetal residues and placental material was not possible and late resorption when distinct macroscopic discrimination between fetal residues and placental material was possible. Tissues and carcasses of dams were then discarded.



#### 3. Fetal examinations

All data were recorded without knowledge of treatment group. After internal examination of the neck, the head of fetuses from approximately half of each litter was immersed in Bouin's fluid and the internal structures examined after fixation. The bodies of all fetuses were dissected for soft tissue abnormalities and sexed. Then the fetuses were fixed in absolute ethanol before staining. A modification of the Stappes and Schnell staining technique was used and a subsequent skeletal examination was performed.

#### 4. Statistics

Means and standard deviations for all maternal and little parameters were calculated for each group. Statistical analyses were performed on the following variables using TERATEST Phase 1. Version 12, TERATEST Phase 4, Version 4 or SAS programs (Version 8.2).

Maternal endpoints: body weight change calculated according to time periods, calculated corrected body weight change, average food consumption calculated according to time periods, liver weight.

Litter based and fetal endpoints: number of Corpora lutea, humber of implantation sites, number of resorption (early and late), pre- and post-implantation loss percentages, fetal sex, fetal death fatus, fetal body weight.

Homogeneity of variances between control and treated groups was evaluated using the Bartlett test. If not significant, means were compared using the analysis of variance (ANOVA) which was followed by the Dunnett test (2-sided) if ANOVA indicated significance.

If the Bartlett test was significant (for body weight change, corrected body weight change, number of corpora lutea, number of implantation sites or number of resortions parameters), group means were compared using the non parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance.

If the Bartlett test was significant, a tog transformation (for food consumption, fiver weight or fetal body weight) or an arcsine root transformation for pressor post-implantation loss percentages) was performed. If the Bartlett test on transformed data was not significant, means were compared using the analysis of variance (ANOVA), which was followed by the Dunnett test (2-sided) if ANOVA indicated significance. In the Bartlett test on transformed data was significant, group means were compared using the non parametric Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed by the Dunn test (2-sided) if the Kruskal-Wall's test, which was followed

If the Bartlett test was significant for fetal sex (male vs. female fetuses) and fetal death status (live vs. dead fetuses) endpoints, control group and each exposed group were compared using the Chi-square test (for fetal sex parameter) or the Esher Exact test (2-sided) (for fetal death status parameter). Death status was analyzed both using the fetus as the statistical onit and using the litter as the statistical unit.

If one or more group variance(s) equale 0, means were compared using non parametric procedures.

## II. Results and discussion

#### A. Mortality

There was one mortality on GP 21 at 75 mg/kg/day and one female was sacrificed due to accidental trauma on GD 15 in the control group. Both deaths were attributable to a gavage error. The macroscopic observation showed hemorthaging in the gung of both females together with hemorrhaging and foam in the traches of one female and a traches filled with fluid for the other female. In addition, one female was killed for humane reason on GD 23 at 25 mg/kg/day, following a slight loss in body weight and a reduction in food consumption between GD 20 and 22. Clinical signs in this female consisted of a limited use of the right hindlimb, in association with massive subcutaneous hemorrhaging and a distal epiphysal femoral disjunction. The condition of this animal was considered to be due to accidental trauma.



#### **B.** Abortions

There were no abortions throughout the study.

#### C. Clinical signs

There were no treatment-related clinical signs in any group.

#### D. Pregnancy rate

There was no treatment-related effect on pregnancy rate. The pregnancy rate was 96% invall gro

#### E. Bodyweight

At 75 mg/kg/day, mean body weight gain was reduced between GD 14 and 18 (0.02 kg  $\ll$  0.00 kg for controls, p≤0.01) and between GD 18 and 22 (0.02 kg vs. 0.07 kg for controls, b≤0.01), in comparison to controls. Thereafter, mean body weight gain was similar to the controls, resulting in an overall bedy weight gain between GD 6 and 29 of 0.20 kg compared to 0.31 kg for the controls, though the effect was not statistically significant.

Mean body weight change at 25 and 10 mg/kg/day was comparable with the controls.

Maternal corrected body weight change (i.e., maternal body weight change independent of the interine weight) was more pronounced at 75 mg/kg/ca (-0.25 kg) compared with the controls (0.17 kg), though the effect was not statistically significant.

Maternal corrected body weight change at 25 and 10 mg/kg/day was comparable with the controls.

#### F. Food consumption

At 75 mg/kg/day, mean maternal food consumption was reduced by between 22 to 34% ( $p\leq0.01$ ) for all intervals between GD 14 to 26 in comparison to condrols.

Mean maternal food consumption at 25 and 10 mg/kg/day was similar to the controls.

#### G. Litter data

At 75 mg/kg/day, mean fetal body weight for the combined sexes and for the individual sexes were 11% lower than the controls ( $p \le 0.01$  for combined sexes and males  $p \le 0.05$  for females).

There was no effect on mean fetal body weight at 25 and 10 mg/kg/day.

Other litter parameters, including number of live setuses, early or late resorptions, fetal death status and percentage of male fetuses were unaffected by freatment at all dose levels tested.

### H. Fetal necropsy findings

There were two fetuses noted with malformations, but as they occurred as isolated incidences at 10 mg/kg/day and in the control group, they were considered to be fortuitous.

The number of 'runt fetuses (BW<28.0g)', classed as a variation, was increased at 75 mg/kg/day, where the mean percentage of fetuses classified as runts was 12.5% and the percentage of litters affected was 47.6%, compared with 3.6% and 23.8%, respectively, in the control group. At 25 and 10 mg/kg/day, the mean percentage of 'runt fetuses' and percentage of litter affected were very similar to the control values.

### 2. Fetal evaluation: visceral observations

At 75 mg/kg/day, there were two fetuses from separate litters with the malformation 'gall bladder absent' compared to no instance in the current control group. However considering the low incidence of this finding and that it has already been observed at similar incidence in previous studies conducted in-house and in addition, one case of 'gall bladder absent' was observed in a dam (PR3F0651) at the mid-dose (25 mg/kg/day), this finding was considered not to be treatment-related. All other malformations occurred as isolated findings or at a similar frequency across the dose groups including the controls and were considered to have occurred by chance.



There was no treatment-related effect on variations at the visceral examination.

#### 3. Fetal evaluation: skeletal observations

The few malformations which were observed at the skeletal examination either occurred as isolated incidences or at a higher incidence in the controls and were therefore considered to be chance indings.

There was no treatment-related effect on variations at the skeletal examination

#### I. Deficiencies

No deficiencies

#### III. Conclusions

In conclusion, a dose level of 75 mg/kg/day of AE €656948 administered to the pregnant female New Zealand White rabbit caused maternal toxicity, as evidenced by reduced mean body weight gains and food consumption. Fetal toxicity at this dose level consisted of a reduced body weight in both sec.

A dose level of 25 mg/kg/day of AE C656948 was considered to be a OOEL both in the data and in terms of fetal development in the New Zealand White rabbit

#### Assessment and conclusion by applicant

The study has been carried out in line with the OEO 414 of quirements in force at the time it was conducted.

At the a dose level of 75 mg/kg/dax of AEC 656948 administered to the pregnant female New Zealand White rabbit caused maternal toxicity as evidenced by reduced mean body weight gains and food consumption. Fetal toxicity at this dose level considered of a reduced body weight in both sexes.

A dose level of 25 mg/kg/day of AE C656948 was considered to be NOEL both in the dam and in terms of fetal development in the New Zealand White rabbig

## CA 5.7

## Neurotoxicity studies

All studies presented in this section were conducted between 2006 and 2008 and complied with the EU, OECD, US EPA and JMAFF testing guidelines and Good Laboratory Practice (GLP) standards.

In an acute neurotoxicity study, technical grade fluopyram (AE C656948) was administered by gavage in a single dose to nonfasted young-adult Wistarrats Four dose groups (12 rats/sex/dose level) were administered the test substance conominal doses of 0 ovehicle), 125, 500 or 2000 mg/kg for both sexes. Since there were compound-related effects on measures of motor and locomotor activity at 125 mg/kg in females, a follow no study was conducted under the same conditions at nominal doses of 0 (vehicle), 25, 50 or 100 mg/kg to establish an overall No Observed-Adverse-Effect Level (NOAEL) in females. Compound-related effects following a single oral dose of the test substance in the initial study were limited to mid- and high-dose males and females and low-dose females. Effects in males and/or females consisted of docreased motor and locomotor activity on the day of treatment, urine stain, decreased body temperature and a lower of an under the gross or microscopic lesions at the high dose of 2000 mg/kg. A follow-up study established a NOAEL for measures of motor and locomotor activity in females, with no compound-related effects at the two lowest dose levels. Slight decreases in measures of notor and locomotor activity were evident in females treated at 100 mg/kg. These results establish a NOAEL of 125 mg/kg and 50 mg/kg for males and females, respectively.



In a 90-day neurotoxicity study, no evidence of neurotoxicity was observed at any treatment level. Treatment-related findings of general toxicty at the high dose consisted of decreased body weight, total body weight gain and food consumption in males and females, increased cholesterol and triglycerde levels in males and/or females and decreased terminal body weight in females. Also, liver and kidney 

Doses tested: ppm mg/kg bw/d m	NOAEL ppm ng/kg bw/d 25 in males nd < 125 in females 50, 50, 50, 50, 50, 50, 50, 50,	LOAEL pfm mg/kg bw/d 500 in mores and <125 in females 7 100 7 1000 7 10	Main findings		Reference         M-289073         E         M-289073-01-2         M-289073-01-2         M-299110-01-1
Doses tested: ppm mg/kg bw/d m	ppm 1g/kg bw/d	ppm mg/kg bw/d		comotor rsigns and 1 re 2 re	A A co
mg/kg bw/d m	ng/kg bw/d	mg/kg bw/d	↓ motor and loc activity elinica body temperation ↓ motor and loc activity of activity of activity of activity of activity of activity of activity of activity of activity of activity of activity of activity of activity of	comotor r signs and ↓ re comotor re comotor re comotor re comotor re comotor re comotor re comotor re comotor re comotor re comotor re comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comotor comot	<u>M-289073-01-2</u> <u>M-289073-01-2</u> <u>M-289073-01-2</u> <u>M-299110-01-1</u>
		500 in motes 500 in motes 50	↓ motor and loc activity elinica body temperation ↓ thotor and loc activity of activity of activity of activity of activity of activity of activity of activity of activity of activity of activity of activity of activity of activity of activity o	comotor A signs and ↓ ife anototor anototor y endpoints anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anotototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anototor anotototor anotototor anotototor anototototor anototototototototototototototototototot	M-299073-01-2 M-299073-01-2 M-299110-01-1
Acute neurotoxicity in the rat (initial study) 0, 125, 500 and 2000 mg/kg bw Acute neurotoxicity in the rat (follow-up study in females) 0, 25, 50 and 100 mg/kg bw 90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 14.2 / 0, 8.05, 41.2, 197.1, 0 mg/kg bw/ eay in M/F	50, 50, 50, 50, 50, 50, 50, 50,	Adv in finders and <125 in females 7 100~ 2 10~ 2 10	Androi and loc activity elinica body temperation ↓ thotor and loc activity None based off neurotoxic tog	y endpoints of	<u>M-299073 91-2</u> E <u>M-289073-01-2</u>
Acute neurotoxicity in the rat (follow-up study in females) 0, 25, 50 and 100 mg/kg bw 90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 194.2 / 0, 8.05, 41.2, 197.1, 0 mg/kg bw/ day in M/F	females 50, 0 50, 0, 0 50, 0 5	and (22) in females 7 100~7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7	None based off neurotoxic tilog	y endpoints	<u>M-299073 91-2</u> E <u>M-289073-01-2</u>
0, 123, 300 and 2000 mg/kg bw Acute neurotoxicity in the rat (follow-up study in females) 0, 25, 50 and 100 mg/kg bw 90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 44.2 / 0, 8.05, 41.2, 197.1, 6 mg/kg bw/ eay in M/F	50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50,	~ 100~ ~ 100~ ~ 2 ~ 2 ~ 2 ~ 2 ~ 2 ~ 2 ~ 2 ~ 2 ~ 2 ~	None based off neurotoxic tog	y endpoints	M-299110-01-1
Acute neurotoxicity in the rat (follow-up study in females) 0, 25, 50 and 100 mg/kg bw 90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 64.2 / 0, 8.05, 41.2, 197.1, 0 mg/kg bw/ day in M/F	50,0 Q Q y y 4/197 (4/197 (4/197 (4/197 (4/197) (4/197) (4/197) (4/197) (4/197) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1) (4/1)	2 2 2 2 2 2 2 2 2 2 2 2 2 2	Vonecoased off neurotoxic@og	y endpoints	<u>M-299110-01-1</u>
Acute neurotoxicity in the rat (follow-up study in females) 0, 25, 50 and 100 mg/kg bw 90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 64.2 / mg/kg bw/ day in M/F	50, C Q y y 4/4/197 (A) (A) (A) (A) (A) (A) (A) (A)		Vonecoased off neurotoxic@g	y endpoints	E <u>M-289073-01-2</u> M-299110-01-1
the rat (follow-up study in females) 0, 25, 50 and 100 mg/kg bw 90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 44.2 / 0, 8.05, 41.2, 197.1, 0 mg/kg bw/ eay in M/F			None based off neurotoxic log	y endpoints	<u>M-289073-01-2</u> M-299110-01-1
(follow-up study in females) 0, 25, 50 and 100 mg/kg bw 90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 44.2 / 0, 8.05, 41.2, 197.1, 0 mg/kg bw/ day in M/F			Nonetbased off neutotoxicalog	y endpoints	<u>M-289073-01-2</u>
females) 0, 25, 50 and 100 mg/kg <u>bw</u> 90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 44.2 / 0, 8.05, 41.2, 197.1, 0 mg/kg bw/ day in M/F			Nonetbased off- neurotoxictlog	y endpoints	<u>M-299110-01-1</u>
0, 25, 50 and 100 mg/kg <u>bw</u> 90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 44.2 / 0, 8.05, 41.2, 197.1, 0 mg/kg bw/ day in M/F			Nonerbased off neurotoxictog	y endpoints	<u>M-299110-01-1</u>
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90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 44.2 / 0, 8.05, 41.2, 197.1, 0 mg/kg bw/ day in M/F			None based off neurotoxic to g	y endpoints of	<u>M-299110-01-1</u>
the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 44.2 / 0, 8.05, 41.2, 197.1, 0 mg/kg bw/ day in M/F				y endpoints of	<u>M-299110-01-1</u>
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Data Point:	KCA 5.7.1/01
Report Author:	
Report Year:	2007
Report Title:	An acute Oral Neurotoxicity Screening Study with Technical Grade AE 656945
	in Wistar Rats
Report No:	
Document No:	<u>M-289073-01-2</u>
Guideline(s) followed in	OECD 424 (1997); EPA Health Affects Test Guideline (OPPTS \$70.6200; 1998); M A F E, in Japan polifications 2 Nousan N°8047 (2000) guidelines
study:	
Deviations from current	Current guideline: OECD 424, 1997
test guideline:	No deviations
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially	Yes, conducted under GLP/Officially becognized testing facilities
recognized testing	
facilities:	
Acceptability/Reliability:	Yes X X X A O X

#### CA 5.7.1 Neurotoxicity studies in rodents

#### **Executive Summary**

Technical grade AE C656948 was administered by gavage in a single dose to non-asted young-adult Wistar rats. Four dose groups (12 rats/sex/dose level) were administered the test substance at nominal doses of 0 (vehicle), 125, 500 2000 mg/kg for both sexes. Since there were compound related effects on measures of motor and locomotor activity at 125 mg/kg in females, a followoup study was conducted under the same conditions at nominal doses of (vehicle), 25, 50 or 100 mg/kg to establish an overall No-Observed-Adverse-Effect Level (NOAEL) in females. The test substance was administered in 2% (v/v) Cremophor EL in deion zed water at a dosing volume of 10 mL/kg. Dose concentrations, as well as the homogeneity and stability of AE C656948 in the dosing suspensions, were confirmed. All animals (12/sex/dose level) were used for neurobehavioral evaluation, with six/sex/dose level from the initial study used for pheropathology. For the initial study, observations for moribundity and mortality were performed at bast once dail (unless otherwise noted) detailed clinical observations for each animal were performed daily throughout the study and body weight measurements (measured weekly as a component of the functional observational battery) were performed. Also, a functional observational battery (POB) and automated measurements of acovity (figure-eight maze) were conducted during the week prior to treatment and on days 0 (day of treatment at the time of peak effect), 7 and 14. Lastly, all animals from the initial study were subjected to a gross necropsy. Selected animals (six/sex/dose level) were perfused, the brain was weighe Pand skeletal muscles peripheral nerves, eyes (with optic nerves) and tissues from the central Gervons system were examined microscopically for lesions. Observations and measurements included in the follow-up study consisted of observations for moribundity and mortality performed at least once daily (unless otherwise noted), detailed clinical observations for each animal performed daily throughout the study and body weight measurements (measured weekly as a component of the functional observational battery (FOB) and automated measurements of activity (figure ofght maze) were conducted during the week prior to treatment and or day 0 (day of treatment, at the time of peak effect). Animals from the follow-up study were sacrificed once all signs of toxicity were no longer evident. It should be noted that animals from the follow-up study were reated the same as animals from the initial study for consistency. The NOAEL for clinical observations FOB and body weight had already been established in the initial study.

Based on analytical results, the actual doses of AE C656948 for the initial study were 0, 126, 498 and 1840 mg/kg for makes and remales and for the follow-up study actual doses were 0, 25, 51, 100 mg/kg for remales.

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Effects attributed to exposure to AE C656948 were as follows:

#### **Initial Study:**

**2000 mg/kg**. Measures of motor and locomotor activity were statistically significantly reduced in males (71% and 73%, respectively) and females (72% and 77%, respectively) on day 0.5 emales had decreased body temperature and a lower incidence in the number of animals that vocalized during removal from the home cage. Also urine stain was evident in males. All signs of toxicity resolved by next test occasion, which was seven days after treatment.

*500 mg/kg*. Measures of motor and locomotor activity were statistically significantly reduced in motes (51% and 49%, respectively) and females (53% and 58%, respectively) on day 0 Also, temales had o decreased body temperature. All signs of toxicity resolved by day 74

125 mg/kg. Measures of motor and locomotor activity were statistically renificantly reduced in females (26% and 31%, respectively) on day 0. All signs of toxicity resolved by day 7.

#### Follow-up Study (Females only):

100 mg/kg. Measures of motor and locomotor activity were non-statistically significantly rediced (18%, each) on day 0.

50 mg/kg. There were no compound Bated effects

25 mg/kg. There were no compound-related effects.

**Conclusions:** In the initial study, compound related effects following a single oral dose of the test substance were limited to mid and high-dose males and females and low dose females. Effects in males and/or females consisted of decreased motor and locomotor activity on the day of treatment, with recovery by the next test occasion, urine stain which resolved within five days after treatment, decreased body temperature and a lower incidence in the number of animals that vocalized during removal from the home cage. There were no compound related gross of microscopic esions at the high dose of 2000 mg/kg. A follow-up study established a NOAEL for measures of motor and locomotor activity were evident in females treated at 100 mg/kg. Based on these results, doses of 125 mg/kg and 50 mg/kg are the overall NOAEL for males and females, respectively.

I. Materials and method	S S JS J
A. Materials 5 5	
\$' 4' \$	
A. Materials	AE C656948 Light beige powder Mix batch 985280002 94,7% (May, 2005, certified through May, 2007)
Description	Quight beige mowder
Lot / Batch #:	Mix batch 08528 0002
Purity	94,7% (May, 2005, certified through May, 2007)
	$0.500000553-4^{\circ}$
Stability of test compound:	Stable in the dosing vehicle at the room temperature for 8 days
2. Wehicle and / or positive	2% w/vo remonhor EL in deionized water
control:	
3. Test animals: 🔬 🔪 🖉	Stable in the dosing vehicle at the room temperature for 8 days 2% (w/y) Cremophor EL in deionized water
Species Species	rat Ø
control: 3. Test animals: Species Strain Age: Noight do Train	Wistar HAN CRL: WI (HAN)
Agen a j	At least 9 weeks
	Initial Study: 252.2 to 315.3 g for the males; 167.0 – 206.9 g for
Weight at dosing:	the females
	Follow-Up Study: 166.6 – 214.7 g for females
Source:	
	Initial Study – 8 days
Acclimation period:	Follow-Up Study – 7 days
	ronow-op study – / days



Diet:	Purina Mills Rodent Lab Chow 5002 in meal form provided for <i>ad libitum</i> consumption during the acclimation period and	
	throughout the study except during neurobehavioral testing.	
Water:	Tap water, ad libitum except during neurobehavioral testing	
Housing:	Animals were caged individually in suspended stainless seel wire-mesh cages.	
Environmental conditions:		
Temperature:	$22 \pm 2^{\circ}C$ $50 \pm 70\%$ Minimum daily average of 10.13 changes per hour 12 hours dark / 12 hours light	
Humidity:	$50 \pm 70\%$ $\swarrow$ $\checkmark$ $\checkmark$ $\checkmark$ $\checkmark$	
Air changes:	Minimum daily average of 10.13 changes per bour 2 2	
Photoperiod:	12 hours dark / $\frac{12}{2}$ hours light $\sqrt{2}$	
B. Study design	Minimum daily average of 10.13 changes per bour $3^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$ $4^{-1}$	
In life dates: 25 April 2	$2006 - 18$ August 2006 $3^{\circ}$ $2^{\circ}$ $3^{\circ}$ $3^{\circ}$ $3^{\circ}$ $3^{\circ}$	
Animal assignment and treatm		
andomization procedures utilized s	software from INSTEM Computer Systems. Following acclimation,	
a animals for the initial and follow	w undertudias wara-waigher and trace with had waights the wara	

1. In life dates:

2. Animal assignment and treatment

Randomization procedures utilized software from INSTEM Computer Systems. Following acclimation, the animals for the initial and follow-up studies were weighed and those with body weights that were more or less than 20% of the mean weight for each sex were rejected. The remaining animals were randomly assigned to a control group or one of six dose levels (i.e. 25, 50, 100, 325, 500 or 2000 mg/kg) in order that, for each sex, groups had comparable body weights when the treatment was administered. This was done to facilitate comparisons following treatment. Extra animals that remained following randomization and rejected animals were sacrificed without necropsy. However, alternative uses for the animals were explored prior to their sacrifie. Appenals were randomly assigned to the test groups.

Each rat was identified by cage card and tak mark with a number that did not reveal the animal's treatment group. In addition, animals were assigned an identification number that specified the rat's sex, treatment group, and cage number and identified it with the study.  $\bigcirc$ 

The oral route of posurowas employed in accordance with the test guideline. The rat was selected due to its general acceptance and suitability as a rodent species for to cological testing of this type, as well as the availability of historical database on the Wistar strain. The gudy design for the initial study required a total of 96 rats (48 makes and 48 females) and an additional 48 females were utilized for the follow-up study to establish a NOAEC for motor and locomotor activity. These animals were at least nine weeks of age when the reatment was administered. An additional 24 animals (8 males and 8 females, each for the initial study and & females for the follow-up study) were tested (FOB and motor activity) during the pre-treatment week and reserved for use on case they were needed to replace animals (e.g. if mis-dosed) that were assigned to the study and the second s

For the initial study, four dose groups (by rats/sex/dogo level) were administered the test substance at nominal doses of 0 (vehicle), 125, 500 or 2000 mg/kg (actual doses, 126, 498 and 1840 mg/kg) for both sexes. For the follow-up study in females only, four dose groups (12 rats/dose level) were administered the test substance at sominal doses of 0 (pehicle), 25, 50 and 100 mg/kg (actual doses, 25, 51 and 100 mg/kg). The test substance was administered by gavage as a single dose in 2% Cremophor EL in deionized water at a dosing volume of 10 mk/kg. Animals were not fasted overnight before treatment.

For the initial study, all animals were observed daily for 14 days and weighed weekly as a component of the FOPS Twelve rate sex/dose level were used for neurobehavioral testing, with half used for micropathology and were sacrificed 14 or 15 days after exposure. For the follow-up study, all 12 female Horse level were used in the same manner as animals from the initial study, except they were sacrified two or three days after treatment and were not subjected to a gross necropsy or micropathology. Dosing and neurobehavioral testing was staggered over two days for each sex to accomposite the schedule. The following observations and measurements were also included in the study: clinical observations, mortality and body weight. In addition, brain weight (6 rats/sex/dose), and gross necropsy were performed in the initial study. Micropathology was performed on selected tissues



collected from 6 rats/sex/dose group from the control and high-dose groups in the initial study. The table below summarizes the study design.

Table 5.7.1-1 Stu	idy design an	d animai	assignmei	nt		~			O ^Y
Experimental	Initial S	tudy - Do	se Group	(mg/kg	Follow-Up	Study I	<b>Jose Grou</b>	p@mg/kg	]
Parameter		bv	v)		t	w)Fema	les Only	× , Ç*	
	Control	125	500	2000	Control	<u> </u>	50	<b>A000</b>	Ô
Total number of	12	12	12	10%	11-12 ²	ر» 12	×12 /	× 12 ~	ŕ
animals/sex/group	12	12	12		11-12	12		³ 12	, O
Behavioral Testing				Å.	Ő	4			Ô
(FOB, Motor	12/sex	12/sex	12/sex (	2/sex	1 K 1 2 ²	12_0	12%		ĺ∕ _
Activity) ^a				*			Å.		1
Neuropathology ^b	6/sex	0/sex	0/sex	6/sex		×			]

Table 5.7.1-1	Study design and	l animal assignment
---------------	------------------	---------------------

a FOB and motor activity were assessed prior to dosing (both studies) and again diving day 0, 7 and 14 (Initial study) or during day 0 (Follow-up study).

b Tissues from the mid- and low-dose groups were not examined, as no related neuropathologo was noted at they highest dose level.

2 One control female was found dead after dosing

The rationale for dose selection was based on the results of an acute oral toxicity study in young-adult female Wistar rats. In that study, six tasted female Wistar rats were administered an acute oral (gavage) dose of 2000 mg/kg as an aqueous suspension in 2% Gemophor EL in tap vater, at a dosing volume of 10 mL/kg. Animals were observed for mortality and clinical signs of rat least 14 days after treatment. The test substance produced no mortality and no clinical signs of foxicit? The only finding evident was an increase in water intake at the limit dose. These results support the use of a limit dose (2000 mg/kg) in the neurotoxicity study, but provided no information to establish the time of peak effect.

Thus, preliminary results from a study using radio Abeled AE C656948 were examined to estimate the time of peak effect. In that study, adult male and remale Wistar rats (4 sex/dose) received a single oral (gavage) dose of 5 mg/kg AE C656948 in 0.5% aqueous tragacanth, at a dosing volume of 10 mL/kg. The tmax in plasma occurred 40-60 minutes after treatment in males and 2-3 hours after treatment in females following administration of 5 mg/kg.

Based on these collective results, the closes offected for the initial study were 0, 125, 500 and 2000 mg/kg for both sexes. The 2000 mg/kg dose was selected as a limit dose that may produce slight evidence of toxicity, the middle dose was selected to produce minimal or no effect and the low dose was expected to be an overall NOAEL in both sexes. Based on the initial findings, it was necessary to conduct a follow-up study to establish a NOAEL for measures of activity in females. In that study, doses selected were 0, 25, 50 and 100 mg/kg. The too mg/kg dose was selected to either reproduce the findings in the initial study of possibly establish an overall NOAEL at a dose level slightly lower than the lowest dose tested in the initial study (i.e., 105 mg/kg). The remaining treated doses were selected to establish an overall NOAEL if 100 mg/kg as proven to produce treatment-related effects. Based on the estimated time of peak blood concentrations at these dose ranges, the FOB began approximately one hour (minimum) following dose administration, with the automated test of activity concluding at about 4 hours after treatment

## 3. Dose preparation and analysis

Doses were prepared by Dispending the test substance in 2% (v/v) Cremophor EL in deionized water. The concentration of AE C656948 in the vehicle was measured using high- performance liquid chromatographic/ultra violet (HPLC/UV) analysis. The homogeneity and stability of AE C656948 in the vehicle were established using samples at nominal concentrations (2.5, 5 and 200 mg/mL) that either bracketed or included the range of concentrations used in the present study (nominal 2.5 and 200 mg/mL). Homogeneity was accepted if the percent relative standard deviation (%RSD) was <5%. Each dosing suspension was also analyzed to measure the concentration of AE C656948.



Homogeneity Analysis: Homogeneity of the test substance in the vehicle was accepted for the range of concentrations used here, as the 2.5, 5 and 200 mg/mL concentrations had percent relative standard deviations (%RSD) of 1.2%, 1.9% and 1.7%, respectively.

Stability Analysis: The stability (at room temperature conditions) of AE C656948 in the vehicle was established, with no appreciable decrease in concentration with eight days at room temperature storage for nominal concentrations of 2.5, 5 or 200 mg/mL, respectively (equivalent to doses of 25, 50 or 2000 mg/kg, respectively).

Concentration Analysis: For the initial study, doses of 0, 125, 500 and 2000 mg/kg for males and females ranged from 92% to 101% of the nominal concentrations. Based on these sesults, the analytically-confirmed doses for males and females were 0, 126, 498 and 1840 mg/kg. For the follows up study, doses of 0, 25, 50 and 100 mg/kg for females ranged from 100% to 102% of the from half concentrations. Based on these results, the analytically-confirmed doses for female on the following study were 0, 25, 51 and 100 mg/kg.

#### 4. Statistics

4. Statistics Statistical evaluations were generally performed using software from either INSTEM Computer Systems or SAS. The level of significance was set at p≥0.05 Group means with equal variances were analyzed further using an Analysis of Variance (ANOVA). followed by a Dunnet's test a significant F-value was determined in the ANOVA. For the FOB continuous data were first analyzed using a Repeated-Measures ANOVA, followed by a one-way ANOVA if there was a significant interaction between dose group and test day. For days on which there was a significant treatment effect, Dunnett's test was applied to determine which groups if any, were significantly different from the control group. Categorical data collected in the FOB was analyzed in a similar mannet jusing General Linear Modeling and Categorical Modeling (CATMOD) Procedures, with post-hoc comparisons using Dunnett's test and ŕ an Analysis of Contrasts, respectively, Ő C

Motor and locomotor activity (total session activity and activity for each 10-minute interval) was analyzed using ANOVA procedures. Session activity data were analyzed asing a Repeated-Measures ANOVA, followed by a one-way ANOVA if there was a significant interaction with test occasion. For days on which there is a significant meatment effect, Dunnett's test was used to determine which, if any, groups were significantly different from the control group. Interval data were subjected to a two-way Repeated Measures ANOVA, using both test interval and test occasion as repeated measures, followed by a Repeated Measures ANOVA to determine or which days there was a significant treatment by interval interaction. For those days, the data for each interval were subjected to analysis using a oneway ANOVA to determine at which intervals there was significant treatment effect. For those intervals, Dunnett's test was used to determine which groups, if any were significantly different from the control group.

Continuous pathology date (e.g. Orain weight) was initially evaluated using Bartlett's test to analyze for homogeneity of variance among groups. Groups with homogeneous variances were analyzed further using an ANOVA followed by Dunnet's test for pair-wise comparisons. In the event of nonhomogeneous variances, continuous date were analyzed using the nonparametric Kruskal-Wallis test followed by a Mann-Whitney test for pair wise comparisons. Micropathology frequency data were evaluated using a Chi-Square procedure, followed by a one-tailed Fischer's Exact Test in cases of significant variation by the Chi-Square analysis. The level of significance was set at p≤0.05, with the exception of Bartlett's test, which was tested at p ≤ 0.001

## C. Methods

## 1. Mortality and Chinical Observations:

Case-side observations were performed at least once daily (once daily on holidays and weekends) for mortality or clinical signs of moribundity. Detailed physical examinations for clinical signs of toxicity were carried out and recorded once each day for 14 days following dosing (initial study) or for 2 or 3 days following dosing (follow-up study).



#### 2. Body weight:

Animals were weighed weekly as a component of the FOB. Additionally, all animals were weighed prior to dosing and study animals from the initial study were weighed on the day of sacrifice for terminal body weight measurement. Animals from the follow-up study were not weighed after day 0 since a NOAEL for body weight had been established in the initial study.

#### **3. Food Consumption:**

Food consumption was not measured in this study.

#### 4. Neurobehavioral Assessment

For the initial study, all animals that were assigned to the study were tested using the FOB and motor activity on four occasions - one week prior to treatment, approximately 1 hour (minimum) after administration of the dose, and again seven and a days following treatment. For the follow-up study, females that were assigned to the study were tested using the SOB and motor activity on two occasions - one week prior to treatment and approximately 1 your (minimum) after administration of the dose. The order of testing and assignment of animals to mazes were done in a semi-random manner such that groups were balanced across test times and test devices, and no animal would be tested more than once in the same maze. On the day of FOB and motor activity testing, the appropriate animals were placed in the sequence that was established for resting on that day. The dose group identification was concealed at that time to ensure that testing would be performed without knowledge of the group assignment. Animals were then transferred to the room where the esting book place and were allowed to acclimate with minimal disturbance for approximately 30 minutes prior to testing. The test room was a standard animal room that was maintained on the same light dark cycle and settings for temperature and relative humidity as the animal room, with tests performed during the light phase. Sets of eight animals (maximum) were evaluated individually using the EOB and then, approximately 30 minutes after the last animal in the set had finished being tested in the FOR, all eight rats were placed individually into the mazes to measure activity. It should be noted that any mals from the Pollow ap study were treated the same as animals from the mitial study for consistency of he NGAEL for clinical observations, FOB and body weight had already been established in the initial study. 2

An additional 4 animals (eight males and eight females for the initial study and eight females for the follow-up study) were tested (FOB and motor activity) during the pretreatment week and reserved for use in case they were needed to replace animals (e.g., if miss dosed) that were assigned to the study.

# a. Functional Observational Battery (FOB):

The FOB closely followed the battery of tests described by Moser (J. Am. Coll. Toxicol., 1989, 8, pp. 85-93) with each animal tested individually. Scoring criteria and explicitly-defined scales were used to rank the severity of observations that do not readily lend themselves to quantitation. The procedures used to determine landing boot spary and grip strength are based on established methods. The technicians who performed the FOB were 'blind' with respect to the animal's group assignment. Observations and measurements (e.g., grip strength and foot splay) for all animals were performed by the same person throughout the study. Inter observer reliability has been established in order to allow a second person to perform either the observations or measurements, ensuring the consistency of the results of each technician. Studies have been conducted with acrylamide, carbaryl and untreated rats to establish the sensitivity, reliability, and validity of these test procedures, the adequacy of training of technical personnel and to serve as historical control.

When applicable, observations were scored on intensity as follows: 1) slight (barely perceptible or infrequent) or moderate to severe. Data were collected while the rats were in their home cage, during handling, and in an open field for 2 minutes (in the center of a flat surface with a perimeter barrier, such as a cart) in addition, reflex and physiologic observations and measurements were made while the animals were sitting on the cart surface following open field observations.



Home cage observations included: posture, piloerection, involuntary motor movements (such as repetitive "chewing" movements of mouth and jaw, tremors, and convulsions), gait abnormalities, vocalizations, decreased activity, repetitive head bobbing, and increased reactivity.

Observations during handling included: ease of removal from cage, reaction to being handled muscle tone, palpebral closure, lacrimation, salivation, nasal discharge, stains (lacrimation, nasal, periartal, uring, oral), alopecia, emaciation, bite marks, exophthalmia, broken teeth/malocclusion, missing toe nations), dehydration, and temperature upon touching (cool-to-touch).

Open field (2 min.) observations included: number of rears, piloerection, respiratory abnormalities, posture, involuntary motor movements, stereotypy (excessive or repetitive behaviour), bizarre behaviour, gait abnormalities, vocalizations, arousal level, and amount of excretion.

Reflex and physiologic observations/measurements included: approach response, touch response, auditory response, tail pinch, pupil size at normal opting, pupil response, righting reflex, grop strength, body weight, body temperature, and landing foot splay. The functional observation parameters evaluated in this study are summarized below. Functional observation parameters (checked (X) parameters examined)

Functional observation p	parameters (checked (X) paramete	rexamined) is in the
HOME CAGE	ANDLING	OPEN FIELD O OBSERVATEONS (?)
UDSERVATIONS	ODSERVATIONS y	ODSERVATIONS
X Posture*		X Rearing+
X Piloerection	X Muscle tone* 0 4	X Piloerection*
X Involuntary motor movements	X Palozbral closure*	X Respiratory abnormalities
e.g.: X Repetitive chewing	Val accimentation *	V Destant
X Repetitive chewing	X Lacrimation C (	X Posture
X Convulsion		X Involuntary motor movements
X Tremore	X NasaPdischarge 🚿	X  Repetitive chewing
	X Red/crusty/deposits (stains)*	
X Gate abnormalities 🖉 🗸	X Red/crusty/deposits (stains)* X Fur appearance, X Emaciation	X Tremors*
X Vocalizations	X Emaciation	X Stereotypic behavior*
X Decreased active X	X Bije marks	X Bizarre behavior*
X Repetitive head bobbing	X Eye prominence*	X Abnormal movements *
X Increased reactivity	Broken teeth maloc Ausion	X Gait abnormalities* / Gait score*
	X Mussing Fee Nails)	X Vocalizations
SENSORY 6	X Dehydration	X Arousal/ general activity level*
OBSERVATONS 7		
X Approach response +	X Cool-to-Touch	X Urination / defecation*
X Touch response+		
X Auditory response	PHÝSIOLOGICAL	NEUROMUSCULAR
X Pain response*	SERVATIONS	OBSERVATIONS
X Pupil cosponse C	X Body weight*	X Forelimb grip strength*
X Pupil size	X Body temperature+	X Hindlimb grip strength*
X Air righting reflex+		X Landing foot splay*
*Required parameters; +Recommende	ed parameters	
Ű		



#### b. Motor and Locomotor Activity:

Motor and locomotor activities were measured approximately 30 minutes after the last animal in the set (8 rats maximum) had finished the FOB. All rats in each set were placed individually into figure eight mazes and activity was measured for a total of 60 minutes. The figure-eight maze was selected as an established and widely-used automated activity-measuring device that can be used to detect both increases and decreases in activity. Each maze consisted of a series of inter-connected alleys, converging on a central arena and was covered by transparent plastic. Eight infrared entiter / detector pairs (three in each of the figure-eight alleys and one in each of the blind alleys) measured activity; each time a beam was interrupted, an activity count was registered. The floor of each maze rested above absorbent paper which was changed at the end of each day. A Columbus Instruments (Columbus, OE) Universal Maze Monitoring System and a personal computer were used for automated data collection. Broad spectrum background noise (approximately 74 dB(A)) was provided throughout the fest to minimize acoustical variations during testing. The uniformaty of light intensity (100  $\pm$ 70 lux) over each of the mazes was verified daily.

Motor and locomotor activities were examined during each of the six ten-minute intervals. Motor activity was measured as the number of beam interruptions that occurred during the test session. Locomotor activity was measured by driminating consecutive counts for a given beam. Thus, for locomotor activity, only one interruption of a given beam was counted until the rad relocated in the maze and interrupted one of the other beams. Habituation was evaluated as a decrement in activity during the test session.

#### 5. Sacrifice and Pathology 🔍

For the initial study, all animals placed of study were subjected to a complete gross necropsy. It was not necessary to subject animals from the follow up study to a complete gross necropsy since a NOAEL was established for gross pathology endpoints in the mitial soldy. The necropsy in folved an examination of all organs, body cavities, get surfaces, external orifices and surfaces. For the initial study on day 14, a minimum of six males and six females of each dose level were selected for perfusion and collection of tissues, with replacement as necessary, if the perfusion was considered inacequate. These animals were deeply anesthetized using an intrapertroneal lose (50 mg/kg) of pentobarbital and then perfused via the left ventricle with a sodium intrite (in phosphate buffer) thush followed by Universal fixative (1% (w/v) glutaraldehode and 4% (w/v) EM grade formate hyde in phosphate buffer. The entire brain and spinal cord, both eyes (with optic nerves) and selected (bitateral peripheral nerves (sciatic, tibial and sural), the gasserian ganglion gastrochemius muscle, both forelimbs, gross lesions in neural tissues or skeletal muscle and physical dentifier were dissected from each animal and post-fixed in 10% buffered formalin. The brain was weighed upon removal from the skull, poior to placement into formalin, and the brain:body weight ratio was alculated. Other animals that survived to term were sacrificed by CO2 asphyxiation without perfusion. For the follow up study, it was not necessary to collect tissues for histological examination since an OAEC had been established in the initial study.

For the initial study, micropathology examinations were performed on a comprehensive battery of neural tissues from perfusion-fixed control and high-dose rats of both sexes, as follows. Eight coronal sections of the brain and sections from three levels of the spinal cord (cervical, thoracic, lumbar) and the cauda equina were embedded in paraffin and examined utilizing hematoxylin and eosin (H&E). Dorsal root ganglia (including dotsal and ventua root fibers) from the cervical and lumbar swellings and gasserian ganglion were embedded in glycol methodylate (GMA). Eyes, optic nerves and gastrocnemius muscle were embedded in gratfin and cut in cross/transverse-section as well as longitudinal section. GMA-embedded tissues were sectioned at 2-3 µm and stained using a modified Lee=s stain. The sciatic nerve was also cross-sectioned at approximately 2-3 µm and stained with a modified Lee=s stain. In addition, histopathology was performed on any gross lesions collected at necropsy. Tissues from perfusion-fixed animals at the low- and mid-dose levels were not subjected to micropathology unless compound-related lesions were present in the high-dose group. For the follow-up study, it was not



Sciatic nerve (bilateral)

Ĉ Sural nerve (bilateral

Lumbar dotsal root ganglig

Lumbar forsal root fibers

Sumbar VentraDoot filers

Cervical dorsal root fibers

Cervical ventra root fibers

Cer dcal dopsal root ganglion

biakper

🕅 Отржк

ve (pilateral)

necessary to prepare tissues for micropathological examination since a NOAEL had been established in the initial study. The tissues collected for all animals in the initial study are shown in the following table. For all control and high-dose animals in the initial study, the tissues shown below were processed and evaluated for microscopic pathological changes. Tissues evaluated by micropathology (checked (X)tissues were evaluated)

Tissues evaluated by micropathology (checked (X) tissues were evaluated) **CENTRAL NERVOUS SYSTEM** PERIPHERAL NERVOUS SCIATIC NERVE BRAIN

- Х Olfactory bulbs
- Х Cerebral cortex
- Х Caudate-putamen/globus pallidus
- Х Hippocampus
- Х Thalamus
- Х Hypothalamus
- Х Midbrain (tectum, tegmentum, and cerebra peduncles)
- Х Cerebellum
- Medulla oblongata Х SPINAL CORD
- Х Cervical swelling
- Х Thoracic swelling
- Х Lumbar swelling
- Х Cauda equina
- Х Gasserian ganglion
- Х Optic nerve
- Х Eye
- Х Gastrocnemius muscle
- II. Results and discussion

#### A. Mortality

For the initial and follow-up studies, there were no compound selate deaths at any dose level in either sex. One control female (EK0102) from the follow-up study was found dead on day 1 of the study. The cause of death was not determined but was not due to treatment, since it occurred in a control animal. Necropsy findings for this animal included mild, bilateral, ditated kidney pelvis, multiple, bilateral, discolored zones of kidneys and abnormal contents in the urinary bladder (i.e. Thickened material).

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#### B. Clinical observations

Ö For the initial study, compound-related clinical signs were limited to urine stain in four high-dose males with none in the control, low- or phid-dose groups. This finding was first evident on day 0 and generally resolved two to five days after treatment. There were no compound-related clinical signs evident in males at lower dose levels or in females at any dose level.

For the follow-up study, there were no compound-related signs at any dose level.

### C. Bodyweight

In the initial study, body weight was not affected by treatment at any dose level in either sex. The body weight data from the initial study are summarized in the table below.



Control Body w 279±17 298±20 314±25	125 eight-Males 288±13 311±16	500 283±10 303+15	2000 282±14° 295±05 295±05
279±17 298±20	288±13 311±16	303-15	0295±09
298±20	311±16	303-15	0295±09
	<u></u>		
314+25	22600		
517-23	32 20	20±19	3115≠22 🔊
Body we	ight-Females	8 x	
190±13	187±8	186±8 O	190
197±15 🔏	199±10 ~	∑ ©194±10 √	O 2000±8 0
208±14 炎		∞ 205₽8 ०	°~216±34
fi	190±13 197±15 208±14 &	197±15         199±10           208±14 &         209±8           rom control@≤0.05         2	190±13     20187±8     186±8       197±15     199±10     2094±10       208±14     209±8     205±8

#### Table 5.7.1-2Body weight – Initial Study ( $g \pm s.d.$ )

For the follow-up study, body weight was not measured beyond day of since a NOAEL for body weight had been established in the initial study. The day 0 body weights for the failes on the faile

weight had been established in the initial study. The day 0 body weights for the temales in the followup study are summarized in the table below.

#### Table 5.7.1-3 Day 0 Body Weight _ Pollow Up Study (g + od.)

T ( D			Dose Level (r	ngakg bw)	$\bigcirc^{\nu}$
	ố O ^V Con	brol ¹	25	** ~~ ** A	⁾ 100
	A	Body weight Fe	manes 🏑	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	
Day 0	8		$\tilde{j}$ $\pm 10$ °	190±10	193±11
Do de avoiglet avag not at mettiogle	e 1:00 All All	and a	ř @.	-	

Body weight was not statistically different from control  $(p \le 0.05)$  N=12 (unless otherwise noted)? I: n=14

#### D. Neurobehavioral Pesults

## 1. Functional observation battery (EOB)

For the **initial study**, treatment-related findings were limited to a statistically significant decrease in mean body temperature in mid- and high-dose females (i.e., 37,4%C and 36.9%C, respectively vs. 37.9%C for controls). Also, there were statistical differences from control involving ease of removal from the home cage in high-dose females with a similar (non-statistical) trend in mid-dose females (i.e., a lower incidence of animals that vocalized compared to those that did not; three mid- and one high-dose vs. six controls). There were no treatment-related findings in males at any dose level or in low-dose females.

For the **follow-up study**, there were no compound-related findings at any dose level. In addition, there were no incidental findings unrelated to tradiment

For the **initial study**, there were a few findings that were statistically different from control but not related to treatment. This included an increased number of urine pools on the open field in males assigned to the high-dose (3 0 vs. 1.0 for controls) and a statistical decrease in mean foot splay (66 vs. 84 for controls) for temales assigned to the mid-dose group. Both of these statistical differences from control occurred prior to the initiation of treatment. Lastly, there was a similar statistical decrease in mean foot splay (61 vs. 79 for controls) for mid-dose females on day 7. This difference from control is not thought to be related to treatment since it occurred 7 days after treatment (not on days 0 or 14), there was no relationship to dose and a similar difference from control also occurred in the same group of animals prior to treatment.

Additional observations considered incidental and unrelated to treatment included areas of hair loss described as alopecia on various occasions in one control male and in one or two control or mid-dose



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females, each. Lastly, one mid-dose female had a dermal lesion described as a scab observed during the pretreatment week. All of these findings were considered incidental and unrelated to treatment because they occurred at a low incidence, the incidence did not increase with dose and, in some cases, occurred prior to treatment or in control as well as treated animals.

			Dose Level (	mg/kg bw/da	y) 29
Males	Observation	Öntrol	125	500	<u>200</u>
Pretreatment	$\frac{\text{Urination}}{\text{Mean} \pm \text{S.D.}}$	1.0±1.0	10¥1.4	1.6+1,6	3.1±23
Day 0	No Findings	12(100) 0(0)			
Day 7	No Findings	12(100) (0)	12(100) 0(0)		(12(100) (00)
	No Findings	× 11(98) 1(8)			
Females		$\sim$ $\sim$		mg/kg w/da	хŶ
remates	Observation	Control	6 125	Č 500 *	200
	Not Observed & & &			10(89) 2(17)	12(100) 0(0)
Pretreatment Week	Scab Present			D11(92)	12(100) 0(0)
	Reflex/Physiologic Observations - Foot Splay	~84±17	\$ <b>4</b> \$16	* 66±14	81±15
	Handling – Ease of Removal:	6(50)	€ 5(42) 7(58)	9(75) 3(25)	* 11(92) 1(8)
Day 0	Minimal@esistance with ocalizations	11(92) 2(8)	12(100) 0(0)	10(83) 2(17)	12(100) 0(0)
~		∂ ⁷ 37.9±0.4	4 37.8±0.3	* 37.4±0.5	* 36.9±0.5
Day 7	Handling, Other Not Opserved A Alopecia, Present	10(83) 2(17)	12(100) 0(0)	10(83) 2(17)	12(100) 0(0)
	Reflex/Physiologic Observations Foot Splay Mean S.D.	79±16	71±15	* 61±11	69±14
Day 04	Handling - Other: No Observed Mopecia, Present The number of Animals and % incidence (in parenth)	10(83) 2(17)	12(100) 0(0)	10(83) 2(17)	12(100) 0(0)

Table 5.7.1-4	Functional observational battery results – Initial study

Values represent the number of animals and % incidence (in parentheses) with observation. * Stanistically afferent from could of  $(p \le 0.05)$ 



#### 2. Motor activity:

A comparison based on the inherent variability of the average pretreatment values in the initial study for motor and locomotor activity among the four groups of males and females provides a measure of the c magnitude of the difference that should be considered biologically significant. For motor activity, the pretreatment values for groups that later received the test substance averaged from 12% low of 17% higher than controls for males and from 7% lower to 22% higher than controls for females (see following table). For locomotor activity, the pretreatment values for groups that later received the states substance averaged from 10% lower to 28% higher than controls for males and from 12% lower to 22% higher than controls for females. In the follow-up study, for motor and locomotor activity the pretreatment values for groups that later received the test substance averaged from 9% to 15%, wer than controls and from 7% to 20% lower than controls, respectively (see the following table) As a general guide, these results confirm that differences of approximately +20% are within the range of pormal variability in this laboratory for groups of 10-12 rats/sex/dosp level and, therefore, are not biologically significant.

For the overall 60-minute test session in the initial study, compound-related effects on session motor activity and locomotor activity (see tables 5.7.1.-5 & 5.7.77) were evident on the day of treatment in both sexes at the mid- and high-dose levels and and in low-dose females. Measures of motor and locomotor activity were not affected by treatment in low-dose males. On day 0, measures of motor and locomotor activity in mid- and high-dose males and females were significantly reduced, compared to controls (in males, an average 51% and 71% (motor) and 49% and 73% (locoprotor) respectively and in females an average 53% and 72% (motor) and 58% and 77% (locomotor), repetitvely. In addition, motor and locomotor activity in females at the lowest dose level was statistically reduced (26% and 31%, respectively), compared to controls. After day 0, there were no compound-relate Deffects in males or females at any dose. On day 0 in the follow-up study, measures of motor and locomotor activity were non-statistically reduced an average 38%, each), compared to controls of females dosed with 100 mg/kg. There were no treatment-related differences from control in motor optocomotor activity in L.  $\bigcirc$ females at lower dosedevels. K) L  $\bigcirc$ 

Other differences from control were more modest and not considered to be related to treatment. For the initial study locomotor activity was slightly outside the range of formal variability (approximately + 20%) on day for high-dose males (22% fower than controls). This difference from control was not ascribed to treatment since it was not statistically significant, occurred 7 days after treatment and was not seen in remales at the same dose level. For the follow-up study, motor activity was slightly outside the range of normal variability on day of for low-does females (22% lower than controls). This difference from control was not ascribed to treatment since the difference was small and not dose-related.

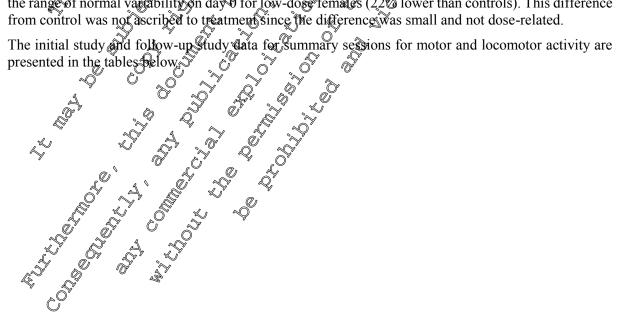


Table 5.7.1-5 Summary Session Motor Activity Results - Initial Study (Percent Difference from Control)^a

Control			
Track Data		Dose Level (mg/ kg bw)	
Test Day	125	500	2000 05
	Males		
Pretreatment	17	-12	
Day 0	-5	-51*	~71*~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Day 7	4	-14 V	
Day 14	6		
	Females	Ŷ, ô	
Pretreatment	13		
Day 0	-26*& &		<i>Q</i>
Day 7			0 ~ 19 ~ ~ ~ °
Day 14		Y ~ 17 A 6	
a Percent greater $(+)$ or less $(-)$ that	concurrent Jontrol for N=12		

a Percent greater (+) or less (-) than concurrent control for N=12

^{*} Summary session motor activity was statistically different from control of 20.05; ANOVA).

Test Day     Dose Lexel (mg/kg bw)       Test Day     25       Sector     50       Females     3       Pretreatment     3			
Itst Day         0         25         50         7         9         100           Females         7         9         7         -15	Test Der	Dose Lexel (mg/kg bw)	
Pretreatment     P	Test Day		
		Females & a v &	
	Pretreatment		
Day 0	Day 0		

a Percent greater (+) or less (-) than concurrent control for N= V2. Summary session rotor activity was not statistically ofference from control (p=005; ANOVA). Table 5.7.1-0 Summary Session Locomotor Activity Results – Initial Study (Percent Difference from Control) *

	<u> </u>	
	& Dose level (mg/ kg bw)	
Test Dax	ື່ 🥎 500	2000
Mates	- A	
Pretreatment Day 0 A 28 A 4 A 4 A 4 A 4 A 4 A 4 A 4 A 4 A 4 A	-8	-10
	-49*	-73*
Day 0 7 5 7 7 4 7 7 7 12 7 7 7 12 7 7 7 12 7 7 7 12 7 7 7 7	-9	-22
$Dax 44$ $\sqrt{2}$ $\sqrt{2}$ $\sqrt{3}$ $\sqrt{3}$	-3	-15
Female	2S	
Pretreatment of the second sec	-12	22
Day 0 & 11*	-58*	-77*
Day 0         31*           Day 7         3*           Day 7         3*           Day 7         3*           Top 7         3*           Top 7         3*           Top 7         3*           Top 7         3*	-16	12
Day 4 7 7	7	11

a Percent greater (+) or less (3) than concurrent control for N=12.

* Summary session locomotor activity was statistically different from control ( $p \le 0.05$ ; ANOVA).

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 Table 5.7.1-8
 Summary Session Locomotor Activity Results – Follow-Up Study (Percent Difference from Control) ^a

T 4 D		Dose Level (mg	g/kg bw)	
Test Day	25	50	\$	100 0
	Females			K . Q
Pretreatment	-11	-7	.1	\$20 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Day 0	-14	-18		× -38 × ×
a Percent greater (+) or less (-) that	n concurrent control for $N=12$ .		0,×	<u>a</u> a o

Summary session locomotor activity was not statistically different from control ( $p \le 0$ ); ANOVA).

For the initial study, motor and locomotor activity data were subjected to further analysis at each merval on each test day. Measures of motor and locomotor activity for mid- and high dose males and females and low-dose females were statistically different from control on day 0 and are ascribed to treatment. On day 0, the high-dose males exhibited statistically lower levels of motor and locomotor activity during intervals 1 through 6 (35%, 73%, 85%, 83%, 92% and 86%, respectively, for motor and 32%, 78%, 91%, 87%, 94% and 89%, respectively, for locomotor). Also on day 01 the high-dose temales exhibited statistically lower levels of motor and logomotor activity during intervals 1 through 6 (44%, 72% 9%, 85%, 82% and 84%, respectively for motor and 46%, 79%, 88%, 95%, 90%, and 00%, respectively for locomotor). For mid-dose males, interval protor and locomoto activity was statistically lower than controls during intervals 2 through 3 or 6 (47%, 62%, 73%, 76% and 94%, despectively for motor and 47%, 65%, 76% and 79%, respectively for focomotor). For mid-dose females interval motor and locomotor activity was statistically lower than controls during intervals 1 or 2 through 6 (25%, 45%, 57%, 60%, 76% and 70%, respectively for motor and 50%, 68%, 72% \$3% and 84%, respectively for locomotor). Lastly, for low-dose females, interval motor and locomotor activity was statistically lower than controls during intervals 3, 5 and 6 (29%, 53% and 57% respectively) and during intervals 3 through 6, respectively (43%, 45%, 56% and 55%, espectively) Therewere prodifferences ascribed to the test substance for males at the lowest cose level on any test occasion.

For the follow-up study, measures of motor and locomotor activity were not statistically different from control at any interval on any test occasion. However, differences from control in interval motor and locomotor activity were evident in females that received the 100 mg/kg dose level and were considered to be related to treatment. These differences from control in females treated with 100 mg/kg included lower interval motor and locomotor activity during intervals 1 of 2 through 6 (23%, 32%, 31%, 48%, 42% and 68%; respectively for motor and 30%, 32%, 48%, 50% and 75%, respectively for locomotor).

For the initial study, there were a few additional statistical differences from control for measures of interval motor and locomotor activity for males and temales, none of which were attributed to treatment. For high-dose males, motor and locomotor activity was statistically decreased on day 7 for interval 3 and motor activity was statistically increased on day 7 for interval 6 in high-dose females, compared to control. These differences from control are not attributed to treatment because there were no patterns associated with either dose or time, relative to dose administration.

Habituation was not affected by treatment with AE C656948 at any dose level in either sex.

The initial study and follow-up study data symmarized by total activity counts/sessions for motor and locomotor activity are presented in the tables below.

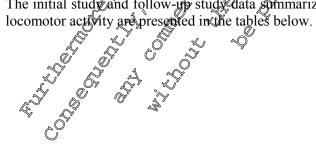




Table 5.7.1-9	Motor Activity -	- Initial Study (to	tal activity counts	for session)
1 abic 5.7.1-7	Motor Activity -	- Initial Study (to	tal activity counts	ioi sessionj

		Ø		
Test Day	Control	125	500	2000
		Males	ð	
Pretreatment	493±130	579±152	432±130	464¥155
Day 0	480±130	454±148	237 * 91	639 * ±35
Day 7	496±110	518±14@%	42 <b>9</b> ≠126	≾ ⁷ 398±94
Day 14	433±82	457±140	\$95±120	∑ 39a ±94 ~~ ⊘ 39a ±91 ~~
		Females		
Pretreatment	435±181	<b>690</b> ±111	40 ³ 2±127.Q	530±171
Day 0	521±132	385 * ±79 _0		148 * ±66
Day 7	480±115	0 489 ± 98	∯ 42 <b>6⊕</b> 189 ∯	573±114
Day 14	447±129	@75±10@	Q 525±212	O 4849100 0

activity was statistical				, L			×,
					÷ č	Ű	Ő,
Table 5.7.1-10	Motor Activity	-Follow Pp	Study (to	tal activity	y counts for	session)	

Values represent mean =	s.d. for 1:00:00 (hh:mm/55) Test-Session/n=12, * Sommary Fession motor & Signature for the session motor of the session for the session motor of the session for the session f	Ž
activity was statistically	ifferent from control (2005; ANOVA). The second secon	
T-11.57110		
<b>Table 5.7.1-10</b>	lotor Activity - gollow or Study (totar activity counts for session)	
	ifferent from control (0.05; ANOVA) ifferent fr	
Test Day		
Test Day	25 × 0 100	
	Bemales A A A A	
Pretreatment	±108 455±115 457±19 429±138	
Day 0	\$539 <b>±</b> 92 \$23±185 \$\$430±69\$\$ 335±93	
Values represent mean -	Vd for 1 hr Test Session the mm st n=1 Sounless	

Values represent mean + (d. for 1 hr Tex Session (th:mm:S)) n = 12 nullessotherwise noted. $a: n=11 Summary session under activity war not statistically different from control (<math>p \le 0.05$ ; ANOVA). Table 5.7.1-11 If comotor Activity – Initial Study (total activity counts for session)

	K K A					
Arest Day		<b>Dose Cevel</b>	(mg/kg bw)			
Fest Day	Control 🔗	125	500	2000		
Maleo Contractione accurate						
Pretreatment	Q	AP5±140	299±118	292±121		
Day 0 🔷 🗸	335⊉112 ∽	×3224120	171 * ±72	92 * ±33		
Day 7			295±109	251±68		
Day 14	287±65	23±108	277±90	243±76		
Image: Second						
Pretreatment	284±131	[©] [*] 330±96	251±86	347±114		
Day 0 🖌 🔬	× 2 362€¥08 < <	251 * ±66	151 * ±57	82 * ±36		
Day 7 🖉 💭	311±87	333±77	261±148	348±88		
Day 14 0 0	© 281±87	300±89	301±116	313±76		
Values northesenter han + and	$f_{-} = \lambda(0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,$	F ( G ·				

â

Values representation  $\pm \Delta d$ , for 100:00 (hh:mm:ss) Test Session n=12 Summer session locomotor activity was statistically different from control (p $\leq 0.05$ ; ANOVA).



Table 5.7.1-12	Locomotor Activit	v – Follow-U	n Study (total	activity count	s for session)
1 able 5.7.1-12	Locomotor Activit	y – ronow-oj	p Study (total	activity count	5 101 56551011)

Test Des		<u></u>		
Test Day	Control ^a	25	50	100
		Females	ð	
Pretreatment	322±89	286±76	300±80	256±97
Day 0	353±94	302±142	289+65	0 [°] 218±84
Values represent mean $\pm s$	.d. for 1 n=12 unless othe	erwise noted.		

a: N=11 Summary session locomotor activity was not statisticating different from Control ( $p \le 0.05$ ; CANO)

#### E. Sacrifice and pathology

#### 1. Gross pathology:

There were no compound-related gross lesions evident at terminal sacrifice in mates or females at any dose level. It was not necessary to perform these procedures on animals in the follow-up study since NOAELs for these endpoints were established in the initial study

#### 2. Terminal Body and Brain Weights

There was no compound-related effection terminal body weight or absolute or relative brain weight in perfused males or females at any level of exposure. The brain weight data from the initial study is summarized in the table below.

Ô

Weights (g)	Dose Level (mg/kg/bw)			
	<b>O</b> ontrol	A25 X	Õ <b>500</b> ž	2000
Body wt ^a		Males		
Body wt ^a	312.6±23.6	✓ Maleso → 328.4 19.8 ~	\$16.1±17.4	307.3±21.1
Brain wt ^b	1.826±0.078	√ 1.803±0.05	³ 1.80 <b>€</b> ≠0.038	1.866±0.052
Brain/body wt b		0547±0.019 @	0.265±0.023	0.619±0.046
		Fen@le 🗸	, O ^y	
Body wt ^a	\$09.1±(5.2	207.7±16.5	204.7±7.6	209.2±7.9
Body wt ^a	A.812±0:081	.≪1.739±0.083	1.750±0.038	1.721±0.025
Brain/body wt	0378±.045 "	0 0.849±0.0560	0.841±0.012	0.828±0.016

Table 5.7.1-13 Absolute and Relative Brain Weights - Initial Study @

a N=12 Measurements were not datistically different from the coupol ( $p \le 0.05$ ).

#### *b N=6*

#### 3. Neuropathology

There were no compound related microscopic lesions in the high-dose males or females. Therefore, tissues from animals that received a lower dose of AE C656948 were not examined.

#### F. Deficien des 🔨

#### None

#### III. Concelusions

In conclusion, compound related effects following a single oral dose of the test substance in the initial study were limited to mid- and high-dose males and females and low-dose females. Effects in males and/or temales consisted of decreased motor and locomotor activity on the day of treatment, urine stain, decreased body temperature and a lower incidence in the number of animals that vocalized during



removal from the home cage. There were no compound-related gross or microscopic lesions at the high dose of 2000 mg/kg. A follow-up study established a NOAEL for measures of motor and locomotor activity in females, with no compound-related effects at the two lowest dose levels. Slight decreases in measures of motor and locomotor activity were evident in females treated at 100 mg/kg. These results establish a NOAEL of 125 mg/kg and 50 mg/kg for males and females, respectively.

#### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013.

There were no compound-related gross or microscopic lesions at the high dose of 2000 mg/kg follow-up study established a NOAEL for measures of motor and locomotor activity in females with no compound-related effects at the two lowest dose levels. Slight decreases in measures of motor and locomotor activity were evident in females treated at 100 mg/kg. These results stablist a NOAEL of 125 mg/kg and 50 mg/kg for males and females, respectively,

Data Point:	KCA 5.7.1/02
Report Author:	
Report Year:	
Report Title:	2008     A Subchronic Neurotoxicity Screening Study with Technical Chade AE C656948       in Winter Pate     A Subchronic Neurotoxicity Screening Study with Technical Chade AE C656948
	III WIStal Katson was a constructed of the construction of the con
Report No:	
Document No:	<u>M€299110-01-1</u> ~
Guideline(s) followed in	QECD JE 424 (1997); SEPAGealth Effects Test Guideling OPPTS
study:	870.6200; 1998); M. & F.F. in Japan notification 12 Nousan 8 8147 (2000)
	guide times.
Deviations from current	Corrent guideline: OECD 424, 1997 O
test guideline:	No devisions
Previous evaluation	Yes, evaluated and accepted of the DAR (2011).
GLP/Official	Yes, conducted under GLP/Officially recognised testing facilities
facilities:	$\begin{array}{cccccccccccccccccccccccccccccccccccc$
Acceptability/Reliability	
· ·	
~ ³⁷ 4	

#### **Executive Summary**

Technical grade AE C656948 was administered in the diet for 13 weeks to young-adult Wistar rats (11-12/sex/dietary level), using nominal concentrations of 7, 100, 500 and 2500 ppm for males and females. All test diets (including ontrol) were provided for ad libitum consumption throughout the study except during neurobehavioral testing. Concentration in the diet, as well as the homogeneity and stability of AE Co56948 in the dietary ration was confirmed. Ten to twelve rats/sex/dietary level were used for neurobehavioral evaluation, with micropathology performed on selected tissues from 6 rats/sex from control and high-dose groups, Body weight and food consumption determinations, as well as detailed clinical observations, were conducted weekly throughout the study. Observations for moribundity and mortality were performed at least once Gaily. Automated measurements of activity (figure-eight maze) and a functional observational battery (FOB) were conducted the week prior to treatment and during weeks 2, 4, 8 and 13 Ophthe imologic examinations were conducted on all animals prior to shipment release and then again on all study animals during week 12. All animals placed on study were subjected to agross decropsy. For selected animals, the brain was weighed in order to calculate the brain:body weight and skeletal muscle, peripheral nerves, eyes (with optic nerves) and tissues from the central nervous system were also examined microscopically for lesions. On the day of sacrifice, known target organs (liver, kidney and thyroid) were collected and weighed and hematologic and serum chemistry



evaluations were performed on surviving non-perfused animals (four to six/sex/dietary level), for reference to verify that a sufficiently toxic dose was tested and for comparison with other studies at these dietary levels.

The mean daily intake of the test substance over approximately 13 weeks at nominal retary concentrations of 100, 500 and 2500 ppm, respectively, was 6.69, 33.2 and 1642 mg/kg/day for mates and 8.05, 41.2 and 197.1 mg/kg/day for females.

The highest dose tested produced clear evidence of systemic toxicity, but no evidence of neurotoxicity. The neurobevioral tests and the neuropathological examinations revealed no evidence of neurotoxicity at any treatment level.

Conclusions After approximately 13 weeks of continuous dietary exposure to the test substance treatment-related findings at the high dose consisted of decreased body weight total body weight gain and food consumption in males and females, increased cholester and the lowers in males and/or females and decreased terminal body weight in females. Also, liver and kinney weights tabsolite and relative) were increased in high-dose males and liver weight (absolute and relative) was increased in high-dose females. The only other finding at the mail-dose was decreased food consumption of females, which was not associated with any effect on body weight.

Based on neurotoxicology endpoints a NQAEL of 2500 ppm was established for mates and remales (specifically, 164.2 and 197.1 mg AF C656948/kg/day for male and female rats, respectively).
 I. Materials and methods
 A. Materials

Materials and methods

1	Test material:	
1.	· •	Daiga natidar (1)
	Description	Beige powder $O$ $O$ $A$
	é V	Mix-batch 08 28/0902
	Purity:	
	CAS # 5 0' 5'	658066-35-4 5 and 5000 ppm at the room temperature for
	Stability Ptest compound:	Stable in the diet at 5 and 5000 ppm at the room temperature for
	• • • • • • • • • • • • • • • • • • • •	7 days or at freezer storage conditions for 28 days.
	2. Vehicle and or positive?	Acetone served as a solvent in the diet preparation process and
	control:	was allowed to evaporate prior to administration
3.		
	Species: $\sqrt[3]{4}$	$\operatorname{Rat}^{O'}$ if is a first second s
	Strain:	Wistar HAN CRE: WIGHAN)
	Age:	Approximately 8 weeks of age
	Weight at dosing:	219:6 – 2839 g range for males and 142.9 – 174.9 g range for
	weight at dosing:	females of a
	Source:	
	Source: Acclimation period: Diet:	$10  \mathrm{daws}$ $\infty'$
		Purfue Mills Rodent Lab Chow 5002 in meal form provided for
	Diet:	ad libitum consumption during the acclimation period and
		frougbout the study except during neurobehavioral testing.
	Water:	Tap-water, <i>ad libitum</i>
		Aquinals were caged individually in suspended stainless steel
	Housing: S S S	wire-mesh cages.
	Environmental conditions:	whe-mesh eages.
	Environmental conditions:	22 + 40C
lī.	Temperature:	$22 \pm 4^{\circ}\mathrm{C}$
R	Humidity:	50-20%
	Air Changes:	Approximately 10 changes per hour
	Photoperiod:	12 hours dark / 12 hours light



#### B. Study design

**1. In life dates:** 26 February 2007–01 June 2007

#### 2. Animal assignment and treatment

ñ

Randomization procedures utilized software from INSTEM Computer Systems, Following accumation, the animals were weighed and those with body weights that were more or less than 20% of the mean weight for each sex were rejected. The remaining animals were randomly assigned to a control group or one of three dietary levels in order that, for each sex, groups had comparable body weights when treatment was initiated. This was done to facilitate comparisons following treatment. Animals were randomly assigned to the test groups noted in Table 5.7.1.-14 below.

Each rat was identified by cage card and tail tattor with a number that did not reveal the animals treatment group. In addition, animals were assigned an identification number that specified the rat's sex, treatment group, cage number and identified it with the study of the second second

The oral route of exposure was employed in accordance with the test guideline. For 13 weeks, four dose groups (10-12 rats/sex/dietary level) were administered the test substance in the diet at nominal concentrations of 0, 100, 500 and 2500 ppm for males (6,69, 35/2 and 164.2 mg/kg/day mg/kg/day) and females (8.05, 41.2 and 197.1 mg/kg/day). All 10-12 rats/sex/dietary level were used for neurobehavioral testing, with six/sex/level/used for micropathology. The following deservations and measurements were also included in the study ophthalmic examinations, clinical observations, mortality, body weight, food consumption, brain weight (6 rats/sex/dose), and gross necropsy. Micropathology was performed on selected tissues from 6 rats/sex/irom control and high-dose groups. Hematologic and serum chemistry evaluations were performed on selected animals were collected and weighed for reference to verify that a sufficiently toxic dose was tested and for comparison with other studies at these dietary levels. The study design is outlined in Table 5.7. h. 14 before.

Table 5.7.1-14 Study Design				
		Dose Group ppn	n (mg/kg bw/day	7)
Experimental Parameter 6		√√ √ (∂6.69) ↔	500	2500
			(♂ 33.2) (♀ 41.2)	(♂ 164.2) (♀ 197.1)
Total number of animals/sex/group	0 ⁵ 12 ⁵	× 12×	12	12
Behavioral Testing (FOB, Motor )	7 1042/sex	0 [°] 1012/sex	10-12/sex	10-12/sex
Clinical Chernostry and Tissue	4-6/ <b>&amp;</b>	© 5-6/sex	5-6/sex	5-6/sex
Neuropathology ^b	sex sex	0/sex	0/sex	6/sex
Ophthalmic Examination ^c A	@1-12/sex	11-12/sex	11-12/sex	11-12/sex

a FOB/and motor activity was pressed prior to desing and again during weeks 2, 4, 8 and 13.

b Neuropathology tissues from the mid- and low-dose groups were not examined, as no treatment-related neuropathology was evident at the highest distary lovel.

c Ophthalmic exons were performed prior to dosing and at week 12.

The rationale for close selection was based primarily on the results of a 90-day toxicity study, with the test substance administered via the diet at nominal concentrations of 0, 50, 200, 1000 and 3200 ppm to male and female Wistar rate (10/sex/dietary level).

The preliminary pre-mating phase results of a two-generation reproductive toxicity study in Wistar rats were also considered for dose selection.



Based on the results of these studies, the doses selected for the present subchronic neurotoxicity study were 0, 100, 500 and 2500 ppm for males and females. The 2500 ppm dietary level was selected as a maximum-tolerated dose (MTD) in both sexes following subchronic exposure. The 100 ppm dietary level was selected to produce no evidence of toxicity for endpoints measured in this neurotoxicity study and the 500 ppm dietary concentration was selected as an intermediate dietary level.

#### 3. Diet preparation and analysis

The diet was prepared every other week. Acetone served as a solvent in the diet preparation process and was allowed to evaporate. The control diet was prepared the same way, excluding the test substance A sample of each batch of feed mixed was taken and retained in the frequer (daily average of 22.62 to - 24.74°C) until the study was complete and the analytical data deemed satisfactory. Feed was available for ad libitum consumption for a period of one week prior to changing, at which time any uncaten feed was collected and disposed of by incineration.

The concentration of AE C656948 in the ration was measured by LC-MS/MS analysis. The stability [following both room temperature (~ 22°C) and freezer (~  $-23^{\circ}$ C) exposure and homogeneity of the test substance in the feed were established by analysis of samples at nominal concentrations of 2 and 5000 ppm. The concentration of the test substance in the ration was measured for the ration that was used during all weeks of the study.

**Homogeneity Analysis**: Homogeneity of the test substance in the ration was within the acceptable range for concentrations that bracket these used in this study. These concentrations of 5 and 5000 ppm had percent relative standard deviations (% BSD) \$2.7% and 1,8%, respectively.

Stability Analysis: The stability of AE C656948 in the ration was established at room temperature at dietary concentrations of 5 and 5000 ppt, with no appreciable decrease in concentration with seven days of storage. AE C656948 was stable at freezer conditions for 28 days with no appreciable decrease in concentration at 5 and 5000 ppm.

**Concentration Analysis:** Actual (analytically-determined) concentrations of the active ingredient in the 100, 500 and 2500 pprodictary levels used in this study averaged 98% to 102% of the nominal concentrations. Based on these results, the mean analytically-confirmed dietary levels for this study were 100, 512 and 2458 ppn.

#### 4. Statistics

Statistical evaluations were performed using software from either INSTEM Computer Systems or SAS. With the exception of Bartlett's test, which was tested at  $p \le 0.001$ , the level used to establish statistical significance was  $p \ge 0.05$ .

Continuous data were analyzed using an Analysis of Variance (ANOVA), followed by a Dunnett's test if a significant F-value was determined in the ANOVA. For the FOB, continuous data were first analyzed using a Repeated-Measures ANOVA followed by a one-way ANOVA if there was a significant interaction between dose group and test week. For weeks in which there was a significant treatment effect, Dunnett's test was applied to determine which groups, if any, were significantly different from the control group. Categorical data collected in the FOB were analyzed in a similar manner, using General Linear Modeling (GLM) and Categorical Modeling (CATMOD) Procedures, with post-hoc comparisons using Dannett's est and an Analysis of Contrasts, respectively.

Motor and become activity (activity for the entire session and activity for each 10-minute interval) were analyzed using ANOVA procedures. Session activity data were first analyzed using a Repeated-Measures ANOVA, followed by a one-way ANOVA if there was a significant interaction with test occasion. For weeks in which there was a significant treatment effect, Dunnett's test was used to determine which groups, if any, were significantly different from the control group. Interval data were subjected to a two-way Repeated-Measures ANOVA, using both test interval and test occasion as the repeated measures, followed by a Repeated Measures ANOVA to determine on which weeks there was a significant treatment by interval interaction. For those weeks, the data for each interval were subjected



to analysis using a one-way ANOVA to determine at which intervals there was a significant treatment effect. For those intervals, Dunnett's test was used to determine which groups, if any, were significantly different from the control group.

For pathology, continuous data were evaluated initially using Bartlett's Test to analyze for homogeneity of variances among groups. Homogeneous data were further analyzed using an Analysis of Variance (ANOVA) followed by Dunnett's Test for pair-wise comparisons. In the event of non-homogeneous data, statistical analysis was performed using the non-parametric Kruskal Wallis Test for pair-wise comparisons.

Micropathology frequency data were analyzed using a Chi-Square Test followed by one-tailed Fisher's Exact Test in cases of significant variation by the Chi-Square analysis. A probability value of  $\mathbb{E}^2 0.03$  was accepted as significant for all statistical tests with the exception of Bartlett's Test in which a probability value of  $\mathbb{P}^2 0.01$  was used.

#### C. Methods / observations

#### 1. Mortality and Clinical Observations:

Cage-side observations were conducted twice daily (once daily on holdays and weekends) for mortality or clinical signs of moribundity. Detailed physical examinations for clinical signs of toxicity were carried out and recorded once each week.

#### 2. Body weight:

Individual body weight determinations were made weekly Additionally, all animals were weighed on the day of sacrifice for terminal body weight measurement.

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#### 3. Food Consumption:

Individual food consumption was measured week. Daily food consumption was averaged over the duration of the study based on perception weight.

The average daily intake of the active ingredient (A.P.) (mgA.I./kg body weight/day) was calculated using weekly body weight and food consumption data. The general relationship used for this calculation was: [AI in feed (ppr)/1,009] x [feed consumed g/g/g body weday)] = mg AI/kg body wt/day. Using this formula, the average consumption of Ad. for moles and females that received diets containing analytically determined nominal concentrations of 0, 100, 500 and 2500 ppm AE C656948 was as follows

0, 8.05, 410 and 97.1 reg/kg/day, respectively for females.

### 4. Neurobehavioral Assessment

All animate that were assigned to the study were tested using the FOB and motor activity on five occasions - once during the week prior to initiating the exposure and again during weeks 2, 4, 8 and 13. The order of testing and assignment of arimals to mazes were done in a semi-random manner, such that groups were balanced across test times and test devices, and no animal would be tested more than once in the same maze. On the day prior to testing on that day. Animals were placed in the correct sequence that had been established for testing on that day. Animals were then transferred to the room where testing took place and allowed to acclimate with minimal disturbance until testing on the following day. The dost group identification was concealed prior to testing to ensure that testing would be conducted without knowledge of the group assignment. The test room was a standard animal room that was maintained on the same light:dark cycle and settings for temperature and relative humidity as the animal foom, with tests conducted during the light phase. Sets of eight animals (maximum) were evaluated individually using the FOB and then, approximately 30 minutes after the last animal in the set had finished being tested in the FOB, all eight rats were placed individually into the mazes to measure activity.



Each week, testing was staggered over two days for each sex to accommodate the schedule for behavioral testing. Males and females were tested on separate days, with the open field and parzes cleaned during the ensuing interval to reduce the residual scent from the other sex.

#### a. Functional Observational Battery (FOB):

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This FOB closely follows the battery of tests described by Moser (J. Am. Coll. Toxicol. 1989, 5, pp. 85-93), with each animal tested individually. Scoring criteria and explicitly defined scales were used to rank the severity of observations that do not readily lengthemselves to quantitation. The proceedures used to determine landing foot splay and grip strength are based on established methods. The echnicians who conducted the FOB were "blind" with respect to the animal's group assignment. Inter-observer reliability has been established in order to allow multiple person to perform either the observations and/or measurements, ensuring the consistency of the results of each technician. The FOB parameters evaluated are summarized below.

When applicable, observations were scored on intensity as follows: 1) Slight Barely, perceptible or infrequent) or 2) moderate to severe. Data were corrected while the rats were in their home case, during handling, and in an open field for 2 minutes (in the center of a that surface will a perimeter barrier such as a cart). In addition, reflex and physiologic observations and measurements were made while the animals were sitting on the cart surface following open field observations.

Home cage observations included: posture, piloerection, prolour motor movements (such as repetitive "chewing" movements of mouth and javy tremors, and conversions, gait abnormalities, vocalizations, decreased activity, repetitive head bobbing, and in meased reactivity.

Observations during handling included ease removal from cage reaction to being handled, muscle tone, palpebral closure, la rimation, salivation nasal rischarge, stains (lacrimal pasal, perianal, urine, oral), alopecia, emaciation, bite marks, exophthaliora, broken teeth/malocclusion, missing toe nail(s), dehydration, and temperature upor touching (coop to-touch).  $\cap$ 

Open field (2 min.) observations included. number of rears, pilor rection, respiratory abnormalities, posture, involuntary motor movements, stereotypy (excessive or repetitive behavior), bizarre behavior, gait abnormal@ies, vocalizations, arousal level, and amount of excretion.

Reflex and physiologic observations measurements included; approach response, touch response, auditory response, tail pinch, pipil size at normal lighting pupil response, righting reflex, grip strength [Chatillon, Model DF15-10 digital strain gauges (50 kg capacity), which were both equipped with a grid system attached to an extension arm], body weight, body temperature, and landing foot splay. FOB Parameters Evaluated (checked (X) parameters evaluated)  $\sim 0$ , Ø °~

	FOB Parameters Evaluate	ed (checked (X) pacameters eva	aluat	ed)
	HOMOE CAGE	@HANDLING_~~		OPEN FIELD
	OBSERVATIONS	∧ OBSERVAQIONS		OBSERVATIONS
Х	Posture*	0X Reactivity	Х	Rearing+
Х	Piloerection 6	X Muscle One*	Х	Piloerection*
Х	Involuntary motor movements	X Palperral closure*	Х	Respiratory abnormalities+
	e.g.:			
Х	e.g.: Repetitive chewing	X Laerimation* /	Х	Posture*
		chromodacryorrhea		
Х	Conversions	X Salivation*	Х	Involuntary motor movements
				e.g.:
Х	Tremors &	X Nasal discharge	Х	Repetitive chewing
X «	Abnormal movements	X Red/crusty deposits (stains)*	Х	Convulsions*
Х	Gate@bnormalities	X Fur appearance	Х	Tremors*
Х	Vocalizations	X Emaciation	Х	Stereotypic behavior*
Х	Decreased activity	X Bite marks	Х	Bizarre behavior*



- Repetitive head bobbing Х
- Х Increased reactivity

#### SENSORY OBSERVATIONS X Dehydration

- Х Approach response+
- Х Touch response+
- Х Auditory response*
- Pain response* Х
- Х Pupil response*
- Х Pupil size
- X Air righting reflex+

*Required parameters; +Recommended parameters

#### X Eye prominence*

- X Broken teeth/malocclusion
- Х Missing Toe Nail(s)
- X Cool-to-Touch

#### PHYSIOLOGICAL **OBSERVATIONS**

- X Body weight*
- X Body temperature+

- X Abnormal movements*
- Х Gate abnormalities / Gait score*
- Vocalizations Х
- Arousal/ general activity level Х
- X Urination / defecation*
- **NEUROMUSCULAR OBSERVATIONS**
- X Forelimb grip strength* X Hindlimb grip strength
- XQ Landing foot play*

#### b. Motor and Locomotor Activity:

Motor and locomotor activity were evaluated approximately 30 minutes after the last animal in the set (8 rats maximum) had finished the FOB. All eight rate in each set were placed individually into figureeight mazes and activity was measured for a total of 60 minutes. The figure-eight maze was selected as an established and widely-used automated activity-measuring device that can be used to detect both increases and decreases in activity. Each maze consisted of a series of infer-connected alleys, converging on a central arena and was covered by transparent plastic. Fight infrared emitter detector pairs (three in each of the figure-eight alleys and one in each of the blind alleys) measured activity, each time a beam was interrupted, an activity count was registered. The floor of each maze rested above absorbent paper which was changed at the end of each day. A columbus Instruments (Colombus @H) Universal Maze Monitoring System and a personal computer were used for automated data collection Broad-spectrum background noise (74±2 dB(A)) was provided throughout the test to minimize acoustical variations during testing. The uniformaty of light intensity (100±70 lux) over each of the mazes was verified daily.

Motor and locomotor activity were examined during each of the six, teneminute intervals. Motor activity was measured as the number of beam interruptions that occurred during the test session. Locomotor activity was measured by climinating consecutive cours for a given beam. Thus, for locomotor activity, only one interruption of a given beam was counted until the rat repocated in the maze and interrupted one of the other beam Phabituation was evaluated as a decrement in activity during the test session. L.

### 5. Ophthalmology

Pre-exposure and pre-to-minal week 2) ophthalmed examinations were conducted on study animals in a semi-darkened room. The pre-exposure examination was used to select animals which did not have ophthalmological defects that could interfere with the interpretation of study results. Animals with such defects were sacrificed without necropsy. The pupillary reflex was tested using a penlight or transilluminator with more (Welch Allyo, Inc. Skane reles Falls, NY), and then a mydriatic agent was applied to each eye to dilate the pupil. After modriasis, the conjunctiva, cornea and lens were examined with a slit lamp microscope Kowa \$L-15 Kowa Company, Ltd., 20001 So. Vermont Ave. Tollerance, CA 90502, USA) and the vitreous hypor, retina, choroid, and optic disc were examined using an indirect ophthalmoscope [PEINE OMEGA (Heine USA, LTD, One Washington Center, Suite 555, One Washington Street, Dover, NIQ03820, USA)].

### 6. Clinical Chemistry

Hematologic and servin chemistry evaluations were performed on all surviving **non-perfused** animals (four to six sex/dictary level) on the day of sacrifice after 13-weeks of treatment. Blood samples were obtained from fasted (overnight) non-perfused males and females (four to six/sex/dose level) via the retroorbital sinus white under light anesthesia (IsoFlo7; Isoflurane).

### 7. Sacrifice and Pathology

All animals placed on study were subjected to a complete gross necropsy. The necropsy involved an examination of all organs, body cavities, cut surfaces, external orifices and surfaces. The first six males and six females at each dietary level were selected for perfusion and collection of tissues, with



replacement, as necessary, if the perfusion was considered inadequate. These animals were deeply anesthetized using an intraperitoneal dose (50 mg/kg) of pentobarbital and then perfused via the left ventricle with a sodium nitrite (in phosphate buffer) flush followed by Universal fixative (1% (%/v) glutaraldehyde and 4% (w/v) EM-grade formaldehyde) in phosphate buffer. The entire brain and spinal cord, both eyes (with optic nerves) and selected (bilateral) peripheral nerves (sciatic, tibial and sural), the gasserian ganglion, gastrocnemius muscle, both forelimbs, gross lesions in peural tissues or skeletal muscle and physical identifier were dissected from each animal and post-fixed in 10% buffered formalin. The brain was weighed upon removal from the skull, prior to placement into formalin, and the brain:body weight ratio was calculated. The remaining animals, including ones that were sacrificed proor to study termination, were sacrificed by carbon dioxide asphyxiation and necropsied. For all **sonperfused** animals (four to six/sex/dietary level) that remained on study until termination, the fiver kidneys and thyroid were collected, weighed and preserved in 00% buffered formalin for possible histopathologic evaluation. Terminal body weight were performed immediately prior to recropsive to allow for calculation of organ to body weight ratios.

Micropathology examinations were conducted on a comprehensive selection of neutal tissues from perfusion-fixed control and high-dose rats of both sexes, Eight coronal sections of the brain and sections from three levels of the spinal cord (cervical, theracic, lymbar and the cauda equina were embedded in paraffin and stained with hematoxylipand cosin (H&E). Dorsal poot ganglia pricluding docal and ventral root fibers) from the cervicaband lupibar swellings and gasserian gangfron were embedded in glycol methacrylate (GMA). Eyes optic nerves and gastrocneppius muscle were entbedded in paraffin and stained using H&E. Peripheral nerves (scianc, tiber and oural) were embedded in GMA and cut in cross/transverse-section, as well as longitudinal section. GMA-entipedded tissue@were sectioned at 2-3 µm and stained using a modified Lee's stain. The sciatic nerve was also cut in cross-section at approximately 2-3 µm and stained with a medified Lee's stain. In addition, instopathology was performed on any gross lesion collected at new opsy Tissues from perfusion-fixed animals at the lowand mid-dose levels were not subjected to micropathology unless a compound-related lesion was present in the high-dose group? For the non-perfused animals, the liver, kidneys and thyroid at all dietary levels were trimmed, processed and sectioned according to standard procedures for light microscopy (paraffin with H&E stain) if deemed necessary by the study director. The tissues examined to evaluate potential pathological effects on the norvous system are summarized in berow. [°]~

		, M	
		R	
	KNS and PNS Tissues Evaluated (checked (X	Wie .	ues@valuated)
		J~435	
	CENTRAL NERVOUS SYSTEM	/	[▲] PERIPHERAL NERVOUS SYSTEM
	BRAIN W W	Ô	<b>SCIATIC NERVE</b>
Х	Olfactory bulbs Q S S S	X	Sciatic nerve (bilateral)
Х	Cerebra cortex	"O"	
Х	Caudate-putamen/globus pallfdus Hippocampus Thalamus	1	
Х	Hippocampus S St &		
Х	Toralamus 👾 🦓		
Х 🔬			OTHER
X	Midbrain (tectum, tegmentum, and Gerebra	Х	Tibial nerve (bilateral)
	peduncles)		
Х	Cerebeltum A N Q A Q	Х	Sural nerve (bilateral)
Х	Meduta oblongata		
	Covical svelling		
Х		Х	Lumbar dorsal root ganglion
Х	Thoracioswelling	Х	Lumbar dorsal root fibers
X	Lumbar swelling	Х	Lumbar ventral root fibers
XÌ	Cauda equina		
¥	OTHER	Х	Cervical dorsal root ganglion
X	Gasserian ganglion	Х	Cervical dorsal root fibers
Х	Optic nerve	Х	Cervical ventral root fibers
Х	Eye		



20 20

Х Gastrocnemius muscle

#### 8. Positive Controls

This study did not include concurrent positive controls, but previous studies conducted at the laboratory served as positive controls. For the Functional Observational Battery (FOB), studies were conducted with acrylamide, carbaryl and untreated rats to establish the sensitivity, reliability, and validity of these test procedures, the adequacy of training of technical personnel and to serve as a historical control. To assess motor activity, studies with untreated animals and with rats treated with reference substances that? increase (triadimefon) and decrease (chlorpromazine) motor activity have established the sensitivity, reliability and validity of the test procedures used. Studies performed at this laboratory with thim the state of the stat and acrylamide established the sensitivity and reliability of the micropathology procedures for detecting lesions in peripheral nerves and the central nervous system.

#### II. **Results and discussion**

#### A. Mortality

There were no treatment-related deaths in males of temples at any dietar

#### **B.** Clinical observations

There were no treatment-related clinical observations seen a any detary level in other sex. The findings summarized in Table 5.7.1-15 befow were considered incidental and unrelated to treatment. These findings were malocclusion on days 74,388 and red lacrimal staining on days 74, and 88 and red nasal staining on day 74 all in one high-dose female and areas of hair loss in one control and high-dose male each on days 4-88.

Table 5.7.1-15 Character Observations (If		<b>gs</b> ) o ^{rg} (sy		
Observation	1 ² 2	Dos@Level.ppm	(mg/kg bw/day	<i>'</i> )
			\$ 500	2500
	Control	(36.69)	(ී <b>33.2</b> )	(ð <b>164.2</b> )
	Nabes Nabes			
Hair, Alopecia Forelimb-Both Thorax-Ventrato				
	× 1,912	<u>≫</u> 0/12	0/12	0/12
Thorax-Ventra	×0/12 ×	≈0/12	0/12	1/12
Thorax-Ventrato	📈 Females ,	ð		
Observation Observation		100	500	2500
	Control-	(♀ <b>8.05</b> )	(♀ <b>41.2</b> )	(♀ <b>197.1</b> )
Lacrimal Stain, Red	0/14-12	0/11-12	0/11-12	1/11-12
Nasal Stain, Red	0 M-12	0/11-12	0/11-12	1/11-12
Malocclusion-Both Upper neisors	0/11-12	0/11-12	0/11-12	1/11-12

Table 5.7.1-15	Clinical	Observ	ations	(Incidenta) Findings)	×,
	<u>.</u>	Con	SK 1		(( ))

Numbers represent to al number of animale exhibiting sign at least once/number of animals in each group

## C. Bodyweight and bodyweight gain @

For high-dose makes, body weight was slightly (non-statistical) decreased (6-7%) on days 35 through days 56 In addition, there was a trend for a slight decrease (maximum 5%) in body weight on days 21-28 and again on day 63, continuing until study termination. For high-dose females, body weight was statistically reduced (7-12%) on day 21 and on days 42 through study termination. Lastly, body weight was non-Statistically reduced (5-6%) on days 28-35 in high-dose females. Body weight was not affected by treatment in males or females at lower dietary levels.



In high-dose females, total body weight gain was statistically decreased (26%), compared to controls. In high-dose males, total body weight gain was slightly (non-statistically) reduced (10%). Total body weight gain was not different from controls in low- and mid-dose males and females. These date are summarized Tables 5.7.1-16 and 5.7.1-17 below for males and females, respectively. 

D N		. 5 ⁴ . 5 ⁴ . 19		
Day No.	Control	100 (6.69)	509 (33.2)	2500 (Y64.2)
Day 0	244.3±13.5 12	244.4±19.0 12	0245.6±9.2 12	247 2±8.7 12
Day 21	325.7±23.6 12 4	325.3±34.8 12	3273±12.912	∂09.8⇒13.5 12 [°]
Day 28	347.8±24.8 12	304.5±36912	5 347. <b>3</b> 10.8 15	331.1±13.8 12
Day 35	365.5≠2 <b>3</b> .9 %	7438 1747	₹ <b>6</b> 6.8±1 <b>0</b> .8 12∢	, 344.9±14.7,02
Day 42	370,2±29.3 120	369.8₽\$7.9 120	3750±12.3512	53.6±47.2 12
Day 49	³⁸⁸ <b>@</b> 30.4	380.6±40712	5∕387.6€16.3,1¢	360.9±17.3 12
Day 56	≪398.5±29.2	391.4≇41.6 12	400.0±17,2 12	373.5±18.2 12
Day 63 L	405.9±30	3999.9 <b>±</b> € ³ .7 12 [€]	410.8±18:3 92	384.2±20.0 12
Day 70 5	×41476-32.5	409.8±4209.12	420.4 <b>@</b> 19.2 12	394.7±19.9 12
Day 77 2 20	₫20.3≠3¥.8 12	413,0244.8	426.3±18.0 12	399.5±21.7 12
Day 84	4266±32.2 12	\$21.9±\$7.1 12	₩ 431.1±19.0 12	408.0±21.0 12
Day 90	5 432.9 9 34.5 √ √12 √	428.7±44.8 12	437.8±22.2 12	416.2±22.7 12
Total Body weight ganv (Das) 0-99) -Males	€\$8.6±26.3	425.7±44.8 12 184.3±31.1 12	192.3±18.3 12	169.0±19.3 12
Values represent mean $\pm$ s.d., n. $\mathcal{Q}$		, N N		
Values represent mean ± s.d., n.				
	)			
Ċ ^O ´				

#### Body Weight and Body Weight Gain (Mean (g) ± s.d.) for Mates Table 5.7.1-16



Dar: Na	Dose Level ppm (mg/kg bw/day)				
Day No.	Control	100 (8.05)	500 (41.2)	2500 (197.1)	
Day 0	156.0±9.0 12	158.3±9.4 12	152.4±5.8 🝞	155.8±0,7 12	
Day 21	196.7±14.7 11	196.4±16.4 11	189.8±7 <b>®</b> 11	182.4±7.6*•1	
Day 28	205.6±16.2 11	206.7±18.5 11	199.948.7 11	19 <b>.</b> 7±7.6°11	
Day 35	213.7±18.2 11	213.2-06.7 11	20 <b>%</b> 9±7.8 11	201.4±10.3*115	
Day 42	217.5±17.3 11	216.1≚17.0 11	209.1±8.0 11	197.2 12.3**M	
Day 49	222.9±15.9 11	209.4±17.4 11	✓ 214.1±9.5 10	199.9±15.9 11	
Day 56	226.4±15.5 11	@224.9±19.8 11	2199±10.211	308.9±10.0* 1	
Day 63	231.1±19.5 11	229.5±18.4@1	<b>2</b> 26.1 <b>±9</b> .7 11∂	211,3¥10.4\$¥1	
Day 70	236.2±18.801	293.1±20.3 11	∑ 226.99±9.3 b	212.1±121*11	
Day 77	236.4±1,47 11.	©236,4 <u>₽</u> 22.2 11	229.0±1109 11	208.4 2.6* 10	
Day 84	240.8418.1.11	239.9±21,811	234.1 € 2.8 1	2167±13.6€11	
Day 91	24508±2023 11	243.2±22.1 11×	2390±11.4	223.2±11.6* 11	
Fotal Body weight gain (Day 0- 91)Females	Q89.3±13.4 11	85 L±12.7 DI	86.8±85 11	66:≨∓10.2* 11	
lues represent mean $\pm$ s.d., n. p $\leq$ 0.05, compared to control.				0 ⁴	

Table 5.7.1-17	Body Weight and Body Weight Gain (Mean (g) ± s.d.) for Females
----------------	----------------------------------------------------------------

## D. Food consumption 🦃

For high-dose males, food consumption was statistically significantly decreased (5%) on day 21. Also, food consumption was non-statistically significantly decreased (6-8%) in high-dose males on day 35 through day 49. For high-dose females, food consumption was statistically significantly decreased (13-24%) beginning on day 21 and continuing for all remaining weeks measured. In addition, food consumption was statistically significantly decreased (7-42%) in mid-dose females beginning on day 21 through day 42, day 63 through day 70 and again on day 91.0 ood, consumption was not affected by treatment in mid-dose males or in low-dose males of females. These data are summarized in Tables 5.7.1-18 and Table 5.7 C+19 below for males and febrales, fespectively.

Table 5.7.1-18 Second Consumption (g/animal.day $\pm$ s.d.) for Males					
Day No O	Dose Kevel ppm (mg/kg bw/day)				
	🗡 Centrol 🖉	100 (6.69))	500 (33.2)	2500 (164.2)	
Day 7 5	22@4±1.83,12	<u>\$</u> 23.60±2.99 12	22.88±1.74 12	25.27±5.12 12	
	23.69±1.67 12	♀ 24.21±2.53 12	23.20±1.64 12	20.12±2.34* 12	
5 Day 35 5	/ 24.07 1.59	24.06±2.74 12	23.89±1.88 12	22.38±1.47 12	
D <b>ay</b> 42	23@7±1.92/12	23.73±2.28 12	23.64±2.22 12	22.47±1.75 12	
Day 49	*23.43≠1.78 12	23.21±2.35 12	23.16±2.08 12	21.62±1.86 12	
$\begin{array}{c c c c c c c c c c c c c c c c c c c $					

Qean Phod Consumption (g/astimal/thay + s th) for Males Table 5 7 1-18



Dose Level ppm (mg/kg bw/day) 🖉 🔗							
Control	100 (8.05)	500 (41.2)	2500 (197:4)				
18.19±4.45 12	16.08±1.55 12	16.09±1.34 12	20.88±5.65				
18.19±2.20 11	16.72±1.35 11	16.29±1.04* 11	0 14.56±0.63*11				
17.61±1.65 11	16.48±0.93 11	16.12±0.61* 11	↓ 15.07±©08*13				
18.22±1.72 11	17.09±1.15 11	16036±1.05* 11	15.43⊭1.07* ¥1 🔗				
18.53±1.71 11	17.10±1.38 11	16.54±1.07* 11Q	1@66±1.42* 11				
17.96±2.55 11	16.45±1.41 11	√ [™] 16.40±1.23 M	014.32±1.67* 19				
17.73±2.24 11	16.12±1.23 11	0 16.23±1,20 11	Q 15.09±1.56*11				
18.99±3.13 11	17.07±1.00 11	16.79±0.31* 11	☞ @4.63±1,23*11				
18.35±2.25 11	16.85±1.15 11 0	[©] 16.23±1.19€11 ~	@13.91±1.24*¶1				
17.68±1.82 11	17.37±1.43 14	Ø √8.36±1.36 11	13.98±2.30°11				
17.23±1.55 11	16.83±1.18 11	€ 16.08 1.30 11	× 35.01±4,12* 14				
17.86±2.31 11	16.60±137 11	1601±1.20° 11 Û	£ 14.70≠0.95*11				
alues represent mean $\pm s.d.$	, n *=p≤0.05, compare@to cont	trol. S					

Table 5.7.1-19	Mean Food Consumption (g/animal/day ± s.d.) for Females
	fitual 1 obu Consumption (grammar day = s.u.) for 1 chares

## E. Neurobehavioral results

## 1. Functional observation Pattery (FOB)

The data from Functional Observational revealed po evidence of treatment-related neurobevioral changes. Compound-related effects observed in the absence of neurobehavioral changes included statistical significantly decreases in body weight for high dose females during weeks 8 and 13 (9% and 10%, respectively). Compound-related findings were not apparent in high-dose males or in either gender at lower dietary levels.

1

Remaining observations considered preidental and inrelated to treatment included areas of hair loss in one control (all test preeks including pretreatment week) and one high-dose male (weeks 4 and 8) and broken teeth or malocclusion in one high-dose female (week 13). The body weight data and incidental findings discussed above are summarized in Table \$7.1-20

	Josef Valional Datect y Nest	~			
Males			ose Level p <u>p</u>	om (mg/kg b	w/day)
	bservation in a		100 (6.69)	500 (33.2)	2500 (164.2)
Pretreatment Not Observed (0	Alopecia (severity):	11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)
Week 2 Not Observed (		11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)
Week 4 Not Observeed	Àlopecia (severity): )) Present	11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	11(92) 1(8)
Weel 8 Not Observed (( (1))	- <u>Alopecia (severity)</u> : )) Present	11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	11(92) 1(8)

Table 5.7.1-20 Qunctional Observational Battery Results



Week 13	<u>Handling – Alopecia (severity)</u> : Not Observed (0) Present (1)	11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	
		D	ose Level pp	om (mg/kg l	bw/day
Females	Observation	Control	100 (8.05)	500 (41.2	2500 (19% IN
Pretreatment	No Findings	12(100)			
Week 2	No Findings	12(100)	12(100)	1200	
Week 4	No Findings	11(100)	Öř1(100)	41(100	0) 11(0°00) 0
Week 8	Body Weight (Mean ±S.D.)	224±18	Q 2 <u>20</u> ±20	215 <u>≭</u> ]	0 Q03±8*
Week 13	Handling – Broken Teeth/Malocckosion (severity): Not Observed (0) Present (1)	11(190) 59(0)	×11(100) ×11(100) ×0(0)	°r ∖oʻ	10091)
	Body Weight (Mean ±S.D.) →	240-19	38±20	233±1	3 216 22*

*= $p\leq 0.05$ , compared with controls Severity: 0=Not Observed, 1=Slight, 2=Moderate to Severe  $\sqrt{2}$ 

#### 2. Motor activity:

Summary session (60-minute) motor and locomotor activity measurements are presented in Tables 5.7.1-21 and 5.7.1-22, respectively. An examination of inherent variability, using the average pretreatment values among the four groups of males and females, provides measure of the magnitude of the difference that should be considered biologically significant. For motor activity, the pretreatment values for groups that later received the test substance averaged from 12% to 18% higher than animals assigned to the control group for males and from 13% lower to 8% higher than controls for females. For locomotor activity, the pretreatment values for groups that later received the test substance averaged from 5% to 14% higher than controls for males and from 12% lower to 10% higher than controls for females. As a general guide these results confirm that differences of approximately  $\pm$  20% are within the range of normal variability in this laboratory for groups of 10-12 rats/sex/dietary level and, therefore, are not biologically significant.

For the overall 60-minute test session, motor and locomotor activity was not affected by treatment at any dietary level in either sex (see Tables 5.7.1.23 and 5.7.1.24).

For males, there were of the occasions when measures of motor (week 2: 23% and 28% higher at the low- and high-flose, respectively) and locomotor (week 2: 28%, 21% and 25% higher at the low-, midand high-flose, respectively) and locomotor (week 2: 28%, 21% and 25% higher at the low-, midand high-flose, respectively, week 4: 25% higher at the low-dose; week 8: 35% higher at the low-dose; week 13: 39% higher at the high-dose activity were above the reference (approximately +20%) range of normal variability By comparison, locomotor activity for high-dose females was 24% lower and outside the reference range of normal variability during week 8, with no similar trend on other test occasions. These relatively modest differences from control are not ascribed to treatment since they were not statistically significant and there was no consistent relationship with dose or in the nature of the difference from control (e.g., relatively higher in males at various dietary levels on various test occasions and relatively lower in high-dose females during week 8 only).

occasions and relatively lower in high-dose females during week 8 only).



	Dose Level ppm (mg/kg bw/day)					
Week No.	100	500	day)         2500         5            (∂ 164,29)         5			
	(♂ <b>6.69</b> )	(ී <b>33.2</b> )	→ (♂ 164,29)			
	Μ	ales ^b	9 4 ₄ 9			
Pretreatment	+12	+18				
Week 2	+23	+19	, * ¥ + 28 💫 💦			
Week 4	+20	+5 V +5				
Week 8	+16	-10 [×]				
Week 13	+8					
	Fem	ales ^c 500 5 500 5 5 (2011.2) 5				
Week No.	100 % % ( <b>♀ 8.05</b> )	500 ⁻ 50 ⁻ <u>5</u> (241.2) 5 ⁻	2500 2500 2 4(2 1974)			
	(♀ <b>8.05</b> ) √	(2041.2)	© ∢⊊ 197∰) <u>°</u>			
Pretreatment ^b		~~-13 <u>~</u> ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	× +&			
Week 2						
Week 4						
Week 8		-14.0	$\begin{array}{c} & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & & \\ & & & & & \\$			
Week 13	that Concurrent control					



	Dose Level ppm (mg/kg bw/day)				
Week No.	100 (♂ 6.69)	500 (♂ 33.2)	2500		
	Mal	es ^b	× 4 . 4		
Pretreatment	+5	+14	\$14 ×		
Week 2	+28	+21 2	× +25°×		
Week 4	+25	+21 +13			
Week 8	+35	+104	A Q13		
Week 13	+11	420 °	× +29 °C		
	Fema	les ^c 20 0°			
Week No.	100 (° 8.05)	les ^c 500 (41.2)	2500		
Pretreatment ^b		× ~ -12 ~ ~	+10		
Week 2	@ -12°7 @		S ~-4 ~		
Week 4		\$ \$ \$ 17 \$ \$	-76 2 -76 2 -724		
Week 8		~ -160 Å	8 °~74		
Week 13			S & -9		

Table 5.7.1-22	Summary Session Locomotor Ac	ctivity Results (Percent Difference from Control) ^a

a Percent greater (+) or less (-) then concurrent concept.

a Percent greater (+) or less (-) then concurrent control. bN=12 for pretreatment week, week 2, week 8 and week 13 N=10 for week 4. c N=12 for pretreatment week and week 2, N=11 for week 4 and week 8; N=10 for week 13 control unimals and N=11 for remaining dietary levels. Summary session locomotor activity was not statistically different from control  $W \leq 0.03$ , ANOVA, at any time for any dietary level. Ś L) S level. L Ø Ì

Motor and locomotor activity data were also analyzed for differences at each 10-minute interval of each test session. For frales and females, interval motor and locomotor activity were not affected by treatment at any dietary evel in either Sex. Habituation was not affected by treatment with AE C65694& in mates or females at any dietary level.

. Š 8.5 Ô

Bose Level ppm (	(mg/kg bw/day)			
Test Week	500 (♂ 33.2)	2500 (♂ 164.2)		
Pretreatment 542215 & 602207	640±165	626±136		
Week2 547±166 0 672±206	653±134	698±160		
Week 4 0 558±003 0 670±319	586±167	552±165		
Week 8 500±136 \$ \$ 587±173	504±133	521±197		
Week 13 $\mathcal{A}$ $\mathcal{A}$ $\mathcal{A}$ $\mathcal{A}$ $\mathcal{A}$ $\mathcal{A}$ $\mathcal{A}$ $\mathcal{A}$ $\mathcal{A}$	452±142	491±234		
Week 13				



		Females ^b		
Test Week	Control	100 (♀ 8.05)	500 (♀ 41.2)	2500
Pretreatment ^a	824±309	830±270	716±275	
Week 2	882±299	712±238	813±317	9214,122
Week 4	759±233	704±205	692±200	7 <b>82</b> ±187
Week 8	798±202	798±196	690±163	\$697±162
Week 13	641±185	644±260 😵	674 332	C 662 168 V

Values represent mean  $\pm$  s.d. for 1:00:00 (hh:mm:ss) Test Session

a N=12 for pretreatment week, week 2, week 8 and week 13;  $N = 0^{2}$  for week 4. b N=12 for pretreatment week and week 2; N=11 for week 4 and week 8; N=10 for week 3 control animals and N=11remaining dietary levels. ()

ANOVAJON any me for any dieta Summary session motor activity was not statistically different from control  $g \ge 0.05$ level.

Table 5.7.1-24	Locomotor	Activity	(total activit	counts for	session)
----------------	-----------	----------	----------------	------------	----------

	(C)		$\sim$ $\sim$ $\sim$	
	Į į	Dose Level ppm	(mg/kg bw/day)	
Test Week	Control	<b>Bose Level ppm</b> <b>100</b> (5 6.69)		۲ <u>۲</u> 2500 ک (ک≰164.2)
	a, ""	Malec /		) <u>«</u> .
Pretreatment	332-137	348±142 [√]	3784108	© [♥] 377±88
Week 2	≥90±100 ×	300±101	~051±45~~	2 362±66
Week 4	*√276 <u>≠6</u> 2	\$344±674	312±93	ž 274±97
Week 8	220 ^{9±66} «	296±87	0 244≠82 5	248±118
Week 13	£177±76	2 197±55	212±62	229±127
Ő		Females ^b 5		
Co		\$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$	500 41.2)	2500 (♀ 197.1)
Pretreatmenta	88 37 °	427	→342±150	428±126
Week 2	44.9±153	-\$° 369±138	387±202	404±63
Week 4	361±157 >>	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	301±132	337±87
Week 8 🖉	<u>∂</u> 36€95 ~0°	°∼ 372⊕96 <	302±88	275±73
Week 12	86±85 ×	294±119	300±185	260±65

Values represent mean ± s.d. foo1:00:00(hh:mmss) Test Session

a N=12 for pretreatment week, week 2 week 5 and week 13; N=10 for week 4. b N=12 for pretreatment week and week 2; N=11 for week 4 and week 8; N=10 for week 13 control animals and N=11 for remaining dietary levels,

was for state field with the form control ( $p \le 0.05$ ; ANOVA) at any time for any dietary Summary session locomotor activit level.

#### F. Opthalmology

No treatment-related ophthalmologic findings were seen in either sex.

#### G. Climeal pathology

## 1. Clinical Chemist

Cholester (males and females) and triglyceride (females only) levels were increased in high-dose males and/or females. There were no treatment-related findings in low- or mid-dose males or females. These findings are summarized in Table 5.7.1-25.



Clinical		Dose Level ppm (mg/kg bw/day)			
Chemistry Parameters	Control	100 (♂ 6.69)	500 (♂ 33.2)	2500 5 ( 164,2)	
		Males ^a		×	
Cholesterol	57±14	58±11	110±123	\$2±14\$\$	
·		Females ^b	Ľ,		
Weights (g)	Control	100 𝒞 (♀ 8.05),	500 Q 41.2)	2500 - (5197.1) - (51)	
Cholesterol	51±3	50=10	Q 65±16	\$ <u>86±1</u>	
Trigyceride	30±8	<b>32</b> 2)≓11		57\$26*	

a N = 6

*b N* = 4-5

* Statistically different from the control ( $p \leq 0.05 A n \overline{q} v a + D u n n ett's v a$ \$ Statistically different from the control (p ≤0.05 Kruskal-Wallis

#### 2. Hematology

level in oither, There were no compound-related bematology change at ăny

#### E. Sacrifice and pathology

#### **1. Gross Pathology**

There were no compound related gross desions or males or formales at observations evident at terminal sacrifice in O males or females at any dietary level

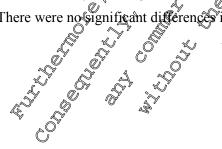
## 2. Terminal Body and Organ Weights @

Mean body weight data and mean organ weight (absolute and relative) data for perfused and nonperfused animal sacrificed at the end of the study are summarized in Fable 5.7.2-15 below. Terminal body weights were statistically decreased (10%, and 14%, respectively) in perfused and non-perfused high-dose females compared to controls. Terminal bod@weights were not different from control in males at any dietary level or in low-or mid-dose females. S  $\bigcirc$ 

Compound-related organ weight changes relative to control increased liver and kidney weights (absolute and relative) in high-dose makes and increased liver weights (absolute and relative) in high-dose females.

Other mean organ weights for males and temales that were statistically different from controls included an increase in relative liver weights in mid-dose males and females. These changes were considered not to be compound-related for one or more of the following reasons: (1) the changes were not statistically significant in both absolute and relative weights and/or (2) the changes were due to the decrease in terminal body weights. These organ weights were not different from control in low-dose males or females and thyroid weight was not affected ineither gender at any dietary level.

There were no significant differences in bran weights between control and treated perfused rats.





	Dose Level ppm (mg/kg bw/day)							
Weights (g)	Control	100 (♂ 6.69, ♀ 8.05)	500 (♂ 33.2, ♀ 41.2)	2500 (ð 164.2, 097.1)				
		Perfused Males	Ŕ	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~				
Body Wt. ^a	419.7±39.2	413.4±49.3	440.3±2009	418.1±23.0				
Brain Wt. ^b	1.916±0.107	1.906±0.075	1.918±0.069	1.825±0,139				
Brain/Body Wt. ^b	0.436±0.027	0.439±0.035	0.445±0.021	0.42 0.04 k				
	•	Non-Perfused Males	L ^O Y C					
Body Wt. ^b	399.2±34.1	391.1456.0	417.4018.1	∛375.1±8.3 ⊀				
Liver Wt. ^b	14.937±0.755	15.9% ±2.618	18.183±3.361	21.357±1.080*				
Liver/Body Wt ^b .	3.752±0.162	4079±0,203	4351±0722* \$	\$_5,695±0,265*				
Kidney Wt. ^b	2.817±0.170	2.841 0.427	Q3.573=4.171	3.362±0.289\$ √				
Kidney/Body Wt. ^b	0.711±0.085	0.727±0.032	0.853±0.258	∠ 0.897±0.085				
Thyroid Wt. ^b	0.025±0.004	(0,026±0,006 )	.0.031±0.008 @	0033±0006				
Thyroid/Body Wt. b	0.0063±0.0641	0.0067±0.0016	0.0070±0.00€	50.0087±0.0015				
		Perfused Females						
Body Wt. ^c	248.4±19,3	244,4±22.6	∠ 238,2€10.3	222.6±11.5*				
Brain Wt. ^b	1.994±0.101	1,781±0.075	1.632±0.279	\$ 1.769±0.124				
Brain/Body Wt.b	0.762 0.046	€.726 <b>±</b> €.067 0	& 0.674±0.159	0.775±0.063				
Ő		Non-Perfused Females						
Body Wt. d	225.8±19.0 🖉	220.1±1\$6	21.0.0±12/1	193.3±15.9*				
Liver Wt. do	7.612±1.033€	7.595±9.457	€8.497±0.992	10.729±0.487*				
Liver/Body Wt. d	3.271±0.358	∛ 3,460±0.202	© 4.04⊗±0.402*	5.569±0.359*				
Kidney Wt. d	<u>∢1</u> .736±0,152	) 10781±0.116	10686±0.157	1.656±0.066				
Kidner Body Wt. d	0.760-0.042	<u>0.811</u> €0.035√	©0.802±0.052	0.860±0.055				
Thyroid Wt. d	04020±0,005	0.020±0.005	≤ 0.019±0.003	0.023±0.004				
Thyroid/Body W.	0.0091 0.0020 0.0091 0.0020	0,0091±0.0015	0.0090±0.0011	0.0120±0.0023				

Values represen Qnean ±Qd

a N= 12; b N₹ 6

cN=10-11  $dS \neq 4-5$ * Statistically different from the control ( $p \leq 0.05$  Anexa + Dutitlett's tests). \$ Statistically different from the control ( $p \geq 0.05$  Keyskal-Wallis Anova + Mann-Whitney U-tests). Q,

### 3. Micropathology

There were no treatment-related findings in geural and/or non-neural tissues from perfusion-fixed highdose males opiemates that were related to administration of the test substance. Tissues from animals at lower dose Devels were, Derefore, not examined. Liver, kidney and thyroid tissues were not examined microscopically because the microscopic findings that are associated with the measured changes in tissue weight have been established in other studies.

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# H. Deficiencies

There were no deficiencies .



#### III. Conclusions

There were no compound-related effects evident by clinical observations, FOB, measures of activity or neuropathology in males or females at any dietary level. Thus, the highest dietary level tested, 2500 ppm, was the neurotoxicity NOAEL. Treatment-related findings of general toxicity observed in the absence of neurotoxicity included decreases in body weight, total body, weight gain, and food consumption in high-dose males and females. Food consumption was decreased in mid-dose females, but there was no associated effect on body weight. Systemic toxicity was demonstrated by increased cholesterol and triglyceride levels in high-dose males and/or females, as well as, increased liver and kidney weights (absolute and relative) in high-dose males and increased liver weight (absolute and relative) in high-dose females. There were no compound-related effects evident by clinical observations, FOB, measures of activity or neuropathology in males or females at any dietary level.

These results establish a NOAEL for neurotoxicology endpoints of 1642 mg/kg/day in males and 197.1 mg/kg/day in females.

#### Assessment and conclusion by application

Study meets the current guidance and the requirements in 283/2010

Thirteen weeks exposure to the test substance, resulted in treatment-related findings at the high dose consisted of decreased body weight, total body weight gain and food consumption in males and females, increased cholesterol and trig vcerice levels in males and/or females an decreased terminal body weight in females. Also, fiver and kidney weights (absolute and relative) were increased in highdose males and liver weight (absolute and relative) was increased in high-dose females. The only other finding at the mid-dose was decreased food consumption in ternales, which was not associated with any effect on body weight.

Based on neurotoxicology endpoints, a NOAEL of 2500 ppm was established for males and females (specifically, 164, and 197.1 mg/AE, 656948/kg/do) for male and female rats, respectively).

### CA 5.7.2 🔊

### Delayed polyneuropathy studies

Fluopyram is not an organophysphate compound, therefore a delayed polyneuropathy is not an expected effect, so that respective studies on such a potential were not necessary and not conducted.

# CA 5.8 Other toxicological studies

## CA 5.8.1 Toxicity studies of metabolit

For the definition of residues for risk ascessment of fluopyram it is required to assess the genotoxic potential of plant and lovestock metabolites Since not all the metabolites have genotoxicity data available an in silico assessment way conducted. The in silico assessment has been conducted using Derek Nexus Jeadscope and Toxtree software (expert and rules based predictive software respectively) (M-763978-01-1)

In addition to the in silice predictions a read-across analysis for the assessment of structural similarities of the substances was done using the following OECD QSAR Toolbox (version 4.4) profilers for genotexicity and functional groups.

Experimental data of fluopyram and of those metabolites for which experimental data exist were used for read across and therefore included in this in silico assessment.

Based on the prediction results and read across and expected exposure there is no concern that the plant and livestock metabolites of fluopyram.



In addition, to support the residue definition and the non-relevance of metabolites predicted to leachate in groundwater at concentration above 0.1 µg/L a series of toxicity studies have been carried out on the metabolite trifluoroacetic acid (TFA). Trifluoroacetic acid is a fluoparam groundwater metabolite which exceeds the groundwater PECgw threshold of 0.75 µg/L, consequently, genotoxicity and general toxicity testing are triggered. TFA is a common metabolite for several other active substances and an Ames test, an *in vitro* Mammalian Cell Gene Mutation test (mouse lymphoma L5178Y cells) and Mammalian Chromosome Aberration test (human lymphocytes) are available which were all negative, indicating that TFA does not have a genotoxic potential. Testing for general foxicity is currently ongoing. The completed studies, include an acute oral toxicity study in the rat. These studies will be submitted when available with an updated risk assessment.

A summary of available studies with TFA is presented in the table betow

Study	Concentrations of	Result A	Reference &
	[Substance] fested		
In vitro assay			
Ames mutagenesis	Without and with 89:	Negative 2	2005
	1.6, 8, 40, 200, 1000 and 5000 ug/plate		<u>M-256628-01-1</u>
Micronucleus assay	Without and with \$9:	No. Start Start	2005
using human lymphocyte cultures	340. 680 and. 1560 μg/ml	Negotive The Street	<u>M-260807-01-1</u>
Mutation at the	Without and with S9:	Negative	2005
Thymidine Kinase (to	42.5, 859, 170, 40		M-260699-01-1
locus of mouse	680, and 1360 µg/mL		<u>Ivi-200099-01-1</u>
lymphoma L51787 cells			
(MLA)			
In vivo assay			
Acute oral toxicity	2000 mg/kay bw	LD50 2000 mg/kw bw	
Wistar rat (5 females)			<u>M-444479-01-1</u>
14-day dietary toxicity	0, 600, \$200 ånd 2400	Inver findings (increased	;
study in the Wistor rat	ppm v	organ weight in	2001
(8/sex/ control and top)	(42.63, 84090 and 69.68		<u>M-202165-01-1</u>
dose group and O	mg/kg.bŵ/day 🕉	hepatocellular	<u>NI-202103-01-1</u>
5/sex/ low and mid dose	(0, 45,41, 91,08, 189.59	hypertrophy, increased	
group)	mg��g bw/day ♀), 🗞 🎽	eytochrome P-450, lauric acid hydroxylation	
		activity, specific and	
		total palmitoyl-CoA	
	mgQg bw/day ♀), A A A A A A A A A A A A A A A A A A A	oxidation activities).	
S A	V S Q	NOAEL 600 ppm	
		42.63 mg/kg bw/day ♂	
		45.41 mg/kg bw/day ♀	
28-day Gretary toxicity	0,600, 1800, 5400 and	No adverse effects	
study in the Wistar rat	46000 ppm	observed up to the top	2005
(5/sex/group)	(0, 50, 149, 436 and	dose.	M-259106-01-1
	1315 mg/kg bw/day ♂)	Changes in few clinical	
Ũ	(0, 52, 157, 457 and 1344	chemistry parameters	
	mg/kg bw/day ♀)	were not accompanied	

Table 5.8-1 Smmary of toxicology studies with Trefluoroacetic acid (TFA)



		by changes in the	
		correlated organs	
		liver weight changes	Q A
		were not correlated with	Ž Ž
		histopathological effects	
		liver enzyme activities	
		were not measured	
		NOAEL 16000 ppm	
		1315 mg/kg bw/day 🖧 🎾	
		1344 mg/kg bw/day	
90-day dietary toxicity	0, 160, 1600 and 16000	1600 ppm	2000 <u>N-283094-0151</u>
study in the Wistar rat	ppm	©nanges in	
(10/sex/ group)	(0, 9.9, 98, 1043 mg/kg	nematological and	<u>10-283094-01-1</u>
	bw/day ♂) 🖓	clinical chemistry	<u>19-20074-001</u>
	(0, 12.2, 123, 1216	hematological and clinical chemistry. parameters, organ weights and	
	$m\sigma/k\sigma hw/dav \cup $		P & A .
		weights and histopathological liver	
	40 ° ~ ~ ~	findings 🔪 🖂	
		NOAEL 600 ppm	
		≪9.9 mg/txg bw/day ∂	
		12.2 mg/kg bw/dag	
	<u> </u>		
Effect of TEA on renro	ductive and development	attoxicily of O	~ ⁰ %,
ence of the on tepto	uucure anu ac veropment	an univers	U .*

Information on potential effect of  $\overrightarrow{DFA}$  on development is available from public literature and from GLP studies published on the ECHA website. a) in a 1996, a peer-review publication (<u>M-765251-01-1</u>) presents the results of a comparative study in Repatic and renal effects of the human anesthetic halothane and its main metabolite TFA were investigated.

Halothane or its oxidative metabolite trifluoroacetic acid (PFAA) were given to Sprague-Dawley rats on gestational days 10–20. Balothane was administered by inhalation at concentrations of 50 or 500 ppm/ 6 h/ day, and TFAA was administered by gavage at doses of 75 or 150 mg/kg bw/ day

The exposed offsprings were xamined on postnatal days 3, 12, or 49 for hepatic and renal biochemistry and/or function through measurements of Serura and usinary parameters.

Neither halothane nor TO AA treatments had statistically significant effect on litter size, neonatal survival or postnatal growth.

Both prenaral halothane and TFAA exposure produced changes in liver biochemistry of newborns, as indicated by significant increases in the serum activities of glutamate dehydrogenase and aspartate aminotransferase. In addition, TFAA caused a functional deficit of the proximal tubule in newborns, as evidenced by the significant increase in the arinary excretion of b2-microglobulin. However, these hepatic and renar alterations were restricted to the early postnatal period and were no longer observed by postnatal day 49. It is concluded that prenatal exposure to relatively low levels of halothane can cause slight and transient changes in the neonbal rat liver.

b) The results of toxicity studies submitted to REACH registration dossier ECHA may be retrieved at the ECHA website. According to the summary of a GLP compliant prenatal developmental toxicity study with Trifluoroacette acid was conducted in rats according to OECD Guideline 414, there was no evidence of concern for developmental toxicity up to the highest dose of 150 mg/kg bw/day.



Dosing was well-tolerated by all females, and doses up to 150 mg/kg bw/day had no adverse effect on body weight, body weight gain, food consumption, pregnancy, c-section parameters, fetal, placental, and uterine weights, organ weights, or fetal abnormalities, variations or ossification parameters. In conclusion, under the conditions of this study, the maternal and the embryo-fetal no-observed-adverse effect-level (NOAEL) were established at 150 mg/kg bw/day TFA. Due to the non-adverse, test article-related organ weight increases, the maternal and embryo-fetal no-observed-effect-levels (NOEL) were established at 75 mg/kg bw/day (maternal) and 150 mg/kg bw/day (embryo₃ fetal).

Therefore, based on the available toxicological data on TKA there are no concerns for developmentation toxicity studies in the rat.

However, The European Chemicals Agency (ECHA) had requested an extended 1-Generation study (EOGRTS) and a developmental toxicity study of the rabbits under the regulation of Registration, Evaluation, Authorization and Restriction of Chemicals (REACH)? Since these two studies once available, would also be considered for the evaluation of pesticides, Bayer decided to cooperate with the REACH lead registrant to conduct the studies as co-owners. Within the experimental place of the developmental toxicity study in rabbits new results regarding TFA became available, which may influence the outcome of future risk assessments and which where communicated by Bayer proactively to the Commission and all European authorities where pesticides potentially forming TFA are registered. The data is preliminary, and a preliminary evaluation does not indicate an immediate concern for human health or the environment.

# Conclusion on Toxicological relevance of the metabolite TFA

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The TFA toxicological database has been evaluated by EFSA in 2016, and the current acceptable daily intake (ADI) is 90.05 mg/kg by/day based on the 90-day rat study (macertainty factor (UF) 200 for the extrapolation from sub chronic to chronic). No acute reference dose (ARfD) is needed on the basis of the available toxicological studies.

The toxicological properties of two metabolites; XE C650948-Pyridyl-Carboxylic-Acid (AE C657188) and AE C656948-Methyl Sulfoxide (AE 1344122) of Buopyram were investigated in a series of studies carried out according to relevant testing guidelines, except for the 28-day rat study on AE C657188, which was nevertheless based on the OECD testing guideline 409. bOTH

### a. Toxicity studies on AE C636948 Pyridyf-Carboxylic-Acid (AE C657188)

AE C656948-Pyridyl-Carboxetic-Acta (AE C657088) was identified in plant metabolism studies (grapes, potatees, bears, red bell peppers) and confined rotational crops and was included as part of the plant residue definition in the original dossier submission, but is no longer part of the plant residue definition. This also known to be a metabolite of another BCS fungicide (Fluopicolide, AE C638206). Hereafter are presented the toxicological data generated on this metabolite to demonstrate its non-relevance in the original theory and submission and are part of the baseline dossier. This includes an acute/oral toxicity study *3 in vitro* genotoxicity tests and a 28-day oral toxicity study in the rat. In addition, since an *in vitro* micronucleus test (M-673693-01-1) was conducted for fluopicolide to upgrade the genotoxicity package, its also included in this dossier for AIR. These studies demonstrated that AE C657188 was not genotoxic and not toxin after an acute or subacute oral administration.



Data Point:	KCA 5.8.1/01
Report Author:	
Report Year:	2000
Report Title:	2000       Image: Constraint of the constrai
	C657188 00 1B99 0002
Report No:	C008168
Document No:	Report includes Trial Nos.: TOX20044
	<u>M-197257-01-1</u>
Guideline(s) followed in	EU (=EEC): 96/54/EECB1 tris; OECD: 423
study:	EU (=EEC): 96/54/EECB1 tris; OECD: 423
Deviations from current	current guideline: Current Guideline: OECD 423, 2001
test guideline:	No deviations
Previous evaluation:	Yes, evaluated and accepted in the DAR (201). O
GLP/Officially	Yes, conducted under Go Officially recognise Clesting facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes a contract of the second s

#### **Executive Summary**

In an acute oral toxicity study, in a preliminary assay, one male and one temale rat dosed at 4000 mg/kg bw died following treatment. Doselevels for the main study were based on these findings

In the main study, two groups of three fasted, sound adult sprague Dawley rats (Hsd:Sprague-Dawley(CD))/sex were giverka single oral dose of AE C657188 (batch number RAW 244045/1, 99.7% purity) in 1% aqueous methylcellind se at 500 or 2000 mg/kg bw and were observed for 14 days.

Both dose levels were tolerated without more and no gross pathological findings. The acute lethal or dose of AE 0657188 in rats was greated than 2000 mg/kg. Therefore, no labeling is required according to Commission Directive 93/21/EEC Both dose levels were tolerated without more lities minimal clinical signs and effects on weight gain,

- A. Materials AE 665718 1. Test material Description Off-white erystatine solid and a Lot / Batch #? BAW 244045/4 ©99.7 % Purity: CAS # Stability of test compound Notreported Stable for the duration of the dosing phase 2. Vehicle and / or positive 1% adjeous methylcellulose control: 3. Test animals: Rat **Species:** Hsd:Sprague-Dawley(CD) Strain: 8 - A weeks approximately Age: Male/Female Sex: Weight at dosing: 202 to 232 g (main study) Source: Ő Acclimation period At least 6 days (main study) Special Diet Services RM1 (E) SQC expanded pellets, ad Diet libitum Water: Tap water, ad libitum



Housing:	Animals were housed in groups of the same s treatment groups, in metal stainless steel cage floors (main study)	
<b>Environmental conditions:</b>		
Temperature:	22 ± 3°C	
Humidity:	30 - 70%	4 . 4
Air changes:	Not reported	
Photoperiod:	Alternating 12-hour light and dark cxcles	
B. Study design and methods		

#### 1. In life dates: 03 May - 01 June 2000

#### 2. Animal assignment and treatment

The test substance was tested in a preliminary study at 4000 mg/kg body woight to one male and one female rat. On the basis of the results from this preliminary study, the test substance was tested at 500 and 2000 mg/kg body weight to groups consisting of three male and tomale rats. The animals were assigned to their groups without conscious bias. Following overnight fasting, each groups eccived a single dose of 500 or 2000 mg/kg of Af C657 88 (99.7% purity) by gavage. The test substance was administered in 1% w/v aqueous metholicelluhose at a volume of 20 mL/kg bw 44000 and 500 mg/kg and 10 mL/kg bw at 2000 mg/kg. Chical signs and mortality rates were determined several times on the day of administration and subsequently at least once daily for apobservation period of at least 14 days. Body weights were recorded on days 1,8 and 5. Onday 15 survioing and mals were sacrificed and all animals were necropsied and examined for gross pathological changes. 

#### 3. Statistics

The data did not warrant statistical analysis.

#### Results and discussion П.

#### A. Mortality

In the preliminary study, both rats were found dead on Day 3. In the main study, no mortalities occurred at 500 or 2000 mg/kg by

Details are provided i

Table 5.8.1-1     Poses, mortality / animals treated	
Dose (ng/kg) w	Males
	2/1
	Ø 0/3
2000 Q & X	0/3
Dose (mgAg bw)	Females
4000 × 4000	1/1
	0/3
	0/3

### B. Clinical observations

In the prelighnary oudy, a range of clinical signs was observed in both animals.

In the man study, clinical signs of reaction to treatment were confined to piloerection on Day 1 observed in males at both dose levels and females at 500 mg/kg. All animals had recovered by Day 2. No clinical signs were observed in females dosed at 2000 mg/kg.



#### C. Body weight

There was no toxicological effect on body weight gain throughout the main study, with the exception of a low body weight gain on Day 15 in two females dosed at 500 mg/kg.

#### **D.** Necropsy

In the preliminary study, macroscopic examination revealed congestion in arange of tissues in both animals. 

No abnormalities were observed at gross necropsy in the main study.

#### E. Deficiencies

No deficiencies are noted.

#### III. Conclusions

The acute lethal oral dose of AE C657188 in rats was greater than 2000 mg/kg

×.

Ø

#### Assessment and conclusion by applicant:

Study master the surrant guidance and the province of	283/2013.	
Study meets the current guidance and the requirements in	203/20015. S	
The acute lethal oral dose of AE C657188 in rats was greater	ater han 2000 r	nekg

45 CA 5 (0.1/02) 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
Bacterial mutation assay AE C657188 (plant merabolite of AE C638206) Code:
$\begin{array}{c c} AE C657188 D9 1B99 0002 \\ \hline C00$169 \\ \hline \end{array} \\ $ \\ \hline \end{array} \\ \\ \hline \end{array} \\ \hline  \\ \hline \end{array} \\ \hline \\ \\ \hline \end{array} \\ \hline \end{array} \\ \hline \\ \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \\ \\ \hline \end{array} \\ \\ \\ \hline \end{array} \\ \\ \hline \end{array}  \\ \hline \\ \\ \\ \\ \\ \end{array} \\ \\ \hline \end{array} \\ \\ \\ \\ \hline \end{array} \\ \\ \\ \\
Report includes Total Nos; Tox20045
₩-197258-01-1 [™] SQ S [™] O . C
EU (= BEC): 92/69/EBC B.13,9B.14; JMAF: 200; OECD: 471; USEPA (= EPA):
OP(5) S 870 5 100
current guideline OECD 971, 2020
Deviation: non v v v
Yes, Evaluated and accepted in the DAR (2011).
Yes conducted under GLROfficially recognised testing facilities
$\begin{array}{c} & & & & \\ & & & & \\ & & & & \\ & & & & $

### Executive Summary

Executive Summary of the metagenic potential of AE C657188 (Batch-No.: R001739, 99.7%) purity), histidine dependent auxotrophic mutants of Salmonella typhimurium, strains TA1535, TA1537, TA98 and To 100, and tryptophan dependent mutant of Escherichia coli, strain WP2uvrA/pKM101 were exposed to AE Co3718 up to 5900 µg/plate, diluted in dimethyl sulfoxide (DMSO). For each bacterial strain and dose evel, triplicate plates were used in both the presence and absence of an Aroclor 1254induced rat liver metabolic activation system (S9 mix). DMSO was also used as a negative control. Specific positive controls were used for each strain. After 72 hours of incubation at 37°C, the numbers of revertant colonies were scored using an automated colony counter.



There was no indication of a bacteriotoxic effect of AE C657188 at any dose up to and including 5000 µg/plate. AE C657188 did not cause any significant increase in the number of revertant colonies in either the presence or absence of metabolic activation.

All the positive control compounds produced expected increases in the number of revertant colonies, thereby demonstrating the sensitivity of the assay and the efficacy of the S9 mix

Therefore, AE C657188 was non-mutagenic with or without S9 mix in the plate incorporation as in the pre-incubation modification of the Bacterial Mutation Assay.

- I. Materials and methods
- A. Materials
- 1. Test material: Description Lot / Batch #: **Purity:** CAS# **Stability of test compound:**
- 2. Control materials:

Ated ⁵ μg/plate 3. Test organisms Salmonella typerimurium and Escherichia coli Species: Histidine-dependent auxomophic mutants of Salmonella typhimuritum: TA1335, TA100, TA1537 & TA98 Strain: Typtophan dependent mutants of Escherichia coli: WP24 A/pK 101 Strains of \$. Ayphimurium were obtained from Prof. Bruce Appes, University of California, USA. The strain of E. coli was Source Betained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland. 4. Test compound concentrations: For all strains with or without S9 mix: 0, 5, 15, 50, 150, 500, Plate incorporation as 1500 and 5000 µg/plate For all strains with or without S9 mix: 0, 50, 150, 500, 1500 and Pre-incubation assa 5000 µg/plate The S9 fraction was isolated from the livers of Aroclor 1254 5. Metabolie activatio induced rats. Study design and methods 1. In life dates: 28 April - 08 May 2000



#### 2. Plate incorporation assay

The test substance was added to cultures of the five tester strains at seven concentrations separated by ca half-log10 intervals. The highest concentration of AE C657188 tested was 50 mg/mL in the closen solvent, which provided a final concentration of 5000 µg/plate. This is the standard limit concentration of recommended in the regulatory guidelines. The negative control was the chosen solvent. Gimetbyl sulfoxide. The appropriate positive controls were also included.

An aliquot of 0.1 ml of a 10 hour bacterial culture and 0.5 ml S9 mix or 0.5 ml 0.1 M sodum physiphate buffer (pH 7.4) were placed in glass bottles. An aliquot of  $100 \,\mu$ l of the test solution was added followed immediately by 2 ml of molten agar containing 0.05 mM histine/bioon/tryptophan. The maxture was thoroughly shaken and overlaid onto previously prepared Petri dishes containing 25 ml minimal agar. Each Petri dish was individually labeled with a unique code corresponding to a sheet, identifying the dish's contents. Three Petri dishes were used for each dose level. Plates were also prepared without the addition of bacteria in order to assess the sterility of the test substance, S9 mix and sodium phosphate buffer. All plates were incubated at 37°C for *ca* 77 hours. After this period the appearance of the background bacterial lawn was examined and revertant colonies counted using a Section automated colony counter.

Any toxic effects of the test substance would be detected by a substantial teduction in revertant colony counts or by the absence of a complete background bacterial lawn. In the absence of any toxic effects the top concentration normally used in the second test would be the same as that used in the first. If toxic effects were observed a lower concentration may be chosen. It should be ensured that if a lower concentration was chosen, signs of bacterial inhibition are present at the top concentration. Ideally a minimum of three non-toxic concentrations should be obtained.

#### 3. Pre-incubation assay:

As a clear negative response vars obtained in the first test, a variation to the test procedure was used for the second. The variation used was the ore-incubation assay in which the bottles, which contained mixtures of bacteria, buffer or \$9 mix and test solution, were incubated at 37°C for 30 minutes with shaking before the addition of the agar overlay, 5000 µg/plate was gain chosen as the top concentration, but only five dose levels were used as it was known from the first test that the test substance was nontoxic.

#### 4. Acceptance criteria

The mean of the solvent control revertant colony numbers for each strain should lie within the 99% confidence limits of the in-house historical control range. The positive control compounds must cause at least a doubling of mean revertant colony numbers over the negative control.

#### 5. Assessment criteria

**a**. If treatment with a test substance produces an increase in revertant colony numbers of at least twice the concurrent solvent controls, with some evidence of a positive dose-relationship, in two separate experiments, with any bacterial strain either in the presence or absence of S9 mix, it is considered to show evidence of mutagenee activity in this test system.

**b**. If treatment with a test substance does not produce reproducible increases of at least 1.5 times the concurrent solvent controls in either mutation test, it is considered to show no evidence of mutagenic activity in this system.

c. If the results obtained fail to satisfy the criteria for a clear "positive" or "negative" response given in paragraphs a and b additional testing may be performed in order to resolve the issue of the test substance's nutagenic activity in the test system. Should an increase in revertant colony numbers then be observed which satisfies paragraph a, the substance is considered to show evidence of mutagenic activity in this test system.



If no clear "positive" response can be obtained, the test data may be subjected to analysis to determine the statistical significance of any observed increases in revertant colony numbers.

#### **Results and discussion** II.

0 No signs of toxicity were observed towards the tester strains in either mutation test. No prespitation was observed. No evidence of mutagenic activity was seen at any dose level of AE C657188 in either mutation test.

The concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activi of the liver preparations.

Results are presented in the following tables:

Table 5.8.1-2 Revertant colony counts obtained per plate using S. typhimurlum strains T 100, TA 1535 and TA 1537 and E. coli strain P2uvrA/pKM101 experiment 1

		×.	r O	<u></u> ~*		-~~	
Treatment	Concentration	Metaboli		Mean re	evertant g	colony co	ints in strains
	(µg/plate)	activation	ŤХ	<b>PA</b>		TA	WP2uvrA/oKM161
		+/~\$9	`~y98 _'	≫100≫	1535	1537	
AE C657188	5000	- 2	23 a		15	Ĵ~Ĩ	C 1200 2
	1500		25	Ĵ21	<b>KJ</b> 7	چ 9 گ	184 ^O
	500		°~25	≪ <b>)9</b> 7 ≈		0 9 r 115	× 184 × 199
	150 Q	×	22 🔊	, 98 S	16	L.	~~~~ <b>\\$</b> 84
	50 🥡	- < ?	27.0	9 <b>5</b> ,7	43	M1 🔊	Õ 💪 200
	15 🔊		20	4∳3	£¥3 ∂	10 C	198
	5 [%] (k	~ Č	£27	//p ·	S 13 S	1Ø	187
Solvent control	<b>, ()</b> O	- 8	21	105	1,21,5	A A	× 197
	Y A.	A A		<i>S</i>		KV A	×
AE C657188	≪ 5000	¢ 0	26	×120	[≫] 13 &	. 8 %	182
	2 1500 ×	v+ 0	\$26	×111	140	12/	192
Å	N° 5400 ∿. U)		250	113	16	<i>2</i> 12	179
Ô	150 √√	¢ v	24	123	A6	S 11	200
	50		×27	<u>م</u> ا08 ه	\$ 14 ·	<b>10</b>	185
		+ .	[©] 23 🔏	🎽 105 🗘	130	11	203
		+	27 0	112	<u>,</u> 106	9	202
Solvence ontrol		+	ZG	<b>Å</b>	15	13	192
k~y°		, S	°~~ .		Ý		
Sodium azide 🌋	ര് മം ര്	- 🍡 🕺	V NA	\$ 558	197	NA	NA
AF-2	A0.05	2	NA	NOT	NA	NA	2134
9-Aminoacridine			ха	ŇA	NA	117	NA
2-nitrofluorene		- ~	×400 ~	ŇA	NA	NA	NA
Benzo[a]pyrene	O5 a0″	+	257	804	NA	72	NA
200	<u>2</u> - 10*5		NA	NA	133	NA	1866
aminoarthracene			$\sim$				
AE-2: 2.(2-Furyl)-3-(5	more 2 feed 1) across	imide 🔊	'N				

AF-2: 2/(2-Furyl)-3-(5/n)tro-2-foryl) acropamide @ NA . not applicable

NA fnot applicable *: 2 µg/plate for TA 1\$35, and 10 µg/plate for WP2uvrA



= 0.1

**T** . . . . . . . .

Treatment	Concentration	Metabolic		Mean re	evertant c	olony co	unts in strains 🖉
	(µg/plate)	activation	TA	ТА	ТА	TA	WP2uvrA/pKM101
		+/- S9	98	100	1535	1537	
AE C657188	5000	-	22	105	14	8	189 5
	1500	-	23	98	10	₄ 8	Q25
	500	-	23	113	14	×71	
	150	-	21	<b>()</b> 98	12	§ 12	L 201 × 201
	50	-	24	<b>%1</b> 11	8	8	
Solvent control	0	-	24 🔍	111	l¶0∛	9	
				-	X	(	
AE C657188	5000	+	<u></u> @3 [%]	94 😞	13	?7.Q"	0 [×] 198
	1500	+	[~] 28	_ 104 _	/ 11~y	12	213 ~
	500	+ &	24 👰		j5	≪13 ,	× ×218
	150	+ 0	23	ØÃ		≥°13 °C	
	50	+	<u>`</u> 26	<b>Q</b> 15	[©] 12 ₄	100	
Solvent control	0	+ ~ ~	× ⁹ 28 ~	y ⁷ 98 Ö	15	$\hat{Q}$	× 196 5
		<u> </u>			<u> </u>	<u>s á</u>	
Sodium azide	0.5	ôy yy	N A	~\$02	A 168	U NAS	O'NA
AF-2	0.05	y- 0'	ŇA		y NAS	NA	<u>\$</u> 22,95
9-Aminoacridine	30 🚿	- 6) (	D NA Ĉ	r NAS	ŊĄ	۵0	Ŭ [™] NA
2-nitrofluorene	1	<i>₹</i> , 0°	166	ŊŴ.	<b>A</b>	NA ô	NA NA
Benzo[a]pyrene	5	+ ~ ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	250	630	NA C	85	O NA
2-	2 - 10*	V+ 50 5	NA	ØΝΑ	140	MA	l 1939
aminoanthracene		ampide 5			$\swarrow'$	~~ ~	°₽ ≽

NA : not applicable *: 2 µg/plate for TA 153 and 10 µg/plate for WP24VrA

#### III. Conclusions

It is concluded that APC65788 showed to evidence of onutagenic activity in this *in vitro* bacterial system. Ś Ø

Assessment and conclusion by applicant: Study meets the current guidance and the requirements in 283/2013. It is concluded that AEC657188 showed no evidence of mutagenic activity in this *in vitro* bacterial

the showed not evidence



Data Point:	KCA 5.8.1/03
Report Author:	
Report Year:	2003
Report Title:	AE C657188 (metabolite of AE C638206): Induction of chromosome aberrations
	in cultured human peripheral blood lymphocytes 🔬 🔊
Report No:	C034337
Document No:	Report includes Trial Nos.: 2014/68
	<u>M-234744-01-1</u>
Guideline(s) followed in	ICH: S 2 A; OECD: 473 (1997)
study:	
Deviations from current	Current guideline: OECD 473, 2016 Deviation: 200 instead of 300 metaphases were scored. This deviation is not
test guideline:	
	considered to impact the integrity of the strey.
Previous evaluation:	Yes, evaluated and accepted in the DAR $(2011)$ $0^{\circ}$ $\sqrt{2}$ $\sqrt{2}$
GLP/Officially	Yes, conducted under GLP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes the second s

#### **Executive Summary**

In this *in vitro* assessment of the classogenic potential of AE C65/188 (brtch OP2150091, putity 99.1%), the test compound was tested in an *in vitro* extogenetics as ay using duplicate human lymphocyte cultures prepared from the posted blood of three remale donors in two independent experiments. Treatments covering a broad range of doses separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S9). The test article was dissolved in sterile anhydrous analytical grade dimethyl sulfoxide (DMSOs and the highest dose level used, 2256  $\mu$ g/mL, was equivalent to 10 mM.

Treatment of cultures with AE C657188 on the absence and the presence of S-9 resulted in frequencies of cells with structural aberrations which were similar to those in concurrent negative controls. Numbers of aberrant cells (exclusing gaps) in all treated cultures fell within historical negative control ranges.

No increases in the frequency of cells with numerical aborrations, which exceeded the historical negative control range, were observed in cultures treated with AE C657188 in the absence and presence of S-9.

AE C657188 did not induce thromosome aberrations in cultured human peripheral blood lymphocytes, when tested to a maximum concentration of 10 mM (following 3+17 hour treatments in the absence and presence of S-9) of to its limit of cytotoxicity (following 20+0 hour treatment in the absence of S-9). AE C657188 was therefore considered not to be clastogence for mammalian cells *in vitro*.

- I. Materials and methods
- A. Materials
- 1. Test material: AE-6571 White powder Description Lot / Batch# OP2150091 99.1% **Purity:** Newreported CAS # Stability of test compound: Stable for the duration of the study 2. Control materials: Negative: Culture medium Solvent: DMSO Positive: 4-Nitroquinoline 1-oxide (NQO), Supplier: Aldrich Chemical Co., Gillingham, UK, in the absence of liver S-9 Cyclophosphamide (CPA), Supplier: Sigma Chemical Co., Poole, UK, in the presence of liver S-9



3. Test organisms:	
Cell line	Human lymphocyte cultures
Source	Pooled blood from three female donors
4. Test compound	
concentrations:	
Experiment 1:	without S9 mix (3 + 17 h) at 739.2, 1444 2256µg/mL with S9 mix (3 + 17 h) at 378.5, 924.1, 2256 µg/mL without S9 mix (20 + 0 h) at 320.9, 377.5, 723.2 µg/mL
Experiment 1.	with S9 mix $(3 + 17 \text{ h})$ at 378.5, 924.1, 2256 µg/mL $\sim$
<b>Experiment 2:</b>	with S9 mix $(3 + 17 h)$ at 378.5, 924.1, 2256 µg/mL without S9 mix $(20 + 0 h)$ at 320.9, 377.5, 723.2 µg/mL
Experiment 2:	with S9 mix $(3 + 17)$ at 1001, 1385, 2256 µg/mL $(3 + 17)$

#### **B.** Study design and methods

10 March - 29 April 2003 1. In life dates:

The in vitro cytogenetic test is a mutagenicity test system for the detection of chromosome aberrations in cultured mammalian cells. The test is designed to detect structural aberrations (chromatid and Ŕ chromosome aberrations) in cells at their first post-treatment mitosis. Ø

AE C657188 (batch OP2150091, purity 99.1%) was tested in an in vitro Sytogenetics assay using duplicate human lymphocyte cultures prepared from the pooled blood of three female donors in two independent experiments. Treatments overing a bread range of doses, separated by parrow intervals, were performed both in the absence and presence of metabolic activation (S). The test article was dissolved in sterile anhydrous analytical grade dimethyl sulfoxide (DMSO) and the highest dose level used, 2256 µg/mL, was equivalent to 10 mM.

In Experiment 1, treatment in the absence and presence of S-9 was for 3 hours followed by a 17-hour recovery period prior to hapyest (1)17). The S-Quised was prepared from a tat liver post-mitochondrial fraction (S-9) from Aroclor 1254 induced animals. The test article dose levels for chromosome analysis were selected by evaluating the effect of AEC657 08 on pritotic index. Chromosome aberrations were analyzed at three dose levels. The highest concentration chosen for analysis, 2256 µg/mL, induced approximately 34% and 37% mitodic inhibition (reduction in mitotic index) in the absence and presence of S-9 respectively.

lable	5.8.1-4 Perperiment 1 Treatment details	
S-9	Treatment + recovery Vehicle Concentration (µg/mL) (hours) vehicle AE C657188	Positive control
_	3+D7 0° 0° 739.Q 1444 2256	NQO, 5.00 μg/mL
+	3+17 2 256	CPA, 6.25 μg/mL

^a: Vehicle confoil was DMS Conly: NOO: 4-Nitroquitroline 1-oxide; CPA: cyclophosphamide

In Experiment 2, treatment in the absence of S-9 was continuous for 20 hours. Treatment in the presence of S-9 was for 3 hours only followed by a 17 hour recovery period prior to harvest (3+17). Chromosome aberrations were analyzed at three dose levels and the highest concentrations chosen for analysis, 723.2 µg/mL and 2256 µg/mL induced approximately 47% and 20% mitotic inhibition in the absence and presence of S-9 respectively.

Table 5.8.1-5	Experi	ment 2 Tr	eatment	details
		v <u>s</u>	~Q	

S-9 Steatment + recovery	^y Vehicle control	Concentration (µg/mL) AE C657188	Positive control
20 <del>-0</del> 20-0 2	$0^{\mathrm{a}}$	320.9, 377.5, 723.2	NQO, 2.50 μg/mL
+ 3+17	0 ^a	1001, 1385, 2256	CPA, 3.125 μg/L

^a: Vehicle control was DMSO only; NQO: 4-Nitroquinoline 1-oxide; CPA: cyclophosphamide



#### II. **Results and discussion**

The proportion of cells with structural aberrations in these cultures fell within historical solvent control ranges. 4-Nitroquinoline 1-oxide (NQO) and cyclophosphamide (CPA) were employed as positive control chemicals in the absence and presence of liver S-9 respectively. Cells receiving these positive? controls were sampled in each experiment, 20 hours after the start of treatment; both compound induced statistically significant increases in the proportion of cells with structural abercations.

Treatment of cultures with AE C657188 in the absence and the presence of 8-9 resulted m frequencies of cells with structural aberrations which were similar to those in concurrent negative controls. Numbers of aberrant cells (excluding gaps) in all treated cultures fell within historical negative control ranges.

No increases in the frequency of cells with numerical aperrations, which exceeded the historical period control range, were observed in cultures treated with AE C657188 in the absence and presence of S-9 , Ó

Table 5.8.1-6:	Mean mitotic indices and nu	mber of ab	berrant h	uman lyn	phocytes,	including	and
	excluding gaps – Experime@	t <b>`1</b> `		â s	ð ð	e	1

( $\mu g/mL$ )       vactivation       ( $\pi me th$ )	Treatment	Concentration	Metabolic	Tratmont	Mitotiy	Abarmant	Aber
ALL C03/103 $137.2$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$	Ireatment	Concentration (ug/mL)	Antivation	Treatment		* Aderrant	Ader
ALL C03/103 $137.2$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$		(µg/mL)			Omnex	Including	ExQu
ALL C03/103 $137.2$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$ $-6$		, Ô ^v	\$ . S			gaps	la gan
144       -       2       8.5       3       1         2256       -       - $3$ $75$ $2$ 1         Solvent control       0       - $3$ $75$ $2$ 1         AE C657188 $38.5$ +       - $4$ $75$ $2$ $2$ AE C657188 $38.5$ + $ 75$ $3$ $11.4$ $2$ $2$ AE C657188 $38.5$ + $ 75$ $3$ $11.4$ $ 10.4$ $1$ $1$ $2256$ + $ 3$ $11.1$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ $0$ </td <td>AE C657188</td> <td>739.2</td> <td>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~</td> <td></td> <td></td> <td></td> <td>55 //</td>	AE C657188	739.2	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~				55 //
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$			ř – (	S 20			1
AE C657188 $348.5$ $924.1$ $+$ $ 36$ $12.4$ $ 1$ $1$ $924.1$ $                                                                                            -$ <		2256 %	D - 4			\$ <u>2</u>	1
AE C657188 $338.5$ $9$ $4$ $124$ $1124$ $1$ $1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ $924.1$ <td>Solvent control</td> <td>$\sim 0_{\rm s}$</td> <td></td> <td>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~</td> <td>\$11.4°S</td> <td>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~</td> <td>2</td>	Solvent control	$\sim 0_{\rm s}$		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	\$11.4°S	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	2
$\frac{2256}{14.8} = \frac{14.8}{2} = \frac{2}{1}$	AE C657188	× 378.5 ×	. 🔊		12.4	× 1	1
Solvent control $\sqrt[6]{9}$ $0$ $\sqrt[6]{9}$ $\sqrt[6]$	, ů	924.1	\$ + ^{\$}	∑ 3 <u>©</u>	M.1	[♥] 0	0
Solvent control $\sqrt[6]{9}$ $0$ $\sqrt[6]{9}$ $\sqrt[6]$		<u>○ 2256</u>		Y 33	Ø 9.3 S	3	2
NQ $\phi$ $5$ $\phi$ $A$ $\phi$ $7$ $0$ $3$ $\phi$ $-$ 27 19*	Solvent control		<u> </u>	<u>~</u> 3 2	14.8	2	1
<b>CPA C 6 S C + C - 61</b> 45 [×] ** p< 0.001 statistically/significantly different from controls Fisher's test IQO : 4-Nitroquinoling - oxide PA : Cyclophosphatoide	NQQ	5, 9	A A	° 3	~- ~	27	19**
** p< 0:001 statistically significantly different from controls Fisher's test IQO : 4-Nitroquinoling -oxide PA : Cyclophosphagide	<b>≈C</b> ₽A	U 695 &	,+ <i>\$</i>	, <u>`</u>	× -	61	45**
				Ĩ			



Ŵ

No.

Table 5.8.1-7	Mean mitotic indices and number of aberrant human lymphocytes, including and	
	excluding gaps – Experiment 2	

						a, [°]
Treatment	Concentration (µg/mL)	Metabolic activation +/- S9	Treatment time (h)	Mitotic index	Aberrant cells brcluding gaps	Aberfant colls Excluding gaps
AE C657188	320.9	-	20	7.6	0 . Ć	
	377.5	-	<u>Č</u> O	4	2	
	723.2	-	20	A20	10	
Solvent control	0	- 4	© 20	Q 7.5 。		
AE C657188	1001	+ 0	3 🕎	_1 <b>2</b> A	Q" 30"	
	1385	-Ky	\$° 3 \$	×9.4 ×		
	2256			8.20		2 2
Solvent control	0	× + ×	3	ÂŶ9.4 (		
NQO	2.5		Ø 20 ⁵		<i>3</i> 6 <i>×</i>	3⊕***
СРА	3.125	×+ ~~	~3~~		S 41 C	<i>\$</i> 36***

< 0.001 statistically signific NQO: 4-Nitroquinoline 1-oxide Ŷ O

CPA : Cyclophosphamide

III. Conclusions AE C657188 did not induce chromosome abeatations in cultured human peripheral blood lymphocytes, when tested to a maximum concentration of 10 mMC following 3-17 hour treatments in the absence and presence of S-9) or to its limit of Stotox wity (following 20+0 hour reatment in the absence of S-9). AE C657188 was therefore considered not to be clastogenic for matomalia@ cells in vitro.

## Assessment and conclusion by applicant:

Study preets the current guidance and the requirements in 283/2013.

Study meets the current guidance and the requirements in 283/2013. AE C657188 did not induce chromosome aberrations in cultured human peripheral blood lymphocytes and is therefore considered not to be clastogenic for mammalian cells *in vitro*.



Data Point:	KCA 5.8.1/04
Report Author:	
Report Year:	2019
Report Title:	AE C657188: Micronucleus test in human lymphocytes In vitro
Report No:	1969602
Document No:	<u>M-673693-01-1</u>
Guideline(s) followed in	OECD Test Guideline No. 487 (July 2016)
study:	
Deviations from current	None
test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially	Yes, conducted under GLP/Officially recognised testing facilities
recognised testing	Yes, conducted under GLP/Officially recognised testing fabilities
facilities:	
Acceptability/Reliability:	Yes of the stand o

#### **Executive summary:**

AE C657188 was evaluated for its potential to induce micronucles in human lymphosytes *in vitro*, in two independent experiments; Experiment comprised a four hour exposure period in the presence and absence of metabolic activation (provided by \$9) whilst experiment II provided 20-hour exposure period in the absence of \$9. Concentrations ap to 2000 ag mL overe tested in duplicate and 1000 binucleated cells/culture were examined for sytogenetic damage

In experiment I, no cytotoxicity or precipitation was observed (+f-S9) up to the highest evaluated concentration. Similarly, in experiment II (-S9), no cytotoxicity or precipitation was observed at the highest tested concentration. Therefore, concentrations up to the limit concentration of 2000 µg/mL were evaluated.

No relevant increases in the numbers of micronucleated cells were observed following treatment with the test item either in the presence of absence of S9 nix. However, in experiment I in the absence of S9, a dose dependency, tested via trend test, was observed ( $p \neq 0.030$ ). Since none of the values exceeded the historical control data or were statistically significantly increased, this finding is considered to be biologically irrelevant.

Appropriate reference substances wave the expected statistically significant increase in the number of micronucleated cells.

The test substance ABC65D88 can be considered neither clastogenic nor an eugenic under the conditions of this *in Stro* micronucleus test, when tested up to the highest possible concentration, both in the presence and absence of motabolic activation.

Materials and methods A. Materials 1. Test material AEC657188 ∼99.9 % (w/w) BCOO 6709-1-1 Batch no.: 16th October 2020 Expiry date: 2. VehiCle and/or positive Vehicle: DMSO control Positive controls:



#### <u>-S9</u>

Mitomycin C (MMC), 0.8  $\mu$ g/mL (98% purity, dissolved in deionized . water)

Demecolcine, 100 ng/mL (purity ≥98%, dissolved in deionized water) +S9

Cyclophosphamide (CPA), 15 μg/ml (purity 97 303%, dissolved in saline)

#### 3. Activation:

Metabolic activation was provided by S9-mix derived from phenobarbral/B-naphthoffavone induced rat liver homogenate; each batch of prepared S9 has been routinely svaluated for its ability to be ivate known mutagens.

An appropriate quantity of S9 was thawed and mixed with S9 coractor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. So mix contained MgCl₂ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium orthors hosphate-buffer (100 mM  $_{\odot}$ H 7.45  $_{\odot}$ 

The protein concentration of the S9 preparation used for this stordy was 30.4 mg/mk.

#### 4. Cell cultures and media:

#### Cells

Blood was drawn from healthy non-smoking conors, not receiving medication. Blood from a female donor (27 years old) and a male donor (22 years old) were used in experiments 1 and 1, respectively. The lymphocytes have been shown to respond well to PHA and positive control substances; furthermore, both donors have an established low incidence of micronuclei in perpherak blood symphocytes.

#### Media

Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hours following collection. The culture medium was Dulbeeco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1), supplemented with 200 mM GlutaMAXO. The medium was further supplemented with penicillin streptomycin (100 b/mL/100  $\mu$ g/mL), the mitogen PHA (3  $\mu$ g/mL), 10 % FBS (foetal bovine serum), 10 mM HEPES and the ampcoagulant heparin (125 U.S.P.-U/mL).

All incubations were carried out at 37°C with 5.5 % CO2 in hundidified air.

### 5. Test compound concentrations used

Test concentrations for the first experiment were 0 (solvent control), 15.2, 26.5, 46.6, 81.2, 142, 249, 435, 762, 1333 & 2006 µg/mL, both with and without S9 mix.

In the second experiment continuous (20 how) treatment was used in the absence of S9 mix at test concentrations of 0 (solvent control),  $(42, 249, 435, 462, 1333 \& 2000 \,\mu\text{g/mL})$ , in the absence of S9 mix.

### B. Test Performance

Experimental phase: 34th August 2019 to 7th September 2019

### 1. Preliminary assay

A preliminate cytotoxicite test was performed to determine the concentrations to be used in the main experiment. Cytotoxicite (characterized by the percentages of reduction in the CBPI in comparison to the controls and expressed as % cytostasis) was determined from 500 cells/culture. The experimental conditions in this pre-experimental phase were identical to those described for the mutagenicity assay.

The pre-test was performed with 10 concentrations of the test item separated by no more than a factor of  $\sqrt{10}$  and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hours (with and without S9 mix). The preparation interval was 40 hours after start of the exposure.



This preliminary test was designated Experiment I since the cultures fulfilled the criteria for cytogenetic evaluation and appropriate concentrations could be selected for evaluation.

#### 2. Cytogenetic experiment

Cells were subject to either 4 hours pulse exposure (with and without S9; experiment I) or the hours continuous exposure (without S9; experiment II). The succeeding procedure for cell preparation was the same for both exposure periods.

#### Pulse exposure

Approximately 48 hours following seeding, two blood cultures of 10 mL each, were set up in parallel in 25 cm² cell culture flasks (for each test item concentration). The culture medium was replaced with serum-free medium containing the test item. For the freatment with metabolic activation 50 µL 89 mix per mL culture medium was added. After 4 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells resuspended in and washed with "same G (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • HZO, 192 mg/L Na2HPO4 • 2 H2O and 150 mg/L KH2PO4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 40 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

#### Continuous exposure

Approximately 48 hours following seeding, two blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test nem. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells were spunded in and washed with "saline G". The vashing procedure was repeated once as described. Following washing, the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4  $\mu$ g/mL) was added and the cells were cultured for a further 20 hours until preparation.

The activation as a was performed independently with an identical procedure, except for the addition of the S9-mix. In these experiments 9 mL this tead of 20 mL cubure medium and additionally 1 mL of S9-mix were added to the thasks for the treatment period, resulting in a concentration of 5% S9 mix in the cultures. The number of 64 G resistant mutants and evability were determined as in the nonactivation assay.

#### Preparation of cells

Following the precedures above (approximately 40 hours from the start of treatment), the cultures were harvested by contribution. The cells were spundown by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. They the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at \$7 °C for 20 minutes. I mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope since. The cells were stained with Giemsa.

### 3. Acceptance Criteria

The micronucles assay is decined acceptable if it fulfils the following criteria:

- The concurrent solvent control will normally be within the laboratory historical solvent control data range. The average mutant frequency of the vehicle controls should not exceed 25x10⁻⁶ constant.
- The concurrent positive controls should produce a statistically significant increase in the micronucleus frequency and should be within the laboratory historical positive control data



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range. The positive controls should induce a mutant frequency at least 3 times that of the controls

- Cell proliferation criteria in the solvent control are considered acceptable.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- The quality of the slides should allow the evaluation of an adequate number of concentrations
- The criteria for the selection of top concentrations should be met

Any mutant frequencies not meeting these criteria were excluded from the statistical anal

#### 4. Assessment criteria

Providing that all of the acceptability criteria are forfilled, a test item is considered to be clearly negative Ŋ if, in all of the experimental conditions examined:

- None of the test item concentrations Okhibit Da statistically significant increase compared with the concurrent solvent control Õ
- There is no concentration-related increase •
- The results in all evaluated test them concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered mable to induce chromosome breaks and or gain or loss in this test ¢, system. Ô

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control  $^{\bigcirc}$
- The increase is concentration-related in a least one experimental condition
- The results are outside the range of the laboratory historical solver control data (95% control limit realized as 95% confidence interval

When all of the criteria are met, the test tem is then considered able to induce chromosome breaks and/or gain@r loss in this test system. m

There is no requirement for verification of a clear positive or negative response.

In case the response neither clearly negative for clearly positive as described above and/or in order to assist in establishing the biological relevance of a result the data should be evaluated by expert judgement and/or further investigations. Scoting additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. narrow concentration spacing. other metabolic activation conditions, ite S9 concentration or S9 origin) could be useful.

#### 5. Statistical analysis

Statistical significance was confirmed by the Chy square test (p < 0.05), using a validated test script of "R", a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those galues that indicated an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression was performed using a validated test script of "R", to assess a possible dose dependency in the rates of more roucleated cells. The number of micronucleated cells obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p value (probability value) is below 0.05.

Both, bi@ogical and statistical significance were considered together.



#### II. **Results and discussion**

In experiment I neither precipitation nor cytotoxicity was observed up to the highest applied concentration, either in the presence or absence of S9, and no relevant influence on osmolarity of pH was observed. Similarly, in experiment II, no precipitation or cytotoxicity was observed up to the highest applied concentration. The highest concentration applied (2000µg/ml) was therefore the limit concentration.

concentration. The results of both experiments, with and without metabolic activation, are summarised in the table? below:

					Ň	O	* a ⁰ .
Exp.	Preparation	Test item	Proliferation		Micronucleated		control data
	interval	concentration	index 📿	5 [°] in %*	∕≫ cell∮	95% Ctrl	Min - Max
		in µg/mL	CBPI 🎸		9 in 29** 7	lionit 💊	
		Exposure	e period 4 brs w	vith@ut S9 m	tix 🖄 🔊		. 1
Ι	40 hrs	Solvent control ¹	1.80		0.20 U	0.01 - 1.20	0.50 - 1.55
		Positive control ²	k69 ^~	14.1	∑ [∞] 11. <b>30</b> ^S	2.66 - 22.74	\$95-28.60
		762	<b>∦</b> .79 √	Ø.9 Å	() <b>(£375</b> , `>		
		1333	Q 1.78	2.6	×0.35 č		Ö
		2000	0 [°] 1.95 [°] 2	n.c.	0.505		, ÔQ
	Exposure period 20 hrs without S9 mix 2 2 2						
II	40 hrs	Solvent control ¹	× 1.83 m		2 \$30 8	0.00-1.14	0.05 - 1.60
		Positive control ³	>>> 1.43	048.5	€¥.20 ^s	1.95 - 6,44	1.95 - 8.80
		762 📎 🐇	1.82	0.A	~ 0.25 °	Ô _e	
		133 <i>3</i>	ب ب ب ب ب ب ب ب	9.7	. 0.20 .~		
		2000	1.62	<b>2</b> 5.6	0.20		
		Softvent control	re period 4 hrs	with S9-mi	x X &		
Ι	40 hrs	Solvent control	× 1002 2	22	0.30	\$0.00 − 1.24	0.10 - 1.30
	Å	Positive control?	×.35	.~ <b>50</b> .8	<b>3</b> (75 ^s (0)	1.01 - 7.34	1.80 - 8.85
	Ô		1.65	×8.7 ×	<b>20</b> .50		
	<u></u>	P333	1459		0.40		
		∂ ² 2000€	1.62	140	O Q 50		
	Ŵ			"0" a		•	•

Table 5.8.1-8	Summary o	of results o	of experiment	Land II
1 4010 0.001 0	Summary	or results o	i experiment	I and II

For the positive control groups and the terr item treatment groups the values are related to the solvent controls *

The number of micromicleated cells was determined in Sample of 2000 Dinucleated cells **

S The number of micronucleated cells is statistically significantly higher than corresponding control values

n.c Not calculated as the CBPI's equação or higher than the solvent control value

- 1 DMSO 0.5 % (v/v)
- 2 MMC 0.&@g/mL
- 3 Demecolcine 00°ng/mIC

4 ľ5 μg≱n£l CPA

In both independent experiments, nonelevant increases in the numbers of micronucleated cells were observed following treatment with the test them ether in the presence or absence of S9 mix. However, in experiment 1 in the absence of 89, a dose dependency tested via trend test was observed (p=0.039). Since none of the values exceeded the historic control data or were statistically significantly increased. this finding is considered to be biologically or relevant.

Ø)

Demecolcine (100 mg/mL) MMC (0.8 µg/mL) and CPA (15 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei, thus confirming the validity of the assay to detect clastogens/aneugens



#### III. Conclusions

The test substance AE C657188 did not induce micronuclei in this *in vitro* micronucleus test in human lymphocytes, when tested up to precipitative concentrations.

Therefore AE C657188 is considered to be neither clastogenic nor aneugenic under the conditions this assay.

#### Assessment and conclusion by applicant:

The study was conducted according to OECD TG 487 and is value and acceptable to assess the clastogenicity of AE C657188 *in vitro*. AE C6571886's neither clastogenic nor meugenic under the conditions of this study.

Data Point:	KCA581/05 O W S S S O O A
<b>Report Author:</b>	
<b>Report Year:</b>	
Report Title:	AE C657188 V79/PPRT-test in vitre for the detection of pauced forward
	mutations & & & & & & & & & & & & & & & & & & &
Report No:	AE C657188@V79/f4PRT-test in vitro for the detection of induced forwards C034731
Document No:	Report includes Trial Bos.: A 600551 C O
Guideline(s) followed in	EU = EEC): 2000/32/EC; OECD: 476; USEPA (#EPA); OPPTS@70.5300
study:	
Deviations from	Current guidebare: OECD 476, 2016 V V
current test guideline:	Deviation: None.
Previous evaluation: 🔊	Current guidebne: OECD 476, 2016 Deviațion: None. Yest evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GDP/Officially recognised testing facilities
recognised testing	
facilities: 🖉 🔊	
Acceptability Reliabilit	$\begin{array}{cccccccccccccccccccccccccccccccccccc$
y: O ^y	

#### Executive Summary

The purpose of the study was to assess the point mutagenic potential of AE C657188 (Batch-No.: D0526, 97.7 to 99.1 % purity) at the hypoxanthine guarante phosphoribosyl transferase (HPRT) locus in V79 cells.

AE C657188 was tested at concentrations up to 5000  $\mu$ g/mL with or without metabolic activation. Without and with S9 mix, AE C657088 induced no decreases in survival to treatment or in relative population growth. However, AE C657088 was tested up to its limit of solubility under culture conditions. Precipitation occurred in the culture medium at 4000  $\mu$ g/mL and above, so that at 5000  $\mu$ g/mL no further evaluation was possible. Adequate positive controls (ethyl methanesulfonate and dimethylbenzapthracene) were used for each experiment.

With and without \$9, mix there was no biologically relevant increase in mutant frequency above that of the vehicle controls. Ethyl methanesulfonate and dimethylbenzanthracene induced clear mutagenic effects demonstrating the sensitivity of the test system and the activity of the S9 mix.

Based on these results, AF C657188 was considered to be non-mutagenic in the V79/HPRT forward mutation assay, both with and without metabolic activation.

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Ø)

#### I. Materials and methods

#### A. Materials

	AE C657188 Light grey powder D0526 97.7 to 99.1% 80194-68-9 Stable for the duration of the study Negative: Culture medium [Eagle's minimal essential medium supplemented with 1% L-glutamine, 1% MEM-vitamins, 9% MEM NEAA, 1% penicillin/streptomycin and 10% fetal calf serum (FCS)
1. Test material:	AE C657188
Description	Light grev powder
Lot / Batch #:	D0526
Purity:	97.7 to 99.1%
CAS #	80194-68-9
Stability of test compound:	Stable for the duration of the stude $\mathcal{O}$ $\mathcal{O}$ $\mathcal{O}$ $\mathcal{O}$
2. Control materials:	Negative: Culture medium [Eagle's minimal essential medium , O
	supplemented with 1% L-glutamine, 1% MICM-vitamins, 2%
	MEM NEAA 1% penicillin/streptonycin and 10% fetal calf
	serum (FCS)
	Solvent: DMSO for AE C657 188 and Dimetry Iben 2 anthracene
	not exceeding 1% (v/c) in the culture medium. No solven,
	needed for effort methanesultionate as it is a liquid as a liquid
	Positive: Ethyl methanesulfonate (EMS), a diffectly alkylating
	agent, used at a final concentration of 900 µg/mL in non- O
	activation trials. No no so so a
, and a second se	activation trials. Dimethylbenzanthracene (DMBA), promutagen requiring a
() ()	metabolic activation, used at a that cancentration of 20 µg/mL
, Î	how trials with So mixed and a second s
3. Test organisms:	For trials with S9 mix Chinese manster V79 bing cells Cells obtained from Prof. C Speik University of Ulm, Cerrenny, These cells have since the profession of the maintain
3. Test organisms:	
Cell line:	Chinese mamster V /9 bing cells
	Cells obtained from throf. C Spent University of Ulm,
Source:	Gernany. These cells have since been recloned to maintain
	katyotypic stability. They have a model chromosome number of
	22 and arrapid population doubling time (10 to 14 hours)
Culture condition:	Olncultation performed at $37\%$ in a humidified atmosphere with about 5% $602$ .
	AP C657488 was used at concentrations ranging from 1 to 5000
4. Test compound 🖉 🛒	fig/mL in the lonal sytotox weity assay and from 16 to 5000
concentrations: 🔊 🚀 🧹	$\mu g/mL$ in the mutagenic assays.
	The S9 fraction was isolated from the livers of Aroclor 1254
	aduced male Sprague Dawley rats. The preparation dated from
	February 04, 2003 (protein content 26.4 mg/mL) and was kept
5. Metabolic activation	frogen at - 30°C. The batch was tested for contamination and
	stotoxicity prior to use in the first study. Cofactors were
	freshly dissolved in sodium phosphate buffer (150 mM, pH 7.4)
B Stindy design and mathings	
Culture condition: 4. Test compound concentrations: 5. Metabolic activation: B. Study design and methods 1. In life dates: 20 May	
B. Study design and methods 1. In life dates: 20 May -	17 June 2003

The selection of V79 forward mutations is based on the resistance of induced mutants to the purine analogue 6 thiographine (6 TG). This resistance is a result of a mutation at the X-chromosome-linked HPRT locus rendering the cells unable to use 6-TG for DNA synthesis. Therefore, cell colonies formed in the presence of 6-TG are considered to represent mutants at the HPRT gene.

# 2. Determination of cytotoxicity

Exponentially growing V79 cells were plated in 20 mL culture medium in a 275 mL flask (4x106 cells per flasks). For each concentration, one culture was available. After attachment (16 to 24 ours later), cells were exposed without S9 mix to vehicle alone or to a range of concentrations of the test substance



for 5 hours in 20 mL medium containing 2% FCS. In experiments with metabolic activation 1 mL of medium was replaced by 1 mL of S9 mix. Thereafter, each cell monolayer was washed with PBS, trypsinized and replated in 5 mL culture medium at a density of 200 cells into 3 Petri dishes (diameter of 60 mm). These dishes were incubated for 6 to 8 days to allow colony development. Thereafter, colonies were fixed with 95% methanol, stained with Giemsa (Merck; stock solution diluted 1.5 with deionized water) and counted automatically using an Artek counter, when there was no interference by precipitation on the plates or colouration of the plates. Cytotoxicity was expressed by comparison of colonies in treated cultures versus vehicle control cultures (relative cloning efficiency).

#### 3. Treatment protocol without metabolic activation::

Exponentially growing V79 cells were plated in 20 mL culture medium in two 75 cm2 flass per concentration (4x106 cells per flask) including all control groups. After attachment (16 to 24 hours later), the cells were exposed to vehicle alone or to a range of concentrations of the test substance for 5 hours in 20 mL culture medium with reduced serum content (2%). Thereatter, cell monorayers were washed with PBS, trypsinized and replated in 20 mL culture medium using 1.5x906 cells per 75 cm2 flask and in 5 mL culture medium using 200 cells per Petr dish chameter of 60 mm. One flask and 3 Petri dishes were used per culture. These dishes were incubated for 6 to 8 days to allow colony development and to determine the cytotoxicity associated with each fest substance directly after treatment (survival to treatment).

Cells in 75 cm2 flasks were incubated to permit growth and expression of induced mutations. Cells were subcultured (= count 1, normally after 3 days) by rescaling  $15 \times 106$  cells into 20 mL of medium in 75 cm2 flasks. At the end of the expression period (=count 2, normally a total of 6 days), cultures were reseeded in Petri dishes (diameter of 100 mm) at  $3\times105$  cells per dish (8 dishes per culture) in 20 mL culture medium without hypoxamine but containing 10 µg/mL 6 flG for selection of mutants. In addition, 200 cells per dish (diameter of 60 pm, 3 dishes per culture) were seeded in 5 mL culture medium to determine the absolute cloning efficiency for each concentration. After incubation for 6 to 8 days, the colonies were fixed, stained with Giensa and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.

Two trials were performed

### 4. Parameters assessed

The parameter "survival to treatment" in % was determined on the basis of the following calculation:

Mean number of colonies (treated cultures)×100

```
Mean number of Polonies (vehicle control cultures)
```

The "absolute population growth" was valculated using the following formula: Absolute population growth (for each culture) = cell count 1 x cell count 2 The parameter "relative population growth" shows the cumulative growth of the treated cell populations, relative to the vehicle control.

Absolute population growth treated culture×100

Absolute population gowth of corresponding vehicle control culture

The ability of cells to form colonies at the time of mutant selection is measured by the parameter "absolute coloning efficiency". It is expressed in %.

 $\underbrace{\underbrace{}_{k}}_{k} \underbrace{\underbrace{}_{k}}_{k} \underbrace{\underbrace{}_{k}}_{k} \underbrace{Mean number of colonies per dish \times 100}_{200}$ 



0

Dy Stranger

The "mutant frequency" is calculated for each group by dividing the total number of mutant colonies by the number of cells seeded (usually 8-10 plates at  $3x10^5$  cells per plate), corrected for the absolute cloning efficiency. The mutant frequency is expressed as 6-TG resistant mutants par  $10^6$  clonable cells.

Total number of mutant colonies×100

Number of evaluated dishes  $\times 3 \times 10^5 \times C.E$ 

#### 5. Acceptance criteria

- The average cloning efficiency of the negative and vehicle controls should be at least 50%

- The average mutant frequency of the vehicle control should not exceed 25 x  $10^{50}$  cells

- The mutant frequency of the two cultures of the volticle and /or the negative control should differ only to an acceptable extent. As a rule of thumb, the difference of mutant frequencies should not be greater than  $5 \ge 10-6$ .

- The positive control should induce an average mutant requency of a least three that of the vehicle control.

- If not limited by the solubility of the test substance in the vehicle the highest concentration should induce cytotoxicity of about 80 to 90% or should be a concentration where precipitation occurs in the medium. The survival at the lowest concentration should be in the rappe of the negative control.

- For the calculation of an acceptable mutant frequency at least 5 dishes per culture should be available and relative survival to treatment, relative population growth and absolute cloning efficiency should be 10% or greater.

However, these criteria may be overruled by good scientific judgment.

### 6. Assessment criterio.

- Mutant frequencies were only used for assessment, of at least 5 dishes per culture were available and relative survival to treatment, relative population growth and absolute cloning efficiency were 10% or greater.

- A trial was considered positive if a concentration-related and in parallel cultures reproducible increase in mutant frequencies was observed. To be relevant the increase in mutant frequencies should be at least two to three times that of the highest negative or vehicle control value observed in the respective trial. If this result was reproduced in a second trial, the test substance was considered to be mutagenic.

- Despite these criteria a positive result was only considered relevant, if no significant change in osmolality compared to the vehicle control was observed. Otherwise, unphysiological culture conditions may be the reason for the positive result of the vehicle control was observed.

- A test substance was judged as equivocal if there was no strictly concentration related increase in mutation frequencies but if one or more concentrations induced a reproducible and biologically relevant increase in mutant frequencies in all trials.

- An assay was considered negative if no reproducible and relevant increases of mutant frequencies were observed.

However, these criteria may be overruled by good scientific judgment.

## 7. Statistical analysis:

The statistical analysis relied on the mutant frequencies which were submitted to a weighted analysis of variance as well as to a weighted recursive regression, both with Poisson derived weights.



The two mutant frequency values obtained per group were, although somewhat related, considered as independent measurements thus increasing the power of the statistical tests applied. Since the protocol of the HPRT assay required at least two independent trials, the overall analysis without respectively with activation was the most important one for classifying substances into mutagens and non-mutagens However, separate analyses were run for each trial in order to examine the consistency of the results.

All acceptable groups were included in the weighted analysis of variance followed by pairwise comparisons to the vehicle control on a nominal significance level of p = 0.05 using the Dunnet test. The regression analysis part was performed on the basis of the actual concentrations thereby omitting the positive, negative and vehicle controls. If there was significant concentration related increase of the mutant frequency (p = 0.05) in the main analysis the highest concentration was dropped and the  $\bigcirc$ analysis repeated. This procedure was repeated untite p > 0.05. In that way elipsimated conceptrations were flagged correspondingly.

II. Results and discussion A. General remarks: In the absence of S9 mix Chinese hamster 779 cells were exposed to AE C657188 at concentrations of up to and including 5000 µg/mL. Substance precipitation occurred in the medium at the concentration of 4000 µg/mL and above. Therefore, the test without \$9 mix was no longer interpretable at 5000 µg/mL. With S9 mix cells were exposed to concentrations of up to and including 5000 w/mL Substance precipitation occurred in the medium at the concentration of \$000 µg/mL. Therefore, the test with S9 mix was no longer interpretable at 5000 µg/ml Å

Good cloning conditions were demonstrated by the absolute cloning efficiency for the vehicle controls ranging from 59.5% to 112.3% and from 54,0% to 87.3% without and with metabolic activation, respectively.

#### **B.** Mutation assay:

**B. Mutation assay:** The test system proved to be sensitive of both experimental conditions (activation and non-activation) since treatment with the positive controls caused a biologically relevant increase in mutant frequencies

There was no relevant increase in mutant frequencies after treatment with AE C657188 at any concentration (up to the highest dose  $\sqrt{5000}$  µg/mL) either with  $\frac{1}{2}$  without metabolic activation.

the second secon



Table 5.8.1-9	Relative survival and mean mutation frequency (mutant colonies per 1 millions	cells)	
	– Experiment 1-without S9 mix	°	

Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency
AE C657188	5000	ND	ND 5 P
	1600	164.9	1.70 ( S
	500	158.5	0° 2.25° 2°
	160	139.8	
	50	<u>ک</u> 96.6 کې	L.1.75 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
	16	V 137.2 Q	
Negative control	0	م ۲7.9 م	0000 0000 00 00 00 00 00 00 00 00 00 00
Solvent control	0	100. <del>0</del> °	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
EMS	900	43,9 . 0	
AE C657188	4000 🕵	_@°\$P22.5 🖉 📣	v 000 √v
	2000	130AF ~~	Q 2.50 A
	1000	74.6	
	500	\$1.4 × ×	1,80
	160	× × 88.3 ×	Q ^Y Q.35 O
	<u>190</u> ~ ~	× ~ 78,9 ~ ~	5 5 1.35 5 1'30
	Q 16 0	\$6.2 O O	Č 1°30
Negative control	Q B O	96.20 ^Y O	~~~ & <b>2</b> .05
Solvent control			© 1.15
EMS		14.8	× 329.2
D : not determined MS : ethylmethanesulfonate			

Relative surviver and mean mutation frequency (mutant colonies per 1 millions cells) Table 5.8.1-10:

Treatment	Concentration & mL	Relative survival (%)	
Treatment S	Concentration (ug/mL)	Relative sprvival (%)	Mutation frequency
AE 0005/188	, <u>2</u> 5000, <u>5</u> <u>5</u> 1800		ND
AE @57188	× 4800	S 107 2	0.60
	L 500 0 L	<u>لارم 203.1</u>	0.70
AE 0657188	3 $4$ $500$ $3$ $1$ $4$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $500$ $3$ $1$ $1$ $500$ $3$ $1$ $1$ $500$ $3$ $1$ $1$ $1$ $1$ $1$ $1$ $1$ $1$ $1$ $1$	0° 0° 99.1	1.80
		¥ 6 84.65	1.85
4		O 82 5	1.25
Negative control		103.4	1.40
		100.0	0.90
DMBA Q	20 20	63.3	80.75
AE C657988	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	128.85	1.15
	2000	129.65	1.65
	2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 200 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2000 2	95.1	1.15
	× 500	90.3	2.65
	160	93.5	2.15
AE C657488	50	112.3	2.25
$\bigcirc$	16	96.35	3.35



Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency	
Negative control	0	115.6	0.70 Q°	
Solvent control	0	100.0	3.40	
DMBA	20	47.6	Ž 71.00 C &	
ND : not determined			Q	
DMBA : dimethylbenzanthrace			I mutation assay.	
III. Conclusions	red to be non mutagenic in	Č A		
AE C657188 was conside	red to be non mutagenic in	n this \$79/HPRT forward	I mutation assay.	
Assessment and equal	sion by applicants			
Assessment and conclu	sion by applicant:			
Study meets the current	guidance and the requiren	nendos in 283/20139 🗸		
AE C657188 was consid	lered to be non mutagenic	in this 79/HPRT forwar	rd mutation assay. 🧳	
Data Point: Report Author:	KCA 5.8.1/06			
Report Year:	2001 2 6 6			
Report Title:		ninary 28 day toxicity study	in the rat by dietary	
neport me.	administration Version 2			
Report No:	C034882 0 4			
Document No:	<u>91-204933-03</u>			
Guideline(s) followed in	Preliminary study based on	ØECD 07 (1995)		
study:			×	
Deviations from current	Current guideling. none		^r Y	
test guideline:		<u>~~~~~~~~~~~</u>		
Previous evaluation:	Yes, evaluated and accepte	d in the DAR (2911).		
Previous evaluation:				
GLP/Officially $\mathcal{O}^{*}$ Set conducted under GLP/Officially recognized testing facilities				
recognised lesting				
facilities to the second se				
Acceptability/Reliability: Ves and a second se				
Executive Summary		Ŭ ô		
	y a.v (020 a. ~ (			

AE C657188 (batch number D 9526, 99.1 % parity) was administered continuously via the diet to groups of Sprague-Dawley rats () sex/group) for 28 days at concentrations of 20, 200, 2000 and 20000 ppm. A similarly constituted group received untreated diet and acted as a control. Animals were observed daily for mortality and clinical stens. Body weight and food consumption were recorded once weekly. During the acclinicatization phase all anomals were subjected to an ophthalmic examination. All animals at 0 and 20000 ppm were re-examined at the end of Week 3. Hematology, plasma chemistry and urine parameters were determined at the end of the study. All animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically. The only treatment-related tinding occurred at the high dose of 20000 ppm and consisted of a slight decrease in food consumption in females ()9% on weeks 2 and 4, and 15% ( $p \le 0.05$ ) on week 3, and a slight decrease in inorganic obosphorus concentration of 12% ( $p \le 0.01$ ) in males, compared to the controls. In the absence of any other corresponding findings, these findings were considered to be non-adverse.

# Conclusion:

The No Observed Adverse Effect Level (NOAEL) was considered to be 20000 ppm (equivalent to 1574 and 1581 mg/kg/day in males and females, respectively).



#### I. Materials and methods

AE C657188

Not reported

D 0526

99.1%

none

Rat

A white powder

- A. Materials
- Test material: Description Lot / Batch #: Purity: CAS # Stability of test compound:
   Vehicle and / or positive control:
   Test animals: Species: Strain:

Age: Weight at dosing: Source: Acclimation period:

Diet:

Water:

**Housing:** 

Temperature: Humidity:

Air changes:

**Photoperio** 

Environmental conditions

40-70% Approximately 40-15 air changes per bour Alternating 12-four light and dark cycles (7 am- 7 pm)

Stable in rodent diet for a period covering the stady

220 to 243 g tor the males A161 to 198 g for the Temales

o days of the rodent powdered and irradiated the AQC-10, P1 from

U.A.R. (Usine d'Alimentation Rationnelle, Villemoisson-sur-

suspended stainless steel

Sprague Rawley@rl: CDrSD)IGS

6 to 7 weeks approximately

Orge, France), addibitum ( Municipal tap Water, ad libitum

wire mesh eage

-24°/

Animal's were caged individually in

- B. Study design
- 1. In life dates:
- 📣 4 June 11 Auty 20
- 2. Animal assignment and treatment

There were 5 animals of each second dose group. Animals were assigned to dose groups randomly by body weight. AE C657188 was administered in the diet dor 28 days to Sprague Dawley rats at the following dose – 0, 20, 200 2000 and 20000 ppm (equating to 1.50, 15.0, 149 and 1574 mg/kg/day in males and 1.63, 15.9, 162 and 1581 mg/kg/day in females). A negative control group received plain diet. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service National Institute of Health, NIH publication N°86-23, revised 1985) and "La Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE au 24 Novembre 1986".

### 3. Diet preparation and analysis

AE C657188 was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. The stability was demonstrated during the course of the study at concentrations of 20 and 20000 ppm for a time which covered the period of usage and storage for the study. Homogeneity at the lowest and highest dietary concentrations and concentration checks at all dose levels were within the range 86-108% of nominal concentrations.



Test group	Concentration in diet (ppm)	Dose per animal (study averages)		Animals	assigned
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Mate	Ferstale
1	0	0	0	4 ⁰⁰ 5	
2	20	1.5	1.63	\$ 5	
3	200	15.0	15.9	Î 5 E	×5 0°
4	2000	149	لَمْ 162 أَنْ	5 🏑	5 5 Q 0
5	20000	1574	1581 Q	° 520	
Statistics			x ~ .	<del>v</del> ^Q , Ó	

#### 4. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous, a modified t-test was performed. Group means were compared at the 5% and 1% revels of significance. Statistical analyses were carried out using the Path/Tox data collection system.

#### C. Methods

#### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs overe recorded at least once daily focall animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

An ophthalmic examination was performed on all animals during the acclimatization phase and on control and high bose goup animals at the end of Week 3. After institution of an atropinic agent (Mydriaticum, Merck Sharp and Dohme) each eye was examined by means of an indirect ophthalmoscope.

#### 2. Body weight

Body weights were recorded at least once during the acclimate ation phase, on the first day of test substance administration, then at weekly intervals throughout the treatment periods. Diet-fasted animals were weighed before negropsy.

#### 3. Food consumption

Food consumption was recorded weekly the weekly mean achieved dosage intake for each week and for weeks to 4 was calculated for each sex.

## 4. Clinical pathology

On study days 22 or 23, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet-fasted overnight prior to blood sampling and anesthetized by inhabition of Isofturane. Blood was collected on EDTA for hematology, on lithium heparin for plasma and slot activator for serum for clinical chemistry and on sodium citrate for coagulation parameters.

The following bematology parameters were assayed using a Technicon H1 (Bayer Diagnostics, Puteaux, France): red blood cell coant, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count and differential count evaluation and platelet count. A blood smear of Wright's stain was examined when results of Advia 120 determinations were abnormal. Prothrombin time was assayed on an ACL 3000 (Instrumentation Laboratory, Paris, France).



A blood smear was prepared and stained with Wright stain. It was examined when the results of Technicon H1 were abnormal.

Reticulocytes were stained with brilliant cresyl blue. A smear was prepared but not examined since no significant red blood cell changes were observed.

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, chloride, sodium, potassium, calcium, inorganic phosphorus, total cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase activities, total protein and albumin concentrations were assayed on serum samples using an Hutachi 91 (Roche Diagnostics, Meylan, France).

On study days 29 or 30, overnight urine samples were collected from all animals Feed and water were not accessible during urine collection. Any significant change in the general appearance of the urine was recorded. Urinary volume was measured, pH was assayed using a Cliffitek 200+ and Ames Multistix dipsticks (Bayer Diagnostics, Puteaux, Francey Urinary refractive index was measured using an Atago clinical refractometer (Bioblock Scientific, Ilfkirich, France). Gluosse, bifrubin ketone bodies, occult blood, protein and urobilinogen were assayed using a Cliffitek 200+ and Ames Multistic dipsticks (Bayer Diagnostics, Puteaux, France). Microscopic examination of the urinary sediment was performed after centrifugation of the urine. The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was graded.

#### 6. Sacrifice and pathology

On study day 29 a complete necrops, was performed on all surveying animals Animals were deeply anesthetized by Isoflurane initialation, then examplinated before necropsy. All animals were diet-fasted prior to scheduled sacrifice. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded sampled and exampled microscopically. Adrenal gland, brain, kidney fiver, wary, spleen destis, thyroid gland (with parathyroid gland) were weighed fresh at scheduled sacrifice only daired organs were weighed together.

The following organs or dissues were sampled, adrenal glafd, aorfa, articular surface (femoro-tibial), bone (sternum) bone marrow (sternum), brain, epididymis, oesophagus, exorbital (lachrymal) gland, eye and optic nerve, Harderian gland, heart, imestine (duodenum, jejunum, ileum, caecum, colon, rectum), kieney, larynx/pharynx, faver, lung, longh nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracie, lumbar), spiten, stomach, submaxillary (salivary) gland, testis, thymus, thyroid gland (with parathyroid) tongue, trachea, urinary bladder, uterus (with cervix), vagina. A bone marrow smear was prepared from femur, stomed with May-Grünwald Giemsa, but not examined. Tissues samples were fixed by immersion for neutral buffered 10% formalin with the exception of the eye optic nerve, Harderian gland, porstate spleen, seminal vesicles, testis, thymus, hyroid gland, uterus and vagina from fall the animals in the control and high dose groups. Significant macroscopic findings were also examined in all dose groups.

### II. Results and discussion

# A. Clinical signs and morgality

## 1. Clinical signs of toxicity

There were no reatmont-related clinical signs in any group.

## 2. Mortality

There was no mortality in any group.



#### 3. Ophthalmology examination

No ocular abnormalities were induced by treatment.

#### B. Body weight and body weight gain

No relevant changes in mean body weight or mean body weight gain were observed at any dose either sex.

#### C. Food consumption and compound intake

At 20000 ppm in females, food intake was reduced by \$% on weeks and 4, but the effect was about statistically significant, and by 15% ( $p\leq0.05$ ) on week 3 compared to control values. There was n impact on food consumption in males at any dose level or in females at 2000, 200 r 20 ppm. 0

Compound intake details are presented in Table

#### D. Hematology, clinical chemistry, and urigalysis

#### 1. Hematology

Hematological examination showed no treatment related finding

 $\bigcirc$ 

#### 2. Clinical Chemistry

At 20000 ppm in males, clinical chemistry changes consisted of a 12%  $p \le 0.0^{10}$  reduction in inorganic 200 or 20 ppm/in mates or at any dose level in females. phosphorus concentration, when compared to controls

Ø

No relevant changes were  $ob_{s} e_{v} e_{v} at 2000_{s}$ 

#### 3. Urinalysis

Urinalysis revealed no treatment-related finding

### E. Sacrifice and pastology

There were no changes inferminal body weights or organ weights at any dose in either sex.

There were no meatment-related macroscope at any dose in either sex of microscopic changes at 20000 ppm in either Sex.

### F. Deficiencies

thisstudy No specific deficiencies ed in

#### Conclusions III.

y 200 202 4 be 20000 ppm (equivalent to 1574 and 1581 mg/kg/day in males and The NOAEL was considered to females, respectively).

O

## Assessment and conclusion by applicant

Study meets the current guidance and the requirements in 283.

The NOAEL was considered to be 20000 ppm (equivalent to 1574 and 1581 mg/kg/day in males and females, respectively ~Q



#### b. <u>Toxicity studies on AE C656948-Methyl Sulfoxide (AE 1344122)</u>

AE C656948-Methyl Sulfoxide (AE 1344122) was identified in low amounts in a confined rotational crop study, but is no longer part of the plant residue definition for fluopyram. It is also known to be a metabolite of another BCS fungicide (Fluopicolide, AE C638206). Hereafter are presented the toxicological data generated on this metabolite to demonstrate its non-relevance that were included on the original fluopyram submission and form part of the baseline dossier. This includes an acute oral toxicity study, 3 *in vitro* genotoxicity tests and a 28-day oral toxicity study in the rat. In addition since an *in vitro* micronucleus test (M-673685-01-1) has been conducted for fluopyram dossier for AfR. These studies demonstrate that AE 1344122 was not genotoxic, and not toxic after acute or subacute, oral administration.

Data Point:	KCA 5 8 1/07 % & & & &
Report Author:	
Report Year:	
Report Title:	Acute toxicity in the rat after oral administration AE 1044122 Project AE
Report No:	
Document No:	Report includes Torial Nos AT00486 N AT A A
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Guideline(s) followed in	EU ( EEC): 67/548/DEC , 2506.1967, OE D: No. 23, 1792.2001; USEPA
study:	(=EPA): OPPTS \$79.1100, August 1998, 2 0
Deviations from current	current guideline Current/Guid@ine: OECD 423/2001
test guideline:	No deviations
Previous evaluation:	Yes evaluated and secepted in the BAR (2011).
GLP/Officially recognised testing	Yes conducted under GLP Officially recognise (stesting facilities
recognised testing	
Acceptability/Rehability.	

#### Executive Summary

In an acefee oral toxicity study two groups of three disted, young adult Wistar female rats (HsdCpb:Wu) were given a single oral dose of AF 1344-22 (bach number YG3228, 98.8% purity) in demineralized water with the aid of 2% Cremophor EL at 2009 mg/kg bw and were observed for 14 days.

This dose level was to bated without nortalities, conical agens and effects on weight gain, and no gross pathological ondings

According to OECD guideline 423, the LDS0 cut off of AE 1344122 is higher than 5000 mg/kg (category 5/unclassified in the Globally Hamonizer Classification System).

J. Materials and method	
A. Materials	
1 Toot a Governor a	$\sum_{j=0}^{N} \alpha_{j}$
1. Test material S	AT\$\$1344122
1. Test material Description Let/Batch #:	White powder
Let Batch #: A	YG3228
Rurity;	98.8 %
CAS A	Not reported
Stability of test compound:	Stable for the duration of the dosing period
2. Vehicle and / or positive control:	Demineralized water with the aid of 2% Cremophor EL



3. Test animals: **Species:** Rat HsdCpb:Wu Strain: 9 to 10 weeks approximately Age: 148 to 166 g (main study) Weight at dosing: Source: **Acclimation period:** At least 5 days Provimi Kliba 3883.0.15 Maus/Ratte Paltung, Kias Pau **Diet:** Switzerland, ad libitum Tap water, ad libitum Water: Animals were group housed cor **Housing:** cages **Environmental conditions: Temperature:** 20 - 24 **Humidity:** 50 - 60% Air changes: Approximately and dạr k cy **Photoperiod:** Alternatir **B.** Study design and methods Aprál 1. In life dates: 04 April - 24

#### 2. Animal assignment and treatment

The test substance was tested, bing a stepwise procedure, each step using three animals of the same sex (females). The test substance was initially 2000 ng/kg/body weight to a group consisting three female rats. Based on the results from this first group a second group consisting three female rats was also dosed at 2000 mg/kg body weight. The animals were assigned to their groups by randomization. Following overnight fasting, oach group received single dose of 2000 mg/kg of AE 1344122 (98.8% purity) by gavage. The test substance was administered in demineralized water with the aid of 2% Cremophor EL at a volume of 10mL/kg bw Chinical signs and mortality rates were determined several times on the day of administration and subsequently at least once daily for an observation period of at least 14 days. Body weights were recorded on days 1, 8 and 5. Or day 15, surviving animals were sacrificed and all animals were peropsied and examined for gross pathological changes.

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### 3. Statistics

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The data did not wat ant statistical ana
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#### Results and discussion II.

### A. Mortality

In the main study, no mortalities oc 00 mg/kg bw.

#### Table 5.8.1-12 Doses mortality / animals torated

Dose (mg/kg/bw)	Females
2000 (1 step)	0/3
2000@2 nd step)	0/3

## B. Climical observations

Nochnical signs were observed.

## C. Body weight

There was no toxicological effect on body weight or body weight gain throughout the study.



#### **D.** Necropsy

In the preliminary study, macroscopic examination revealed congestion in a range of tissues in both animals.

No treatment-related abnormalities were observed at gross necropsy for the main study.

#### E. Deficiencies

No deficiencies are noted.

#### III. Conclusions

According to OECD guideline 423, the LD50 cut off of AE 13441220s higher than 5000 m

#### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013. According to OECD guideline 423, the kD50 cor off of AE 4344122 is higher than 5000 mg/kg

Data Point:	KCA 5.84/08 0 7 7 7 8 8 8
Report Author:	
Report Year:	
Report Title:	Salpionella/microsome test - Plate incorporation and preincubation method Code:
	AE 134 122 ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~
Report No:	
Document No:	Report includes Trial Nos WT00500 4
	$M_{2}$ 1825 $J_{1-1}$ $J_{2}$
Guideline(s) follower in	EU (=ECC): 2000/32/EC B.13/04; OECD: 471; USEPA (=EPA): OPPTS
study:	870.5400 Curfent guddeline: OECD 471, 1997 DeviationOnone Yes, evaluated and accepted in the DAR (2014)
study: Deviations from current test guideline	Curfent gudeline: DECD 471, 1997
test guideline	Deviation Dione & O &
Previous evaluation."	Yes, evaluated and accepted in the DAR (2014)
GLP/Officially	Yes, evaluated and accepted in the DAR (2014) Yes, conducted under GLP/Officially recognised testing facilities
recognised testing	
identities.	
Acceptability/Reliability:	Yes y y y y
	$\begin{array}{cccccccccccccccccccccccccccccccccccc$

#### Executive Summary

In this *in staro* assessment of the mutagenic potential of AE 1344122 (Batch-No.: YG3228, 98.8% purity), histidine dependent anxotrophic mutants of Salmonella typhimurium, strains TA 1535, TA 1537, TA 98, TA 100 and TA 102 were exposed to AE 1344122 up to 5000  $\mu$ g/plate, diluted in dimethyl sulfacted (DMSO). For each bacterial strain and dose level, triplicate plates were used in both the presence and absence of an Aroctor 1254-induced rat liver metabolic activation system (S9 mix). DMSO was also used as a negative control. Specific positive controls were used for each strain. After 48 hours of incubation at 37° , the numbers of revoltant colonies were scored using an automated colony counter.

There was no indication of a bacteriotoxic effect of AE 1344122 at any dose up to and including 158  $\mu$ g/plate At higher doses, the substance had only a weak, strain-specific bacteriotoxic effect. Due to the weakness of this effect this range could nevertheless be used up to 5000  $\mu$ g/plate for assessment purposes.

AE 13 122 did not cause any significant increase in the number of revertant colonies in either the presence or absence of metabolic activation.



All the positive control compounds produced expected increases in the number of revertant colonies, thereby demonstrating the sensitivity of the assay and the efficacy of the S9 mix. Additional supporting positive control data are given in document KIIA 5.8 /13; :: 2009; M-344996-01-1 (Check of S9 metabolizing capacity S9 fraction, batch of February 4, 2003).

- S9 metabolizing capacity S9 fraction, batch of February 4, 2003).
  Therefore, AE 1344122 was non-mutagenic with or without S9 mix in the plate precorporation as wellows in the pre-incubation modification of the Salmonella/microsome test.
  I. Materials and methods
  A. Materials
  A. Materials
  A. Material:

  Description
  Lot / Batch #:
  YG3228
  Purity:
  CAS #
  Stability of test compound:

  Control materials:

  Negative:
  Solvent / final DMSO Sedium azide (Serva) for TA 535 aO10 µg plate, ( Solvent / final ∕DMSØ concentration: Nitroforantoin (Sigma) for TA 100at 0.2 µg/plate 4-Nigo-1,2 phenylene diamine (Merck Schuchardt) for TA 1567 at 10 ug/plate and JA 98 at 0.5 ug/plates Mitomycin C (Fluka) for T & 102 at 0.2 µg ate only in plate **Positive:** Uncorporation plate ( Custon hydroperoxide (Sigma) for TA 102 in pre-incubation trials only at 50 yag/plate, () 2-Aminoanthracene (Aldrich) for the activating effect of the S9 3. Test organisms: Specific mix in all stearins and µg/plate. Salmonella typprimurium LT2/mutants Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA Strain: 98 and TA 102 Strains obtained from Prof. Bruce Ames in 1997 and stored in Source 4. Metabolic activation The laboratory since fren The \$9 fraction was isolated from the livers of Aroclor 1254 Acst concentrations: Preliminary cytoroxicity assay (+/-S9) and number induced male Sprague Dawley rats. 5. Test concentrations: assay pre-For all strams with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 ag/plate For all strains with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 µg/plate Q



#### **B.** Test performance

#### **1. In life dates:** 22 July 2003 – 28 July 2003

The Salmonella/microsome test is a screening method which detects point mutations caused by chemical agents *in vitro*. Auxotrophic mutants of Salmonella typhimurium are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups.

#### 2. Plate incorporation assays

AE 1344122 or the positive control material was dissolved in 0.1 mL of DMSO. DMSO (0.1 mL) containing AE 1344122 or positive controls were added to glass vessels with 0.1 mL of bacterial cultures grown overnight, 0.5 mL of S9 mix or buffer and 2 mL of soft agar. The mixture was placed in a waterbath at 45°C for 30 seconds, shaken and overland onto Petri dishes containing solid agar. After 48 hours of incubation at 37°C, the numbers of revertant colonies were scored using an automated colony counter. Three plates were used, both with and without S9 mix for each strain and cose. The doses for the first trial were routinely determined on the basis of a standard protocol with a maximum dose of 5000 µg/plate and at least 5 additional doses. If less than three doses were used for assessment, at least two repeats were performed.

#### 3. Pre-incubation assay

An independent repeat test was performed as pre-incubation of the previously described mixture in a water bath at 37°C for 20 minuted At the end of the preincubation period 2 mL of molten soft agar were added to the tubes, the content mixed and plated onto petri dishes with solid agar. After 48 hours of incubation at 37°C, the numbers of revertant colonies were also scored using an automated colony counter.

#### 4. Acceptance criteria: 🏷

The negative controls had to be within the expected range. The positive controls had to show sufficient effects. Title determinations had to demonstrate sufficient bacterial density in the suspension. The title was determined by the total bacterial counts taken on two plates for each concentration studied with S9 mix.

#### 5. Assessment criteria

A reproducible and dose related increase in mutant colonies of at least one strain was considered to be positive. For TA 1535/7 A 100 and TA 98, this increase should be about twice that of negative controls, whereas for TA 1537, at least a threefold increase should be reached. For TA 102 and increase of about 100 mutants should be reached. Otherwise, the result was considered as negative.

# II. Results and discussion of

There was no indication of a bacteriotocic effect of AP 1344122 at any dose up to and including 158  $\mu$ g/plate. Aigher doses had only a weak, strain-specific bacteriotoxic effect. Therefore they could nevertheless be used for assessment purposes up to the highest dose of 5000  $\mu$ g/plate.

Results are presented in the tables below. There was no relevant increase in mutant counts at any concentration of AE 1344122 compared to the negative controls both with and without metabolic activation (S9 mix). Those results were confirmed in the 2nd experiment with a pre-incubation step. The positive controls caused a significant increase in the number of revertant colonies compared to the controls depionstrating the sensitivity of the system.

E ST C ST ST



Table 5.8.1-13	Revertant colony counts obtained per plate using S. typhimurium strains TA 98, TA	
	100, TA 1535 and TA 1537 and TA 102 – experiment 1.	~

Treatment AE 1344122 Solvent control AE 1344122 AE 1344122 Solvent control Solvent control Solvent control Solvent control Controluorene MMC 2- aminoanthracene 4-NPDA : 4-Nitro-1,2 MC : Mitomycin C	Concentration (μg/plate) 5000 1581 500 158 50 16 0 5000 1581 500 1581 500 158 50 16 0 0 158 50 16 0 0 2 0.2 0.2		35 30 29 26 NA 17 NA NA	TA         100         152         173         165         063         159         158         165         145         1977         108         214         1937         1938         214         1937         1938         214         1830         1937         1938         214         1937         1938         214         1830         1937         099         1938         2099         1938         209         1830         1837         0         1938	TA 1535 16 16 16 16 16 16 16 16 16 16 16 16 16	TA 1537 6 6 8 7 6 7 6 7 6 7 6 7 6 7 7 7 7 7 7 7 7 7 7 7 7 7	274 274 274 260 264 264
Solvent control AE 1344122 Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       5000 \\       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       5000 \\       1581 \\       500 \\       158 \\       500 \\       158 \\       50 \\       16 \\       0 \\       0 \\       10 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.$	+/- S9 - - - - - - - - - - - - -	98 20 22 16 18 17 13 ( 22 26 26 26 29 26 29 26 29 26 29 26 29 26 29 26 29 26 20 29 26 20 20 20 20 20 20 20 20 20 20	100 152 173 165 063 159 158 165 145 097 098 214 183 099 197 197 197 197 197 197 197 1	1535 16 16 16 20 15 0 8 15 0 8 17 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 9 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 7 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 0 0 0 0 0 0 0 0 0 0 0 0	1537 6 8 8 8 7 6 7 6 7 8 7 6 7 8 7 6 7 9 7 9 7 9 7 9 7 9 7 9 7 9 7 9 7 9 7 9 7 9 7 9 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 10 10 10 10 10 10 10 10 10	$ \begin{array}{c} 234 & 5^{\prime\prime} \\ 304 & 7^{\prime\prime} \\ 224 & 7^{\prime} \\ 297 & 7^{\prime} \\ 205 & 7^{\prime} \\ 205 & 7^{\prime} \\ 207 & $
Solvent control AE 1344122 Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       5000 \\       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       $		20 22 16 18 17 13 ( 22 20 26 23 26 23 26 23 26 29 26 29 26 29 26 29 26 29 20 29 20 29 20 20 20 20 20 20 20 20 20 20 20 20 20	152 173 165 063 159 158 165 145 197 798 214 183 098 214 183 099 209 209 209 209 209 209 209 209 209	16 16 20 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 15 15 15 15 15 15 15 15 15	6 6 8 8 7 6 7 6 7 8 7 6 7 9 7 9 7 10 7 9 7 10 7 9 7 10 7 9 7 10 7 8 7 9 7 10 7 8 7 9 8 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 10 7 7 7 7 7 7 7 7 7 7 7 7 7	$ \begin{array}{c} 234 & 5^{\prime\prime} \\ 304 & 7^{\prime\prime} \\ 224 & 7^{\prime} \\ 297 & 7^{\prime} \\ 205 & 7^{\prime} \\ 205 & 7^{\prime} \\ 207 & $
Solvent control AE 1344122 Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       5000 \\       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       $		22 16 18 17 13 4 22 23 26 4 23 26 4 23 26 4 29 1 29 1 26 4 29 1 26 4 29 1 29 1 26 4 29 1 29 1 20 20 20 20 20 20 20 20 20 20	173 165 165 159 158 165 145 197 197 198 214 183 197 198 214 183 197 197 198 214 183 197 198 214 183 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 197 198 214 198 209 198 209 198 209 198 209 198 198 198 198 198 198 198 19	16 16 20 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 0 15 15 15 15 15 15 15 15 15 15	6 8 8 7 6 7 6 7 9 9 9 9 9 9 9 0 9 0 9 0 9 0 9 0 9 0 10 9 0 0 10 0 0 0 10 0 0 0 0 0 0 0 0 0 0 0 0 0	204 224 224 297 205 205 205 205 205 205 207 207 207 207 207 207 207 207
AE 1344122 Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       500 \\       158 \\       50 \\       16 \\       0 \\       5000 \\       1581 \\       500 \\       158 \\       50 \\       158 \\       50 \\       16 \\       0 \\       0 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 $		16 18 17 13 4 22 26 23 26 29 26 29 26 29 26 29 26 29 26 29 26 29 26 29 26 29 26 29 20 20 20 20 20 20 20 20 20 20	165 063 159 158 165 145 197 197 198 214 183 194 194 194 194 194 194 194 194	16 20 15 15 20 15 20 15 20 20 20 20 20 20 20 20 20 20	6 8 8 7 6 7 6 7 9 9 9 9 9 9 9 0 9 0 9 0 9 0 9 0 9 0 10 9 0 0 10 0 0 0 10 0 0 0 0 0 0 0 0 0 0 0 0 0	
AE 1344122 Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       158 \\       50 \\       16 \\       0 \\       5000 \\       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       10 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.$		18 17 13 4 22 23 26 26 29 26 29 26 29 26 29 26 29 26 20 29 26 20 20 20 20 20 20 20 20 20 20	063 159 158 165 145 197 198 214 183 198 209 NAS NAS NAS NAS	20 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15,0 15	<ul> <li>8</li> <li>7</li> <li>6</li> <li>7</li> <li>8</li> <li>7</li> <li>6</li> <li>7</li> <li>2</li> <li>2</li> <li>2</li> <li>7</li> <li>6</li> <li>2</li> <li>2</li> <li>9</li> <li>2</li> <li>9</li> <li>2</li> <li>9</li> <li>2</li> <li>0</li> <li>4</li> <li>9</li> <li>2</li> <li>0</li> <li>4</li> <li>4</li></ul>	
AE 1344122 Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       50 \\       16 \\       0 \\       5000 \\       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       0 \\       10 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 $		17 13 4 22 22 20 22 26 20 25 26 29 26 29 26 29 26 29 26 29 26 20 29 20 20 29 20 20 29 20 20 20 20 20 20 20 20 20 20 20 20 20	×159 158 165 145 197 197 198 214 183 192 192 192 193 192 193 193 193 193 193 193 193 193	15.0 15.0 15.0 15.0 15.0 16.0 16.0 17.5 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0	<ul> <li>✓ 8</li> <li>7</li> <li>6</li> <li>7</li> <li>2</li> <li>2</li> <li>2</li> <li>3</li> <li>7</li> <li>2</li> <li>2</li> <li>3</li> <li>7</li> <li>8</li> <li>7</li> <li>7</li> <li>8</li> <li>7</li> <li>7</li> <li>8</li> <li>7</li> <li>8</li> <li>7</li> <li>8</li> <li>7</li> <li>8</li> <li>7</li> <li>8</li> <li>7</li> <li>8</li> <li>8</li> <li>8</li> <li>7</li> <li>8</li> <li>8</li> <li>7</li> <li>8</li> <li>8</li> <li>8</li> <li>9</li> <li>8</li> <li>8</li> <li>9</li> <li>8</li> <li>9</li> <li>8</li> <li>9</li> <li< td=""><td></td></li<></ul>	
AE 1344122 Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       50 \\       16 \\       0 \\       5000 \\       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       0 \\       10 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 $		17 13 4 22 22 20 22 26 20 25 26 29 26 29 26 29 26 29 26 29 26 20 29 20 20 29 20 20 29 20 20 20 20 20 20 20 20 20 20 20 20 20	×159 158 165 145 197 197 198 214 183 192 192 192 193 192 193 193 193 193 193 193 193 193	15.0 15.0 15.0 15.0 15.0 16.0 16.0 17.5 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0 16.0	6 . 7 2 2 2 2 2 2 2 2 7 2 3 2 7 2 3 2 7 2 3 2 7 2 3 3 3 4 9 2 3 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 1 1 1 1 1 1 1 1 1 1 1 1 1	
AE 1344122 Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$ \begin{array}{r} 16\\ 0\\ \hline 5000\\ 1581\\ 500\\ 158\\ 50\\ 16\\ 0\\ \hline 10\\ 0.5 - 10*@\\ 0.2\\ \hline 0.2\\$		13 22 23 26 26 29 29 26 29 26 29 26 20 29 20 20 20 20 20 20 20 20 20 20	158 165 145 197 197 198 214 183 199 199 199 199 199 199 199 199 199 19	20 28 27 27 27 27 27 27 27 27 27 27	6 . 7 2 2 2 2 2 2 2 2 7 2 3 2 7 2 3 2 7 2 3 2 7 2 3 3 3 4 9 2 3 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 3 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 9 4 1 1 1 1 1 1 1 1 1 1 1 1 1	244 244 244 274 260 264 264 264 264 264 264 264 264 264 264
AE 1344122 Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$\begin{array}{c} 0 \\ 5000 \\ 1581 \\ 500 \\ 158 \\ 50 \\ 16 \\ 0 \\ \hline \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0$		220 23 26 0 29 ~ 29 ~ 26 0 29 ~ 26 0 29 ~ 26 0 29 ~ 26 0 29 ~ 20 20 20 20 20 20 20 20 20 20	165 145 1977 198 214 1830 199 199 NAS NAS	2 8 7 1 1 2 3 2 7 10 7 10 1 4 7 7 5 2 7 5 2 7 5 2 7 5 2 7 5 2 7 5 2 7 5 7 5 7 7 5 7 7 7 7 7 7 7 7 7 7 7 7 7	. 7 2 2 2 3 3 3 7 7 10 5 7 7 7 10 5 7 7 10 5 7 7 7 7 7 7 7 7 7 7 7 7 7	244 244 244 274 260 264 264 264 264 264 264 264 264 264 264
AE 1344122 Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       5000 \\       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       0 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\      $		26 26 35 00 26 29 26 20 26 20 26 20 20 20 20 20 20 20 20 20 20 20 20 20	145 1977 1977 198 214 1830 198 199 199 NA 387	2 11 3 2 10 14 2 2 10 14 2 2 3 2 3 2 3 2 3 2 3 2 3 3 2 3 3 3 3 3 3 3 3 3 3 3 3 3	2 2 3 3 3 3 7 7 10 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 9 2 1 1 1 1 1 1 1 1 1 1 1 1 1	244 244 244 274 260 264 264 264 264 264 264 264 264 264 264
Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       \hline       10 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       \hline       0       \end{array} $		23 26 35 30 29 29 26 20 26 20 20 20 20 20 20 20 20 20 20 20 20 20	1977 198 214 1830 1927 209 209 209 209 209 209 209 209 209 209	11-y 07 07 0 10 14 0 10 759 0 A 0 NA 0 NA	<ul> <li>29</li> <li>7</li> <li>0</li> <li>7</li> <li>0</li> <li>7</li> <li>0</li> <li>7</li> <li>0</li> <li>0</li> <li>9</li> <li>0</li> <li></li></ul>	244 244 244 274 260 264 264 264 264 264 264 264 264 264 264
Solvent control Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       1581 \\       500 \\       158 \\       50 \\       16 \\       0 \\       \hline       10 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       \hline       0       \end{array} $		26 0 35 00 29 26 0 NA 0 172 NA 0 NA 0	1977 198 214 1830 1927 209 209 209 209 209 209 209 209 209 209	9 07 10 14 02 0 10 0 10 0 10 0 10 0 0 0 0 0 0 0 0 0 0 0 0 0	10 0 10 0 10 0 9 0 0 0 0 0 0 0 0 0 0 0 0 0	244 244 244 274 260 264 264 264 264 264 264 264 264 264 264
Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       500 \\       158 \\       50 \\       16 \\       0 \\       0 \\       0.5 - 10 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0.2 \\       0$	$+ 0^{2}$ $+ 4^{2}$ $0^{2} + 4^{2}$ $0^{2} + 4^{2}$ $0^{2} + 4^{2}$ $0^{2} - 0^{2}$ $- 0^{2}$ $- 0^{2}$	25 29 26 NA 1727 NA NA	198 214 1830 199 2009 NAS NA 387 0 NA	27 10 14 2 10 759 28 10 759 28 4 8 759 28 4 8 759 28 759 28 759 28 759 28 759 28 759 28 759 28 759 759 759 759 759 759 759 759	10 0 10 0 10 0 9 0 0 0 0 0 0 0 0 0 0 0 0 0	244 244 244 274 260 264 264 264 264 264 264 264 264 264 264
Sodium azide 4-NPDA 2-nitrofluorene MMC	$     \begin{array}{r}       158 \\       50 \\       16 \\       0 \\       \hline       0 \\       0.5 - 10^{\circ} \\       0.2 \\       0.2 \\       0.2 \\       \hline       0       2       7       7       7       7       7       $		© 0 29 26 26 17 2 NA 0 NA 0 NA	214 1830 194 2009 NAS NA 387 0"NA	14 0 10 759 0 NA 0 NA	10 0 10 0 10 0 9 0 0 0 0 0 0 0 0 0 0 0 0 0	274 274 260 264 XA XA XA
Sodium azide 4-NPDA 2-nitrofluorene MMC			29 26 26 NA 172 NA NA	1830 1929 2009 NAS NA 387 O'NA	14 0 10 759 0 NA 0 NA	© 2 9 5 9 5 0 9 5 0	D NA C NA C NA
Sodium azide 4-NPDA 2-nitrofluorene MMC	$   \begin{array}{r}     16 \\     0 \\     \hline     0 \\     \hline     0.5 - 10^{*} \\     0.2 \\     0.2 \\   \end{array} $		26 37 NA C 17 NA	1924 2009 NAS NA 387 O'NA	2 710 759 0 A A A A A A A A A A A A A	× '9 9 0 0 0 109 2 NA	D NA C NA C NA
Sodium azide 4-NPDA 2-nitrofluorene MMC	$ \begin{array}{c} 0 \\ 10 \\ 0.5 - 10^{*} \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \\ 0.2 $		NA C NA	2009 * NAS NA 387 Ø NA	2 710 759 0 A A A A A A A A A A A A A	× '9 9 0 0 0 109 2 NA	D NA C NA C NA
Sodium azide 4-NPDA 2-nitrofluorene MMC	$   \begin{array}{r}     10 \\     0.5 - 10 \\     0.2 \\     0.2 \\     0.2   \end{array} $		NA C NA	2009 * NAS NA 387 Ø NA	759 DA WA NA	NA NA	D NA C NA C NA
4-NPDA 2-nitrofluorene MMC	$0.5 - 10^{*}$		172 NA	NA 387 O'NA	759 QĂ CNA C	NA NA	D NA C NA C NA
4-NPDA 2-nitrofluorene MMC	$0.5 - 10^{*}$		172 NA	NA 387 O'NA	NA NA	NA NA	
2-nitrofluorene MMC	0.2		NĂ	387 Ø'NA	NA NA	NA NA	
2-nitrofluorene	0.2		NA	ØΝΑ	NA Q	NA NA	
MMC	0.2		NA	ØΝΑ	NA		
2- aminoanthracene 4-NPDA : 4-Nitro-1,2 IMC : Mitomycin C	-phenylene dramin		1272	1375		NĂ	گي 592
aminoanthracene 4-NPDA : 4-Nitro-1,2 MC : Mitomycin C	e-phonylene@famine				185	~\$54	694
4-NPDA : 4-Nitro-1,2 MC : Mitomycin C	e-phonylene@raming	ig o		1375 [\]	100	10 ° 1	
A A				7			
	V S .	~ Ç					



#### Table 5.8.1-14 evertant colony counts obtained per plate using S. typhimurium strains TA 98, TA 100, TA 1535 and TA 1537 and TA 102 – Experiment 2.

				-			<i>a</i> . •	
Treatment								
	(µg/plate)	activation	ТА	ТА	TA	TA	TA 102	
		+/ <b>- S9</b>	98	100	1535	1537		
AE 1344122	5000	-	5	70	6	-5	107	
	1581	-	23	143	18	¹⁰	Q62	
	500	-	25	136	20	\$77	<u></u>	
	158	-	27	<b>B</b> 31	19	§ 6	L 270 S	
	50	-	22	\$133	19,0	10		
	16	-	28 🎣	141	1,Ø [♥]	8	x 3292 x 40	
Solvent control	0	-	22 ⁰¹	140	Ĵ ³ 5	。7		
			Ĩ,	~		?Q	AU3 55	
AE 1344122	5000	+	®14	106 🕡	r 9 🕎	í de se	AU3 ~~	
	1581	+ 🐇	505	150	jy j	چٽ ا	∞ ≫303 ≫	
	500	+ 0	50	155	A6 7	<b>9</b> 0		
	158	+2	<u>م</u> 70	<b>462</b>	🍣 16 🧹	9Q	0 [*] 30 [*] 0 [*]	
	50	Ľ.	¥45 🗠	1580	14	, Q	× 3ľ4 ×	
	16	\$\$\vee\$+\vee\$	50 <i>©</i>	162	Q	×14	282	
Solvent control	0	<i>6</i> ∛ + <i>€</i> ∛	<b>A *</b>	~ <b>Q6</b> 8	@13 .«	Ç 11 Ç	<u> </u>	
	~	$\langle \circ \rangle$	$\sim$		y ô		1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1	
Sodium azide	10 👋	6-0	NA O	r NAS	739	<u>_</u> ®Ă	NA NA	
4-NPDA	0.5 - 10*	<u> </u>	17	N/X	<b>D</b> A	⁰ 129 %	NA NA	
Nitrofurantoin	0.2	″- <u>~</u> ℃	NĂ	433	NA Q	NA	© _{NA}	
Cumene	50		NA	Ø'NA	NA	NA	<i>©</i> 493	
hydroperoxide	NA A	Q- 5		Ĉ	N°			
2-	3	\$ + 0	14,91	1007	\$227	570 Ô	641	
aminoanthracene		K° a	Å.	×,~	O' Å			
4 NUND 4 AND 1 4		Yoz Uli	a ¥	110				

Sand 10 ug/plate for TA 1537 * 4-NPDA : 4-Nitro-1,2-phenylene diamine - 0.5 m/plate for

#### A. Deficiencies

No deficiencio were dentified

#### III. Conclusion

vice remutagenic activity in this in vitro bacterial It is concluded that 0 system. A

#### and conclusion by applicant Assessment

showed no evidence of mutagenic activity in this *in vitro* bacterial

0

Length of the state of the stat



Data Point:	KCA 5.8.1/09
Report Author:	
Report Year:	
Report Title:	AE 1344122 (metabolite of AE C638206): Induction of chromosome aberrations
	in cultured human peripheral blood lymphocytes
Report No:	C034338
Document No:	Report includes Trial Nos.: 2014/67
	<u>M-234746-01-1</u>
Guideline(s) followed in	ICH: S 2 A; OECD: 473 (1997)
study:	
Deviations from current	Current guideline: OECD 473, 2016 Deviation: 200 instead of 306 metaphases were scored. This deviation is not
test guideline:	Deviation: 200 instead of 306 metaphases were scored. This deviation is no
	considered to impact the integrity of the story.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially	Yes, conducted under GLP/Officially secognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes A S A

#### **Executive Summary**

In this *in vitro* assessment of the distogenic potential of AE 1844122 (batch YG3228, purity 98.8%), the test compound was tested in an *in vitro* eytogenetics assay doing doplicate human lymphocyte cultures prepared from the pooled blood of three female donors in two independent experiments. Treatments covering a broad range of doses, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9). The test article was dissolved in sterile anhydrous analytical grade dimethyl sulfaxide (DMSC) and the highest dose level used, 2532  $\mu$ g/mL, was equivalent to 10 mM.

Treatment of cultures with AE 1994122 in the absence and presence of S-9 resulted in frequencies of cells with structural aberrations which were generally similar to those in concurrent negative controls, with one exception. Following 20+6 hour treatment in the absence of S-9 in Experiment 2, a small but significant increase in aberrant cell frequency was observed at the highest concentration analyzed (1123  $\mu$ g/mL). Af this concentration a single culture exhibited an aberrant cell frequency that marginally exceeded the historical negative control (normal) range. However, the aberrant cell frequency of the replicate culture, and all other treated cultures in both experiments, fell within the normal range, therefore this observation was not considered biologically significant.

AE 1344122 did not induce chromosome aberrations in criticured human peripheral blood lymphocytes, when tested to a maximum concentration of 10 mM (following 3+17 hour treatments in the absence and presence of S-9) or to its mit of cytotoxicity following 20+0 hour treatment in the absence of S-9). AE 1344122 was therefore considered not to be classogenic for mammalian cells *in vitro*.

I. Materials and methods	
A. Materials of by	
K A Q A	ý vy
1. Test material	AE@344122
Description >	Fine white powder
Description	YG3228
Ppreity: 5 2	98.8 %
SAS # O X	Not reported
Stability of test compound:	Stable for the duration of the study
2. Control materials:	
Negative:	None (Culture medium was used as the negative control)



Solvent / final concentration:	DMSO	0				
Positive:	4-Nitroquinoline 1-oxide (NQO), Sup Co., Gillingham, UK, in the absence of Cyclophosphamide (CPA), Supplier: S Poole, UK, in the presence of liver S-9					
	Poole. UK. in the presence of liver S-9					
3. Test organisms:	·····					
Cell line:	Human lymphocyte cultures					
Source:	Pooled blood from three female donor	s s a s				
4. Test concentrations:						
Experiment 1:	without S9 mix (3+17 h) at 1296, 202 with S9 mix (3+17 h) at 1620, 2026;	26, 25,82 µg/m0				
Positive:Co., Gillingham, UK, in the absence of liver S-9 Cyclophosphamide (CPA), Supplier: Sigma Chemical Co Poole, UK, in the presence of liver S-93. Test organisms: Cell line: Source:Human lymphocyte cultures Pooled blood from three female dotors4. Test concentrations: Experiment 1: B. Test performancewithout S9 mix (3+17 h) at 1296, 2026, 2532 µg/md with S9 mix (3+17 h) at 1620, 2026, 2532 µg/md with S9 mix (3+17 h) at 1620, 2026, 2532 µg/md with S9 mix (3+17 h) at 306, b, 498.5, 4123 µg/mL with S9 mix (3+17 h) at 7829, 2152, 2532 µg/mL with S9 mix (3+17 h) at 7829, 2152, 2532 µg/mLB. Test performanceOf March 2003, Of May 2003I. In life dates:05 March 2003, Of May 2003The experimental phase of the study was performed from March 5th te May 1st, 2003 at Govance Laboratories, Harrogate, North Yorks UK.						
B. Test performance		o L A co				
1. In life dates: 05 Mai	rch 2003 201 May 2003					
The experimental phase of the Laboratories Harrogate North N	rch 2003 201 May 2003 study was performed from March Sth to orks UK.	May 1st, 2003 at Svance				
I he <i>in vitro</i> cytogenetic test is a	mutagenicity test system for the detection	of chromosome aberrations				
n cultureu manimanan cens. @	The test is designed to detect structural s at their first post-treatment mitains.	abelliquons actinomatic and				
AE 1344122 (batch YG322% pu	ricy 98.8%) was dested in an in vitro stoge	metics assay using duplicate				
	1  f  g  f  f  f  f  f  f  f  f					
uman lymphocyte cultures prep	ared from the pooled blood of three female	domers in two independent				
numan lymphocyte cultures prep experiments. Treatments covered	ared from the pooled blood of three female g a broad range of doses, separated by marro	donsits in two independent wintervals, were performed				
numan lymphocyte cultures prep experiments. Treatments covering both in the absence and presence	pared from the pooled blood of three female g a broad range of tosses, separated by marro e of metabolic activation (S-9). The test art	domers in two independent w intervals, were performed ticke was dissolved in sterile				
human lymphocyte cultures prep experiments. Treatments covern both in the absence and presence anhydrous analytical grade dime	ared from the pooled blood of three female g a broad range of doses, separated by marro	domers in two independent w intervals, were performed ticke was dissolved in sterile				
numan lymphocyte cultures prep experiments. Treatments covern both in the absence and presence anhydrous analytical grade dime was equivalent to 10 mM.	pared from the pooled blood of three female g a broad range of coses, separated by marro e of metabolic activation (S-9). The test art (By) sufficience (DMSO) and the highest do	donots in two independent w intervals, were performed ticke was dissolved in sterile se level used, 2532 $\mu$ g/mL,				
numan lymphocyte cultures prep experiments. Treatments covern both in the absence and presence anhydrous analytical grade dime was equivalent to 10 mM.	ared from the pooled blood of three female g a broad range of coses, separated by marro e of metabolic activation (S-9). The test art (Byl suffoxide (DMSO) and the highest do e absence and presence of S-9 was for 3 h	domets in two independent w intervals, were performed ticke was dissolved in sterile be level used, 2532 $\mu$ g/mL, nours followed by a 17-hour				
numan lymphocyte cultures prep experiments. Treatments covern both in the absence and presence unhydrous analytical grade dime was equivalent to 10 mM. In Experiment of the threatment of the recovery period prior to harvest.	ared from the pooled blood of three female g a broad range of coses, separated by marro e of metabolic activation (S-9). The test art (Byl suffixide (DMSO) and the highest do e absence and presence of S-9 was for 3 h (3+17). The S y used was prepared from a	domets in two independent w intervals, were performed ticke was dissolved in sterile be level used, 2532 $\mu$ g/mL, nours followed by a 17-hour rat liver post-mitochondrial				
numan lymphocyte cultures prep experiments. Treatments covering both in the absence and presence inhydrous analytical grade dime was equivalent to 10 mM. In Experimental, treatment of the ecovery period prior to harvest raction (\$9) from Arcelor 1254	ared from the pooled blood of three female g a broad range of coses, separated by narro e of metabolic activation (S-9). The test art (Byl suffoxide (DMSO) and the highest do e absence and presence of S-9 was for 3 h (3+17). The S-9 used was prepared from a induced animals. The test article dose level	domets in two independent w intervals, were performed ticke was dissolved in sterile be level used, 2532 μg/mL, hours followed by a 17-hour rat liver post-mitochondrial els for chromosome analysis				
numan lymphocyte cultures prep experiments. Treatments covering both in the absence and presence anhydrous analytical grade dime was equivalent to 10 mM. In Experiment 1, treatment on the recovery period prior to harvest fraction (\$9) from Arcelor 1254 were selected by evaluating the	ared from the pooled blood of three female g a broad range of coses, separated by marro e of metabolic activation (S-9). The test art myl sulfoxide (DMSO) and the highest do absence and presence of S-9 was for 3 h (3+17). The S 9 used was prepared from a induced animals. The test article cose leve effect of AE 1344922 on mitotic index. Ch	domets in two independent w intervals, were performed ticke was dissolved in sterile be level used, 2532 $\mu$ g/mL, nours followed by a 17-hour rat liver post-mitochondrial els for chromosome analysis romosome aberrations were				
numan lymphocyte cultures prep experiments. Treatments covern both in the absence and presence inhydrous analytical grade dime was equivalent to 10 mM. In Experiment 1, treatment in the ecovery period prior to harvest raction (SP) from Arcelor 1254 were selected by evaluating the malyzed at three dose levels (see	ared from the pooled blood of three female g a broad range of coses, separated by marro e of metabolic activation (S-9). The test art thyl sulfoxide (DMSO) and the highest do e absence and presence of S-9 was for 3 h (3+17). The So used was prepared from a induced animals. The test article cose leve effect of AE 1344922 on mitotic index. Ch e Table 58.1-15). The trighest concentration	domets in two independent winervals, were performed ticke was dissolved in sterile be level used, 2532 $\mu$ g/mL, nours followed by a 17-hour rat liver post-mitochondrial els for chromosome analysis romosome aberrations were on chosen for analysis, 2532				
numan lymphocyte cultures prep experiments. Treatments covern both in the absence and presence anhydrous analytical grade dime was equivalent to 10 mM. In Experiment 1, treatment in the recovery period prior to harvest fraction (\$9) from Arcelor 1254 were selected by evaluating the analyzed at three dose levels (see ug/mL, induced approximately	ared from the pooled blood of three female g a broad range of coses, separated by marro e of metabolic activation (S-9). The test art (Byl suffixide (DMSO) and the highest do e absence and presence of S-9 was for 3 h (3+17). The S y used was prepared from a induced animals. The test article toose level effect of AE 1344 22 on mitotic index. Ch Table 98.1-15). The trighest concentration 27% and 20% mitotic infibition (reducti	domets in two independent winervals, were performed ticke was dissolved in sterile be level used, 2532 $\mu$ g/mL, nours followed by a 17-hour rat liver post-mitochondrial els for chromosome analysis romosome aberrations were on chosen for analysis, 2532				
numan lymphocyte cultures prep experiments. Treatments covering ooth in the absence and presence unhydrous analytical grade dime was equivalent to 10 mM. In Experiment 1, treatment of the recovery period prior to harvest raction (\$59) from Arcelor 1254 were selected by evaluating the unalyzed at three dose levels (sec ug/mL, induced approximately ubsence and presence of \$-9 rosp	ared from the pooled blood of three female g a broad range of coses, separated by narro e of metabolic activation (S-9). The test art (Byl suffixide (DMSO) and the highest do e absence and presence of S-9 was for 3 h (3+17). The S y used was prepared from a induced animals. The test article toose level effect of AE 1344 22 on mitotic index. Ch Table 98.1-15). The trighest concentration 27% and 20% mitotic inhibition (reduction pectively.	domets in two independent winervals, were performed ticke was dissolved in sterile be level used, 2532 $\mu$ g/mL, nours followed by a 17-hour rat liver post-mitochondrial els for chromosome analysis romosome aberrations were on chosen for analysis, 2532				
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human lymphocyte cultures prep experiments. Treatments covern both in the absence and presence anhydrous analytical grade dime was equivalent to 10 mM. In Experiment 1, treatment of the recovery period prior to harvest fraction (\$9) from Arcelor 1254 were selected by evaluating the analyzed at three dose levels (see ug/mL, induced approximately absence and presence of \$-9 resp Table 5.8-15 Experiment 1	ared from the pooled blood of three female g a broad range of coses, separated by narro e of metabolic activation (S-9). The test art thyl sulfoxide (DMSO) and the highest do e absence and presence of S-6 was for 3 h (3+17). The Soused was prepared from a induced animals. The test article cose level effect of AE 1344922 on mitotic index. Ch able 58.1-15). The trighest concentratic 27% and 20% mitotic inhibition (reduction bectively.	domets in two independent winervals, were performed ticke was dissolved in sterile be level used, 2532 $\mu$ g/mL, nours followed by a 17-hour rat liver post-mitochondrial els for chromosome analysis romosome aberrations were on chosen for analysis, 2532				
numan lymphocyte cultures prep experiments. Treatments covering both in the absence and presence inhydrous analytical grade dime was equivalent to 10 mM. In Experiment 1, treatment in the ecovery period prior to harvest raction (\$29) from Arcelor 1254 were selected by evaluating the malyzed at three dose levels (see ug/mL, induced approximately absence and presence of \$-9 rosp Table 5.8-15 Experiment 1 Treatment + recovery	ared from the pooled blood of three female g a broad range of coses, separated by marro e of metabolic activation (S-9). The test art (Byl suffixed (DMSO) and the highest do e absence and presence of S-9 was for 3 h (3+17). The S-9 used was prepared from a induced animals. The test article close level effect of AE 1344/22 on mitotic index. Ch table 58.1-15). The trighest concentration (27% and 20% mitotic infibition (reduction pectively. Treatment details Venicle antrol (metabolite of AE C638206)	domets in two independent winervals, were performed ticke was dissolved in sterile be level used, 2532 $\mu$ g/mL, hours followed by a 17-hour rat liver post-mitochondrial els for chromosome analysis romosome aberrations were on chosen for analysis, 2532				
numan lymphocyte cultures prep experiments. Treatments covering both in the absence and presence anhydrous analytical grade dime was equivalent to 10 mM. In Experiment 1, treatment in the recovery period prior to harvest fraction (\$29) from Aroclor 1254 were selected by evaluating the malyzed at three dose levels (see ug/mL, induced approximately absence and presence of \$-9 rosp Table 5.8-15 Experiment 1 Treatment + recovery	ared from the pooled blood of three female g a broad range of coses, separated by marro e of metabolic activation (S-9). The test art (Byl suffixed (DMSO) and the highest do e absence and presence of S-9 was for 3 h (3+17). The S-9 used was prepared from a induced animals. The test article close level effect of AE 1344/22 on mitotic index. Ch table 58.1-15). The trighest concentration (27% and 20% mitotic infibition (reduction pectively. Treatment details Venicle antrol (metabolite of AE C638206)	domets in two independent w intervals, were performed ticke was dissolved in sterile be level used, 2532 $\mu$ g/mL, hours followed by a 17-hour rat liver post-mitochondrial els for chromosome analysis romosome aberrations were on chosen for analysis, 2532 on in mitotic index) in the				
numan lymphocyte cultures prep experiments. Treatments covern both in the absence and presence anhydrous analytical grade dime was equivalent to 10 mM. In Experiment 1, treatment of the recovery period prior to harvest fraction (\$9) from Aroclor 1254 were selected by evaluating the analyzed at three dose levels (see ug/mL, induced approximately absence and presence of \$-9 rosp Table 5.8-15 Experiment 4 S-9 Treatment + recovery (hours)	ared from the pooled blood of three female g a broad range of coses, separated by marro e of metabolic activation (S-9). The test art (Byl suffixide (DMSO) and the highest do e absence and presence of S-9 was for 3 h (3+17). The S-9 used was prepared from a induced animals. Fae test article close leve effect of AE 1344/22 on mitotic index. Ch a table 58.1-15). The trighest concentration (7% and 20% mitotic infibition (reduction pectively.	domets in two independent w incrvals, were performed ticke was dissolved in sterile be level used, 2532 $\mu$ g/mL, hours followed by a 17-hour rat liver post-mitochondrial els for chromosome analysis romosome aberrations were on chosen for analysis, 2532 on in mitotic index) in the <b>Positive control</b>				

ŀ

of S-2 was for 3 hours only followed by a 17-hour recovery period prior to harvest (3+17). Chromosome aberrations were analyzed at three dose levels (see Table 5.8.-16) and the highest concentrations chosen for analosis, 1123 and 2532  $\mu$ g/mL, induced approximately 48% and 44% mitotic inhibition in the absence and presence of S-9, respectively.



S-9	Treatment + recovery (hours)	Vehicle control	Concentration (µg/mL) AE 1344122 (metabolite of AE C638206)	Posit	ive control
-	20+0	0 ^a	306.1, 498.5, 1123	×QO,	5.00 µg/mL
+	3+17	$0^{a}$	1829, 2152, 2532	CPA,	6.25 µg/mb
a : Veh	nicle control was DMSO only:	4-Nitroquinol	ine 1-oxide; CPA: cxclophosphamide.	Š.	

II. Results and discussion

The proportion of cells with structural aberrations in these cultures fell within historical solvent Control ranges. 4-Nitroquinoline 1-oxide (NQO) and cyclophosphamide (CPA) were employed as positive control chemicals in the absence and presence of liver s-9 respectively. Cells receiving these were sampled in each experiment, 20 hours after the start of treatment; both compounds induced statistically significant increases in the proportion of cells with spuctural aberrations

Treatment of cultures with AE 1344122 in the absence and presence of S-9 resulted in frequencies of cells with structural aberrations which were generally similar to those in concurrent negative controls, with one exception. Following 20+0 reatment in the absence of S-9 in experiment 2, a small but significant increase in aberrant cell frequency was observed at the highest concentration analyzed (1123  $\mu$ g/ml). At this concentration, a ongle culture exhibited an aberrant cell frequency that marginally exceeded the historical negative control (normal) range. However, the aberrant cell frequency of the replicate culture, and all other treated cultures in both experiments, fell within the normal range, therefore this observation was not considered biologically significant.

Table 5.8.1-17	Mean mitotresindices and mimber of aberrant human lymphocetes, including and	I
	exeluding sans - Fineriment 1	
	excluding/gaps – Experiment 1	

	y when the second		N N	0″ ″	Ç.	
Treatment	Concentration (µg/mL) (	Metabolic activation	Treatment time(h)	Mitotiv index	Aberrant cells Including gaps	Aberrant cells Excluding gaps
	× 1288 0	ð,		11.2	3	2
AEX344122 %	© <u>\$</u> \$\$26	~-~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		9.4	6	6
	2532		0 3	9.6	3	3
Solvent control		\$ \$ \$		13.1	3	2
~~ Č	1620 ×	× + ×	3	9.8	0	0
AE 1344122	2020		<b>9</b> 3	8.6	3	1
	2582	10 + ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	3	7.8	5	3
Sofvent control			3	9.8	3	3
NQO			3	-	51	49***
CPA CPA	6.25	+	3	-	66	63***

*** p< 0.001 chitistically significantly different from controls Fisher'stest

NQO : 4-Nitrequinoffice 1-oxide CPA : Cyclophosoptamide



Table 5.8.1-18	Mean mitotic indices and number of aberrant human lymphocytes, including and
	excluding gaps – Experiment 2

Treatment	Concentration (µg/mL)	Metabolic activation +/- S9	Treatment time (h)	Mitotic index	Aberrant cells focluding gaps	Aberzant cells Excluding gaps
	306.1	-	20	8.0	10	\$* <b>4</b> \$ [*] &
AE 1344122	498.5	-	20	5.8 🖋 🎽	7 🍾	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
	1123	-	Z0	46	9 0	× 8 Ø
Solvent control	0	-	20	08.8	Å é	
	1829	+ 4	<b>©</b> ″3	0 [×] 11.7 。	⁰ 4	
AE 1344122	2152	+ 0	3	112	$\mathcal{Q}^{*}$ 3 $\mathcal{O}^{*}$	<u> </u>
	2532	+	<u>。3</u>	<b>\$</b> .9 7	r (\$` .	K 29
Solvent control	0	Ċ,	N 30	£ 12.4	\$ ⁴	4 4
NQO	2.5	A - m			45 0	\$7*** <i>\$</i>
СРА	3.125	× + ×	~ 3 ~	A.	× 66	58***

CrA: Cyclophosphamide No increases in the frequency of cells with numerical aberrations, which exceeded the historical negative control range, were observed in cultures treated with AE 1344122 (metabolite of AE (638206)) in the absence and presence of S-9.4 A. Deficiencies No deficiencies were identified III. Conclusions AE 1344122 did not induce chromosome aberrations in a large set of the se

when tested to Omaxipum concentration of 0 mM (following 3%17 hour treatments in the absence and presence of S-9) or the its limit of cytotoxicity (tollowing 20+0 hour freatment in the absence of S-9). AE 1344122 was therefore considered for to be clastogenic for manimalian cells in vitro.

## Assessment and conclusion by applicant: 7

Study meets the current guidance and the requirements in 283/2013.

AE 1344122 did hot induce chromosome aberrations in cultured human peripheral blood lymphocytes, and was therefore considered not to be clastogenic for mammalian cells *in vitro*.

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Data Point:	KCA 5.8.1/10	
Report Author:		
Report Year:	2019	ð
Report Title:	AE 1344122: Micronucleus test in human lymphocytes In vitro	Ş
Report No:	1969603	•
Document No:	<u>M-673685-01-1</u>	
Guideline(s) followed in	OECD Test Guideline No. 487 (July 2016)	
study:		)
Deviations from current	None Ca da a	
test guideline:	None & & & & & & & & & & & & & & & & & & &	e C
Previous evaluation:	No, not previously submitted	Š
		,
GLP/Officially	Yes, conducted under GLP/Officially recognised testing facilities	
recognised testing	Yes, conducted under GLP/Officially recognised testing facilities of the second	
facilities:		
Acceptability/Reliability:	Yes O O V V V O V	

#### **Executive summary:**

AE 1344122 was evaluated for its potential to induce micronucler in human lymphosytes *in vitro*, in two independent experiments; Experiment comprised a four hour exposure period in the presence and absence of metabolic activation (provided by \$9) whilst experiment II provided 20-hour exposure period in the absence of \$9. Concentrations up to 2024 ag mL overe tested in duplicate and 1000 binucleated cells/culture were examined for sytogenetic damage

In experiment I, no cytotoxicity or precipitation was observed (+f-S9) up to the highest evaluated concentration. Similarly, in experiment II (-S9), no cytotoxicity or precipitation was observed at the highest tested concentration. Therefore, concentrations up to the limit concentration of 2024  $\mu$ g/mL were evaluated.

No relevant increases in the numbers of micronucleated cells were observed following treatment with the test item either in the presence of absence of \$9 mix in either experiment.

Appropriate reference substances gave the expected statistically significant increase in the number of micronucteated cells.

The test substance can be considered neither clastogenic nor an ugenic under the conditions of this *in vitro* micronucleus est, when tested up to the highest possible concentration, both in the presence and absence of metabolic activation

# I. Maternals and methods

A. Materials

Ô AE 1344122 the the eport 98.8% (w/&) 1. Test material Test substance: 



2. Vehicle and/or positive control	Vehicle: DMSO
	Positive controls:
	Mitomycin C (MMC), 0.8 $\mu$ g/mL (98% purity, dissolved in deionized water)
	Demecolcine, 75 ng/mL (purity $\geq$ 98%, dissolved in deionized water) $\pm$ S9 Cyclophosphamide (CPA), 17 $\approx$ µg/ml (purite 97-103%, desolved in $\sqrt{2}$
	saline)

#### 3. Activation:

Metabolic activation was provided by S9-mix derived from phenobarbital/B-naphthoNavone induced rat liver homogenate; each batch of prepared Sy has been routinely evaluated for its ability to activate known mutagens.

An appropriate quantity of S9 was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained MgG² (8 mM), KCI (33 mM), ghicose-6-phosphate (5 mM) and NADP (4 mQ) in sodium ortho-phosphate-buffer (100 mM, gH 7.4).

The protein concentration of the SS preparation used for this study wa 30.9 mg/mL

#### 4. Cell cultures and media:

Cells

Blood was drawn from healthy non-smoking conorst not receiving medication. Blood from a female donor (29 years old) and a mate donor (29 years old) were used in experiments) and II, respectively. The lymphocytes have been shown to respond welco PHA and positive control substances; furthermore, both donors have an established low incidence of micronucleion peripheral blood lymphocytes.

#### <u>Media</u>

Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hours following collection. The calture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/E12, mixture 0:1), applemented with 200 mM GlutaMAXTM. The medium was further supplemented with pencillin streptomycin (100 UmL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (foetal bovine Serum), 10 mM HEPPS and the anticoagutant heparin (125 U.S.P.-U/mL).

All incubations were carried out at 37%C with 5.5 % CO2 in humidified air.

### 5. Test compound concentration's used:

Test concentrations for the first experiment (4 hour purse treatment) were 0 (solvent control), 15.3, 26.8, 47, 82.2, 44, 252, 441, 771, 4949 & 2024 cc/mL both with and without S9 mix.

In the second experiment a continuous (26 hour) treatment was used at test concentrations of 0 (solvent control), 144, 252, 441,  $\frac{10}{10}$ , 1349 & 2024 µgmL, in the absence of S9 mix.

#### B. Test Performance

Experimental phase. August 2009 to 18th October 2019

### 1. Preliminary assay

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment? Cytotoxicity characterized by the percentages of reduction in the CBPI in comparison to the controls and expressed as % cytostasis) was determined from 500 cells/culture. The experimental conditions in this pre-experimental phase were identical to those described for the mutagenicity assay.



The pre-test was performed with 10 concentrations of the test item separated by no more than a factor of  $\sqrt{10}$  and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hours (with and without S9 mix). The preparation interval was 40 hours after start of the exposure?

This preliminary test was designated Experiment I since the cultures fulfilled the criteria for cytogenetic evaluation and appropriate concentrations could be selected for evaluation.

#### 2. Cytogenetic experiment

Cells were subject to either 4 hours pulse exposure (with and without \$9, experiment) or 20 hour continuous exposure (without S9; experiment II). The succeeding procedure for cell preparation was the same for both exposure periods.

#### **Pulse exposure**

Approximately 48 hours following seeding, two bood cultures of 10 m each, were set up in parallel in 25 cm² cell culture flasks (for each test item (concentration)). The sulture medium was replaced with serum-free medium containing the test item. For the freatment with metabolic advation 50 µL S9 mix per mL culture medium was added. After 4 hours the cell@were spun down by gentle centrif@ation for 5 minutes. The supernatant was discarded, and the cells resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L K@l, 1100 mg/L glucose • H2O, 192 mg/L Na2HBO4 • 2 H2O and 150 mg/L KH2PO4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10% FBS (var and outured for a 16-hour recovery period. After this period Cytochalasin Bat µg/naL) was added and the cellowere cultured for a further 20 hours until preparation.

#### **Continuous exposure**

Approximately 48 hours following seeding, two blogst cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test tem. After 200 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells re-suspended in and washed with "sahine G'\ The washing procedure was repeated once as described. Following washing, the cells were suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4  $\mu g/mL$ ) was added and the cells were cultured for a further 20 hours until preparation.

The activation assay was performed independently with an identical procedure, except for the addition of the so-mix. In these experiments 19 mL instea of 20 mL culture medium and additionally 1 mL of S9-mix were added to the basks for the treatment period, resulting in a concentration of 5% S9 mix in the cultures. The number of 60 G resistant mutants and viability were determined as in the nonactivation assay

### Preparation of cells

ð, Following the procedures aboy approximately 40 hours from the start of treatment), the cultures were harvester by centrifugation. The cells were spure down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrol ugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at \$7 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19) parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

## 3. Acceptance Criteria

The miconucleus assay is deemed acceptable if it fulfils the following criteria:



Ø

- The concurrent solvent control will normally be within the laboratory historical solvent control data range. The average mutant frequency of the vehicle controls should not exceed 25x10⁻⁶ cells
- The concurrent positive controls should produce a statistically significant increase in the micronucleus frequency and should be within the laboratory historical positive compol data range. The positive controls should induce a mutant frequency at least 3 times that of the controls
- Cell proliferation criteria in the solvent control are considered acceptable.
- All experimental conditions described in section Experimental performance wer one exposure condition resulted in a clearly positive result.
- The quality of the slides should allow the evaluation of an adequate number cells concentrations
- The criteria for the selection of top concentrations should be met

Any mutant frequencies not meeting these criteria were excluded from the statistical analyse

#### 4. Assessment criteria

Providing that all of the acceptability criteria are fulfilled, a test iten os considered to be clearly per ative if, in all of the experimental conditions examined:

- None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase
- L The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control Cata

The test item is then considered unable to induce from of the breaks and/or of in or loss in this test Ś system. O  $\bigcirc$ 

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control ñ
- The increase is concentration collated in at least one experimental condition
- The results are outside the range of the laboratory historical solvent control data (95% control limit realized as 95% confidence ofterval L,

When all of the criteria are mer, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system

There is no requirement for verification of a clear positive or negative response.

In case the response is wither of early negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. narrow concentration spacing, other metabolic activation conditions i.e. So concentration or S9 origin) could be useful.

## 5. Statistical analysis

Statistical significance was confirmed by the Chi square test (p < 0.05), using a validated test script of "R" a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those values that indicated an increase in the number of cells with microrfuelei compared to the concurrent solvent control.



A linear regression was performed using a validated test script of "R", to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Both, biological and statistical significance were considered together.

#### II. Results and discussion

In experiment I neither precipitation nor cytotoxicity was observed up to the highest applied concentration, either in the presence or absence of S9, and no relevant influence on osmolarity or  $\vec{\rho}$  is applied concentration. The highest concentration or cytotoxicity was observed up to the highest of applied concentration. The highest concentration applied (2024µg/ml) was therefore the limit concentration.

The results of both experiments, with and without metabolic activation, are sumparised in the table below:

							ľ "K
Exp.	Preparation	Test item	Proliferation	Cy@stasis*	Micronucleated	Historical	ontrof data
	interval	concentration	Q index	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	~cells ©	25% Ctr	Min - Max
		in µg/mL 🍦	C CBAPI ²		vin %	95% Cter Simil	
		Exposure	period 4 hrs v	vithont S9 n	nt o o		Y
Ι	40 hrs	Solvent control	ू≪1.73 <i>°</i> °	S O	ð 25 Ö	0.09 - 1.20	0.00 - 1.55
		Positive control ²	≫ 1.26	"n.c 🕅	. <b>06.45^s</b>	2.66 - 22074	3.95 - 28.60
		771	× 1,98 °	⊻ n@r	0,30		
		1349	Ø.79 🔊	An.c	N 60.45 5		
		2024	<u><u> </u></u>	n.c. O	& 0.45 <u>,</u>	, Ö	
	•	Exposure	period 20 hrs	without \$9 r	nix ^O	()	
II	40 hrs	solvent control	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		Q 0.20	0.00 - 1.14	0.05 - 1.60
	, Â	Positice control ³	[™] 1.82 [™]	°~n.c	2:85 ^s	1.15 - 6.44	1.95 - 8.80
		371	1.65	12,2	£~0.30 √v″		
	ð	\$1349 ⁰		4	0.30		
	Ô	2024 🔍	A1.74 X	10 ²⁵	0.20		
		Exposu	re period 4 hrs	with S9.m.	x ~		
Ι	40 hrs	Soferent cooprol1	1.92	$\bigcirc$ $\checkmark$	<b>0.30</b>	0.00 - 1.24	0.10 - 1.30
		Positive control*	` ^۲ ۵٫۲۲ √	15.5	گ ^۳ 2.85 ^s	1.01 - 7.34	1.80 - 8.85
		771	× 7.85 0	Q.9 🚿	0.40		
	Q	1549	<u></u> 1.84	R 8.4 R	0.15		
		1549 5 Q024 0 5	J.84 💊	8.4 8.8 8.8	0.35		
	·····			^o	•		

Table 5.8.1-19	Summary of results	ofexperiment	I and I
----------------	--------------------	--------------	---------

* For the positive control groups and the test them treatment groups the values are related to the solvent controls

** The non-ber of micronocleated cells was determined in a sample of 2000 binucleated cells

⁸ Theorem of microsucleated vells is statistically significantly higher than corresponding control values

^{n.c} Not calculated as the CBPL is equal to or higher than the solvent control value  $\frac{1}{2}$ 

¹ ΔDMSO 0.5 % (Δβ) ² MMC 0.8 μg/mL ³ Demecolcine 75 ng/mL ⁴ CPA 1745 μg/mL

In both independent experiments, neither a statistically significant nor a biologically relevant increase in the number of nicronucleated cells were observed following treatment with the test item either in the presence of absence of S9 mx.

Demecolcine (100 fig/mL), MMC (0.8  $\mu$ g/mL) and CPA (15  $\mu$ g/mL) were used as positive controls and showed distinct increases in cells with micronuclei, thus confirming the validity of the assay to detect clastogens/aneugens.



#### III. Conclusions

The test substance AE 1344122 did not induce micronuclei in this *in vitro* micronucleus test in human lymphocytes, when tested up to precipitative concentrations.

Therefore M-05 is considered to be neither clastogenic nor aneugenic under the conditions of the

#### Assessment and conclusion by applicant:

The study was conducted according to OECD TG 48, and is valid and acceptable to assess the clastogenicity of AE 1344122 *in vitro* AE 1344122 is neither clastogenic nor an agenic under the conditions of this study.

Data Point:	
Report Author:	
Report Year:	
Report Title:	V79/HPRT-test in vitro for the detection of induced forward indutations Code
	$AE 1.344122$ senetaboline of $AE U0$ solution $\sqrt{2}$ $\sqrt{2}$ $\sqrt{2}$ $\sqrt{2}$
Report No:	C035061 Report includes Iffial Nos:: AT00591 M 218160 01 20
Document No:	Report japiudes Ifial Nos.: AT00591 N O C N
	<u>M-218169-0140</u> & O O O V
Guideline(s) followed in	EU (EEC); 2000/32/EC; OCD: 4/6; USE A (=EPA): OPTS \$70.5300
study:	
<b>Deviations from</b>	Current mideline. OECD 476, 2016
0	Deviation: Note. S & S
Previous evaluation: 🔬	Deviation: Note. Yes evaluated and accepted in the OAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLOVOfficially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliabilit	
<u>y: &amp; §</u>	
<i>6</i>	

### Executive Summary

The purpose of the study was to assess the point nutagenic potential of AE 1344122 (batch YG3228, purity 98.8%) at the hypoxanthing guarage physical physical descent and the provide the physical descent and the provide the physical descent and th

AE 1344122 was tested at concentrations up to and including 2400 µg/mL with or without metabolic activation. Without and with S9 mix, AE 1344122 induced no decreases in survival to treatment or in relative population growth. However, AD 1344022 was tested up to its limit of solubility under culture conditions. Adequate positive controls fethyl methanesulfonate and dimethylbenzanthracene) were used for each experiment.

With and without \$9 mix there was no bologically relevant increase in mutant frequency above that of the vehicle controls. Ethyl methanesulfonate and dimethylbenzanthracene induced clear mutagenic effects demonstrating the sensitivity of the test system and the activity of the \$9 mix.

Based on these results, AS 1344122 was considered to be non-mutagenic in the V79/HPRT forward mutation assay, both with and without metabolic activation.

Materiats and methods AE 1344122 1. Test material: Description White powder



	Lot / Batch #:	YG3228
	Purity:	98.8%
	CAS #	Not reported
	Stability of test compound:	Stable for the duration of the study
2.	<b>Control materials:</b>	Negative: Culture medium [Eagle's minimal essential medium
		supplemented with 1% L-glutamine, 1% (TEM-vitamins, 1%)
		MEM NEAA, 1% penicillin/streptomycin and 10% foetal call
		serum (FCS)] $\sqrt{2}$
		Solvent: DMSO for AE 1344122 and
		Dimethylbenzanthracene not exceeding $1\%(v/v)$ in the
		culture medium, No solvent needed for entry Q
		methanesulfonate as it is a liquid of the state of the st
		Positive: Ethyl methanesultonate (EMS), a directly all plating
		agent, used at a final conventration of 900 µg/mL in non-
		activation trials
		Dimethylbenganthracene (BMBA), promutagen requiring a
		metabolic activation used at a final concentration of 20 ug/rov
		metabolic activation, used at a final concentration of 20 µg/mL foctrials with S9/mix.
3	Test organisms:	
5.	Cell line:	Chinase homster $079$ has cells $0^{-1}$
	Centime:	Calls obtained from Prof. G. Speit University of Alm
		Chanese hamster \$79 lugg cells Cells obtained from Prof. G. Speit, University of \$1m, Germany. These cells have since been recloned to maintain
	Source:	karyotypic Cability. They have a modal chromosome number of
	Culture condition.	Acubation performed at 37 ⁽²⁾ in a humidified atmosphere with
	Culture condition:	about 5% CO2
		AF 344122 was used a concentrations ranging from 1 to 2500
4.	Test compound O	22 and a sapid population doubling trove (1600 14 hours) facubation performed at 37 °C in a humidified atmosphere with about 5% CO2. Al 1344122 was used at concentrations ranging from 1 to 2500 µg/mL in the clonal cytotoxicity assay and from 75 to 2400
co	ncentrations.	jug/nd/In the mutagenic assays.
		The S9 fraction was isolated from the livers of Aroclor 1254
		induced male Sprague Dawle Prats. The preparation dated from
_		February 04, 2003 (protein content 26.4 mg/mL) and was kept
5.	Metabolic activation:	frozen at -80°C. The batch was tested for contamination and
		cytotoxicate priooto use in the first study. Cofactors were
	ý A S	freshly dissolved in socium phosphate buffer (150 mM, pH 7.4)
Ъ	Metabolic activation:	
В. 1	Study design and methods	
1	In life datase 90 Mag	13 $4$ $1$ $20$ $1$ $2$

1. In life dates: 20 May - 03 fully 2003 The selection of V79 forward mutations is based on the resistance of induced mutants to the purine analogue 6-thioguatione (6 JG). This resistance is a result of a mutation at the X-chromosome-linked HPRT locus rendering the cells unable to use 6 TG for DNA synthesis. Therefore, cell colonies formed in the presence of 6-TG are considered to represent mutants at the HPRT gene.

# 2. Determination of cytotexicity

~O Exponentally growing \$79 cells were plated in 20 mL culture medium in a 275 mL flask (4x106 cells per flasks). For each concentration, one culture was available. After attachment (16 to 24 hours later), cells were exposed without \$9 mix to vehicle alone or to a range of concentrations of the test substance for hours in 20 mL medium containing 2% FCS. In experiments with metabolic activation 1 mL of medium was replaced by 1 mL of S9 mix. Thereafter, each cell monolayer was washed with PBS, trypsinized and replated in 5 mL culture medium at a density of 200 cells into 3 Petri dishes (diameter of 60 mm). Thereafter, colonies were fixed with 95% methanol, stained with Giemsa (Merck; stock



solution diluted 1:5 with deionized water) and counted automatically using an Artek counter, when there was no interference by precipitation on the plates or colouration of the plates. Cytotoxicity was expressed by comparison of colonies in treated cultures versus vehicle control cultures (relative closing efficiency).

#### 3. Treatment protocol without metabolic activation::

concentration (4x106 cells per flask) including all control groups. After attachment (16 to 24 hours later), the cells were exposed to vehicle alone or to a range of concentrations of the test substance for 5 hours in 20 mL culture medium with reduced serum content (2%). Thereafter, cell monolavers were washed with PBS, trypsinized and replated in 20 mL culture medium using 1.5x106 cells per 75 om2 flask and in 5 mL culture medium using 200 cells per Petri dish (diameter of 60 mm). One flask and 3 Petri dishes were used per culture. These dishes avere incubated for 6 to 8 days to allow colory development and to determine the cytotoxicity associated with each test substance directly after treatment (survival to treatment).

Cells in 75 cm2 flasks were incubated to permit growth and expression of induced mutations. Cells were subcultured (= count 1, normally after 3 days) by esseeding 1.5x406 cells into 20 mL of medium in 75 cm2 flasks. At the end of the expression period (=count 2, normally a total of 6 days), cultures were reseeded in Petri dishes (diameter of 100 mm) at 3x105 cells per dish (8 dishes per culture) in 20 mL culture medium without hypoxanthine but contaiting 10  $\mu$ g/mL 6-TG for selection of mutants. In addition, 200 cells per dish (diameter of 60 mm, 3 dishes per culture) were seeded in 5 mL culture medium to determine the absolute cloning efficiency for each concentration. After incubation for 6 to 8 days, the colonies were fixed, stained with Giemsa and counted to determine the momber of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.

Two trials were performed.

# 4. Treatment protocol with metabolic activation:

The activation as any was performed independently. The procedure was identical to the non-activation assay except for the addition of S9 mix. In these experiments 19 mL instead of 20 mL culture medium and additionally 1 mL of S9 mix was added to the tasks for the treatment period, resulting in a concentration of 5% S9 mix in the cultures. The number of 6-TG resistant mutants and viability were determined as in the non-activation as ay.

Two trials were performed

### 5. Parameters assessed

The parameter surviver to treatment in Navas deermined on the basis of the following calculation:

Mean number of colonies (reated cultures)×100

@ Mean number of colonies (vehicle control cultures)

The "absolute population growth" was cateulated using the following formula:

Absolute population growth (for each culture)  $\stackrel{\frown}{=}$  cell count 1 x cell count 2

The parameter relative population prowth shows the cumulative growth of the treated cell populations, relative to the vehicle control.

Absolute population growth treated culture×100

Absolute population growth of corresponding vehicle control culture

The ability of cells to form colonies at the time of mutant selection is measured by the parameter "absolute cloning efficiency". It is expressed in %.

Mean number of colonies per dish×100



0

The "mutant frequency" is calculated for each group by dividing the total number of mutant colonies by the number of cells seeded (usually 8-10 plates at  $3x10^5$  cells per plate), corrected for the absolute cloning efficiency. The mutant frequency is expressed as 6-TG resistant mutants par  $10^6$  clonable cells.

Total number of mutant colonies×100

Number of evaluated dishes  $\times 3 \times 10^5 \times C.E$ 

#### 5. Acceptance criteria

- The average cloning efficiency of the negative and vehicle controls should be at least 50%

- The average mutant frequency of the vehicle controls should not exceed 25 x 1050 cells

- The mutant frequency of the two cultures of the volticle and /or the negative control should differ only to an acceptable extent. As a rule of thumb, the difference of mutant frequencies should not be greater than 5 x 10-6.

- The positive control should induce an average mutant requency of at least three that of the vehicle control.

- If not limited by the solubility of the test substance in the vehicle the highest concentration should induce cytotoxicity of about 80 to 90% or should be a concentration where precipitation occurs in the medium. The survival at the lowest concentration should be in the rappe of the negative control.

- For the calculation of an acceptable mutant frequency at least 5 dishes per culture should be available and relative survival to treatment, relative population growth and absolute cloning efficiency should be 10% or greater.

However, these criteria may be overruled by good scientific judgment.

### 6. Assessment criterio.

- Mutant frequencies were only used for assessment, that least 5 dishes per culture were available and relative survival to treatment, relative population growth and absolute cloning efficiency were 10% or greater.

- A trial was considered positive of a concentration-related and in parallel cultures reproducible increase in mutant frequencies was observed. To be relevant the increase in mutant frequencies should be at least two to three times that of the highest negative or vehicle control value observed in the respective trial. If this result was reproduced in a second real, the test substance was considered to be mutagenic.

- Despite these driteria a positive result was only considered relevant, if no significant change in osmolality compared to the vehicle control as observed. Otherwise, unphysiological culture conditions may be the reason for the positive result.

- A test substance was judged as equivocal if there was no strictly concentration related increase in mutation frequencies but if one or more concentrations induced a reproducible and biologically relevant increase in mutant frequencies in all trials.

- An assay was considered negative if no reproducible and relevant increases of mutant frequencies were observed.

However, these criteria may be everruled by good scientific judgment.

### 7. Statistical analysis:

The statistical analysis relied on the mutant frequencies which were submitted to a weighted analysis of variance as well as to a weighted recursive regression, both with Poisson derived weights.

The two mutant frequency values obtained per group were, although somewhat related, considered as independent measurements thus increasing the power of the statistical tests applied. Since the protocol of the HPRT assay required at least two independent trials, the overall analysis without respectively with



activation was the most important one for classifying substances into mutagens and non-mutagens. However, separate analyses were run for each trial in order to examine the consistency of the results.

All acceptable groups were included in the weighted analysis of variance followed by pairwise comparisons to the vehicle control on a nominal significance level of p = 0.05 using the Durrett test. The regression analysis part was performed on the basis of the actual concentrations thereby omitting the positive, negative and vehicle controls. If there was a significant concentration related increase of the mutant frequency (p = 0.05) in the main analysis the highest concentration was dropped and the analysis repeated. This procedure was repeated until p > 0.05. In that way eliminated concentrations were flagged correspondingly.

#### **II. Results and discussion**

#### A. General remarks:

In the absence of S9 mix Chinese hamster V79 cells were exposed to AE 1344122 at concentrations of up to and including 2400 µg/mL. Without and with S9 mix no substance precipitation occurred in the medium.

Good cloning conditions were demonstrated by the absolute doming efficiency for the vehicle controls ranging from 54.5% to 75.3% and from 52.5% to 74.9% with and with pretability activation, respectively.

#### **B.** Mutation assay:

The test system proved to be sensitive on both experimental conditions (activation and non-activation) since treatment with the positive controls caused a biologically relevant increase in mutant frequencies as compared to the corresponding controls.

There was no relevant increase in mutant frequencies ofter treatment with AE 1344122 at any concentration (up to and including a dose level of 2400  $\mu$ g/oL) either with or without metabolic activation.

 Table 5.8.1-20
 Relative survival and mean mutation frequency mutative colonies per 1 millions cells)

 -Experiment 1-without \$9 mix
 0

<b>Oreatment</b>	Contentration (µg/mL)	Relative survival	Mutation
			frequency
AE 1344122	× 2400 × 1200 × 1200 × 500 × 500	<u>97.5</u>	2.00
	× 1200 × 1200	100.6	2.40
AE 12441220		82.4	2.65
AE 344126		91.6	2.15
		81.2	4.20
		105.2	1.95
		94.8	1.20
Solvent control		100.0	5.10
	2400	95.9	2.70
AE 1344122		89.4	0.60
	<b>2</b> 600	95.6	4.20
	300	89.3	2.70
	150	92.0	3.95
AF 1344)22	75	104.7	3.70
AE 1344122	0	115.5	3.40
Solvent control	0	100.0	3.80
EMS	900	13.3	745.15
SMS · ethylmethanesulfonate			

EMS : ethylmethanesulfonate



–Experime	ent 2-with S9 mix		~ °
Treatment	Concentration (µg/mL)	Relative survival	Mutation frequency
		(%)	
	2400	147.3	2.05
	1200	119.3	1.35
AE 1344122	600	107.8	\$00 \$°\$
AE 1544122	300	129.8	× 0.30 × ~
	150 🚿	137	
	75 🗸	100.1	× 200 × 40
Negative control	0 4	Q15.4 °	λ 0.75 Č
Solvent control	0	>> 100.0 €	Q 2.760 Q
AE 1344122	2400 °	5 102.4	D 270 V
	1200 @	¥ 16.6 0	4.85
	A600 0 0	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	0 1.100
	300 ~	0° 108, r . 0	L 3.20 L
		96.1 ×	Ø.95 O
	Q \$75 \$ \$	<u></u>	0.000
Negative control		N 1150 C	2,45
Solvent control		Ø £90.0 O	× 4.00
DMBA 👷		× 70.0 00	Ø 87.70
· not cloned due to cytotoxicity			

Table 5.8.1-21 Relative survival and mean mutation frequency (mutant colonies per 1 millions cells)

N : not cloned due to cytotoxicity DMBA : dimethylbenzanthracene

#### III. Conclusions

HPRT forward mutation assay, both with AE 1344122 was considered to be for mutagenie Ŵ and without metabolic activation

No de la comencia de

# Assessment and conclusion by applicant:

No. Study meets the current gordance and the requirements in 283/2013.

AE 1344122 was considered to be non mutagenic in this V⁴⁹/HPRT forward mutation assay, both with and without metabolic activation



Data Point:	KCA 5.8.1/12	
Report Author:		
Report Year:	2003	ð
Report Title:	28-day toxicity study in the rat by dietary administration Code: AE 1344122	Y
Report No:	C037198	
Document No:	<u>M-222343-01-1</u>	
Guideline(s) followed in study:	EU (=EEC): 92/69 Annex V, B7; OECD: 407	
Deviations from current test guideline:	Current guideline: OECD 407, 2008 Deviations: Study lacks the endowine measurements incorporated into entrent of guideline. These deviations do not impact the acceptability of the study given it is intended to investigate the reapeat dose toxicity of a metabolite.	
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).	
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially secognised testing facilities	
Acceptability/Reliability:	Yes A A A A	

#### **Executive Summary**

AE 1344122 (batch number YG2228, 98.8%, purity) was administered continuously via the diet to groups of Wistar rats (10/sex/group) for 28 days at concentrations of 20, 200, 2,000 and 20000 ppm. A similarly constituted group received untreated diet and acted as a control. Animals were observed daily for mortality and clinical signs. Physical staminations were performed weekly. In addition, grasping, righting, corneal, pupillary auditory startle and head shaking reflexes were examined once during the acclimatization phase and during Week 4 of the Study. Body weight and food consumption were recorded once weekly. During the acclimatization phase all animals were subjected to an ophthalmic examination, all anythals in the sontrol and 20000 ppm groups were re-examined during Week 4. Hematology, clinical chemistry and urine parameters were determined at the end of the study. All animals were necropsied, selected organs weighed and a range of assues were taken, fixed and examined microscopically. Toxicological findings were confined to the highest dose level of 20000 ppm and consisted of scabs around the nos head tegion soiling around the exe and chromodacryorrhea in males and anogenital soiling in Temales. Absolute body weight in males was reduced by 7%, with overall mean body weight gain reduced by 18% by Day 28, compared to the controls. In females, absolute body weight was reduced by 4% on Day 28 whilst overall body weight gain was 17% lower than the control group. Food consumption was reduced by 13% during weekone in males and by 9% over the course of the study in females. Organic phosphorus concentration was reduced by 10% by Day 28 in males. Urinalysis revealed coarsely granuftar casts in the urine of 1/10 males and 9/10 females, slightly lower pH values and ketone levels in males and higher mean urinary in both sexes. Microscopically, minimal to moderate tubular degeneration/regeneration and single cell necrosis of minimal to moderate severity was observed in the Lodneys of 8/40 females. This change was correlated with the coarsely granular casts observed in the uring

#### **Conclusion:**

The NOAEL AE 1344122 in this 28² day rat feeding study was considered to be 2000 ppm in both sexes (equivalent to 152 mg/kg day in males and 167 mg/kg/day in females).

Materials and methods

A. Materiats

1. Testomaterial:	AE 1344122
Description	A white powder
Lot / Batch #:	YG3228



Purity:	98.8%
CAS #	Not reported
Stability of test compound:	Stable in rodent diet for a period covering the study duration
2. Vehicle and / or positive	
control:	none
3. Test animals:	S 4 S
Species:	Rat a start a
Strain:	Wistar Rj: WI (IOPS HAN)
Age:	7 weeks approximately $\sqrt{2}$ $\sqrt{2}$
Weight at dosing:	236 to 273 g for the males – 169 to 204 g for the females
Source:	R. Janvier, Le Génest St Isle, France
Acclimation period:	12 days $A$ $Q$ $a^{\circ}$ $A$ $A$ $Q$ $a^{\circ}$
-	Certified rodent powdered and irradiated thet A 04C-10P1 from
Diet:	S.A.F.E. (Scientific Animal Food and Engineering, Epinay Sur-
	Orge, France), and libitium
Water:	Municipal tap water ad libitim
Housing	Animals were caged individually in suspended stainless steel
Housing:	with mesh cages of the the second sec
<b>Environmental conditions:</b>	
Temperature:	20-24 2 2 2 2 2 2 2
Humidity:	Animals were caged individually in suspended stainless steel with mesh cages 20-24% 40-70% Approximately 20-15 ar changes per hour
Air changes:	Approximately 10-15 au changes perhour 6
Photoperiod:	// Itorn/osting []/ hour light and darl/ avelag [/ am (// nm)
B Study dosign	
<b>B.</b> Study design $\bigcirc$	
<b>1. In life dates:</b> 19 Februar	Frequency of $A_{0}$ and $A_{$
2 Animal assignment and freatment a S S O O S	

# 2. Animal assignment and treatment

There were 10 animals opeach sex per dose group. Apimals were assigned to dose groups randomly by body weight. AE 1344622 was administered in the diet for 28 days to Wistar rats at the following doses -0, 20, 200, 2000 and 2000 ppm (equating to \$.5, 149, 152 and 1495 mg/kg/day in males and 1.7, 16.8, 167 and 1616 mg/kg/day in females). A negative control group received plain diet. Animal housing and husbabdry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NH publication N°86-23, revised 1985) and "Le Guide de Journal Officiel des Communantés Européennes L358, 18 Décembre 1986, N°86/609/CEE da 24 Novembre 1986" 3. Diet preparation and analysis

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AE 1344122 was incorporated into the det by dry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. The stability was demonstrated during the course of the study at concentrations of 20, 200 and 20000 ppm for a time which covered the period of usage and storage for the stud Homogeneity at the lowest and highest dietary concentrations and

ot usage and storage for the studie Homogeneity at the lowest and highest dietary concentrations ar concentration checks at all dose levels were within the range of 85-115% of nominal concentrations.



Test group	Concentration in diet (ppm)	-	nimal (study rages)	Animals assigned		
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Mate	Female	
1	0	0	0	× 10		
2	20	1.5	1.7	× 10	~~~.16°~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	
3	200	14.9	16.8	رم 10 ک		
4	2000	152	لَمْ 167 أَنْ	10 🗶	2 10 C	
5	20000	1495	1616 Q [*]	∘ 10¢		
Statistics		Ď	× × .	o Q (Ô		

### 4. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Durnett test (2-suded) on parameters showing a significant effect by ANOVA. When the data were not homogeneous a Kruskal-Wallis ASOVA was performed followed by the Dunn test (2-sided) to identify statistical significance between groups. For some parameters, when data were not homogeneous they were transformed using a log transformation or square root transformation, then reanalyzed as above.

For urine analysis (pH), group means were compared using the non-parametric Kruskal-Wallis test which was followed by the Durin test Z-sided), if the Kruskal-Wallis test indicated significance.

When one or more group variance (Spequaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using the Path/Tox System V4.2.2 (Modifie Englanced Statistics).

### C. Methods

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed finical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

Neurotoxicity assessment was performed during the acclimatization phase and during Week 4 of the study by testing the following reflexes: Grasping reflex, righting reflex, corneal reflex, papillary reflex, auditory startle reflex and head shaking reflex.

An ophthalmic examination was performed on all animals during the acclimatization phase and on control and high dose group animals during Week 4. After instillation of an atropinic agent (Mydrianeum, Merck Sharp and Dohme) each eye was examined by means of an indirect ophthalmoscope.

### 2. Body weight

Body weights were recorded three times during the acclimatization phase, on the first day of test substance administration, then at weekly atervals throughout the treatment periods. Diet-fasted animals were weighed before necessary.

## 3. Food consumption

Food consumption was recorded weekly; the weekly mean achieved dosage intake for each week and for week to 4 was calculated for each sex.

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### 4. Clinical pathology

On study days 24, 25 or 26, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet-fasted overnight prior to blood sampling and anesthetized by inhalation of Isoflurane. Blood was collected on EDTA for hematology, on orthium heparin for plasma and clot activator for serum for clinical chemistry and on sodium of the tor coagulation parameters.

The following hematology parameters were assayed using an Advia 120 (Bayer Diagnostics, Pateaux, France): red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation and platelet count. A blood smear was prepared using Wright stain and was examined when results of the Advia 120 determinations were abnormal. Prothrombin time was assayed on an ACL 3000 (Instrumentation Laboratory, Paris, France).

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, chloride, sodium, potassium, calcium, inorganic phosphotus, total cholesterol, triglycerides, aspartate aminotransferase, alatine aminotransferase, alkaline plosphatase activities, total protein and albumin concentrations were assayed on serum samples using an Hitach 911 (Roche Diagnostics, Meylan, France).

On study days 29, 30, 31 or 32, overnight prine samples were coffected from all animals for arinalysis. Feed and water were not accessible during urine collection.

Additional urine samples were collected overnight from all animals once peoweek for analysis of metabolites.

Any significant change in the general appearance of the urine was recorded. Urinary volume was measured. pH was assayed using a Clinitek 200+ and Ames Multistix dipstices (Bayer Diagnostics, Puteaux, France). Urinary refractive index was measured using a RFM320 refractometer (Bioblock Scientific, Illkirch, France).

Glucose, bilirubin, ketone bodies, occult blood, protein and probilingen were assayed using a Clinitek 200+ and Ame Multistix dipsticks Bayer Diagnostics, Buteaux France).

Microscopic examination of the trinary sediment was performed after centrifugation of the urine. The presence of red blood cells, while blood cells, epithelial cells, bacteria, casts and crystals was graded.

### 6. Sacrifice and pathology

On study days 29, 30, 3 k or 32, 2 complete necropsy was performed on all surviving animals. Animals were deeply anesthetized by foolurate inhibitation, then examinated before necropsy. All animals were diet-fasted prior to scheduled sacrifice. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abrormatties were recorded, sampled and examined microscopically. Adrenal gland, brain, epictidymetis, heart, kidney, liver, ovary, pituitary gland, prostate, spleen, testis, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

The following organs or tissue, were sampled: adrenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone marro@ (sternum), brain, epididymis, oesophagus, exorbital (lachrymal) gland, eye and optic nerve, Harderian gland, beart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kitney, larynx/pharynx liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, of ary, pancrea@ pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, opinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymas, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagma. Abone marrow smear was prepared from femur, stained with May-Grünwald Giemsa, but not examined. Tissues samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative. Histopathological examinations were performed on all tissues from all the animals in the



control and high dose groups. Kidney, liver, lung and thyroid gland were examined in the intermediate dose groups. Significant macroscopic findings were also examined in all dose groups.

### II. **Results and discussion**

### A. Clinical signs and mortality

### 1. Clinical signs of toxicity

At 20000 ppm, treatment-related clinical signs consisted of scabs around the nose/head region observed in 3/10 males, soiling around the eye in one male and anogenital soiling in one female. At 2000 ppfr, the only finding was scabs around the nose/head region in 2/10 males and nasal so fing noted in one male. In the absence of any other treatment-related effects in either sex at this dose level, these findings were considered to have no toxicological relevance

No treatment-related clinical signs were observed in females at 2000 ppm or at either sex.

### 2. Mortality

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There was no mortality in any group

### 3. Neurotoxicity assessment:

No findings were observed at the neurotox wity There were no treatment-related clinical signs of any group

### 4. Ophthalmology examination

At 20000 ppm, chromodactyorrhea was observed in two males.

No ocular abnormalities, were observed at lower dose levels

## B. Body weight and body weight gain

in two males. veduces by 7% (p≤∞⁶ veduces by 7% (p≤∞⁶ vay 1 and Dav vay 28 vare At 20000 ppm, absolute Body weight in males was reduced by 7% (p≤001) on Day 28, with overall mean body weight gain reduced by 18% between Day 1 and Day 28, compared to the controls. In females, absolute body weight was reduced by 4% on Day 20 whils voverall mean body weight gain was reduced by 17% between Day 1 and Day 28, compared to the controls.

Table 5.8.1-23	Mean	body wei	ight gain	(g) in ma	le and fe	malerats				
		Ň Â		<i>w</i>	Dose lev	æl (ppm)				
Week	Q.		Males			) &.		Females		
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	Ŷ	<u>,</u> ⊘20 ∧	<u>^</u> 200	2000	29000	0	20	200	2000	20000
Overall body weight gain					N N N					
between week	141	«135°	≫137 <i>″</i>	¢ 141¢	116	53	49	57	54	44
1 (study day 1)	×4	\$(96) ×	~137 (97)	(1-00)	(82)	(-)	(92)	(108)	(102)	(83)
and week 4 (% control)										

No relevant changes in mean body weight or mean body weight gain were observed at 2000 ppm or Ô below.

C. Food consumption and compound intake

Food consumption was reduced by 13% (p<0.05) during week one of the treatment period in males whilst over the course of the entire treatment period food consumption was reduced by 9% in females.

There was no impact on food consumption at 2000, 200 or 20 ppm.



Achieved compound intake is presented in Table 5.8-20.

D. Hematology, clinical chemistry, and urinalysis

1. Hematology

Hematological examination showed no treatment-related findings.

2. Clinical Chemistry

At the clinical chemistry examination, organic phosphorus concentration was reduced by 10% $p \le 0.01\%$ by Day 28 in males at 20000 ppm.

No relevant changes were observed at 2000, 200 or 20 upm in males of at any dose level in females

3. Urinalysis

At 20000 ppm, urinalysis revealed coarsely granular casts in the urine of 1/10 males and 9/10 females. In addition slightly lower pH values and ketone levels were noted in males whilst mean urmary volume was higher than control levels for both sexes.

Table 5.8.1-24Mean urinary pH and	dyolumes in	male an	dfemale	skat	S
-----------------------------------	-------------	---------	---------	-------------	---

			R			el (ppm)	, Q	S.	Û ^{\$} a	0
Week			Males	° ` ` ` `		Ň	ð (Females	× 5	
	0	20 🥡	× 200	2000	20000	<i>,</i> , 0	\heartsuit \bigcirc	200	ر 2000	20000
pH values	6.8	7,0	6.9	6.6	6.2*	6.20 ⁵	633	6.2	© 6.2	5.8
Mean urinary volume (ml)	4.2	¢4.5	0 4.7 0		5.9	2.1 ×	\$1.8 \$		2.3	4.2**

* $p \le 0.05$; ** $p \le 0.01$ significantly different from the control using the Kruskal-Wallis of Dunn tests

No relevant changes were observed at 2000, 200 or 20 ppm.

E. Sacrifice and pathology

Terminal body weight and organ weights

Mean terminal body weight was 7% (p 0.01) lower matter at 20000 ppm than in control animals.

At 20000 ppm lower liver weights were observed in males. Nevertheless, without any histopathological relationship, this effect was considered to be owno to weological significance.

No relevant changes were observed at 2000, 200 of 20 ppp

Macroscopy

Black to dark red soiled fur around the nose and eyes or eyes was found in 3/10 males at 20000 ppm.

No treatment-related macroscopic resions were observed at 20000 ppm in females or in either sex at any lower dose levels.

Microscopy

At microscopic examination, minimal to moderate tubular degeneration/regeneration and single cell necrosis of minimal to moderate severity was observed in the kidneys of 8/10 females, compared to no cases in the controls. This charge was correlated with the coarsely granular casts observed in the urine.



Sex			Male					Female		Ø	ð
Dosage level (ppm)	0	20	200	2000	20000	0	20	200	2000	20000	<u>S</u>
Number examined	10	10	10	10	10	10	10	S 10		,10°	ð
			Tubular	degenera	tion/reg	eneration		5		Ý Č	, e
Minimal	0	0	0	0	0	0	Q_{0}	0,0	í 🔊	×2	Ś
Slight	0	0	0	0	¢70	0	0		- P	03	1
Moderate	0	0	0	0	0	\sim^0	Ô	$\sqrt[2]{0}$	0 B	30	
Total	0	0	0	×0 پ	ô	3 ⁰ 0 x	\mathcal{T}_{0}			28	
				Single ce	Lnecrosi	s ô	- A	- P	\$		
Minimal	0	0	0 🖉		<u>_</u> 0y		A0 6	۶ 0 ⁽	0	<u></u>	
Slight	0	0	<u>e</u>	î.		× 0 0			~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	5	
Moderate	0	0	jõt j	<u>کې</u> 0	× 0,5		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	\$0	0 Q	1	
Total	0	0		<u></u>	<i>S</i> 0	$\hat{\mathcal{S}}_{0}$			°0%	8	

Table 5.8.1-25	Incidence of treatment-related lesions in the kidney
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No treatment-related microscopic lesions were observed at 20000 ppm in males or in either sex at any lower dose levels.

F. Deficiencies

No specific deficiencies were poted in this study

III. Conclusions &

The NOAEL AFC 3441 22 in this 28 day rat feeding study was considered to be 2000 ppm in both sexes (equivalent to 22 mg/kg/dao in mates and 67 mg/kg/day in females).

Assessment and conclusion by applicant

Study meets the corrent guidance and the requirements in 283/2013 as it provides information on the effects and target organs of AE 1344122 in the rat

The NOAEL AE 1344122 on this 28-day Pat feeding study was considered to be 2000 ppm in both sexes (equivalent to 152 pg/kg/day in oales and 167 ong/kg/day in females).

Groundwater metabolites

Trifluoroacetic acid (TFA), is a fluoryran groundwater metabolite which exceeds the groundwater PECgw threshold of 0.75 ye L, consequently, genotoxicity and general toxicity testing are triggered. TFA is a common metabolite for several other active substances and an Ames test, an *in vitro* Mammalian Cell Gene Quation test (mouse lymphoma L5178Y cells) and Mammalian Chromosome Aberration test (numan lymphocytes) are available, which were all negative, indicating that TFA does not have a genotoxic potential. Testing for general toxicity is currently ongoing. The currently available studies; 3 *fa vitro* genotoxicity studies, rat acute oral toxity study, 14-day, 28-day and 90-day toxicity rat studies are summarized below. The outstanding developmental toxicity study in the rabbit and extended one generation reproduction study in the rat will be summitted when available.



Data Point:	KCA 5.8.1/49	
Report Author:		
Report Year:	2013 Image: Constraint of the second secon	ð
Report Title:	Sodium Trifluoroacetate - Acute oral toxicity study in rats	Ş
Report No:	12/333-001P	,
Document No:	<u>M-444479-01-1</u>	
Guideline(s) followed in	OECD 425; Commission Regulation (EC) No 440/2008, B.1. TRIS; US-EPA 32-	
<mark>study:</mark>	C-98-190 , OPPTS 870.1100;	2
Deviations from current	Current Guideline: OECD 425, 2008	_
test guideline:		Å
Previous evaluation:		Ŏ ^Ÿ
GLP/Officially	Yes, conducted under GLP/Officially recognized testing facolities	1
recognised testing	Yes, conducted under GLP/(opticially recognised testing faculities	
facilities:		
Acceptability/Reliability:	Yes 4 6° 7 27 7 7° 7° 7° 7°	

Executive summary

The single-dose oral toxicity study with sodium Trifluoroacetare was performed according to the acute toxic class method (OECD TG 425, abopted at 3rd October 2008, Comparission Regulation (DC) No 440/2008; B.1.TRIS; US-EPA 712-098-1997, OPPTS 820-11000

Initially one animal was treated at the limit dose level (2000 mg/kg bw) As this animal survived, additional 4 animals were dosed sequentially. As the sedditional animals survived, the LD 50 was defined to be greater than the limit dose and the test was terminated decording to Test Guidelines OECD 425/OPTTS 870.1100. Ň Ô Ô

A single oral treatment was carried out by gavage for each animal after an overnight food withdrawal. Food was made available again 3 hours after the treatment. Sodium Trifluoroacetate was administered as a solution prepared in distilled water at deonceptration of 200 mg/mD at a dosing volume of 10 mL/kg bw. Clinical observations were performed at 30 minutes, 1, 223, 4 and 6 hours after dosing and daily for 14 days thereafter. Body weight was measured on days -1, 0 and 7 and before necropsy. All animals were subjected to a necropsy and a macroscopic examination. J.O

There was to mortality at a dose revel of 2000 mg/kgobw. Treatment with Sodium Trifluoroacetate at the dose level of 2000 mg/kg by did not cause any gest item related adverse effects during the 14 days observation period. Body weight and body weight gain of Sodium Trifluoroacetate treated animals showed no indication of a freatment-related effect. There was no evidence of observations at a dose level of 2000 mg/kg b@at nectopsy

Under the conditions of this study, the source of LD50 value of the test item Sodium Trifluoroacetate was found to be above 2000 mg/kg bw/in female CRL:(WI) rats.

Sodium Frifluoroacetate is non-toxic after acute and administration at a dose I evel of 2000 mg/kg bw. The study result triggers the following classification/labelling:

- EU Directive 1999/45/BC (as @mended): none
- Regulation (FC) No. 1272/2008 (CP): none ~Q⁰

- GHS (rev. 2011 Junclassified



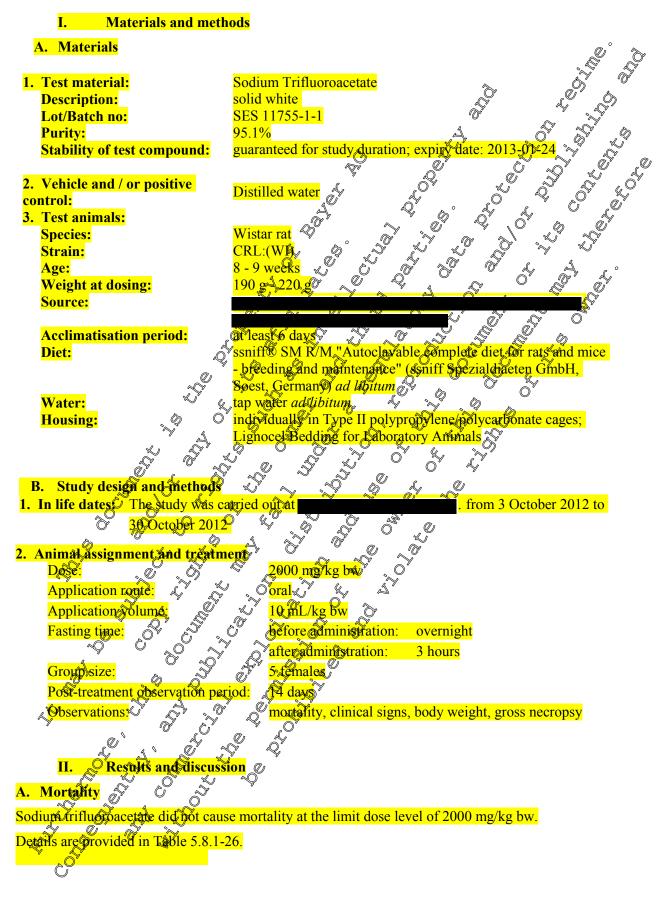




Table 5.8.1-26 D	oses, mortality / anir	nals treated		
<mark>Dose</mark> (mg/kg bw)	Toxicological findings*	Duration of signs	Onset of death after (days)	LD ₅₀ (mg/kg byv)
Group 1 2000	0/0/5		<mark></mark> 🏷	LD ₅₀ >2000
*number of dead ani	mals/number of anii	nals with clinical sign	s/number of amphals	tested.
B. Clinical observa	ations		×,	
No clinical signs we	re observed in femal	les dosed at 2000 mg/	kg.	E A A
C. Body weight				LD ₅₀ >2000 tested.
Body weight and bo	dy weight gain of so	dium trifluoroacetate	treated animals show	wetho indication of
a treatment-related e	ffect.	4. 0° *		
D. Necropsy				dy A de
No abnormalities we	ere observed at gross	Hecropsy.	\rightarrow A δ	
E. Deficiencies	ĺ	odium triffæoroacetate		
No deficiencies are r	noted.			
III. Conc	lusions			
	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~			
Sodium trifluoroace 2000 mg/kg bw in fe	etate is non-texic a	after acute oral adm	nifirstration with an	LD ₅₀ value above
2000 mg/kg Uw m le			`&	×
Assessment and af	Anclusion by applic		O ^Y O ^Y O ^Y	
The study was perfect	ormed under GLP &	anditions in accordance	ce with the current O	ECD guideline 425
		ly is therefore conside	A	
		pacetate $3s > 2000 m_{\odot}$		to the classification
	O Nº A	2008, no class	[∞] ×	
E.		Y Q O D		
~~~~ ( .1				
<u> </u>				
, 40 Å	ST A . O			
		¢ Ö [¢]		
	A` QÎ DÎ			
4, A	L			
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Data Point:	KCA 5.8.1/50
Report Author:	
Report Year:	2005
Report Title:	Trifluoroacetate (TFA): reverse mutation in five histidine-requiring strains of
	Salmonella typhimurium
Report No:	2014/82 <u>2014/82</u>
Document No:	M-256628-01-1
Guideline(s) followed in	OECD 471(1997) ; EEC Annex V, B13/14; UKEMS Quidelines; Japonese MOHW; JMAFF; ICH Harmonised Tripartite Guideline; US-EPA OPPTS 870.5100 (1998);
study:	MOHW; JMAFF; ICH Harmonised Tripartite Guideline; US-EPA OPPTS //
	870.5100 (1998); <u>v</u> <u>Q</u> ' <u>C</u> <u>V</u> <u>Q</u> '
Deviations from current	$\begin{bmatrix} current guideline: OECD 4/1,2020 \\ \bigcirc & \bigcirc$
test guideline:	Deviation: none
Previous evaluation:	Not evaluated
GLP/Officially	Yes, conducted under GDP/Officially recognise desting facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes A A A A A A A A A A A A A A A A A A A

Executive Summary

Trifluoroacetate (TFA) was assaved for mutation in five histidine requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102), both in the absence and in the presence of metabolic activation by an Acoclor 1254-induced fat liver post-mitochondrial fraction (S-9), in two separate experiments.

An initial toxicity Range-Finder Experiment was carried out in strain TA400 only, in the absence and presence of S-9, using final concentrations of TEX at 1.6, 8, 40, 200, 1000 and 5000 μ g/plate, plus negative (solvent) and positive controls. So evidence of toxicity was observed following any of these treatments. As formulations analysis was not performed for this experiment, strain TA100 treatments were included in Experiment 1.

Experiment 1 treatments of all of the test strains in the absence and presence of S-9 retained the same test doses as employed for the Range-Finder Experiment. No evidence of toxicity was observed following any of these reatments.

Experiment 2 treatments of all the tester strains in the absence and presence of S-9 were performed with the maximum test dose of 5000 µg/plate. All treatments in the presence of S-9 were further modified by the inclusion of a pre-incubation step. No evidence of toxicity was observed following any of these treatments.

Negative (solvent) and positive control treatments were included for all strains in both experiments. The mean numbers of revertant exploring on negative control plates all fell within acceptable ranges, and were significantly elevated by positive control treatments.

No dose-related and reproducible increases in revertant numbers were observed following any of the treatments of any of the tester strains in the absence or presence of S-9.

Therefore this study was considered to have provided no evidence of any TFA mutagenic activity.



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1. Materials and methods

A. Materials

1. Test material:	Trifluoroacetate (Sodium Trifluoroacetate) White powder 016911/1 99.1 % 2923-18-4 Stable for the duration of the study Negative: Culture medium Solvent: Sterilised water Positive: Final concentration
Description	White powder
Lot / Batch #:	016911/1
<mark>Purity:</mark>	99.1 % 2923-18-4 Stable for the duration of the study Negative: Culture medium Solvent: Sterilised water
CAS #	2923-18-4 & $\sqrt[4]{2}$
Stability of test compound	: Stable for the duration of the study
2. Control materials:	Negative: Culture medium Solvent: Sterilised water
	Solvent: Sterilised water
	Positive:
Chemical	Positive: Final concentration (µg/plate)
Chemical	(µg/plate) \sim Strain(s) \sim System (s) \sim \sim \sim
2-Nitrofluorene (2NF)	
Sodium azide (NaN ₃)	Q 20 X X X 100, TA100, TA105 X
9-Aminoacridine (AAC)	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
Mitomycin C (MMC)	^Q ₆ 0.2 ₆ ₆ ₆ ⁷ ¹ ¹ ⁰ ₂ ₆ ¹ ² ³ ³
Benzo[a]pyrene (B[a]P)	
2-Aminoanthracene (AAN)	1 $\frac{1}{2}$
Contraction of the second seco	
<u> </u>	$\begin{array}{c c} & \mathbf{TA100}, \mathbf{TA100}, \mathbf{TA103}, \mathbf{TA10} \\ \hline \\ & \mathbf{C}20.0 \\ \mathbf{C} \\ \mathbf{C}$
Activation	

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Apoclor 254 and obtained from Molecular Toxicology Incorporated, USA. Somix was constitued of S9 fraction, sodium phosphate buffer pH 7.4, glucose-6phosphate (disedium), NADP (disedium), magnesium chioride potas sum chloride, L-histidine HCl (in 250 mM MgCl₂), d-biotin and water

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Test organisms:

Five bacterial strang of Salmonetta typh muriting (TA28, TA100, TA1535, TA1537 and TA102) were used in this study All the tester strains, with the exception of strain TA102, were originally obtained from the UK NCTC. Strain TX102 was originally obtained from Glaxo Group Research Limited. For all assays, bacteria were contured for 10 bours at 37±1°C in nutrient broth (containing ampicillin for strains TA98 and TA100 and appicilling and tetracycline for strain TA102). Incubation was carried out in a shaking incubator. Bacteria were taken from wals of frozen cultures, which had been checked for strain characteristics of histidine dependence, un P character, rfa character and resistance to ampicillin (TA98 and TA100) or ampicillin plus tetracycline (TA102).

Test Concentrations

Cytotoxicity/Plate incorporation assay: 1,6,8,40,200,1000 and 5000 µg/plate Pre-incubation as a y: 15 025, 3 \$ 2.5, 629, 1250, 2500 and 5000 μg/plate.

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B. Study design and methods

1. In the dates: The study was carried out at Covance Laboratories Ltd, Otley Road, Harrogate, North Orkshire (UK) from 11 March 2005 and 10 May 2005. Ò

2. Preliminary cytotoxicity/mutation assay



TFA was tested in strain TA100, at 1.6, 8, 40, 200, 1000 and 5000 ug/plate. Triplicate plates without and with S-9 mix were used. Negative (solvent) and positive controls were included in quintuplicate and triplicate respectively, without and with S-9 mix. These platings were achieved by the following sequence of additions to 2.5 mL molten agar at 46±1°C: 0.1 mL bacterial culture, 0.1 mL test article solution or control, 0.5 mL 10% S-9 mix or buffer solution, followed by rapid moving and pouring on to Vogel-Bonner E agar plates. When set, the plates were inverted and incubated at 37 ± 1 °C in the dark for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background bacterial lawn, and where possible revertant colonies were counted.

3. Pre-incubation assay:

Quantities of test article or control solution, bacteria and S-9 mix detailed above, were mixed together and incubated for 1 hour at $37\pm1^{\circ}$ C, with shaking, before the addition of 2.5 ML motion again at $46\pm1^{\circ}$ C. Volumes for positive control treatments were reduced to 9.05 ml. Plating of these treatments then proceeded as for the normal plate-incorporation procedure, Colonies were counted electronically using a Seescan Colony Counter (Seescan Pleyor manually where conformding factors such as split agar affected the accuracy of the automated or unter. The background bacterial lawn was inspected for signs of toxicity.

Statistics The m-statistic was calculated @ check that the data were Poisson distributed, and Dannett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis Ŵ

4. Acceptance criteria:

The mean The assay was considered valid if the following criteria were met:

- 1. the negative Control count fell within the normal ranges
- 2. the positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S&P preparation
- 3. no more than 5% of the plates were lost through contamination of some other unforeseen event. 5. Assessment criteria

The test article was considered to be mutagenic if.

- 1. the assay was valid according the acceptance criteria
- 2. Dunnett's test gave a significant response (p $^{\circ}$ 0.0%) and the data set(s) showed a significant Ø dose correlation Ś

the positive esponses described above we reproducible

Results and discussion II. 🖏

Ø 1 Preliminary cytotoxicity assay

No evidence of particity as would normally be manifest as a thinning of the background bacterial lawn and/or a marked reduction in revertant numbers, was observed following any dose level.

The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments performed.

Results of the formulations malyses demonstrated achieved concentrations within 100±10% of the nominal test article concentrations for all treatment concentrations in each of the main mutation experiments A.

ő Mutation assays



Following treatments of all the tester strains in the absence and in the presence of S-9, only Experiment 2 treatments of strain TA98 in the absence of S-9 resulted in an increase in revertant numbers that was statistically significant when the data were analysed at the 1% level using Dunnett's test. This increase in revertant numbers showed no evidence of a dose-response and was not observed following comparable Experiment 1 treatments. Accordingly, this increase in revertant numbers was considered to have been a chance occurrence, and not a compound related effect. As no other treatments provided any statistically significant increases in revertant numbers, this study was considered to have provided no evidence of any TFA mutagenic activity. 1

The positive controls increased mutant counts to well over those of the negative controls, and thus demonstrated the system's sensitivity and the activity of the S9 mix.

1 able 5.8.1-2/	Summa	ry of mean reve	Core Y	N Q		¥ <u>4</u>
			Salmon	ella typhimurinn	n strains	
	<mark>S-9 mix</mark>	TA98	(TA100)	PA1535	TA1597	TA102
<mark>Dose (µg/plate)</mark>	<mark>(-/+)</mark>		TA100 00	© <mark>mean ≇ SD</mark> ∂		
Experiment 1		×			Â ^r z.	
Solvent control	_	27 ± 6	$1 \frac{100}{100} \pm 14^{\circ}$	$\sqrt{15\pm6}$	∑7 <u>19 ±</u> 4	£ 221 ± 8
TFA 1.6	_	27 ± 8 5 5	€ <mark>103 ± €5</mark>		$\frac{152 \times 8}{\sqrt{8} \pm 3}$	214 ± 20 213 ± 27
8	_	25 * 7	101 ± 7		3 ± 3	213 ± 27
40	_	28 ± 4	91 ± 26	$\mathbb{P}^{1\pm 3}$	~22 ±₫	$\sqrt[6]{219 \pm 16}$
200		$\sqrt{36\pm3}$	$101 \pm 10^{\circ}$	مَرْبَ <mark>13 ∰ک</mark> ې و	, <mark>16₽6</mark> (216 ± 10
1000	_	<mark>34 <u>∔</u>4</mark>	Ç [™] <mark>103,≱46</mark> ⊘	12 ± 5	$\sim 20 \pm 1$	223 ± 6
<mark>5000</mark>	<mark>-</mark> °~	2,5 <u>±</u> 5 ∅	$\frac{164 \pm 5}{2}$	$\frac{13}{12} \pm 5$ $\frac{12}{5} \pm 2$	³ 17 ±3	211 ± 27
Positive controls						
2NF: 5.0	<u></u>	9192 921			- Si	
NaN ₃ : 2.0	\$ ⁷ - , Ó		$666 \pm 26^{\circ}$	$\sqrt{43 \pm 10}$	$\frac{2}{\sqrt{204 \pm 30}}$	
AAC: 50.0		~ ~			204 ± 30	
MMC: 0.20	L <mark>Ş</mark>					643 ± 34
Experiment 2	- -					
Solvent control	<mark>–</mark> Č	20 ± 4	97 ± \$	§ <mark>14 € 8</mark>	19 ± 2	249 ± 17
TFA [《] ¥56.25		°~ ′ <mark>28 ⊯ 7</mark>	³ 103 ≠ 6	1 5≫± 4	16 ± 4	271 ± 29
312.5	S ⁷ - A	² 25 <u>≠</u> 3 √ 9	$1/94 \pm 9$	$\frac{16 \pm 4}{10 \pm 4}$	16 ± 7	234 ± 33
625	* <mark>-</mark> ?"	$3^{3} \pm 8^{4}$	$\sim 103 \pm 2$	$ \begin{array}{c} 16 \pm 4 \\ 18 \pm 4 \\ 13 \pm 5 \end{array} $	23 ± 4	$\frac{208 \pm 27}{210 \pm 27}$
1250 2,500		23 ± 3	93 ± 10	$\frac{13 \pm 5}{10 \pm 3}$	$\frac{17 \pm 4}{20 \pm 1}$	$\frac{219 \pm 37}{248 \pm 40}$
5000		$\frac{29}{100} \pm 2$		10 ± 3 14 ± 4	$\frac{20 \pm 1}{18 \pm 2}$	$\frac{248 \pm 40}{232 \pm 27}$
Positive Controls						
$2NE^{3}$ 5.0	N.	577, ₽ 20				
NaN ₃ : 2.0			38 ± 30	438 ± 30		
0			-Q"		75 ± 12	
MMC:			1			620 ± 8
MMC: 22	, 7 - 0		I	1	I	
J. D		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~				
	Â,	*G ⁻ 7				
E Q		-				

 Table 5.8.1-27
 Summary of mean revertant color





		Salmonella typhimurium strains					
	<mark>S-9 mix</mark>	<mark>ТА98</mark>	TA100	TA1535	TA1537	TA102 。	
Dose (µg/plate)	<mark>(-/+)</mark>			mean ± SD			
Experiment 1					~	6 [°] (
Solvent control	+	30 ± 7	107 ± 16	17 ± 3	2 0 € 5	202 ± 30	
TFA 1.6	+	<mark>36 ± 8</mark>	92 ± 12	13 ± 3	197 ± 3	193 ± 16	
8	+	36 ± 10	<mark>99 ± 19</mark>	14 ± 7	20 ± 1		
40	+	<mark>49 ± 7</mark>	99 ± 5	<u>گ 19 ± 3</u>	13 ± 3	180 ± 14	
200	+	33 ± 8	$\frac{107 \pm 6}{2}$	16 ± 2	18 ± 4	385 ± 20	
1000	+	31 ± 2	88 ± 8	20 ± 2	17 ± 8	Q <mark>189 🎳</mark> 🐇	
5000	<mark>+</mark>	36 ± 15	93 ± 8	<u>15 ±</u> €	° <mark>22∕≨∕8</mark> ,∢	177445	
Positive controls							
B[a]P: 10.0	+	245 ± 32				\sim	
AAN: 5.0	+		977 ±25	∑ <u>196 ±@4</u>	\$≊ <mark>97<i>≌</i>03</mark> _∧		
AAN: 20.0	+	*		A		$2 \pm 10^{\circ}$	
Experiment 2		Ő		. ~ O	N S		
Solvent control	+	41 ± 5 2	√12±2	<mark>17 <u>≠</u>9</mark>	22 <mark>222</mark>	196 ± 34	
TFA 156.25	+	<mark>32 ±∕∮∕0</mark>	‴Cố″ <mark>76 ً⊉∕5</mark> Š	Í <mark>12∞ 1</mark> 🏷	24 ± 7	$1/6 \pm 29$	
312.5	+	<mark>29[°]≇%</mark> ¢	99 ± 5	30 ± 3	$23\pm10^{\circ}$	208 ± 3	
625	+	38 ± 10	69 ± 5	_ر [©] <mark>21 ∡@</mark> `		170 – 2 2	
1250	+	[≪] 41 ≭ 6	<mark>∂⁷⁵∉,6</mark>	1 1 ⊮ ≠ 3	$\sqrt{20 \pm 4}$	212 ± 35	
2500	+ 👡	≥ <mark>26⊕5</mark>	¥ <mark>25 ± 9</mark> ∡ ″	10 ± 3	$\sqrt{21\pm 2}$	246 ± 6	
5000	+ ^{*%}		$390 \pm 40^{\circ}$	³ ² ¹⁵ ≠ 2	20 24	232 ± 18	
Positive controls	Š			Y OY SY	, Č, Š		
B[a]P: 10.0		<mark>3996-62</mark>		(1) n	a		
AAN: 5.0	, ^e v ^o		1038 ± 107				
AAN: 20.0	l Q	0 0 9			*	796 ± 246	

* Dunnett's test, sign Mcant at 1% lexel

O TFA = tributor acetate; 2MF = 2 witrofly rene (2NF); NaN₃= softum azete; AAC = 9-Aminoacridine; MMC = **Doanthracene** \bigcirc mitomy cin C; B[a]P 0

K)

A

III. Conclusion Ø

Trifluoroacetate (TFA) dis not induce motation in five distidine-requiring strains of Salmonella TAJ02) when tested under the conditions of this typhimurium (TA98, A100. TX1535 TA1537 £. study. L.L. Ø

Assessment and conclusion by applicant;

Under the coorditions of the test, wifluor acetate (TFA) did not induce mutation in five histidinerequiring strains of Salmonella typhimutium (TA98, TA100, TA1535, TA1537, TA 102) with and without metabolic activation in the Ames test.

The study was conducted under GLP conditions and according to OECD guideline 471 (1997). There were no deviations when compared to the current OECD guideline 471 2020). The study is therefore considered valid and acceptable.

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Data Point:	KCA 5.8.1/51
Report Author:	
Report Year:	2005
Report Title:	Trifluoroacetate (TFA) - Induction of chromosome aberrations in cultured human
	peripheral blood lymphocytes
Report No:	2014/83-D6172
Document No:	<u>M-260807-01-1</u>
Guideline(s) followed in	OECD 473 (1997), OPPTS 870.5375 (1998), EC directive 2000/32/OC (2007),
<mark>study:</mark>	JMAFF No. 8147 (2000) Current guideline: OECD 473, 2016 Deviation: 200 instead of 300 metaphases were scored. This deviation is not considered to impact the integrity of the study. Acceptability criteria in the study report consider only current historical control data of the testing laboratory but no further information sight as
Deviations from current	Current guideline: OECD 473, 2016
test guideline:	Deviation: 200 instead of 300 metaphases were scored. This deviation is not
	considered to impact the integrity of the study.
	Acceptability criteria in the study report consider only current historical
	control data of the testory laboratory out ho further information stell as
	statistical significance or cell ^o proliferation criteria
Previous evaluation:	Not evaluated O' Q' & A Q' A' A .
GLP/Officially	Yes, conducted under GLAOOfficially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q
	Not evaluated Yes, conducted under GLAOfficially recognised festing facilities Y
Executive Summary	
BACCULIVE Summary	

E.

Ś TFA was tested in an in wiro cytogenetics assor using duplicate https://www.astested.in.an.in.wiro.cytogenetics.assor using duplicate https://www.astested.in.an.in.wiro.cytogenetics.assor using duplicate https://www.astested.in.astest from the pooled blood of three male donors in two independent experiments. Freatments covering a broad range of doses separated by harrow intervals, were performed both in the absence and presence of metabolic activation (S-9). The set article was dissolved in sterile water for injection (purified water) and the highest dose level used in the main experiments, \$360 pg/mL (equivalent to 10 mM) was determined following opreliminary cytotoxieity range-finding experiment.

In Experiment 1, treatment in the absence and presence of \$-9 was for 3 hours followed by a 17-hour recovery period prior to harvest (3+12). The S-9 used was prepared from a rat liver post-mitochondrial fraction (S-9) from Acoclor 1234 induced animals. The test article dose levels for chromosome analysis were selected by exquating the effect of FA on mitoto index. Chromosome aberrations were analysed at 340.0, 680.0, 6360 µg/mL. The highest concentration Grosen for analysis, 1360 µg/mL, induced approximately 16% approvement of the approximation approximately 16% approximately 16% approvement of the approximately 16% approximately of S-9 respectively. ò

Ô In Experiment 2, treatment in the absence of \$29 was continuous for 20 hours. Treatment in the presence of S-9 was for 3 hours only followed by a 19-hoursecovery period prior to harvest (3+17). Chromosome aberrations were analysed at 85.00 170.6, 340.0, 1360 µg/mL (- S9) or 170.0, 340.0, 1360 µg/mL (+S9) and the highest concentration obosen for analysis, 1360 µg/mL, induced approximately 61% and 19% mitotic inhibition in the absence and presence of S-9 respectively.

Exposure to TFA resulted in percentages of chromosome aberrations that were mostly similar to the concurrent vehicle controls in the presence and absence of S-9. There were no increases in the frequency of cells with numerical aberrations in cultures treated with TFA, either in the abserve or presence of \$-9, that were considered to be of any biological relevance.



1. Materials and methods

A. Materials

1. Test material: **Description** Lot / Batch #: **Purity:** CAS # **Stability of test compound:** 2. Control materials: **Negative:**

Vehicle/Solvent **Positive:**

Trifluoroacetate (Sodium Trifluoroacetate) White powder
Trifluoroacetate (Sodium Trifluoroacetate) White powder 016911/1 99.1% 2923-18-4 Stable for the duration of the study
White powder
016911/1
99.1%
2923-18-4 O A S O
Stable for the duration of the study
016911/1 99.1% 2923-18-4 Stable for the duration of the study Culture medium tissue Sterile water non- activation (-S9 mix) 4-Nitroquinorine toxide in
Culture medium tissue
DMSO, final concentration 2,50 µg/ml
with activation (+S9 mix): Syclophosphamide in DMSO final concentration 6 µg/mL
concentration 6 µg/mL
concentration 6 µg/mL

Activation:

The mammalian liver post-mitochondrial fraction (SO) used for metabolic activation was prepared from male Sprague Dawley rats induced with Groclor 125, and Obtained from Molecular Toxicology Incorporated, USA. Glucose 6-phosphate (180 mg/mL), NADP (25 mg/mL), 150 m KCl and rat liver S-9 were mixed in the ratio 1:1: 2. An aliquer of the resulting S-9 mix was added to each cell culture designated for treatment in the presence of S.9 to achieve the required final concentration in a total of 10 mL. The final concentration of liver bomogonate in the test system was 2%. Cultures treated in the absence of S-9 received an equal volume of 150 mM KCC L

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Test cells:

Blood from three healthy, non-smoking make volunteers was used for each experiment. For each experiment an appropriate volume of whole blood was drawn from the peripheral circulation within two days prior to culture mitiation. Blood was stored refrigerated and pooled prior to use.

Culture medium: >

Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL heparinised blood into 8.1 m. Hepes-buffered RPMI medium containing 20% (v/v) foetal calf serum and 50 µg/mL gentamycin. Phytohaernagehrtinin (PHA, reagent grade) as included at a concentration of approximately 2% of culture volume to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37° Qand racked continuously. All cultures were then incubated at 37°C. Ô Ô

Test compounds concentration used (mM);

	Freliminary test	Main test
Non activated condition - 3 h 17h	0.6649, 1.328, 2.656, 5.313, 10.63,	85.00, 170.0, 340.0, 680.0,
	21, 23, 42.50, 85.00, 170.0, 340.0,	<mark>1360 μg/mL</mark>
	<mark>680.0, 1360 μg/mL</mark>	
Non activated conditions 20 h	0 .6641, 1.328, 2.656, 5.313, 10.63,	<mark>85.00, 170.0, 340.0, 680.0,</mark>
	21.25, 42.50, 85.00, 170.0, 340.0,	<mark>1360 μg/mL</mark>
	<mark>680.0, 1360 μg/mL</mark>	
Activated conditions - 3h+17h	<mark>0.6641, 1.328, 2.656, 5.313, 10.63,</mark>	85.00, 170.0, 340.0, 680.0,
Activated conditions - 3h 47h	21.25, 42.50, 85.00, 170.0, 340.0,	<mark>1360 μg/mL</mark>
	<mark>680.0, 1360 μg/mL</mark>	



B. Study design and method

1. In life dates: 04 April 2005 – 24 May 2005

The study was carried out at Covance Laboratories Ltd, Otley Road, Harrogate, North Yorkshire, UK) from 4 April 2005 and 24 May 2005.

Preliminary cytotoxicity Assay (First test)

For the cytotoxicity range-finder experiment, S-9 mix or KCl (0.5 mL) was added appropriately as detailed previously. Duplicate cultures (A, B) were treated with the solvent and single cultures treated with the test article at appropriate concentrations (1.0 mL) per culture) positive control treatments avere not included.

Cytogenetic assay:

For the main experiments, S-9 mix or KCl (0.5 mL) was added appropriately as detailed previously. Quadruplicate cultures (A, B, C and D) were treated with the solvent and duplicate cultures treated with the test article at appropriate concentrations (0.1 mL per culture). Additional duplicate cultures for treatments in the absence of S-9 and in its presence, were treated with 0.1 mL of the positive control chemicals.

All cultures were then incubated at 2 °C. Treatment details are provided in the scheme below.

			à â		,° , °	Č N
	Table 5.8.1-28	Freatmont de	tails S			
	Treatment	× ×	⁷ S-9 ₈	Nur	nber of cult	ures
			⁸⁻⁹	⁰ 3+17*		
	×~	Cytotoxicity			ent S	~C?
	Negative confi					20 74 0 2 9
	fest afficle all oses)		$\frac{1200}{100} = \frac{1000}{100} = 10$			1
		0, 14,0	Laper mice	n T		
	Negative control Negative control Sector article			$\begin{array}{c} & & & & \\ & & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & &$))	
R.Y	•Test articfe (all doses)			y y		
	Call doses (all doses) (alpdoses) (alpdoses)			2 5 7 2		
		N N	Experimer		·	
	Negative contraction		×+ ×	4		<mark>4</mark>
	(all doses)			2		2
V	Positiv@contro (all doses)		Experiment +	2		2
	* Hours treatment * h	ourstecovery				
		× ~				

Treatment media remained on cultures receiving the continuous treatment until sampling, that is, 20 hours after the beginning of treatment. Cultures received pulse treatments (both in the absence and presence of 5-9) for 3 hours only. They were pelleted (approximately 300 x 'g', 10 minutes), washed twice with sterile saline (prewarmed at 37°C) and resuspended in fresh medium containing foetal calf serum and gentamycin. Cultures were incubated for a further 17 hours before harvesting.



Harvesting

Approximately 2 hours prior to harvest, colchicine was added to give a final concentration of approximately 1 µg/mL to arrest dividing cells in metaphase. At the defined sampling time cultures were centrifuged at approximately 300 x 'g' for 10 minutes; the supernatant was carefully removed and cells were resuspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37°C for 15 minutes to allow cell swelling to occur. Cells were fixed by dropping the KCl suspension into an equal volume of fresh, ice-cold methanol/glacial acetic acid (3:1, v/v). The fixative was changed by centrifugation (approximately 300 x 'g', 10 minutes) and resuspension. This procedure was repeated several times (centrifuging at approximately 1250 x 'g', 2-3 minutes) until the cell pellots were clean

Slide preparation:

Lymphocytes were kept in fixative in the refrigerator before slides were prepared but slides were not made on the day of harvest to ensure cells were adequately fixed. Cells were pelleted and resusperded in a minimal amount of fresh fixative (if required) so as to give a milky suspension. Several drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance phromosome spreading, and several drops of suspension were transferred to clean microscope slides labelled with the appropriate study details. Slides were flamed, as necessary, to improve metaphase spreading. After the slides had dried the cells were stained for 5 minutes in 4% 6/v) filtered Gremsa stain in Gurr's pH 6.8 buffer. The slides were rinsed Gried and mounted with coverslips.

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Metaphase analysis:

Where appropriate, 100 metaphases from each code were analysed for chromosome aberrations. Where 10 cells with structural aberrations (excluding gaps) have been noted on a slide, analysis may be terminated. Only cells with 44-46 chromosomes were considered acceptable for analysis of structural aberrations. Any cell with more than 46 chromosomes, that is polyphoid, endoreduplicated and hyperdiploid cells, observed during this search was note and recorded separately. Classification of structural aberrations was based on the scheme described by ISCN. Under this scheme, a gap is defined as a discontinuity less than the width of the chromatid and no evidence of displacement of the fragment and a deletion is defined as a discontinuity greater than the width of the chromatid and/or evidence of displacement of the microscope stage co-ordinates of any aperrant cell.

Evaluation criteria

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The data were evaluated as to whether exposure to the test artigite was associated with:

- 1. The proportion of cells with structural aberrations at one or more concentrations exceeds the historical wehicte control range in both replicate cultures.
- 2. A statistically significant in the proportion of cells with structural aberrations (excluding gaps) occurs at such doses.
- 3. A concentration-related trend in the proportion of cells with structural aberrations (excluding gaps).

A test article would be considered positive in this assay if all of the above criteria were met.

Treatment of data and statistical analysis

After completion of microscopic analysis, data were decoded. The aberrant cells in each culture were categorised as follows:

- 1. cells with structural aberrations including gaps
- 2. Cells with structural oberrations excluding gaps
- 3 polyploid, and or eduplicated or hyperdiploid cells.

The totals for category 2 in negative control cultures were compared with the current laboratory negative control (normal) ranges to determine whether the assay was acceptable or not. The proportion of cells in category 2 in test article treated cultures were also compared with normal ranges. The statistical



significance of any data set was only to be taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentration exceeded the normal range. Under this condition, the statistical method used would be Fisher's exact test. Probability values of $p \le 0.05$ were to be accepted as significant. The proportions of cells in categories 1 and 3 were also examined in relation to historical negative control (normal) ranges and statistical analysis by Fisher's exact test may be used. The proportions of aberrant cells in each replicate were also used to establish acceptable heterogeneity between replicates by means of a binomial dispersion test. Probability values of p ≤ 0.06 were to be accepted as significant.

II. **Results and discussion**

Structural aberrations

Exposure to TFA resulted in percentages of chromosome aberrations that were mostly similar to the concurrent vehicle controls in the presence and absence of S9. There was one exception after a 20hour exposure to TFA in the absence of S9 in Experiment?. There was a small increase in the percentage of cells with structural chromosome aberrations (excluding gaps) exposed at 1360 µg/mL, the highest concentrations of TFA assessed for chromesome damage. The aberrations included two chromosome exchanges in one cell. However, the percentages of cells with aberation fell within the historical vehicle control frequencies. Also, exposure at 1360 to mL was associated with 61% mitotic inhibition in Experiment 2. Numbers of aberrant cells (excluding gaps) in all treated cultures fell within historical negative control ranges. For the reasons mentioned above the small increase mentioned above was not judged to represent a positive response.

Summary of cells with structural aberrations Table 5.8.1-29

			aberrations	
Substance 2		Cells with		Mitotic Index
Dose (μ g/mL)	Secored		Hateluding gans	<mark>(mean)</mark>
Substance Ø Dose (μg/mL) Φ ^{+/-} SP Experiment 1 (Ø hous treated) Ø	itment ≁ 17¢hour i	recovery, #J-S9) 🔊	$\begin{array}{c} 2 \\ 3 \\ 3 \\ 3 \\ 3 \\ 3 \\ 3 \\ 3 \\ 3 \\ 3 \\$	
Solvent 🐎	_ ↓ 0 200 9	$\begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} $	2 . 1	<mark>7.1</mark>
TFA 340 0 +	× <mark>200</mark> .			<mark>7.6</mark>
	ب <mark>ي≲</mark> 200 ج	○ <mark>3</mark>		<mark>7.6</mark>
2 91360				<mark>7.9</mark>
<u>CPA[™] 6.25</u>		5 2 2 2 5 3 4 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	<mark>∢≫ 49ª</mark>	
Solvent S	·» <u>300</u> ~		<u> </u>	<mark>8.3</mark>
TFA 340 🖗 🥁	5 200 200 200	$\begin{array}{c} 5^{2} \\ \hline \end{array} \\ \hline $ \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \\ \hline \\ \hline \end{array} \\ \hline \end{array} \\ \hline \end{array} \\ \\ \hline \end{array} \\ } \\ \end{array} \\ \\ \hline \end{array} \\ \\ \\ \hline \end{array} \\ \end{array} \\ \\ \end{array} \\ \\ \\ \\ \\	2	<mark>8.2</mark>
680 ₀ / 🔊			<mark>3</mark>	<mark>7.2</mark>
1369 ^O - s	<u>v 200</u>		0	<mark>7.0</mark>
NQO 2 ,50 - Experiment 2 (3 hour trea	<mark>\$9186</mark> 4	<u>, </u>	<mark>44ª</mark>	
Experiment 2 (3 hour trea	tment + 17 hour 1	recovery, AS9)		
Solvent 24			1	<mark>7.6</mark>
TFA 340 +	200 200 200 200 200 200 200 200	~\$* <mark>3</mark>	<mark>1</mark>	<mark>7.7</mark>
680 🔬 + "Ü	^ا الم ⁰ 200 م	ی <mark>ر ا</mark>	<mark>0</mark>	<mark>6.1</mark>
680 + 4	2000 ×	<i>Q</i> ′ <u>1</u>	1	<mark>6.2</mark>
CPA 0.20	S <mark>97</mark> a	∼, <mark>48</mark>	<mark>40ª</mark>	
Experiment 2 (20 hour te	atment + 0 hotst 1	recovery, -S9)		
Solvent -	<u>200</u>	1	<mark>0</mark>	<mark>6.1</mark>
TFA 88		<mark>0</mark>	<mark>0</mark>	<mark>5.3</mark>
190 - 2	200 200 200	<mark>0</mark>	0	3.8 3.7
<mark>4 40 _</mark> 340	200 ×	1	<mark>1</mark>	<mark>3.7</mark>
	<mark>200</mark>	<mark>6</mark>	<mark>4</mark>	<mark>2.4</mark>
NQO 2.50 _	102	<mark>40</mark>	34 ^a	
^a statistical significance $n < 1$	0.001		·	

^a statistical significance $p \le 0.001$



Numerical aberrations

No increases in the frequency of cells with numerical aberrations, that exceeded the historical negative control range, were generally observed in cultures treated with TFA in the absence and presence $\mathfrak{A}S9$. The only exception to this was observed in Experiment 1 in a single culture at the lowest concentration analysed following 3+17 hour treatment in the presence of S9. In this culture the numerical aberration frequency marginally exceeded the historical control range. In isolation, this increase is not considered to be of any biological relevance.

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able 5.8.1-30	Sumn	nary of nu	imbers and typ	ວes of nu	merical abe	rrations		S v	4
1				L.		,0×	<u> </u>	<u>y</u>	_ « ⁰
' Substance↔	ب _	Cells [.]			al aberrations		OTotal↔	%with	Ŵ
Dose (µg/mL)¤	+/-∙S9¤	scored¤	hyperdiploid	1 endor	eduplicated	polyploid	abs∳	numabso	Ø
Experiment 1 (3	·hour·trea	tment+17	·hour recovery,	₽QS9) ∙¤					a
Solvent¤	+¤	200¤	0¤ 🐒	, ത്		× 02	0¤ `∧	0∞€	¤
$TFA \rightarrow 340^{\circ}$	+¤	203¤	0¤ 0″		12		3¤	_4.5¤	a,
→ 680¤	+¤	200¤	004		ØF Q.	Qð¤ ∼	3° 3° 00	00 1.00	Ø
→ 1360¤	+¤	202¤	<u></u>		>0¤ 🔊 🎽	<u><u> </u></u>	, 2¤	\$ 1.0	¤
$CPA \rightarrow 6.25^{\circ}$	+¤	168¤			0° (0° 4	02	¤
Solvent¤	-¤	200¤	O ⁰ ¤	\$	0° × «			62	¤
$TFA \rightarrow 340^{\circ}$	-¤	200¤	O ^V 00 ^V	S.			<u> </u>	<i>_©</i> _0¤	¤
→ 680¤	-¤	202¤	2 [°]		0¤ 🔊	O O O	ja a	🔍 1.0¤	a
→ 1360¤	-¤	200¤	🔨 <i>č</i> opa (č		0¤ ()		0° 0°	∞0	¤
$NQO \rightarrow 2.5^{a}$	-¤	186						0 ¤	¤
Experiment 2 (3	·hour·trea		hour recovery,	+ \$ 9)¤			0		¤
Solvent¤	+¤	200¤		d i	0 0 a		<i>0</i> 00	0 ¤	¤
TFA \rightarrow 340 ^{\square}	+¤ 🖇	202¤				~ <u>2</u> ~	2¤	1. 0 ¤	¤
→ 680¤	+¤	2000	o o o		00° 4	0¤	5 °0 ¤	0 ¤	¤
→ 1360¤	+2	200°a		R.			1¤	0.5¤	¤
$CPA \rightarrow 6.25 \alpha$	<u>E</u>	97¤	S &	\$* Š	,0¤	-	0 ¤	0 ¤	¤
Experiment 2 (2	0 bour tre	ament + 1	hour covery,	-S9)p^_	000 00	4 <u>0</u>			a
Solvent¤	-9. \	201			080	l Da	1¤	1. 0 ¤	¤
TFA → 850		200 ¤		K I		0¤	0 ¤	0 ¤	¤
→ 170¤	- Se	≪201¤ "	0d 🗽	\$ _\$	yıa Oʻ	_ 0 ¤	1¤	0.5¤	¤
→ © 40¤	-¤ 🖌	2030	' <u>→</u> 2¤ ∂`	" "0"	0¤0	<mark>∂ l</mark> ¤	3¤	1.5¤	a
' → 360¤	-¤ ©	2000	0° 0¤			0¤	0 ¤	0 ¤	¤
• NQO → 2.5¤	- O	.1 9 20	0	<u>, Oʻ</u>	× 66 ~ 0	0 ¤	0 ¤	0 ¤	¤
abs≔∙aberrations,∙	nwnj⊒∙nu	merical¶_	\vee \cap $'$	5° &	100 · · · · · · · · · · · · · · · · · ·				
-	~¥	· .		\rightarrow \bigcirc	~				

Conclusion Ш.

Trifluoroacetate (TFA) did not produce chromosome aberrations in cultured human peripheral blood lymphocytes when tested up to 1360 µg/mk in either the absence or the presence of a rat liver metabolic activation system (S9). Ò K

Assessment and conclusion by applicant

(R)

Under the conditions of the test, triffuoroacetate (TFA) did not induce chromosome aberrations in cultured human peripheral blood lymphocytes. The study was performed under OLP conditions and in accordance with OECD guideline 473 (1997). When

compared to the currently guideline OECD 473 (2016), a few deviations were noted. Only 200 metaphase cells werefunvestighted, which was the number of metaphases to be recommended by the previous OECD guideline 473 (1997) In addition, acceptance and evaluation criteria of the test were inconsistent with those specified in the current guideline. Acceptability criteria in the study report consider only current historical control data of the testing aboratory but no further information such as statistical significance or cell proliferation criteria. However, these deviations were considered to not compromise the validity of the study. The study is therefore considered valid and acceptable.



Data Point:	KCA 5.8.1/52
Report Author:	
Report Year:	2005
Report Title:	Trifluoroacetate (TFA) - Mutation at the thymidine kinase (tk) locus of mouse
	lymphoma L5178Y cells (MLA) using the Microtitre fluctuation technique
Report No:	2014/89-D6173
Document No:	M-260699-01-1
Guideline(s) followed in	OECD 476 (1997), OPPTS 870.5300 (1998), UKEM (1990)
study:	
Deviations from current	Current guideline: OECD 490, 2016
test guideline:	The study was carried out in compliance with SECD 476 (1997) and herefore a
	few deviations occurred compared to in OECD TG 490 (2016). cytoroxicito was not
	calculated by using the latest formulae for calculating Relative Suspension
	Growth (RSG), and mightion frequency, However, the acceptance critigia were in
	line with what currently required. Star of the second se
Previous evaluation:	Not evaluated of of the two the two is a second of
N	Yes, conducted under GLPOfficially recognised testing facilities
Acceptability/Reliability:	Yes in the A of the A

Executive summary

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TFA was assayed for its ability to induce mutation at the the to cus (Strifluo othyrodine resistance) in mouse lymphoma cells using a fluctuation protocol. The study consisted of a cytoroxicity range-finding experiment followed by two abdependent experiments, each conducted on the absence and presence of metabolic activation by an Aroclor 1254 induced at liver post-mitochondrial fraction (S-9).

A 3 hour treatment incubation period yas used for all experiments performed in the presence of S-9. In the absence of S-9, the range finder was performed using 3 and 24 hour treatment incubation periods, Experiment 1 (and repeat) was performed using a 3-bour treatment incubation and Experiment 2 was performed using a 24 hour treatment incubation. 2 () j ð

6 O In the cytotoxicity range-finding experiment, 3 your treatment, dosed were tested in the absence and presence of \$9-9 ranging from 42,5 to 1360 µg/mL (10 mM). Gells survived all doses of TFA. The highest dose tested (1360 µg/nf) yielded 12% relative total growth (RFG) in the absence of S-9 and 99% RTG in the presence of S-9 S. Š K, K)

°~/ In the cytotoxicits range finding experiment, 24 hour treatment, doses were tested in the absence of S-9 ranging from 5,313 to 360 mg/mL 60 mM. Cells survived all doses of TFA. The highest dose tested ð (1360 µg/mb@yielded 49% RTG Ô Ô

Accordingly, for the first experiment (hour reatment) doses were chosen in the absence and presence of S-9 ranging from 260 to 1360 µg/mL. The highest dose tested (1360 µg/mL) yielded 112% and 93% RTG in the absence and presence of S-prespectively. Due to excessive heterogeneity at the maximum test dose in the absence of S-9 This dose was excluded from mutation assessment, and therefore these toxicity data are from asrepeat experiment. 0

In the second experiment obsets were tested in the absence of S-9 (24 hour treatment) and in the presence of S-9 (Thour Weatment) ranging from 360 to 1360 µg/mL. The highest dose tested (1360 µg/mL) vielded 76% and 104% RTGM the absence and presence of S-9 respectively.

Ø \sim Negative (solvent) and positive control treatments were included in each mutation experiment in the absence and presence of S-9. Mutant frequencies in negative control cultures fell within acceptable ranges, and clear increases in mutation were induced by the positive control chemicals 4-nitroguinoline 1-oxide (without S-9) and benzo(a)pyrene (with S-9). Therefore the study was accepted as valid.



No statistically significant increases in mutant frequency were observed at any dose tested in the absence of S-9. A very small but statistically significant increase in mutant frequency was observed at the intermediate dose of 960 µg/mL in the presence of S-9 in Experiment 1. This increase was sufficiently small in magnitude that it is not considered a biologically relevant response, and furthermore, and vided no evidence of any dose-relationship or reproducibility, as it occurred at a single intermediate dose with no significant linear trend in only one experiment.

1. Materials and methods	Trifluoroacetate (Sodium Difluoroacetate)
A. Materials	
1. Test material:	Initiorogeototo (Nodium (fituorogeototo) a () ()
Description	White powder $(16911/1)$
Lot / Batch #:	White powder 016911/1 99.1% Stable under the test conditions
<mark>Purity:</mark>	99.1% N & X & X
CAS #	2923-18-4 Stable under the test conditions
Stability of test compound:	Stable under the test conditions A A A A A A A A A A A A A A A A A A A
2. Control materials:	
Negative:	Dssue culture predium 2 0 0
· enterer bor vente	Culture medium/Sterile water 8 8 2
Positive:	non-activation (-59 mixes 4-Nucoquingine 1 foxide, ~
Ĵ,	final concentration 0.0 20-0.2 gig/mL & &
	with activation (+S9 mix): benzo(appyrene (BP), and
×	v concentration 2-3 μ@/mL
	concentration 2-3 µ@/mL

Activation:

The mammalian liver post-mitochondria fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats, induced with Aroctor 1254. The S-9 was obtained from Molecular Toxicology Incorporated, SA. Qucose-6-phosphate (#80 mg/mL), NADP (25 mg/mL), 150 mM KCl and rat liver S-9 were mixed in the ratio 1:1:1:2. For all cultures treated in the presence of S-9, a 1 mL aliquot of the mix was added to each cell culture (19mL) or give a total of 20 mL. Cultures treated in the absence of S-9 received 1 mL 150 mM KCl (3 hour treatments only) \bigcirc

Test cells:

L5178Y TK +/- mouse lymphoma cells were stored as tozen stocks in liquid nitrogen, the original cultures were bitainer from Dr Donald Chive, Berroughe Wellcome Co. Each batch of frozen cells was purged of TKC mutatits, checked for spontaneous mutant frequency and that it was mycoplasma free.

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1 Culture medium:

For each experiment, the cells difuted in RPMI \mathfrak{P} media and incubated in a humidified atmosphere of 5% V/v CO₂ in air.

Three types of RPML 1640 medium were prepared as follows:

	RPMA A	RPMI 10	RPMI 20
Horse serum (heat indetivated)		10% v/v	20% v/v
Peniçi îîn / streptomycin	^C 100 units/mL 100 μg/mL	<mark>100 units/mL</mark> 100 μg/mL	<mark>100 units/mL</mark> 100 μg/mL
Amph@ericin B	<mark>2.5 μg/mL</mark>	<mark>2.5 μg/mL</mark>	<mark>2.5 μg/mL</mark>
Pluronic	0.5 mg/mL	0.5 mg/mL	



Ô

Test compounds concent	ration used (µg/ml):		0
	Preliminary toxicity	Test 1	Test 2 🖉 🐎
Non activated conditions	42.50, 85.00, 170.0, 340.0,	250, 500, 1000, 1200,	250, 500, 1000, 1200,
	<mark>680.0, 1360</mark>	<mark>1400, 1500, 1600</mark>	1400, 1500, 1600 ⁰
Activated conditions	42.50, 85.00, 170.0, 340.0,	250, 500, 1000, 1200,	250 , 500, 1000 (1200, 200)
	<mark>680.0, 1360</mark>	1400, 1500, 1600	1400, 1500, <u>1600</u>
		4	
		CA L	
B. Study design and	methods		
. In life dates:	14 March 2005 – 25 May 2	2005	

The study was carried out at from 14 March 2005 and 25 May 2005.

Cell treatment

Treatment of cell cultures for the cytotoxicity range-finding experiment was carried out using sugle cultures only and positive controls were not included Ő 17 Ľ P In the absence of S-9, 3 and 24 hour treatment incubation periods were used, in the presence of S-9 a 3 hour treatment incubation was used. Following 3 hour treatment, cells were washed with tissue culture medium and then resuspended in 50 mL RPMI 10 Following 24 hour treatment, evitures were centrifuged (200 x g) for 5 menutes washed and resuspended on 20 mL RPMI 10% Cultures were incubated at 37°C for 1 day, accounted and where possible diluted to 20x 10⁵ cells/m². Cultures were incubated for a further day, counted and adjusted to 8 cells mL and, for each dose, 0.2 mL was plated into each well of a 96-well microfitre plate for determination of relative total growth. The plates were incubated at 37°C in a humidified incubator gassed with 5% v CO₂ Pair feet 6 to 7 days. Wells containing viable clones were identified by eventsing background illumination and counted. L

. O For Experiment 1 in the absence and presence of S-9/(and Experiment Repeat in the absence of S-9) and in Experiment 2 in the presence of S-903 hour treatments) of least 107 cells in a volume of 17 mL tissue culture medium (cells in RPMI 10 diluted with ROMI AOno section) to give a final concentration of 5% serund were used. For Experiment 2 in the absence of 8-9 (24 bour treatment) at least 4 x 10° cells in a volume of 18 mL RPMI Reverensed. The cell suspensions were placed in a series of appropriate sterile disposable containers, For all treatments 2 mL solvent, test article solution or positive control (comprising 0.2 mL positive control solution plus 1.8 mL purified water) was added. S-9 mix or 150 mM KCl was added, such that each treatment, in the absence or presence of S-9, was in duplicate (single cultures only used for positive control treatments) Ĩ

3 hour treatment: After 3 Hours incubation at $3J \ge 1^{\circ}$ with gentle agitation, cultures were centrifuged (200 x g) for 5 minutes, washed and resuspended in 50 mL fresh RPMI 10 medium. 24 hour treatment: After static incubation at $37\pm1\%$ for 24 hours, cultures were centrifuged (200 x g)

for 5 minutes, wasked and resuspended in fresh RPMI 10 medium (20 mL). Cell densities were determined using a Coulter counter and ovhere sufficient cells survived, adjusted to 2x10⁵ cells/mL. Cells were transferred to fissue culture flasks for growth throughout the expression period. The solubility of the test compound in culture was assessed, by eye, at the beginning and end of treatment. ~

Cultures were maintained in masks for a period of 2 days during which the TK⁻ mutation would be expressed. Subsculturing was performed as required with the aim of not exceeding 1 x 10⁶ cells per mL and, where possible retaining at least 1 x 10⁷ cells/flask. From observations on recovery and growth of the cultures. ĉ

Plating for viability

Expression period



At the end of the expression period, cell concentrations in the selected cultures were determined using a Coulter counter and adjusted to give 1 x 10⁴/mL in readiness for plating for TFT resistance. Samples from these were diluted to 8 cells/mL.

Using an 8-channel pipette, 0.2 mL of concentration C of each culture was placed into each well of two 96-well microtitre plates (192 wells at an average of 1.6 cells per well). The plates were incubated at 37°C in a humidified incubator gassed with 5% v/v CO₂ in air until scoreable (7 to 10 days). Wells containing viable clones were identified by eye using background illumination and counted

Plating for 5-trifluorothymidine (TFT) resistance

At the end of the expression period, the cell densities in the selected cultures were adjusted to 9×10^{4} mL. TFT (300 µg/mL) was diluted 100-fold into these suspensions to give a final concentration of 3 µg/mL Using an eight-channel pipette, 0.2 mL of each suspension was placed into each well of four 96-well microtitre plates (384 wells at 2 x 10³ cells per vell). Plates were incubated at 37°O in a dumidified incubator gassed with 5% v/v CO₂ in air until scoreable (12 days) and wells containing clones were identified as above and counted. In addition, the number of wells containing large colonies and the number containing small colonies were scored for the negative and positive controls and the dose of test article where a statistically significant increase in mutant/frequency was observed.

Statistics

Statistical significance of mutant frequencies (total wells with clones) was carried out according to the UKEMS guidelines. Thus the control log mutant frequency (CMF) was compared with the LMF from each treatment dose based on Dwonett's test for multiple comparisons, and secondly the data was checked for a linear trend in mutant frequency with treatment dose using weighted regression. The test for linear trend is one-tailed, therefore negative trend was not considered significant. These tests required the calculation of the heterogeneity factor to obtain a modified estimate of variance.

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Acceptance criteria

The assay was considered valid it all the following criteria were met:

- 1. the mean mutant frequencies in the negative (solvent) control cultures fell within the normal range (S0 to 120 mutants per 10° viable cells)
- 2. at least one concentration of each of the positive control chemicals induced a clear increase in mutant frequency (the difference between the positive and regative control mutant frequencies was greater than half the historical mean value)
- 3. the mean physing efficiencies of the negative controls from the mutation experiments were between the range 65% to 120% on Day 2
- 4. The mean suspension growth of the negative controls from the mutation experiments was between the tange 8 to 32 tollowing 3 hour treatments
- 5. There was no excessive peterogeneity between replicate cultures.

Evaluation criteria

The test article was considered to be mutagenic of all the following criteria were met:

- 1. the assay was valid according to the acceptance criteria
- 2. the mutant frequence at one for more doses was significantly greater than that of the negative control (p≤0.05)

3. there was a significant dose-relationship as indicated by the linear trend analysis (p<0.05).



II. Results and discussion

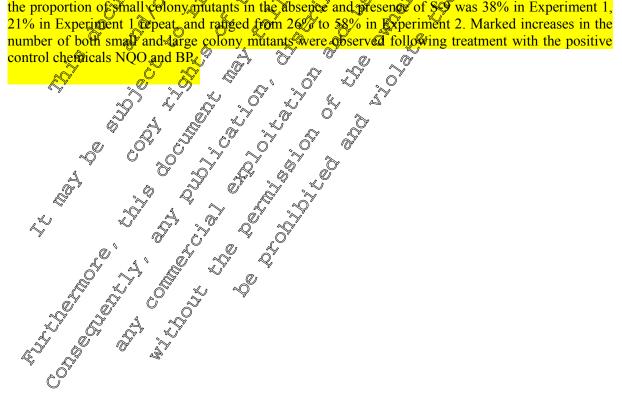
Cytotoxicity

In the cytotoxicity range-finding experiment, 3 hour treatment, six doses of TFA were tested in the absence and presence of S-9, separated by two-fold intervals and ranging from 42.5 to 1360 μ g/mL (10 mM). Cells survived all doses of TFA. The highest dose tested (1360 μ g/mL) yielded 112% relative total growth (RTG) in the absence of S-9 and 99% RTG in the presence of S-9.

In the cytotoxicity range-finding experiment, 24 hour treatment, nine doses of TFA were tested in the absence of S-9, separated by two-fold intervals and ranging from 5.316 to 1360 μ g/mL (10 mM). Cells survived all doses of TFA. The highest dose tested (1360 μ g/mL) yielded 49% RTG. In the second experiment six doses of TFA were tested in the absence and presence of S-9 (24 and 3 hour treatments, respectively), ranging from 366 to 1360 μ g/mL. All doses tested in the absence and presence of S-9 were selected to determine viability and 5-trifftforothymidine (TFT) resistance 2 days after treatment. The highest dose tested (1360 rg/mL) yielded 76% and 104% RTG in the absence and presence of S-9, respectively.

<u>Mutation Assay</u>

No statistically significant increases in mutant frequency were observed at any dose tested in the absence of S-9. A very small but statistically significant increase in mutant frequency was observed at the intermediate dose of 960 μ g/mL in the presence of S-9 in Experiment 1. This increase was sufficiently small in magnitude that it is not considered a biologically relevant response, and urthermore, provided no evidence of any dose-relationship of reproducibility, as it occurred at a single intermediate dose with no significant linear trend in only one experiment. In addition, for the negative and positive controls and dose of test arfiele where a statistically significant increase in mutant frequency was observed the number of wells containing small colonies and the number containing large colonies were scored. Thus the small and large colony mutant frequencies could be estimated and the proportion of small mutant colonies could be calculated. For the negative controls, the proportion of small colony mutants in the absence and presence of S-9 was 38% in Experiment 1, 21% in Experiment 1 when a maged from 26% to 58% in Experiment 2. Marked increases in the





Fable 5.8.1-31	Summary of results			
		<mark>89</mark>		<mark>\$9</mark>
<mark>Dose (μg/mL)</mark>	- <mark>% rel. total growth</mark>	89 mutant frequency [§]	+; <mark>% rel. total growth</mark>	mutant frequency [§]
Experiment 1 (3	hour treatment $+/-S9$)	mature requercy		
TFA 0	100	<mark>58.86</mark>	100	61.07
<mark>360</mark>	<mark>88</mark>	<mark>45.14</mark>	<mark>94</mark> 🔊	<mark>91.37</mark> 、
560	<mark>100</mark>	<mark>45.25</mark>	<mark>90</mark>	<mark>96,29</mark>
760 960	119 122	44.31 51.85 论	78 83	94.48
1160	122	59.43 V	02 · V 103	
1360	120	55.90 <i>L</i>	9 3 0 × ×	83.10
NQO 0.15	<mark>57</mark>	314.40 3		
3	<mark>42</mark>	435.99		
BP 2 3				648,58 9 975.59
\		hour treatment + S9		A A
TFA 0	100	56,490		
360 560	81 82			
760	82	4165		
960	93 L	52. 71 4	83	57.83
<mark>- 1160</mark>	<mark>90</mark> - 🌾		\$ ³ <mark>80</mark> _0 _	79.88
1360	76	<u> <u> </u></u>	100 100 100 100 5 100 100	<mark>% چ_ر 56.79</mark>
NQO 0.05 NQO 0.1		294.33°°° © 398.97 °		
NQO 0.1 BP 2		<u>398,07</u> °0°		
BP 3	- X		ζ ²⁵ ²⁵ ²	\$ 270.80 542.41
[§] 5-TFT (5-triflue	orothymidine resistant	mutants 10 viable cells	2 days after treatment	
* Comparison of	eader treatment with con	ntrol. Dunnett's test (one	-sided), significant at 59	<mark>% level</mark>
(
III. Com	clusion 🕺			
Ŭ k	N N N		0 4	
			s of Lot 78Y mouse ly	ymphoma cells in
ne absence and p	0, -/	metabolic activation's	ystem.	
-			A A A A A A A A A A A A A A A A A A A	
	Conclusion by appl	<mark>oant:</mark> 2	·	
Trifluoroacetate	(TFA) did not indree	mutation at the the boo	cus of L5178Y mouse	lymphoma cells in the
		tabolic activation sys		
			1997) and therefore a	few deviations
occurred compa	red to m OEQD TG 4	90 (2016): cvtotoxicit	<mark>y was not calculated</mark> b	by using the latest
formulae for cal	cutating Relative Sus	pension Growth (RSG	and mutation freque	ency. However, the
acceptance crite	ria were in line with v	what currently required	<mark>1.</mark>	
Therefore the	study is considered	to be acceptable and	<mark>l reliable.</mark>	
	cutating Relative Sus ria were in line with v study is considered			
Ű				



Data Point:	KCA 5.8.1/53
Report Author:	
Report Year:	2001 Image: Constraint of the second sec
Report Title:	Trifluoroacetate - Exploratory 14-day toxicity study in the rat by dietary
	administration
Report No:	SA 01136
Document No:	M-202165-01-1
Guideline(s) followed in	None A O Q
study:	
Deviations from current	Not applicable
<mark>test guideline:</mark>	
Previous evaluation:	Not evaluated
GLP/Officially	No, as it was mechanistic study and not subjected to specific Quality Assurance,
recognised testing	inspections. The study was performed according to SOT's which were previously
facilities:	accepted and periodically inspected by the Quality Assurance Unit XES
Acceptability/Reliability:	Yes O O O O O O O

Executive summarv

The potential systemic toxicity of sediumarifluoroacetate, (TFA, batch number 129013458: a white powder, 98.7% purity), was assessed after dietary administration at concentrations of 0, 600, 1 200 and 2 400 ppm for fourteen days in the rat. A group of 5 rats per sex received with reated diet and acted as a control. A further group of 5 rats per sex received profibrie acid at 5 000 ppm, which was used as a positive control for hepatic peroxisomal proliferation. A satellite subgroup of 3 rats per sex was added to the TFA high dose and control groups for inferim sacrifice after 2 days of treatment to assess liver weight, hepatic cellular proliferation and liver bistopathology only. Ň X)

Animals were observed daily for mortality and clineral signs. Body weight was recorded on Days 1, 7, 14 and before necropsy and food consumption was measured at approximately weekly intervals. Blood samples were taken before that peeropsy for he matology and clinical chemistry determinations. At study termination, brain, kidbeys, liver, ovaries, spleen, tostes and thyroid were weighed; duodenum, kidneys, liver, ovaries spleen, testes and the roid gland were collected from the five remaining males and females in each group (Day 15). Hepatic cellular proliferation was assessed using a specific immunostanting technique. A diver sample was frozen in order to determine hepatic peroxisomal activity. The remaining portions of the liver were homogenized for microsomal preparations in order to determine cytochrome P-450/isoenzyme profile, 3 **%** . S

TFA did not provoke mortalities or clinical signs during the study. Body weight evolution and food consumption were not affected by TAA administration. A 2 400 ppm, no meaningful changes were observed in hagmatology or plasma chemiory parameter except a slight decrease in total white blood cell count in females (39%, relative to confols) Mean liver weights were found statistically significantly higher in males a terminal sacrifice, in correlation with a slight diffuse centrilobular hepatoce ular hypertrophy in Q/5 males. An increased number of hepatocellular mitoses and a higher hepatocellular labeling index were found in makes and females after 3 days of treatment (approximately twice higher than in controls), but not after 14 days. Hepatic total cytochrome P-450 content, lauric acid hydroxylation activity, specific and total pathitoyl-CoA oxidation activities were increased in males reaching 119, 259, 184 and 292% of control, respectively. Only a slight dose-related increase in total cytochrome 2450 content was found in Cemales, representing 114% of control at 2 400 ppm. At 1 200 ppm, mean liver weights were found to be higher in males, in correlation with a slight centrilobular hepatoechular hypertrophy in 2/5 males. At this dose level, hepatic total cytochrome P-450 content, lauric acid bydrox ation activity, specific and total palmitoyl-CoA oxidation activities were significantly increased (117, 225, 145 and 152% of control, respectively) in males but not in females. Treatment with 600 ppm produced slight increases in hepatic lauric acid hydroxylation activity in males and hepatic protein content in females. These increases were considered not toxicologically relevant in



absence of other significant changes in the related parameters. At this dose level, no other statistically significant liver changes were noted in either sex.

Based on the results, trifluoroacetate was only a very weak peroxisome proliferator in male rats at tose levels of 1 200 and 2 400 ppm. The potential responsiveness of the animals used in the study to a peroxisome proliferator was confirmed by the data obtained in male and female rats fed clofibric acid. Marked enzyme induction and histological changes in livers were within expected ranges as reported m the literature.

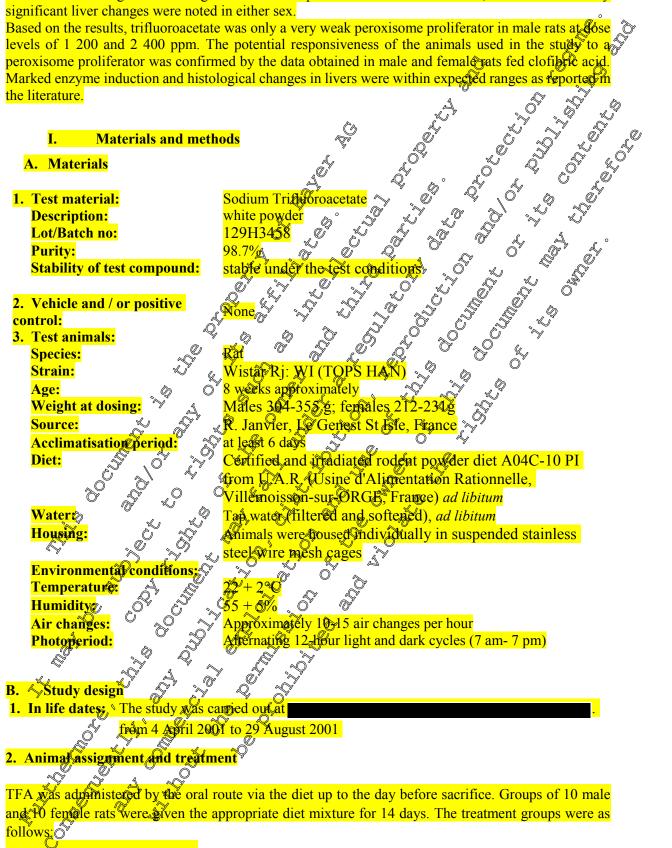




Table 5.8.1-32	Animal assignmen	<mark>t (number of animals/sex)</mark>		
Test group	Test substance	Concentration in diet (ppm)	Sacrifice Day 4	Sacrifice day 15
1	Vehicle	0	<mark>3</mark>	5 5 6
2		<mark>600</mark>	0 🔊	<u>50</u> 7 ~
<mark>3</mark>	Trifluoroacetate	<mark>1200</mark>	0	*
<mark>4</mark>		<mark>2400</mark>	<mark>3</mark> ,	5 5 5 6
<mark>5</mark>	Clofibric acid	<mark>5000</mark> ج	L V	<u>, 7</u> , 5 , 7
			a.	

Diet preparation and analysis

The test substances were incorporated into the diet to provide the required dietary concentrations. The test substances were ground to a fine powder before being incorporated into the diet by dry mixing For each compound, there was one preparation for each concentration. When not fause, the diet formulations were stored at below -15°C.

C. Methods

1. Observations

All animals were checked for morithendity and mortality twice daily (once daily on week-ends or public holidays). Clinical signs were recorded at feast once daily for all sprimals Detailed physical examinations were performed during the acclimatization phase and at least weekly during the treatment period. The nature, onset, severity, teversibility and duration of chinical signs were recorded. Cages and cage-trays were inspected daily for evidence of M-health such as blood or loose faces.

2. Body weight

Each animal was worghed once during the acchinatization period, on the first day of test substance administration, they at weekly intervals throughout the treatment petiod and before necropsy.

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3. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period The weekly mean achieved dosage intake in mg/k@ day was calculated.

4. Clinical pathology

On study Day 15 plood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous pexus. Animats were fasted overagent prior to bleeding and anesthetized by inhalation of Soflurane. Blood was collected on EDTA for haematology (0.5 mL) and lithium heparin for plasma themistry parameters (2.5 mP).

The following haematology parameters were measured red blood cell count, haemoglobin, haematocrit, mean, corpuscular, volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, reticulocy count, white blood cell count and differential count evaluation, and platelet count. A blood genear was prepared and stained with Wright stain.

The following clinical cherostry parameters were measured: total bilirubin, glucose, urea, creatinine, total choloserol, trigly or ides chlorige, sodium, potassium, calcium and inorganic phosphorus, aspartate animatransferase, alanine aminotransferase, alkaline phosphatase and gammaglutany/transferase, total protein, albumin, globulin and albumin/globulin ratio.

5. Sacrifice and pathology

On stud Day 4, three males and three females from the control and TFA high dose groups only were sacrificed. On study Day 15, all surviving animals from all groups were sacrificed. All sacrifices were



performed by exsanguination under deep anaesthesia (pentobarbital, intraperitoneal injection of 50 or 60 mg/kg body weight). Animals were fasted overnight prior to sacrifice.

All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopical At interim sacrifice, only the liver and the brain from three rats/sex in both the control and JFA high dose groups were weighed; the brain was then discarded. Liver and duodenum were sampled and sized in 10% buffered formalin for histological and immunohistochemical assessment. At that say ifice 🖗 brain, kidneys, liver, ovaries, spleen, testes and thyroid glands were weighed fresh. Paired organs were weighed together. Duodenum, kidneys, liver, ovaries, spleen, testes and thyroid glands were sampled. Tissue samples were fixed by immersion in neutral by fered 10% formalin with the exception of testes fixed in Davidson's fixative. Histological sections of the kidners, liver, ovaries, spleen, testes and thyroid glands were prepared for all animals in algroups and stained with haematoxylin and eosin Histopathological examinations were performed on the liver in all suimals from the control and TFA high dose groups sacrificed on study Day 4. The liver, kidneys, spheen, ovaries, 💞 L testes and thyroid glands were examined in TFA high dose and control animals sacrified on Study Day 15 and in animals treated with clofibric acid, in addition, the liver was examined in the intermediate dose groups to identify the no-effect level.

6. Hepatotoxicity testing

At final necropsy, the remaining portions of the liver from all animals were homogenized for microsomal preparations in order to determine total evidence P-450 content and specific cytochrome P-450 isoenzyme profile to check the hegatotoxic potential of the test substance. K) Microsomal preparations were not performed from one make and one female from the clofibric acid treated group due to technical aminitations, and from animals sacrificed at the interim sacrifice date.

Total cytochrome P 50 content in microsomal preparations was determined by spectrophotometry using a reduced Co differential spectrum. One quantification was performed for each sample.

Specific cytoethrome PA50 enzymatic activities were evaluated by spectrofluorimetry using the following substrates - benzoxyresorufin (BROD) - ethoxyresorufin (EROD)

- pentoxyresorufin (PROD)

and by HPLC with fluorimetric detection following derivatization by 4-(bromomethyl)-7methoxycoumarily of 12 hydroxy-lauric acid (fauric acid used as substrate). Ethoxyresorufin is a highly selective substrate for the isoform IA, the Soform IB metabolizes preferentially the O-dealkylation of pentoxyresorutin, while the benzoxyresorutin Ordebengylation is mainly metabolized by the isoform III A. Cytochtome P-450 dependent deally lation of resolution derivatives was followed over a period of 2,

5 or 7 monutes at 37°C Microsomes induced by well known reference compounds (3-naphtoflavone, phenobarbital and clofibric acid were measured at the same time as the study samples to have positive controls for each assay.

7. Cell cycling assessment of a standard of in order to assess cell cyching. A monoconal antibody raised against PCNA (Dako, Trappes, France) was applied to formalin fixed paraffin-embedded, deparaffinized liver sections.

The impunological reaction was amplified by a secondary antibody biotin, (Dako, Trappes, France). After submitting the liver sections to a complex streptavidine-peroxydase, the reaction was revealed using a chromogen, amino-ethyl-carbazol (AEC). The labelling index, expressed as the number of PCNApOsitive hepatocytes per 1 000 (one thousand) randomly selected hepatocytes, was measured for each individual liver sample. The mean group values were calculated. Immunohistochemical staining for PCNA was performed on the liver samples from the control and TFA high dose groups at



interim sacrifice on study Day 4 and from control, TFA high dose and clofibric acid groups at final sacrifice on study Day 15. In addition to the liver, a section of the duodenum was added on each microscopic slide. The duodenum has a high cell proliferation rate and serve as a positive control for the staining.

8. Special testing

At final sacrifice, samples of approximately 2 g of liver of all remaining animals were frozen in liquid nitrogen, in order to determine cyanide-insensitive palmitoyl-CoA oxidation activity. Samples were sent to TNO-BIBRA International, Woodmansterne Road, Carshalton, Survey SMS 4DS, U.K. performed the enzyme activity determination. 1

9. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data work homogeneous, an ANOVA was performed followed by Dunnett's dest on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dump's test if the Kruskal-Wallis was significant. When one or more group variance(s) equated @meanSwere compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4, 2.7. (Medule Enhanced Statistics).

II. **Results and diseussion**

A. Mortality

There were no treatment related mortalityes during the study

There were no treatment-related clinical signs during the study. o s

C. Body weight Store with any body weight gain of sodium triflooroacetate treated aritinals showed no indication of a treatment related aritinal showed no indication of a treatment-related effect. « Ò Õ Ø1

Positive control: During the first freatment wear the rate lost weight males: -14 g; females: -1 g). Lower body weight gain resulted in Yower body weights (males: -19%; females: -10%, p < 0.01), when compared with control mean values on Day 14 of the study.

Summary of mean body weight Table 5.8.1-33.

1 abic 5.0.1-55.	Summar y I III	an Dou y weight						
	Prifluor	oacetate 🍾	Pos.		Trifluor	<mark>oacetate</mark>		Pos.
Dose (ppm)	0 0 600 0	1290 3400	Contr.	0	<mark>600</mark>	<mark>1200</mark>	<mark>2400</mark>	Contr.
Body weight (g)		males 3)		•	females		•
Day I	_~© <mark>\$36</mark> 335	241 338	<mark>336</mark>	<mark>221</mark>	<mark>224</mark>	<mark>223</mark>	<mark>223</mark>	<mark>223</mark>
Day 7	376 373	383 368	<mark>322*</mark>	<mark>239</mark>	<mark>240</mark>	<mark>237</mark>	<mark>235</mark>	<mark>222*</mark>
Day 14	[\] 406 410	2421 408	<mark>327*</mark>	<mark>251</mark>	<mark>249</mark>	<mark>248</mark>	<mark>246</mark>	<mark>227*</mark>

Pos. = positive Contr. + control Ð * statistically different from control $p \le 0.00$

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Table 5.8.1-34: Sur	nmary	of mean	<mark>n body w</mark>	veight g	ain					
		Trifluor	oacetate		Pos.		Trifluo1	coacetate		Pos.
Dose (ppm)	<mark>0</mark>	<mark>600</mark>	<mark>1200</mark>	<mark>2400</mark>	Contr.	<mark>0</mark>	<mark>600</mark>	<mark>1200</mark>	<mark>2400</mark>	Contr.
			males					females		8 °
Body weight gain (g)							d	Ç,	Ĺ	
Day 7	<mark>35</mark>	<mark>38</mark>	<mark>43</mark>	<mark>36</mark>	<mark>-14*</mark>	<mark>17</mark>	<mark>16</mark> 🖉	<mark>14</mark>	12	°~ <mark>}*</mark>
Day 14	<mark>66</mark>	<mark>75</mark>	<mark>80</mark>	<mark>76</mark>	<mark>-9*</mark>	<mark>29</mark>	2 3)	<mark>26</mark> .	0 ²³	6 [×] 4*,
Pos. = positive; Contr. =	control				Č V		a f	, d		
* statistically different f	rom con	trol p ≤ 0).01		- Va	<u> </u>		,¢	Ş.	ž
				, Ø	,Y	Å			Ą.	
D. Food consumption	n			à		, R			۲ ۲	Les than
Trifluoroacetate: Food		nption w	as not af	fected.				* ~/~	, L	~~~~
Positive control: Mean		-	((n ()	intlydec	reased.	more pr	onounce		
in females (-26 and -36									ective	v. 5
Lower food consumpti						ight los			la l	
			Ŭ Ŭ	y _Q			J.	<u> </u>		Ő
Table 5.8.1-35: Sui	nmary		n foot co		tion	Ď	<u> </u>	Č (<u> </u>
			oroacetat	. 4	Pos			roacera	YA .	Pos.
<mark>Dose (ppm)</mark>	<mark>0</mark>		<mark>\$1200</mark>		Contr	<mark>. </mark>	~ <mark>600</mark>	3200		Contr.
Food consumption (g)	Â	Ş.	√ <mark>males</mark>	-	Ş	Ű	Ò.	^O femak	S	
<mark>Day 7</mark>	<mark>27.3</mark>		27 .9	26.6	∕ <mark>⊘20.1*</mark>	~ ~ ~			<mark>18.5</mark>	<mark>16.9</mark>
Day 14	°≈ <mark>2,¶.4</mark>	<mark>27.6</mark>	Q <mark>28.9</mark> 4	28.2 ²	/ <mark>17_6*</mark>	<mark>19/8</mark>	18.5	<mark>\$18.6</mark>	<mark>19.5</mark>	<mark>15.3*</mark>
* statistically differents	from co	(trol p 🖉	<mark>ø.01</mark> 0	Š	°~	No.	¥, °'	<i>0</i> ;		
E. Haematology	, .	Ś		S.	si Si a	, C) 4			
E. Haematology	dency t	owards	lower tot	al white	blood	ell coun	ts mas n	oted in	females	at
2400 ppm (-30% com	ared to	control	s, statisti	cally sig	nificant	p 2 0.05). This s	slight ch	ange w	as
associated with low										
significant $p \le 0.01$). In										
significant change in n		perce								
toxicologically relevan				00.		×	* *			
Positive control: No to			levant el	anges d						
Ŷ.	Ô,	S R) 'N	S.	Å.					
Table 5.8.1-36: Son	nmary	of haen	natelogy	N B	<u> </u>					
	Š		prosectat	Š (-		roacetat		Pos.
Dose (pron)		€ <mark>600</mark>	2 1200	~ 1	<mark>Contr.</mark>	<mark>0</mark>	<mark>600</mark>	1200	<mark>2400</mark>	Contr.
Parameter (unit)	4		males					females		
White blood cell count	15 57	<mark>12/8</mark>	^ <mark>₽3.9</mark>	Å 4.4	<mark>17.3</mark>	<mark>11.9</mark>	<mark>10.9</mark>	<mark>9.9</mark>	<mark>8.3*</mark>	<mark>11.7</mark>
(10 ⁹ /L)										
Neutrophil connt	3.0 0	[•] 2.5	<mark>3.3</mark> ∜	<mark>3.7</mark>	<mark>3.2</mark>	<mark>1.9</mark>	<mark>2.0</mark>	<mark>2.3</mark>	<mark>2.0</mark>	<mark>2.0</mark>
(10 ⁹ /L)		10	۳Q							
Neutrophoe (%)	©20	20 ²⁰	23	26	19	15	18	23	24*	17
Lymphocyte count		″ <mark>9.7</mark>	<mark>9.9</mark>	<mark>9.9</mark>	<mark>13.1</mark>	<mark>9.3</mark>	<mark>8.3</mark>	<mark>7.1</mark>	<mark>5.8**</mark>	<mark>9.0</mark>

 (10,42)
 Statistically different from control p ≤ 0.05
 F. Clinical chemistry ****** statistically different from control $p \le 0.01$

Trifluoroacetate: No treatment-related variation was observed.



Ôr

Positive control: Treatment-related variations (increased aspartate aminotransferase activity, alkaline phosphatase activity, urea concentration and decreased total protein and cholesterol concentrations) were predominantly observed in males. In females, the only noticeable change was a tendency towards higher aspartate aminotransferase activity which was considered not to be toxicologically relevant. Ø

Table 5.8.1-37: Su	<mark>mmary</mark>	of clini	<mark>cal chen</mark>	nistry			Ű		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		
		Trifluo:	roacetate		<mark>Pos.</mark>		Triffuo	roacetate		Pos.	Î.
<mark>Dose (ppm)</mark>	<mark>0</mark>	<mark>600</mark>	<mark>1200</mark>	<mark>2400</mark>	Contr.	0	4 <mark>600</mark>	1200 🔊	2400 [°]	Conte	,
Parameter (unit)			males		- No	Q		females		Ž	Å
Aspartate amino	<mark>50</mark>	<mark>55</mark>	<mark>57</mark>	<mark>57</mark> 🧳	≫ <mark>97</mark>	<mark>53</mark>	<mark>57</mark>	58	Å	64 *	
transferase (IU/L)				A		Q'	ذ		4		
Alkaline phosphatase	<mark>99</mark>	<mark>112</mark>	<mark>109</mark>	1905	<mark>214*</mark> (<mark>≫63</mark> ∘_	🦉 <mark>60</mark> 🔍	* <mark>77</mark> \	63 9	<mark>67</mark>	
(IU/L)							~			₩ ^v	
Urea (mmol/L)	<mark>4.71</mark>	<mark>4.63</mark>	4.69	<mark>5.09</mark>	7.09**	<u>562</u>	<mark>* 9</mark> 4	9.06	\$ <mark>5.20</mark>	<mark>4.84</mark>	>
Protein (g/L)	<mark>63</mark>	<mark>63</mark>	<mark>64</mark> >>	^{\$}		63 🛓	<mark>62</mark>	<mark>8 62</mark>	<mark>65</mark> ©	600°	
Cholesterol	<mark>1.89</mark>	<mark>1.26</mark>	J 46	<mark>)7.44</mark>	0.95**	1.69	1.79	1.55	<mark>₩7</mark> 4	4562	
(mmol/L)		ć	R (× ×	<u></u>		Ø »	_	

* statistically different from control p 20.05 10 statistically different

G. Hepatotoxicity testing Cytochrome P-450

Trifluoroacetate: At 2400 ppm a slightly increased total cytochrome P 450 content reaching 19% and 14% in males and females, respectively, occurred. L, Ł) Positive control: The increase on total extochrome B450 content was pronounced after clofibric acid administration, especially in the males (3.2% increase compared to control mean).

Enzymatic activities

Trifluoroacetate: No significant changes occurred in BROD, EBOD and PROD activities, whereas a significant dose-related increase in laurte acid by drox vation was observed in males reaching 159% increase at 2400 ppm, when compared to controls on the absence of other significant changes in the related parameters (liver weight, histology and peroxisomal activity), the increase in lauric acid hydroxylation observed at 600 ppm in males was considered not toxicologically relevant. Positive control: BROR EROD and PROD activities were not affected by the clofibric acid administration whereas a significant increase in fauric acid hydroxylation was observed in males and females (+363% and +118%, respectively)

🗶 , Čell cycling assessment

Triffboroacetate: After 3 days of treatment, the labelling index was higher in males and females at 2400 ppm, where compared to control. At terminal sacrifice, no effect of treatment on hepatocellular proliferation was noted at 2400 ppm?

Positive control: At terminal sactifice, the labelling index was higher in comparison to control groups in males and fereales.

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Palmitoyl-CoA oxidation activity

Trifluoroacetate: The hepatic whole protein content was not affected in either sex. Specific and total palmitoyl-CoA oxidation activities were increased in male rat \geq 1200 ppm up to 184% and 192% of control, respectively. No statistically significant effects were observed in female rats. Positive control: The whole homogenate protein content was statistically significantly increased to 112% of control in both male and female rats. Hepatic palmitoyl-CoA oxidation activity was statistically significantly induced in both sexes. The specific palmitoyl-CoA oxidation activity was increased by clofibric acid in both sex to 1029 and 503% of control, respectively. For total palmitoyl-CoA oxidation activity the increases were to 1144 and 564% of control, respectively.

Table5.8.1-38: Sum	nary of	hepato	toxicity	assessi	nent	Ŕ,	n° á	5 5	Č	j _L o
		<mark>Trifluo</mark> r	oacetate	¢)	Pos.		Trifluor	oacetate		Røs.
<mark>Dose (ppm)</mark>	<mark>0</mark>	<mark>600</mark>	1200		° Contr.	<u>¢</u> ″	<mark>.600</mark>	AL 2/	°× <mark>2,400</mark>	Contr.
Parameter (unit)			males	<u></u> O`	<u> </u>			females		
Cytochrome P-450 activity	<mark>/</mark>		1	æ.	Ű.	Q '	Ŭ .~	Ŭ,	Ø	
Cytochrome P-450	<mark>1.40</mark>	<mark>1.51</mark>	≱ <mark>¶./64</mark>	<mark>¥.66</mark> 🧹	<mark>¥.89</mark> 🏷	0.95 -	1.05	1,03	1.08	1,02
<mark>(nmol)</mark>		a di		.0	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	Ő		<u></u>	\mathbb{Q}	
Enzymatic activities		Q		Ž	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	Ŵ.	a Ő		y (J
BROD	<mark>14.71</mark>	<mark>20.20</mark>	22:58	° <mark>4∕7.51</mark>	* <mark>42.86</mark> ∧	2 <mark>.99</mark>	3.65 S	4.35	<mark>4,4</mark> \$	<mark>13.31</mark>
(pmol/min/mg protein)		Q'		8	, 2	_0				
EROD	<mark>54.75</mark> @	71.24	23.90°	<mark>24.98</mark>	11.24	<mark>55.95</mark>	<u>387.14</u> #	@1.34	<mark>29.63</mark>	<mark>29.99</mark>
(pmol/min/mg protein)	, S	°∼/		0	Ŷ	ΰ ^y č	h	° o	/	
PROD	<mark>8.31</mark>	<mark>8:47</mark>	1685	\$ <mark>\$%.94</mark>	@ <mark>≱2.32</mark>	∕∕ <mark>4.74</mark> ∕∕γ	3.72	<mark>3.77</mark>	<mark>4.32</mark>	<mark>5.26</mark>
(pmol/min/mg protein)	[©]	0 [.]		Г. Г.				K)		
Lauric acid hydroxylation	<mark>3.20</mark> 🛋	5.85	7.20°	8,28	14.\$2	2.56	2 <u>%10</u>	2 .05	<mark>2.26</mark>	<mark>5.59</mark>
(nmol/min/mg protein)	<u> </u>		0	ð	\sim	6 × %				
Cell cycling	a	S.	¢ ^		V V a.	0	~~			
PCNA positive cells	9 <mark>9.2</mark> y	2 2) <mark></mark> ~,	<mark>20,8</mark> 0	C, <mark>-1</mark>	8.4	_ <mark>@</mark>		<mark>17.4</mark>	
$\frac{1000}{(\text{dav 3})}$	- 4	ų – V.	\sim	L.Y	S'					
PCNA positive cells /1000 (day 14)	<mark>2&</mark>	<mark></mark> 0 [°]	K.	\$ <mark>\$_7</mark>	° <mark>8}₀7</mark> _4	€ ³ .2			<mark>3.3</mark>	<mark>5.8</mark>
<mark>/1000 (day 14)</mark>	Ś	i da	× . ~)> >	\$ <mark>5.2</mark> 				
Palmitoyl-CoA oxidation	<mark>gtivity</mark> k		→ ````````````````````````````````````	"0"	<u></u>	Ň				
Whole protein content	234	238	<mark>247</mark>	246	26¥	<mark>224</mark>	<mark>244</mark>	<mark>236</mark>	<mark>237</mark>	<mark>250</mark>
(mg/protein/g liver)		*		×	* *	7	<mark>**</mark>			<mark>***</mark>
Palmitoyl-CoA oxidation	<mark>4%38</mark>	\$ <mark>\$.39</mark> %	<mark>6.37</mark> 🎇	8.06	45.05	<mark>4.50</mark>	<mark>4.18</mark>	<mark>4.50</mark>	<mark>4.24</mark>	<mark>22.64</mark>
(nmol/min/mg			<mark>**</mark> ≪	**	***					<mark>***</mark>
homogenate protein)	, S	<i>S</i>	Ň,	ð						
Palmitoyl-Cor oxidation	1 <u>.</u> 63	<u>ľ.29</u>	<mark>1,57</mark>	<mark>¥.98</mark>	<mark>_11.78</mark>	<mark>1.00</mark>	1.02	<mark>1.06</mark>	1.01	<mark>5.64</mark>
(µmol/min/g liver)	<u>~</u>		R <mark>**</mark>	** 07	[*] ***					<mark>***</mark>

*, **, *** statistically different from control $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$

-- no data

H. Organ weights

Trifluoroacetate: Body weights in treated animals were not affected at interim as well as at terminal sacrifice. Absolute and relative layer weights were statistically significantly increased in male rat \geq 1200 ppm. There wasno difference of the liver weight in females. Other statistically significant changes were considered incidental and not treatment related since they were lacking dose-response and were not assocrated with any microscopic finding.

Positive control: Mean terminal body weight was statistically significantly lower in males and females. Absolute and relative liver weights were higher in males and females. The increased relative thyroid



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weight was not considered treatment-related since it was not associated with any histopathological finding and the absolute weight was not increased.

Sum Sable 5.8.1-39: Sum	<mark>nary of</mark>		weights		D						5
	0		roacetate		Pos.	0	Trifluor 600	vacetate		Pos	
Dose (ppm)	<mark>0</mark>	<mark>600</mark>	<mark>1200</mark>	<mark>2400</mark>	Contr.	<mark>0</mark>	000 4		<mark>2400</mark> °	Contr.	Po.
Parameter (unit)			males					femaleş	O ^v		2
Interim sacrifice day 3					Ò			×	, s		(
Body weight (g)	<mark>306</mark>		-	<mark>315</mark>	· ¥	203		<mark></mark> 0	196	Z.C.	0
Liver weight (g) - abs.	<mark>9.2</mark>			102 ×		<u>s</u> 9		<u>,0</u> -	∕ <mark>%,7</mark>	,Oʻ ≱	1
Liver weight (g) - rel.	<mark>3.0</mark>			2.2		2.9	Ø <mark></mark>	57 <mark></mark> 6	/ <mark>2.9</mark>		
Terminal sacrifice day 1	<mark>4</mark>			NO .			, w		<u></u>	ŝ	
Body weight (g)	<mark>373</mark>	<mark>374</mark>	<mark>381</mark> %	, <mark>367</mark> ₽	304**	231	<mark>228</mark>	2 26	223	^{213**}	
Liver weight (g) - abs.	<mark>9.9</mark>	<mark>10.7</mark>	<mark>11.7</mark>	<mark>¥¥.7</mark>	1 <mark>9.7**</mark>	6.3	0 <mark>6.1</mark>	6.2 6	/ <mark>6.4</mark>	≫ <mark>8.6</mark> ************************************	
Liver weight (g) - rel.	<mark>2.6</mark>	<mark>2.9</mark>	3 <mark>. ***</mark>	°∕ <mark>≯.2**</mark>	∕> <mark>4.8*</mark> *	[∞] <mark>2.7</mark>	2.7	2 <mark>.7</mark>	<mark>2.9</mark>	<mark>4 **</mark> *	
Thyroid weight (g) -	<mark>0.016</mark>	0.019	<mark>) 0.016</mark>	⁷ 0.020	<mark>0.049</mark>	0015	Q.012	0.014	<mark>Ø:013</mark>	0.015	
<mark>abs.</mark>				2 Z		T T				0	
Thyroid weight (g) - rel.	<mark>0.004</mark>	<mark>g 905</mark>	<mark>09.004</mark>	0.005	0.006**	0.006	0.005	0.006	0,006	<mark>0.007</mark>	
Pos. Comtr. = positive co. ** statistically different fi	ntrol	7. 1	? ? ~~			Å	ð	»Õ /	7		

I. Gross necropsy

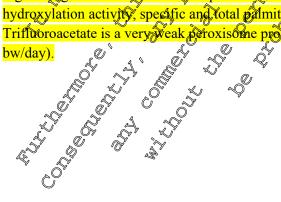
Trifluoroacetate: Only few gross pathology change were poted and considered as incidental findings. Positive control: At Crminal sacrifice larger livers were observed in 2/5 males. < Clark Å

J. Micropathotogy

Trifluoroacetate. At interim sacrifice a slight increase of hepatocellular mitoses was observed in all males and 2/3 females at 2400 ppm. At terminal sacrifice slight diffuse centrilobular hepatocellular hypertrophy was observed in 1/5 and 205 males at 2400 and 200 ppm, respectively. K. All other changes were considered to be incidental in origin and unrelated to the treatment.

Conclusions III. Õ

The NOAEL is 600 ppm (43 /45 mg/kg bw/dag male females) based on liver findings (increased organ weight in correlation with hepatocellular hypertrophy, increased cytochrome P-450, lauric acid hydroxylation activity, specific and total palmiteyr-CoA oxidation activities) in male rats. Trifluoroacetate is a very weak peroxisone profiferator in male rats at doses ≥ 1200 ppm (85 mg/kg





Assessment and conclusion by applicant:

The purpose of this 14-day dietary toxicity study with trifluoroacetate (TFA) was to investigate the effects of this substance on the liver. Results showed that TFA is a very weak peroxisome proliferator in male rats at dose levels of 1 200 and 2 400 ppm. The reliability of the testing procedure and methods used were confirmed by the responses obtained in male and female rats fed clobbric acid as positive control. Marked enzyme induction and histological changes observed in livers of the positive control animals were within expected ranges as reported in the literature. The study was not catried out to meet regulatory purposes but allowed identification of TFA target organs in rat and provided indication for dose section of further short-term/subchrout regulatory studies.

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Data Point:	KCA 5.8.1/54
Report Author:	
Report Year:	
Report Title:	Sodium trifloroacetate (FA) 28-day oxicitostudy in the fat by dietary ?
	administration was a start of a
Report No:	SA 05054 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5
Document No:	SA 05054 M-25910601-1 OECD 407 (1993), Directive 96034/EC, Method B.7 Q
Guideline(s) followed in	OECD 409 (1995), Directive 9654/EC, Method B.7
study:	
Deviations from current	The study lacks the new requirement that were not in Ouded in the previous
<mark>test guideline:</mark>	OFCD 407 (1995) in force when the grady was carried out in particular
(OECD 407, 2008)	Estrous cyclicity, circulating levels of T3 F4, TSA were not measured.
Previous evaluation:	Not evaluated a contract of the second secon
GLP/Officially	Protevaluated v v v v v v v v v v v v v v v v v v v
recognised testing	
facilities:	
Acceptability/Reliability;	Yestives J J J J J J J J J J J J J J J J J J J
Executive summary	
Executive summary	TEAN and antipugation the dist to groups of Wistor rate
Cadiment & Margan a state	TEA) was attriviate and continuous always the dist to snowing of Wiston note

Executive summary

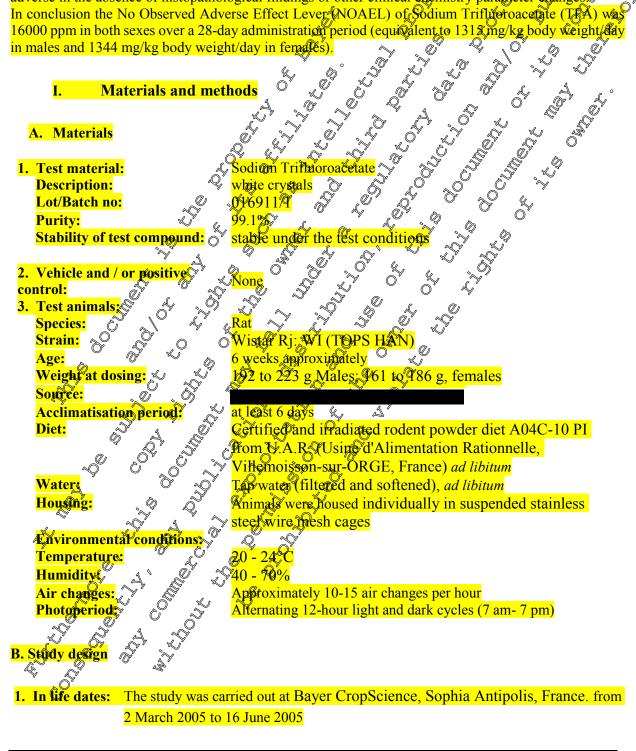
Sodium trifluoroacetate (TFA) was administered continuously wa the diet to groups of Wistar rats (5/sex/group) for 28 days at concentrations of 60001800, \$400 and 16000 ppm (equating approximately to 50, 149, 436 and 1315 mg/kg/day in males and 5% 157, 457 and 1344 mg/kg/day in females). A similarly sonstituted group received untreated diet and acted as a control. Hematology, clinical chemistry and urine parameters were determined at the end of the study. All animals were necropsied, selected organs weighed and a range of tissnes were taken, fixed and examined microscopically.

No mortalities or treatment related clinical signs were noted during the study.

At 16000 ppm, there was no effect on body weight, body weight gain or food consumption. Clinical pathology determinations revealed an increase in the alanine aminotransferase activity of 37 % in males and 230% in females. When compared to the controls, total cholesterol concentration was decreased by 30 % in males and blood glacose concentrations were decreased by 29 % in males and by 30 % in females. Urine analysis revealed higher keton levels in both sexes. At necropsy, mean absolute and relative liver weights were proceed by between 24 to 33% in males and 15 to 24% in females. In addition enlarged livers were observed in 435 makes and 5 females but these changes were not associated with relevant histopathological findings and were therefore considered to be a non-adverse response to the treatment. At 5400 ppm There was negeffect on body weight, body weight gain or food consumption. Clinical pathology determinations revealed a decrease in total cholesterol concentration of 29 % in males and decreased Blood glucose concentrations of 26 % in males and 32 % in females. Urine analysis revealed higher koon levels in both sexes. At necropsy, mean lever to body weight ratios were increased by 19 % in males and 13 % in females. In addition enlarged livers were observed in 3/5 males and 2/5 females but these changes were not associated with relevant histopathological findings and were therefore



considered to be a non-adverse response to the treatment. At 1800 and 600 ppm there was no effect on body weight, body weight gain or food consumption. Clinical pathology determinations revealed decreased blood glucose concentrations of 36 % in males and 16 % in females at 1800 ppm and of 29 % in males and 30 % in females at 600 ppm. Urine analysis revealed higher ketone levels in both sexes. Decreased blood glucose and an increased level of urinary ketones were noted avail doses in both sexes. However, in the absence of other urinalysis changes and histopathological findings associated with these changes these effects were considered not to be adverse over a 28-day administration period. Additionally the slight decrease in blood cholesterol concentration observed in males at 16000 and 5400 ppm and increased alanine aminotransferase activity noted in both sexes at 16000 ppm were considered not be adverse in the absence of histopathological findings or other clinical chemistry parameter changes. In conclusion the No Observed Adverse Effect Lever (NOAEL) of Sodium Trifliperoacetate (TEA) was 16000 ppm in both sexes over a 28-day administration period (equivalent to 1312 mg/kg body weight/day in males and 1344 mg/kg body weight/day in females).





2. Animal assignment and treatment

TFA was administered by the oral route via the diet up to the day before sacrifice. Groups of 5 male and 5 female rats were given the appropriate diet mixture for 28 days. The treatment groups were as follows

	Table 5.8.1-40 Anim	al assignment		
Test group	Concentration in diet (ppm)	Animals assig	ned 0 Female 0	
1 2	0 600		° <mark>5</mark> 🛠 🖧	
3 4	1800 5400	<u>5</u> 57 (7		4
<mark>5</mark>			<u>5</u> 6″	
nd analysis				d V

Diet preparation and analysis

Diet preparation and analysis The test substance was incorporated into the diet to provide the required concentrations. There was one preparation for each concentration. The stability of the test substance was tested at 100 and 20000 ppm and for a time interval that covered the period of usage and confirmed to be stable at room temperature or after freezing over 71 days. The homogeneity of test substance in diet was verified on study preparation at the highest concentration. Dietary level at 600 ppm was checked on samples from the study preparation kept frozen for 71 days Additionally dietary revels of the test substance were verified on the study preparation. Homogeneity and concentration checks of TEAOn the diet were within 88 to 113% of nominal concentration and were therefore considered to be acceptable, as they were within the in-house target range of 85 to 115% of nominal concentration.

C. Methods

1. Observations

All animals were checked for moribundity and mortality wice daily (once daily on week-ends or public holidays). Clinical signs were recorded at beast once date for all animals. Detailed physical examinations were performed during the acclimatization phase an Qat least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body Weight

2. Body weight Each animal was weighed twice during the acclematization period, on the first day of test substance administration, then at weakly intervals throughout the treatment period. Additionally, diet fasted animals were weighed before nechopsy $\hat{\mathcal{O}}$

3. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. The weekly mean achieved dosage intake in mg/kg/day for each week and for Weeks 1 to 13 was calculated.

4. Optainic examination

During the acclimatization period all animals were subjected to an ophthalmic examination. After instillation of an atropinic agent (Mydriaticum, Merck Sharp and Dohme) each eye was examined by



means of an indirect ophthalmoscope. During Week 4 of the treatment period, animals from control and high dose groups were re-examined.

5. Haematology and Clinical pathology

On study Days 29 or 30, blood samples were taken from all surviving animals of all groups by punchare of the retro-orbital venous plexus. An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were fasted overnight prior to bleefing and anesthetized by inhalation of isoflurane. Blood was collected on EDTA for hematology (05 mL) on lithium heparin (for plasma, 2.5 mL) and clot activator (for serum, 0.5 ml) for clinical chemistry and on sodium citrate for coagulation parameters (0.9 mL). sodium citrate for coagulation parameters (0.9 mL). Õ \bigcirc

The following hematology parameters were measured red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemogrobin, mean corpuscular hemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation, platelet count, prothrombin time. A blood smear was prepared and stained with Woight stam. ×, , C

The following clinical chemistry parameters were measured, total by Trubin glucese, urea, creatinine, total 6. Urinalysis
On study Day 25, in the morning, prior to sacrifice overaight upne samples pere collected from all

surviving animals in all groups. An approximately equal number Panimals randomly distributed amongst all groups were sampled on each day. Food and water were not accessible during urine collection. Urine samples were weighed to determine urinery volume. The following parameters were analysed: pH, urinary refractive index, glucose, bilirubin ketone bodies, occult blood, profein and urobilinogen. Microscopic examination of the urineary sediment was performed after centrifugation of the urine. The presence of red blood cells, white plood cells, epithelia cells, pacteria, casts and crystals was graded. C.

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7. Sacrifice and pathology

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On study Days 29 of 30, aff surviving animals from all groups were sacrificed by exsanguination under deep anesthesia (pentobacbital, hatraportoneal njection of @ mg/kg body weight). Animals were fasted overnight prior to sacrifice. All animals, either found dead or folled by design, were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded and sampled. O Ø

Adrenal gland, brain, epididyntis, head, kidney, liver, ovary pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland with parathyroid gland) and uterus (with cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

The following organs or tissues were sampled : adrenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone warrow (sternum), brain, ep(tidymis, esophagus, exorbital (lachrymal) gland, eye and optic nerve, Gall Pladder, Harderian grand, heart, intestine (duodenum, jejunum, ileum, cecum, colon, recturn), kidney, larynx/pbarynx, liver, fung, friph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal ford (cervical, thoracie, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymas, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagina. A bone marrow mear was prepared from femur, stained with May-Grünwald Giemsa, but not examined.

Histological sections stained with hematoxylin and eosin, were prepared for all the organs from all the animals in the control and high dose groups. Additionally, sections from the liver, lung, kidney, thyroid gland, manimary gland and from gross findings observed at necropsy were prepared for all the animals in all intermediate dose groups, sections from the spleen, testis, epididymis, prostate gland and seminal



vesicle were prepared for all the males in intermediate groups and sections from the ovary were prepared for all the females in the intermediate groups. Histopathological examinations were performed for all the sections prepared.

8. Statistics

Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When the or more and discussion group variance(s) equaled 0, means were compared using non-parametric procedure. Group means were

Slightly higher alaning aminotransferase activity (ALAT) was observed at 16000 ppm in both sexes (+37% in males and +23% in females), Decreased lower chalesterol concentration (CHOL) was noted in males 25400 ppm (20% and -29% respectively Increased concentration of glucose (GLUC) was noted in all treated groups in both sexes.

However, in the absence of associated histopathological findings these changes are not considered to be adverse.

Table 5.8.1-41 Summary of clinical chemistr

Parameter	Ő	Dose gr	oup (ppn	n) males			Dose gro	<mark>oup (ppm</mark>) females	
means (unit)	0	600	A 800	≶∕ <mark>5400</mark>	<mark>16000</mark>	<mark>0</mark>	<mark>600</mark>	<mark>1800</mark>	<mark>5400</mark>	<mark>16000</mark>
ALAT (IU/L)	~ <mark>38</mark>	A K	گ <mark>45</mark>	[₡] <mark>43</mark>	<mark>52**</mark>	<mark>35</mark>	<mark>36</mark>	<mark>41</mark>	<mark>40</mark>	<mark>43*</mark>
CHOL (mmol/L)	2.14 Č	[™] 1.69	1.65	<mark>1.51*</mark>	<mark>1.50*</mark>	<mark>1.75</mark>	<mark>1.84</mark>	<mark>1.60</mark>	<mark>1.86</mark>	<mark>2.00</mark>
GLUC (nmol/L)	5.77	4.0**	<mark>3.70**</mark>	<mark>4.25**</mark>	<mark>4.09**</mark>	<mark>6.17</mark>	<mark>4.32**</mark>	<mark>5.19</mark>	<mark>4.18**</mark>	<mark>4.32**</mark>

tatistically different from control $p \le 0.05$, $p \le 0.01$

H. Urimalysis

A dose-related increase of the ketone concentration was noted in all dose groups in both sexes. Higher mean urinary volume was noted at 16000 ppm in males (+65%). However, based on the variability of



individual values in the control group, this isolated difference was not considered toxicologically relevant.

<mark>Parameter</mark>		<mark>Dose gr</mark>	<mark>oup (ppn</mark>	n) males			Dose gro	oup (ppm) females	
<mark>means (unit)</mark>	<mark>0</mark>	<mark>600</mark>	<mark>1800</mark>	<mark>5400</mark>	<mark>16000</mark>	<mark>0</mark>	<mark>600</mark>	1800	<mark>5400</mark>	₽ <mark>160@</mark>)
Ketones								O.		
<mark>0.0 g/L</mark>	<mark>1</mark>	<mark>0</mark>	<mark>0</mark>	<mark>0</mark>	<mark>0</mark>	<mark>0</mark>	1	<mark>0</mark>		
<mark>0.05 g/L</mark>	<mark>3</mark>	<mark>0</mark>	<mark>0</mark>	<mark>0</mark>	<mark>ڻ 0</mark>	<mark>0</mark>	A Contraction	1	K) <mark>2</mark> ~	<mark>0</mark>
<mark>0.15 g/L</mark>	<mark>1</mark>	1	<mark>0</mark>	<mark>0</mark>	<mark>0</mark> 🕅	<mark>0</mark>	Q <mark>2</mark>	<mark>3</mark> 🖉	<mark>, 2</mark> 9	
<mark>0.04 g/L</mark>	<mark>0</mark>	<mark>1</mark>	<mark>0</mark>	<mark>0</mark>	6 <mark>,0</mark>	<mark>م 0</mark>	<mark>o o</mark>	<mark>8</mark>	- Q	
<u>≥ 0.8 g/L</u>	<mark>0</mark>	<mark>3</mark>	<mark>5</mark>	<mark>5</mark>	🔿 <mark>5</mark>	<mark>0</mark>	₿ °	0 0	& <mark>0</mark>	^ک <mark>0</mark> ک
Volume mL	<mark>7.1</mark>	<mark>9.9</mark>	<mark>9.6</mark>	<mark>8.5</mark>	<mark>11.7*</mark>	2.0	°∼ <mark>4.4</mark>	2.5	3 <u>,2</u> ,?	~ <mark>5</mark> ,5

I. Organ weight

At 16000 ppm, mean absolute and relative liver weights were higher and statistically different in both sexes, when compared to controls. AD5400 ppm in both sexes and at 1800 ppm in all s, mean liver to body weight ratios were higher and statistically different, when compared to controls. As these differences were not associated with relevant histopathological findings, they were considered not to be toxicologically relevant.

Table 5.8.1-43: Liver weight changes at terminal sacrifice (% change when compared to controls)

Dose (ppm)	ale S S	, O ⁽ Fer	nale	
	5400 16000		<mark>5400</mark>	<mark>16000</mark>
Mean absolute ver	+9% +2*%	NC NC	<mark>+7%</mark>	<mark>+15%</mark>
weight & NS A	№ № 0.05	NC NC	<mark>NS</mark>	<mark>p≤0.05</mark>
Mean liver to body	+19% +33%	+7%	<mark>+13%</mark>	<mark>+18%</mark>
weightratio	© <mark>p≤0.01</mark> <u>p≤0.01</u>		<mark>p≤0.05</mark>	<mark>p≤0.01</mark>
Mean liver to brain weight ratio	+12%	+10%	<mark>+12%</mark>	<mark>+24%</mark>
Mean liver to brain weight ratio	KNS p≤0,09	NC NS	<mark>NS</mark>	<mark>p≤0.01</mark>

NC: no relevant change CNS: no statist cally significant The other organ weight differences, every if statistically significant were judged to be incidental and

not treatment related.

J. Gross necropsy

A higher incidence of enlarged liver was observed in both sexes at 16000 and 5400 ppm when compared to controls. As this finding was not correlated with any histopathological finding at the microscopic examination, it was considered to be without toxicological significance. All other gross pathology hanges were considered as incidental and not treatment related.

K. Miccopathology

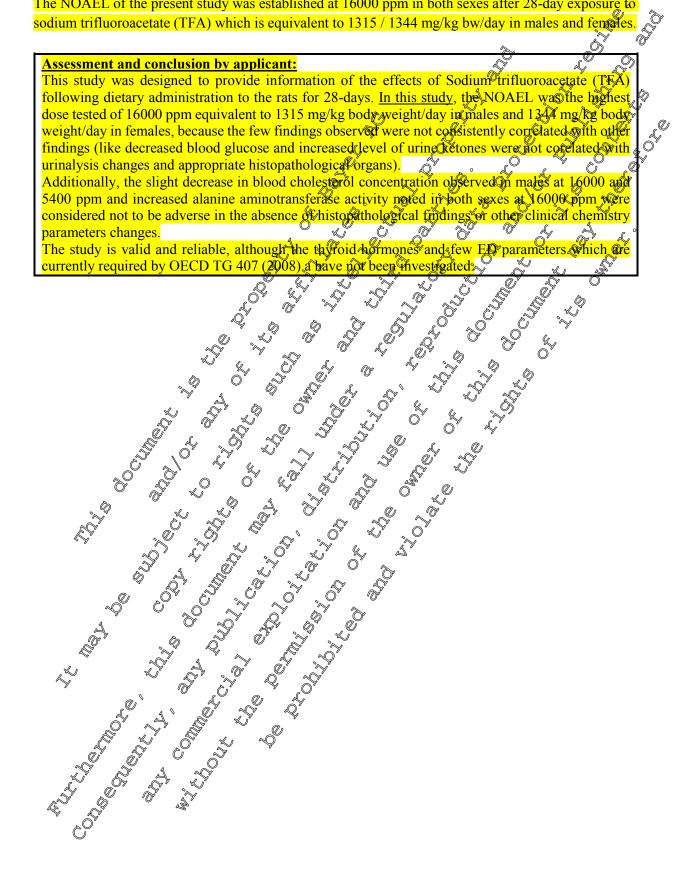
There were no areatment related histopathological changes. All histopathological findings encountered were considered to have arisen spontaneously.

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III. Conclusion



The NOAEL of the present study was established at 16000 ppm in both sexes after 28-day exposure to sodium trifluoroacetate (TFA) which is equivalent to 1315 / 1344 mg/kg bw/day in males and females.





Data Point:	KCA 5.8.1 / 55
Report Author:	
<mark>Report Year:</mark>	2007 & S
Report Title:	Sodium trifloroacetate (TFA) 90-day toxicity study in the rat by dietary
	administration
Report No:	SA 06080
Document No:	M-283994-01-1
Guideline(s) followed in	O.E.C.D. guideline 408 (September 1998)
<mark>study:</mark>	L.L.C. Directive 2001/5//LC Grietilou D.20 // Iugust 2001
	U.S. E.P.A., OPPTS Series 870, Health Effects Testing Suidelines,
	N°870.3100 (August 1998)
	U.S. E.P.A., OPPTS Series 870, Health Effects Testing Suidelines, N°870.3100 (August 1998) M.A.F.F. in Japan 12 Nousan N°8147@November 2000)
Deviations from current	The study lacks the new requirement that wore not included in the previous
test guideline:	OECD 408 (1998), in force when the study was carried out. The following
(OECD 408, 2018)	
	http://www.andlediana.com/andlediana/andle
Previous evaluation:	Not evaluated in the second seco
GLP/Officially	$\frac{\operatorname{Yes}/\operatorname{yes}}{\operatorname{Yes}} \xrightarrow{\mathcal{O}} \mathcal$
recognised testing	
facilities:	Yesives by a by
Acceptability/Reliability:	Yesyyes by O A D O V
8	
Executive summary	

Executive summary

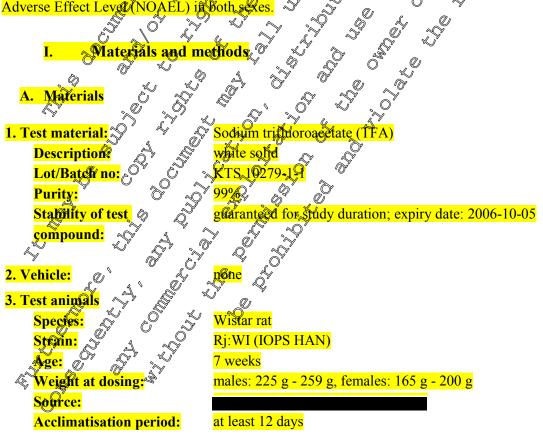
Sodium trifluoroacetate (FEA, batch number KTS 10279-1-1, white solid, 99% purity), was administered continuously via dietary administration to separate groups of Wistarrats (10/sex/group) at concentrations of 160, 1600 and 16000 ppm (equating approximately to 9.9, 98, 1043 mg/kg body weight/day in males and 12.2, 123, 1210 mg/kg body weight/day in females), respectively for at least 90 days. A similarly constituted group of 10 males and 10 females received untreated dief and acted as a control. Clinical signs were recorded daily, body weight and food consumption were measured weekly. A detailed physical examination was performed once during the acclimatization phase and weekly throughout the study. All surviving animals were subjected to a neurotoxicity assessment (exploratory locomotor activity, open field observations, sensory reactivity and grip strength) during Weeks 11 to 12 of the study. Ophmalmological examinations were performed on all animals during the acclimatization phase and on all surviving animals from all groups and grip strength) during the acclimatization phase and on all surviving animals from all groups and grip strength and for hematology and clinical chemistry determinations. All animals were hecropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically.

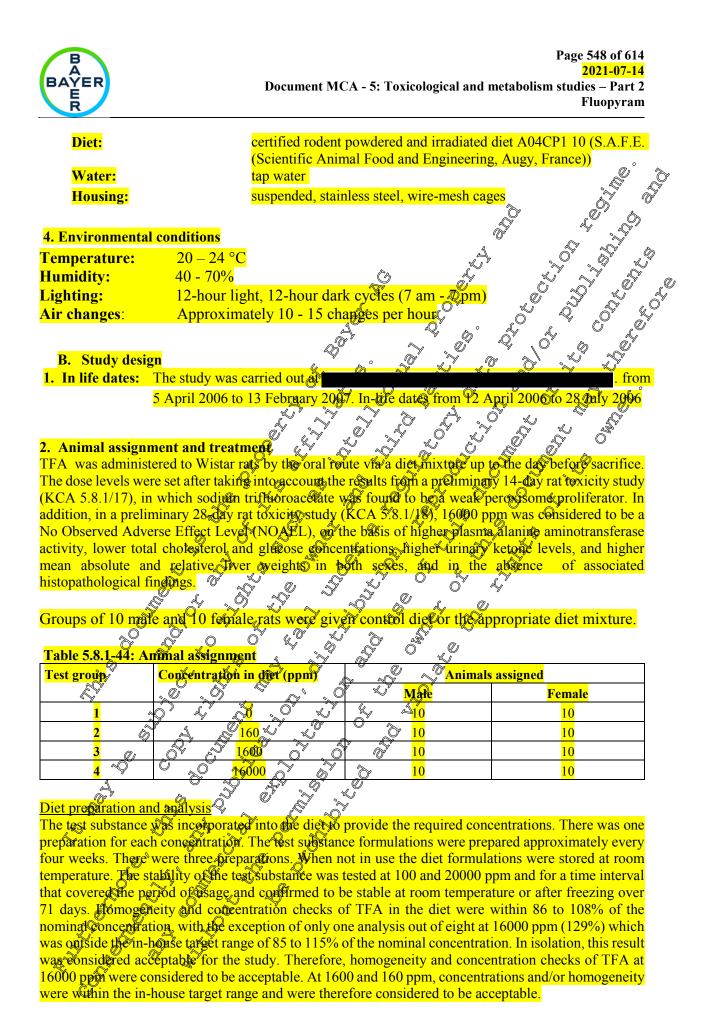
No treatment related mortalities were noted during the study.

At 16000 ppm, mean body weight of makes was reduced by 5 to 11% from study Day 15 onwards, resulting in an overall reduction in mean body weight gain of 17% on Day 92, when compared to controls. In females, mean body weight was reduced by up to 6% during the course of the study, resulting in an overall reduction in mean body weight gain of 14% on Day 92, when compared to controls. Clinical pathology determinations revealed lower mean hemoglobin concentration in females. This slight change was associated with a statistically significantly lower mean corpuscular volume, mean corpuscular hemoglobin and hematocrit. In addition, mean total bilirubin and glucose concentrations were markedly



lower in both sexes. Mean values for alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase activities were higher in males and were correlated with a higher incidence of hepatocellular necrotic foci. At urinalysis, higher ketone levels were noted in both sexes. At necropsy, terminal body weight in males was lower when compared to controls. Mean absolute after relative liver weights were increased by 19 to 33% in males and by 23 to 28% in females. These changes were dose-related and associated with hepatocellular hypertrophy observed at the microscopic examination. All male and 9/10 female rats were noted to have minimal to moderate diffuse centrilobular to panlobular hepatocellular hypertrophy with ground-class appearance of the hepatocellular wtoplasm. In addition, a loss of the periportal hepatocellular vacuolation was observed in both sexes. There was also a higher incidence of hepatocellular necrotic foci in males. Liver findings were considered to be adverse due to the magnitude of the response and the association with clinical pathology changes. At 1600 ppm, there was one death unrelated to peatment. Clinical pathology determinations revealed lower mean hemoglobin concentration and mean corpusculation hemoglobing in females only. In addition, lower mean total bilirubin and glucose concentrations were observed in both sexes and higher mean alkaline phosphatase activities were observed in males only. In addition, aspartate aninotransferace and alanine aminotransferase activities of some individual values in males were togher and were also considered to be treatment related. At orinal wis, higher ketone levels were noted in both sexes At necropsy, mean absolute and relative liver weights were increased by 8 to \$4% in males and by 9 to 13% in females. At the microscopic examination, 5/9 males were noted with minimal to slight diffuse centrilobular to panlobular hepatocellutar hypertrophy. In addition a loss of the periportal hepatocellular vacuolation was observed in males, Liver findings were considered to be adverse due to the magnitude of J.Q. the response and the association with clinical pathology changes. There were no adverse effects at 160 ppm and his dose level (equating approximately to 9.9 mg/kg body weight/day in males and 12,2 mg/kg body weight day in females) is (considered to be a No Observed Adverse Effect Lever (NOAEL) in Both sexes.







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C. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on week-ends or public holidays). Clinical signs were recorded at least once daily for all animals. Detailed physical examinations were performed during the acclimatization phase and at least weekly during the freatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Neurotoxicity assessment

During study Weeks 11 to 12, a neurotoxicity assessment was performed for all surviving animals by & observers who were blind with respect to the dose level. The following investigations were carried of the dose level.

a. Exploratory locomotor activity

Animals were tested individually using an automated photocell recording apparatus (Imetronic) Bordeaux, France) designed to measure quantitatively spontaneous exploratory locomotor activity in a novel environment. Motor activity was recorded for the first 90 minutes with data being collected at 15-minute intervals throughout the session.

b. Open field observation

Changes in gait, posture, as well as presence of clonic of tonic movements. Stereotypic behaviour (e.g. excessive grooming, repetitive circling), bizarre behaviour (e.g. seft-mutilation, walking backward) and other neurological-related changes were recorded for all surviving animals.

c. Sensory reactivity

The following reflexes and responses were recorder.

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- Pupillary redex (by covering the eyes of the mimal for a few seconds and then observing pupillary constriction by focusing a marrow beam of light in the eyes).

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- Surface righting reflex (by parting the animal on its back and measuring its ability/rapidity to reassume a formal standing position).
- Corneal reflex (by touching the medial canthus with a fine object and observing the quick and complete closure of the eyelids).
- Flexor reflexe (by pinching the toes and measuring the presence/strength of the flexor response of each handling).
- Auditory starter response (by measuring the animal response to an auditory stimuli).
- Tail pinch response (by pinching the tail with proceps and measuring the animal reaction).

d. Trip strength Y

The fore- and hind with grip strength of all animals were measured quantitatively using a grip strength tester equipped with one pull of one push strand gauge (Bioseb, Chaville, France). The mean of three successive measurements was noted for both fore- and hindlimb grip strength.

3. Body weight

Each animal was weighed twee during the acclimatization period, on the first day of test substance administration, then at weekly intervals throughout the treatment period. Additionally, diet fasted animals were weighed before necropsy.

4. Food consumption



The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. The weekly mean achieved dosage intake in mg/kg/day for each week and for Weeks 1 to 13 was calculated according to the following form fra:

Dose level (ppm) x Group mean food consumption (g/dax Test substance intake = -Group mean body weight (g) at the end of the food consumption pe (mg/kg/day)

5. Ophthalmic examination

During the acclimatization period all animals were subjected to an oppithalmic examination. After instillation of an atropinic agent (Mydriaticum, Merce Sharp and Dohme) each ever was examined by means of an indirect ophthalmoscope. During Wear 14 of the treatment period animals from contro and high dose groups were re-examined.

6. Clinical pathology

On study Days 93, 94, 95 blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexits. An approximately equabrumber of anymals random distributed amongst all groups were sampled on each day Animals were dasted overnight prior to bleeding and anesthetized by inhalation of coflurarie. Blood was colleged on PDTA for hematology (0.5 mL), on lithium heparin (for plasma 2.5 mb) and lot activator (for serom, 0, Cml) for clinical chemistry and on sodium citrat for coagulation parapheters (0.9 mb). ×

The following hematology parameters were measured red blood cell ount, hemoglobin, hematocrit, mean corpuscular volume mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulogyte count, white blood cell sount and differential count and aluation, platelet count, prothrombin tiple. A blood sinear was prepared and stained with Wright stain.

Ő) The following climical chemistry parameters were measured, total bilirgon, glucose, urea, creatinine, total cholester, trigOycerides, choride, Godium, potassium, calcium and inorganic phosphorus, aspartate animotrapsferase, alanine aminotransferase, Akaline phosphatase and gamma-glutamyltransferase, total protein, albumin, globulin and albamin/globulin ratio.

7. Urinalysis

7. Urinalysis On study Day 87, 27, 88, in the morning prior of sacrifice, overnight urine samples were collected from all surviving animals in all groups. An approximately equal number of animals randomly distributed amongst all groups vere sampled on each day. Food and water were not accessible during urine collection. ~0[×]

Urine samples were weighed to determine urinary volume.

The following parameters were analysed: pre, uring v refractive index, glucose, bilirubin, ketone bodies, occult blood, protein and urobilingen. Meroscopic examination of the urinary sediment was performed after centrifugation of the urine, The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was grade

Ô 8. Sacrifice and pathology

On study Days 3, 94 or 95, all surviving animals from all groups were sacrificed by exsanguination under deep any sthesia (pentoparbital, intraperitoneal injection of 60 mg/kg body weight). Animals were fasted over the proof to sacrifice. All animals, either found dead or killed by design, were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded and sampled.



Adrenal gland, brain, epididymis, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (with parathyroid gland) and uterus (with cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together. The following organs or tissues were sampled : adrenal gland, aorta, articular surface (femoro bial) bone (sternum), bone marrow (sternum), brain, epididymis, esophagus, exorbital (lachrymal) gland, eye and optic nerve, Gall bladder, Harderian gland, heart, intestine (duodenum, jejunum, ileum, cecom, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mannary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, setatic nerve, seminal sesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymus, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, therus (with cervix), vagina. A bone marrow smear was prepared from femur, staned with May-Grünwald Gemsa but not examined. Q Ø Histological sections, stained with hematoxylin and eosin, well prepared forcall the organs from all the animals in the control and high dose groups. Additionally, sections from the liver Jung, kidney, thyroid gland, mammary gland and from gross findings observed of necropsy were prepared for all the animals in all intermediate dose groups, sections from the spleen testis, epididymis, prostate gland and serumal vesicle were prepared for all the males in intermediate groups and sections from the ovary were prepared for all the females in the intermediate groups. Histopathological examinations were performed for all the sections prepared A D Ver No. 9. Statistics â Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous even after transformation, a Kruskal-Wallis ANOVA was performed followed by the Dunn's test if the Kruskal-Wallis was significant. When more group cariance(s) equalled 0, means were compared using nonparametric procedures. Group means were compared apthe 5% and 1% levels of significance. Statistical A. Test substance analysis See section 'Diet preparation and analysis B. Mortality One male from the 1600 ppm group var e noted to have torsion of the imporsion was considered analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). One male from the 1600 ppm goup was found dead on study day 15. At necropsy, this animal was noted to know torsion of the intestine and a dark content within the ileum and jejunum. This intestinal torsion was considered to be the cause of death and not to be treatment related. All other macroscopic findings were related to agonal changes observed at the histopathology examination. In addition, at the histopathological examination degenerative cardiomyopathy was noted. This change is a common finding observed spontaneously in the Wistar rat strain of this age and was considered not to be treatment-related, ñ

C. In-life observations

No tieatment-related clinical signs were observed.

One male from the 16000 ppm group was noted to have ocular discharge in both eyes from study days 78 to 85. As this sign was transient and disappeared before the end of the study, it was considered not to be treatment-related.



D. Neurotoxicological investigations

a. Locomotor activity

At 16000, 1600 and 160 ppm in both sexes, overall mean exploratory locomotor activity was comparable to control values. In addition, the pattern of the locomotor activity over time was similar to controls

b. Open field observation

No treatment-related changes were recorded during the open field observation at any dose level in either sex. The few changes noted were observed in isolation and/or with no dose-relationship and were considered not to be treatment-related

c. Sensory reactivity

All reflexes and responses evaluated were unaffected by the treatment at any dose level in either sex. The increased incidence of exaggerated flexor reflex for both hind paws observed in the high dose females was considered not to be treatment-related, due to the mited magnitude of the change and inter-individual variation of this parameter.

d. Grip strength

The fore- and hind-limb grip strength were unaffected by treatment at any dose tevel in either sex. A slight decrease in forelimb grip strength was observed in high dose females in comparison to controls (-17%, p≤0.01), but it was considered to be fort to us and due to a particularly high mean value in the control group. Furthermore, the mean value observed in the high dose females far this parameter was within the in-house historical control range

E. Ophthalmology

There was no eodencoof treatment related offects up to the highest dose level tested of 16000 ppm. One male from the 16000 ppm group had a corneal opacity in the left eye and another male had anterior synechia in the this of the left ove.

F. Body weight

At 16000 ppm, mean body weight of males was reduced by 5 to 11% from study day 15 onwards, resulting in an overall veduction in mean body weight an of 17% on day 92, when compared to controls. The effect was statistically significant at most time points ($p \le 0.01$ or 0.05). In females, mean body weight was reduced by up to 6% thiring the course of the study, resulting in an overall reduction in mean body weight gain of 4% on Day 2, when compared to controls. The effect was statistically significant on a number of occasions for α invalidities body weight gain (p ≤ 0.01 or 0.05).

Body weight parameters were not affected in other sex at 1600 ppm and at 160 ppm.

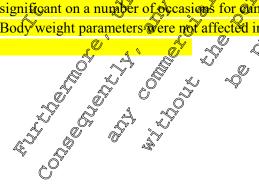




Table	<mark>5.8.1-</mark> 4	45: S	umma	<mark>ry of m</mark>	<mark>lean b</mark> o	<mark>ody we</mark>	<mark>ights (</mark>	<mark>g)</mark>						
							Ma	<mark>ales</mark>					0	
<mark>Dose</mark>	_	_			Me	an bod	<mark>y weig</mark> ł	<mark>nt (g) o</mark>	n study	day				ð
<mark>(ppm)</mark>	<mark>1</mark>	<mark>8</mark>	<mark>15</mark>	<mark>22</mark>	<mark>29</mark>	<mark>36</mark>	<mark>43</mark>	<mark>50</mark>	<mark>57</mark>	<mark>64</mark>	<mark>71</mark>	<mark>78</mark>	85 92	
<mark>0</mark>	<mark>245</mark>	<mark>299</mark>	<mark>348</mark>	<mark>384</mark>	<mark>412</mark>	<mark>442</mark>	<mark>466</mark>	<mark>485</mark>	<mark>503</mark>	<mark>516</mark>	<mark>524</mark>	<mark>535</mark>	543 550	0
<mark>160</mark>	<mark>246</mark>	<mark>298</mark>	<mark>348</mark>	<mark>384</mark>	<mark>410</mark>	<mark>438</mark>	<mark>461</mark>	<mark>480</mark>	<mark>498</mark>	<mark>509</mark>	540	<mark>530</mark>	536 544	\supset
<mark>1600</mark>	<mark>244</mark>	<mark>294</mark>	<mark>342</mark>	<mark>376</mark>	<mark>401</mark>	<mark>431</mark>	<mark>449</mark>	<mark>470</mark>	<mark>483</mark>	<mark>496</mark>	<mark>499</mark> 9	<mark>514</mark>	522 529	
<mark>16000</mark>	<mark>243</mark>	<mark>291</mark>	<mark>332*</mark>	<mark>359+</mark>	<mark>378⁺</mark>	<mark>404⁺</mark>	<mark>421⁺</mark>	<mark>439+</mark>	<mark>450+</mark>	<mark>465⁺</mark> ≉	471 ⁺	482 ⁺	⁴90⁺ 496 ⁺	Q
							Fen	nales		L.				N N
<mark>Dose</mark>					Me	an bod	y weigł	it g) o	n study	day		õ		
<mark>(ppm)</mark>	1	<mark>8</mark>	<mark>15</mark>	<mark>22</mark>	<mark>29</mark>	<mark>36</mark>	<mark>43</mark> ູ	<mark>50</mark>	<mark>57</mark> (6 <mark>4</mark> 64	<mark>71</mark> 、	∦ <mark>∕[®]78</mark>	2 <mark>85</mark> 292	Ő
0	<mark>182</mark>	<mark>204</mark>	220	228	<mark>239</mark>	<mark>249</mark>	260	<mark>264</mark>	269	<mark>271</mark>	274 C	278	280 ج <mark>≎282 %</mark>	
<mark>160</mark>	<mark>181</mark>	<mark>203</mark>	<mark>222</mark>	<mark>230</mark>	<mark>238</mark>	<mark>248</mark>	250	<mark>264</mark>	27Îَ≶	<mark>2765</mark> °	276	27	280 282	7 7
<mark>1600</mark>	<mark>183</mark>	<mark>200</mark>	<mark>217</mark>	<mark>228</mark>	<mark>237</mark>	<mark>244</mark>	$\sqrt{251}$	<mark>257</mark>	2 <mark>262</mark>	2 <u>268</u>	27 1	275	297 284	
<mark>16000</mark>	<mark>183</mark>	<mark>199</mark>	<mark>214</mark>	<mark>223</mark>	<mark>231</mark>	<mark>239</mark>	245 🗞	° <mark>253</mark>	<mark>∲255</mark> ≪	گ <mark>258</mark> √	<mark>@60</mark> 🤇	2 <mark>264</mark>	267 270	
* Statistic	cally si	gnifica	nt differ	ent fror	n contro	ol (p@(<mark>).05)</mark> ©	, second	Ž	~~~	, a	ý (4	

⁺ Statistically significant different from control p < 0.0

In males, there was a dose-related trend wards lower derminal body Oweight when sompared to on trols, the mean terminal body weight was slightly lower at 6000 ppm (not statistically significant).

Ø1 G. Food consumption and compound intake Up to the highest dose level tested food consumption was not affected in eith

Ĩ

Ś Table 5.8.1-46: Achieved intake (m@kg bwday)

Diet concentration (ppm)		500 16000	0 [∞] <mark>160</mark> √	<mark>1600</mark>	<mark>16000</mark>
	🖉 👡 <mark>M</mark>		\$ _ @	Females	
Weeks 1 to 13	9.9 [°]	98 1045	√ <u>12.2</u>	<mark>123</mark>	<mark>1216</mark>
ð á s	4 . Q		, O		

H. Ha@matology

Treatment related changes were noted only in females at 16000 and 1600 ppm. When compared to the controls, lower mean haemoglobin contration (-8%, p≤0.01) was noted at 16000 ppm in females only. This slight change was associated with lower mean corpuscular volume (−6%, p≤0.01), mean corpuscular harmoglobin (−7%, p≤001) and haematocrit (−6%, p≤0.01). \sim Õ

At 1600 ppm, lower mean haenoglobus concentration (-4%, p≤0.05), essentially due to low values noted in two animals, and lower mean corpuscular haemoglobin (-3%, p≤0.01) were also noted.

Ô No freatment-related change was noted to make at any dose level and in females at 160 ppm. , V Ø 1

The few other statistically significant differences were considered to be incidental in view of their The forwest forse occurrence at the towest to se and/or their low magnitude.



	N	Mean ± SD (% change when compared to control)											
<mark>Parameter</mark> Dose (ppm)	Hb (g/dL)	MCV (fl)	Hct (L/L)	MCH (pg)									
<mark>0</mark>	15.6 ± 0.7 ()	52 ± 1 ()	0.462 ± 0.019 ()	7.4 ± 0.4									
<mark>160</mark>	15.6 ± 0.4 (±0%)	51 ± 2 (-2%)	0.467 ± 0.010 (+1%)	17.1 ± 0.5 (-2%)									
<mark>1600</mark>	$14.9 \pm 0.6^{*}$ (-4%)	50 ± 1 (-4%)	0.448 ± 0.018 (-3%)	16.8 ± 0.3 (-3%)									
<mark>16000</mark>	$14.4 \pm 0.4^{**}$ (-8%)	$49 \pm 1^{**}$ (-6%)	$0.435 \pm 0.010^{**}$ ($16.2 \pm 0.9^{**}$									

naemoglobin concentration; I MVH = mean corpuscular haemoglobin

* = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.05$); ** = statistical different from control ($p \le 0.05$); ** = statistical dif

I. Clinical chemistry

()Treatment-related changes were observed at 1,6000 and 1600 opm in both sexes. Mean total bill ubin and glucose concentrations were lower in both Gexes and mean alkaline phosphatase; alanine aminotransferase and aspartate aminotransferase approving were fogher in males only. The slightly lower mean total bilirubin concentration noted ap160 ppm in both sexes was considered not to be treatment-related as the difference to controls was not statistically significant and all individual values were within the in-house historical control data.

Table 5.8.1-4	48: Summary <u>o</u>	Clinical chemis			and/females
		Mean ± SQ(%)	change when com	ared to control)	0.
Parameter	Bili				🖏 <mark>ALT</mark>
	(mmol/L)	(mm@l/L)	<mark>(IU/L)</mark> ∖		/ <mark>(IU/L)</mark>
Dose (ppm)		<u> </u>	<mark>males</mark> &		
<mark>0</mark>	<mark>1.60 ± 0.4</mark>	<mark>≸%7 ± 0.53</mark>	ζ ⁷ <mark>80⁻ ²9</mark> Ο ,	39 ± 37	<mark>47 ± 25</mark> ()
<mark>160</mark>	^{(−3})	[*] √ [*] 5.40 [*] ¥0.64 [*] √ [*] √ [*] √ [*] √	$88 \pm 10^{-15\%}$	∕y <mark>83 21</mark> y <mark>{√7%)</mark>	<mark>47 ± 20</mark> (±0%)
1600	0.5⊊0.1** (−69%)	$\frac{1}{4}$ $\frac{1}{4}$ $\frac{1}{4}$ $\frac{1}{4}$ $\frac{1}{4}$	100 ± 18	46 ± 118 (+64%)	87 ± 84 (+85%)
16000	$\frac{0.3 \pm 0.2^{**}}{(-2^{*})} = 0.3$	$\begin{array}{c} 4.21 \pm 0.44 \\ \bigcirc \\ (-28\%) \\ \hline \\ 4.14 \oplus 0.84^{**} \\ \hline \\ \hline \\ 229\% \\ \hline \\ \end{array}$	(+95%)	$ \frac{100}{111 \pm 24} $ (+25%)	<mark>65 ± 19*</mark> (+38%)
Dose (ppm)		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ر من المحمد ا		
<mark>0</mark>	2.1 ± 0.5 4.2 1.8 ± 0.4 (-14%)	© <mark>5.57(≠0.86</mark> ,)	50 ± 10	$\frac{73 \pm 12}{()}$	<mark>38 ± 9</mark> ()
160 ~ Ç	1.8 ± 0.4 ℃ (-14%)	(-80)		82 ± 17 (+12%)	40 ± 10 (+5%)
1.600	$\frac{1.0 \pm 0.6^{**}}{(-52\%)}$	→ (-88) → 4.19 ± 0.45** (-25%)		<mark>87 ± 16</mark> (+19%)	47 ± 17 (+26%)
رچم <mark>¥6000</mark>	0,5 ¥ 0.3*≰ (−76%)	$\begin{array}{c} 4 & 62 \pm 1 & 1 \\ 6 & 7 & 7 \\ 7 & (-1 & 6) \end{array}$	y ³ 50 ± 12 (±0%)	<mark>85 ± 12</mark> (+16%)	<mark>45 ± 5</mark> (+18%)

Bili = total bilirubin; Gluc = glucose; ALC = alkatine phosphatase; AST = aspartate amino transferase ALP = alanine appino transferase

= statistically orgnificant different from control (p≤0.05); ** = statistically significant different from control (p≤0.01) 1 Ô S

Several males from all treated and control groups had elevated aspartate aminotransferase and alanine amingtransferase activities. These effects were considered to be treatment-related at 16000 and 1600 ppm in males as they were of high magnitude and/or outside the in-house historical control data. There was no effect on these parameters in females at any dose level.

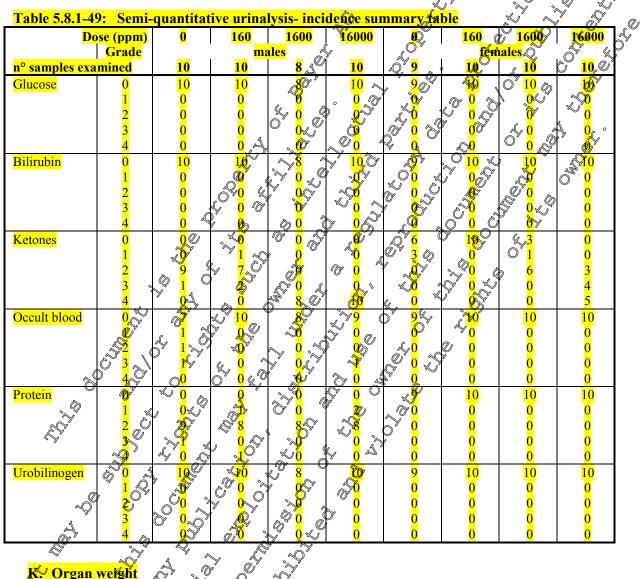
The other statistically significant differences were considered not to be treatment-related in view of the variation of the individual values and/or their low magnitude.



J. Urine analysis

When compared to the control groups, higher ketone levels were noted at 16000 and 1600 ppm in both sexes.

No other treatment-related change was noted for the parameters assayed. The few other statistically significant differences were considered to be incidental.



Mean absolute and relative liver weight were statistically significantly higher in male and female rats at 16000 and 1600 ppm where compared to controls. These changes were dose- and treatment related

at 16000 and 1600 ppm where compared to controls. These changes were dose- and treatment related and associated with repatoellular hypercophy.

All other statistically significant organ weight differences were judged to be incidental in view of their individual variation and in the absence of any correlated histopathological finding.



Table 5.8.1-	50: Summ	<mark>ary of liver we</mark>	<mark>ight data at ter</mark>	<mark>minal sacrifi</mark>	<mark>ce</mark>	
			<mark>1 (% change whe</mark>	en compared to	<mark>o control)</mark>	_ 0
Parameter	Absolute	<mark>liver weight (g)</mark>	Liver to bod	<mark>ly weight ratio</mark>	Liver to bra	in weight ratio
<mark>Dose (ppm)</mark>						
<mark>0</mark>	<mark>12.15</mark>	()	<mark>2.327</mark>	()	<mark>566.930</mark>	()
<mark>160</mark>	<mark>11.61</mark>	(-4%)	<mark>2.258</mark>	(-3%)	546,177	(-4%)
<mark>1600</mark>	<mark>13.25*</mark>	(+9%)	<mark>2.657**</mark>	(+14%)	<mark>613.081</mark>	<mark>(;†8%)</mark> ∑
<mark>16000</mark>	<mark>14.48</mark>	(+19%)	<mark>3.102**</mark>	(+33%)	∛ 701.329**	<mark>0(+24%)</mark>
Dose (ppm)			fen	rales	Å .*	
<mark>0</mark>	<mark>5.96</mark>	()	<mark>2.243</mark>	7 ()	307.108 ⁰	
<mark>160</mark>	<mark>6.25</mark>	(+5%)	<mark>2.343 🏑</mark>	(+4%) O`	^{316.17}	<u>3</u> +3%)
<mark>1600</mark>	<mark>6.71*</mark>	(+13%)	2.520	(+12%)	₀ <mark>334.508</mark>	<mark>(+9%)</mark> @
<mark>16000</mark>	7.36**	(+23%)	2.880**	(+28%)	382Q160**	× (+24%)

L. Gross necropsy findings

a. Unscheduled death

One male was found dead on study day 3. This animal was boted to have forsion and a dark content within the ileum and jejunum. This intesting forsion was considered to be the cause of death and was therefore incidental. All other macroscopic findings were related to agonal changes bund at the histopathology examination and were considered not to be treatment, related

b. Terminal sacrifice

O With the exception of the higher incidence of foci (red or white) within the hver observed in males at efe considered to be incidental and not treatment-related 16000 ppm, all the other changes w

M. Histopatholog

a. Unscheduled death

In addition to agonal changes, degenerative cardiomyopathy was noted. This change is a common spontaneous finding observed in the Wistar rat of this strait and age, it was considered not to be treatment-related. The cause of death was considered to be the intestinal torsion noted at necropsy.

b. Terminal sachtice Ć Ô

Treatment-related histopathological charges were observed in the liver.

In all male and most females at \$6000 apm, as well as in a proportion of males at 1600 ppm, a minimal to moderate diffuse contrilobular to panlobalar hepatocellular hypertrophy with ground-glass appearance of the hepatocellular cytoplasm was observed. This latter observation is usually induced by peroxisome proliferators while change was associated with a loss of the periportal hepatocellular vacuolation observed at 1600 ppm is both sexes and at 1600 ppm in males. The effect was doserelated and correlated with the higher mean liver weight noted in these groups.

There was also a higher jocidence of hepatocellular necrotic foci in males at 16000 ppm when compared to controls, which was considered to be adverse. This finding was correlated with higher individual values of aspartate aminotransferase and alanine aminotransferase activities observed in clinical chemistry evaluation.

A higher neither of minimal to slight degenerative cardiomyopathy was noted in males at 16000 ppm. As this change is a common spontaneous finding observed in the Wistar rat of this strain and age, including in untreated control animals, with a similar severity and incidence, it was considered



not to be treatment-related.

No effect of treatment was seen in any other organ examined microscopically. Some other histopathological findings were noted in animals of all groups but they were considered to be incidental, as they were within the range of expected changes for rats of this age and strain kept inder laboratory conditions.

Table5.8.1-51: Incidence and severity of microscopic changes in the liver, all animals,

terminal s	sacrifice			Ò	Å	/	si a		<i>a</i> i
<mark>Dose (ppm)</mark>	<mark>0</mark>	<mark>160</mark>	<mark>1600</mark>	16000	<mark>0</mark> .Q	<mark>160</mark>	0 <mark>1600</mark>	16000	Ś
Sex		M	<mark>ales</mark> 🧳	Ş		Fen	<mark>fales</mark> Q		J
Number of animals examined	<mark>10</mark>	<mark>10</mark>	<mark>9</mark> 0	10	چ ` <mark>10</mark>		0 ¹⁰		
Centrilobular to panlob	ular hepat	ocellular l	nypertroph	y, diffuse	, K	× č), _v	К ^у	
Minimal	<mark>1</mark>	<mark>0</mark>	0 <mark>3</mark> x	đ	0 ⁰ 0	<mark>С 0</mark> С	<mark>0</mark>	کي <mark>5</mark> _ °	
<mark>Slight</mark>	<mark>0</mark>	<mark>0</mark> 🖉	r ⊳ <mark>2</mark> 0°	~~ <mark>6</mark>	× <mark>0</mark> 4	A	0 🖉	40	
Moderate	<mark>0</mark>	<mark>g</mark> y	<u></u>	<mark>کہ ج</mark>	Å.	^م <mark>0</mark>	₹ <mark>¢</mark> €	<u> IN</u>	
Total	<mark>1</mark>	2 <mark>9</mark> 4	ک <mark>5</mark>	A A	20 0 X		Ø,	<mark>9</mark>	
Periportal hepatocellula	r vacuolat		ie 🚿		y di	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	S V	2	
Minimal	<mark>4</mark>	\$ <mark>3</mark> 2		♥ <mark>0</mark> 6 ↓ ↓	2 S	0 <mark>6</mark> 0	۲ <mark>7</mark> ۳۷	<mark>0</mark>	
Total	A	°~ <mark>3</mark>	<mark>0</mark> 💞	Â,	24 <mark>5</mark>	60°	8 7	<mark>0</mark>	
Hepatocellular necrotic	focus (i), f	acal/mutti	focal 4						
Minimal	<mark>\$ 1</mark> (<mark>0</mark>		<mark>5</mark>	J.	~~ <mark>0</mark> ~~	í <mark>1</mark>	1	
Slight 🔬	k)	<u>6</u> 1	6 ⁵ 18		& <mark>0</mark>	<mark>1</mark> 6	<mark>0</mark>	<mark>0</mark>	
Moderate S	<mark>a</mark> "	2 <u>1</u> a,		<u>ي 1</u>	o <mark>o</mark> os	A.	<mark>0</mark>	<mark>0</mark>	
Total	لم المح الم	25	<mark>3</mark> ~	<u>) 7</u> 90 N	<mark>1</mark>	@, <mark>1</mark>	<mark>1</mark>	1	
Moderate 5 Total 5				, Č,		× ×			

Based on the study results (changes in haematological and clinical chemistry parameters, organ weights and histopythological liver findings) the NOAEL of the present study was established at 160 ppm in both sexes after 90-day exposite to sedium trifluor acetate (TFA) which is equivalent to 10 / 12 mg/kg bw/day in males and females.

Assessment and conclusion by appricant

Conclusion

This study was designed to provide information of the effects of Sodium trifluoroacetate (TFA) following dietary administration to the cats for 90-days. The majority of treatment-related changes seen in clinical chemistry, urinalysis, and effects seen in the liver at the necropsy and microscopic pathology examination are consistent with the mechanism of action of TFA acting as a peroxisome proliferator Additionally, the observed effects were more pronounced in males than in females. The study is varied and reliable, although the ED parameters which are currently required by OECD TG 408 (2018) and other few parameters have not been investigated.



Data Point:	KCA 5.8.2/01
Report Author:	
Report Year:	2010
Report Title:	Fluopyram: 28-day immunotoxicity study in the female Wistar rat by dierary
	administration
Report No:	SA 09357
Document No:	<u>M-387119-01-1</u>
Guideline(s) followed in	EPA OPPTS 870.7800 (1998) 🚿 🖉 🖉 🖉
study:	
Deviations from current	Current guideline: not applicable
test guideline:	
Previous evaluation:	No, not previously submitted.
	No, not previously submitted.
GLP/Officially	Yes (certified laboratory)
recognised testing	
facilities:	
Acceptability/Reliability:	Yes in a construction of the second s

Executive Summary

In this study, fluopyram (AE C656948), was administered continuously via the diet for 28 days to female Wistar rats. Animals (10/females/group) were administered test diet at concentrations of 0, 200, 600 and 1800 ppm (equating approximately to 0, 172, 53.6, 156.3, mg/kg body weight/day). An additional group of 10 female Wistar rats were administered cyclophosphamide (CPS) daily by gayage for at least 28 days at the dose level of 3.5 mg/kg body weight/day and acted as a positive control group.

All rats were immunized withown intravenous injection of sheep red blood cells (SRBC) on study day 24. All animals were enthanized on study day 30.

Animals were observed daily for mortality and clinical signs. Body weight and food consumption were recorded once weekly. A detailed posical examination was performed once during the acclimatization phase and at least weekly throughout the study. Blood samples were collected from the retro-orbital venous plexus of each arimal on day 36 (just before necropsy) for specific anti-SRBC immunoglobulin M (IgM) analysis. All animals were necropsied pross pathology observations were performed and selected organs (spleen and thymus) weighed.

All animals survived the scheduled nearopsy. There were no fluopyram-related clinical observations during the course of the study. A non-statistically significant decrease in bodyweight gain was noted at 1800 ppm. Overall, mean bodyweight gain in females was approximately 12% lower than the controls. No treatment-related effects on bodyweight were noted at the lower dose levels. At 1800 ppm, mean food consumption was reduced by approximately 12% compared to controls on day 29, with a similar tendency (not statistically significant) being noted on day 22. No treatment-related effects on food consumption were noted at the lower dose levels. Macroscopically, atrophic/small spleen was observed in 2/10 females compared to no cases in the control group. There was no treatment-related effect on thymus weight change or macroscopic observations. At 600 ppm, atrophic/small spleen was noted in 1/10 female rats compared to 0/10 in the control group. At such a low incidence and in the absence of a similar finding in the thymus, the atrophic/small spleen observed at 1800 and 600 ppm, were considered to be incidental. No treatment-related change in SRBC-specific IgM concentration was observed after fluopyram advinistration.

For the positive control group, CPS, no clinical signs or change in body weight parameters and mean terminal body weight were noted. Mean food consumption was reduced by approximately 9% during the last week of the study. A marked decrease was noted in the absolute and relative weights for both spleen and thymus, which was considered as statistically significant. Atrophic/small thymus was



found in 7/10 females and atrophic/small spleen in 5/10 females. At 3.5 mg/kg/day, mean anti-SRBC IgM concentration was markedly lower (-89%) when compared to the control group. These effects were consistent with the known immunosuppressant effects and validated the functionality of the assay.

Conclusion:

Under the conditions of this study, fluopyram administered ad libitum in the diet for 28 consecutive days to female Wistar rats at dose levels of 200, 600 and 1800 ppm resulted in a no-obsequed-effectlevel (NOAEL) of 1800 ppm (equivalent to 156 mg/kg bodyweight/day) for the infihunological parameters investigated.

I. Materials and methods

A. Materials

AE (656948 Light beige solid 94.7% w/w Not stated Stable in rodem diet for a period covering the study duration (Expire date; 24 February 2011). 1. Test material: Description Lot / Batch #: **Purity:** CAS# Stability of test compound: (Éxpir) date: 24 February 2011) 2. Vehicle and / or positive O Basal diete cyclophosphamide monohydrate (CPS) control: 3. Test animals: **Species:** Wistar Rj: WI (40PS HAN) Strain: 7 weeks approximatel at staff of dosing Age: Weight at dosing NT70-2099g (females) Source; Acclimation period Ø adays C m Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Epinav-sur-Diet: Orge, France), addlibitum Water: Tap water, ad libitum Rats were boosed individually in suspended stainless-steel wire Housing Environmental conditions Temperature: Humidity: 109,5 air Changes per hour Air changes: 2 hours light, 12 hours dark **Photoperiod:** B. Study design 27 January 2010 – 24 June 2010 1. In life dates 2. Adimal assignment and treatment

There were 10 female animals per dose group. Animals were assigned to dose groups randomly by body weight E C656948 was administered in the diet for 28 days to Wistar rats at the following doses -0, 200, 600 and 1800 ppm (equating approximately to 0, 17.2, 53.6, 156.3 mg/kg body weight/day). A



negative control group received plain diet. A positive control group received the cyclophosphamide formulation by gavage at the dose level of 3.5 mg/kg bodyweight/day and at a dosage volume of 5 mL/kg body weight. The volume administered to each rat was adjusted on the most recently recorded bodyweight. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

3. Diet preparation and analysis

AE C656948 was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. The homogeneity of test substance in diet was verified for the lowest and highest concentrations to demonstrate adequate formulation procedures. Dietary levels of the test substance were verified for each concentration. The stability of the test substance in dietary formulation has been determined during a previous study (M-085510-01M) at concentrations of 20 and 10000 ppm for a time period which covers the period of storage and usage for the current study. The homogeneity and concentration of the fluopyram formulations ranged between 96 and 98% of the nominal concentrations. For the positive control cyclophosphamide, the homogeneity and concentration equipment of the nominal concentration. All these results were therefore within the acceptable in-house target ranges.

n

Table 5.8.2-1	Study desig
1 abit 5.0.2-1	Study desig

1 abic 5.0.2-1		
Test group	Concentration in diet (ppm)	Dose per animal (study averages) Animal Cassigned
1	Q Q	
2	200 4	
3	×600 %	
4	1800	
Test group	Concentration	Dose per animal (study averages) Animals assigned
(CPS)) (mg@kg/day)	
5	∂ 3.5≪	
4. Statistics		

Data were analyzed by the Bartlett's fest for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous, a Kruskal-Wallis ANOVA was performed followed by the Dunn test of the Kruskad Walle was significant. For some parameters, when data were not homogeneous they were transformed (log transformation or square root transformation), then reanalyzed as above. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried outsuing Path/Tox System V4.2.2. (Module Enhanced Statistics).

C. Methods

1. Observations

The animals were observed wice daily for moribundity and mortality (once daily on weekends or public holidays) observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The presence or absence of clinical signs were recorded at least once daily for animals exposed to the immunosuppressive agent cyclophosphamide. The mature, onset, severity, reversibility, and duration of any clinical signs were recorded

Ö



2. Body weight

Each animal was weighed at least weekly during the acclimatization period, on the start of treatment (day 1), then at weekly intervals throughout the treatment period and before necropsy **3. Food consumption and compound intake**

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. The weekly mean achieved dosage intake in mg/kg body weight/day for each week and for Weeks 1 to 4 was calculated (except for the group exposed to the immunosuppressive agent cyclophosphame) using the following formula:

Achieved dosage intake (mg/kg bodyweight/day) = $\frac{\text{Dose level (ppm)} \times \text{Group mean body weight (g) at end of week}}{\text{Group mean body weight (g) at end of week}}$

4. Water consumption

Water consumption was not conducted.

5. Ophthalmological examination

Ophthalmological examination was not conducted

6. IgM antibody analysis:

On the day of injection, Sheep Red Blood Cells were washed of PBS (Phosphate Buffered Saline), counted using a cell counting instrument (Sigmens Advia 220) and diluted in PBS in order to obtain a 5 x 10° cells/mL preparation. On day 26, alkanimals in all groups were immunized by intravenous injection in the fail vein (0.5 mL/animal) with Sheep Red Blood Cell (SRBC) preparation. On day 30, blood Samples were taken from all animals in all groups by puncture of the remo-orbital verous plexus 4 days after SRBC immunization prior to terminal sacrifice. Animals were not dret fasted. Blood (0.5 ml) was placed into tubes with clear activator (for servim preparation). After centrifugation, serum aliquots were frozen (approximately -80° C) until analysis. Enzyme-Linked Immunosorbent Assay (ELISA) was used to determine the level of SRBC specific immunoglobulin M in response to antigen administration.

7. Sacrifice and pathology

On study day 0, a complete necropsy was performed on all surviving animals. Animals were deeply anesthetized by Isofluran inhalmion, then exanguinated before necropsy. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled. Spleen and thymus were weighed

1. Results and discussion

A. Clinical signs and mortality

1. Clinical signs of toxicity

There were no chinical signs exident in any group.

2. Mortality

There was no mortality in any group.

B. Body weight and body weight gain



And a second and a Body weight gain was lower throughout the entire study duration. Overall, mean body weight gain in traitsically significant. No treatment-related effects on body weight parameters were observed at 200 and 600 gmm. Body weight parameters in females were unaffected by treatment with cyclophophamide. Body weight gain was lower throughout the entire study duration. Overall, mean body weight gain in females was approximately 12% lower than the control group. However, this change was not statistically on the permanent of the one of th



Table 5.8.2-2	Body weight and weight gains (kg) in the 28-day immunotoxicity study with AE
	C656948 (mean ± SD)

	,	,			
Dosage level (ppm)	0	200	600	1800	3.5 mg/kg/day
Sex			Females	ð	
BW Day 1	187±11	189±8	188±9	189	187±10
BW Day 8	208±11	209±10	212±12	207±9	205±10
BW Day 15	226±13	224±17	229±11	√22 8±8	221+06
BW Day 22	242±15	241±17	2 3 9±15	√238±9 ×	<u>239¥18</u>
BW Day 29	253±18	251±16	238±9	246±11	239 ± 18
		Body wei	ght gain	,0 ^v , v	
BWG Day 8	2.9±0.9	2.8±0.7	√ [©] 3.3±1.0 ,	2.6±0,6	2.7
BWG Day 15	2.5±0.5	2.2±1.2	2.4±0.7	2.1±Q5	2,4±1.3
BWG Day 22	2.4±0.7	2.4±0.7	3.1±15	2.2+0.7	\$2.6±0.7
BWG Day 29	1.5±0.9	1.5±0.7%	\$ 1.2±0.8	₩±0.6	™1.2±0.8
W: body weight:]	BWG: body weight	t gain 🔍 🗸			L A o

BW: body weight; BWG: body weight gain

C. Food consumption and compound intake *

At 1800 ppm, mean food consumption was reduced by approximately 12% on day 29 with a similar tendency (not statistically significant) being noted on day 22. No treatment selated effects on food consumption was reduced by approximately 9% on day 29.

The achieved doses in the study were 17.2, 53.6, 156.3 mg/kg body

D. Ophthalmoscopic examination

D. Ophthalmoscopic examination Ophthalmoscopic examination was not conducted in this study E. SRBC-specific kgM response

E. SRBC-specific an response

A high inter-individual variability was noted in all the groups exposed to fluopyram as well as in the control group, Despite this variability, the figh mean anti-SRBC IgM concentration observed in the control group confirmed the sensitization of the affirmals No relevant treatment-related change in SRBCspecific IgMoconcentration was observed after Huopyram administration.

In the positive control group (OPS), at 3.5 mg/kg/do mean anti-SRBC IgM concentration was markedly lower (-89%) when compared to the contol group. **%**

Š Mean T-cell dependent antibody responses in the 28-day immunotoxicity study with Table 5.8.2-3 AJOC656948 (mean ± SIO) °, O

Ø

.4	AFC 6569 8 Dosge level (p	pm)	CPS
Parameter		1800	(3.5 mg/kg/day)
IgMa (u/mL)	\$706 ±₹\$20 719©± 4510 11918 ± 112	218 8097 ± 3737	$933 \pm 399*$
*: p≤0%05			

CPS - cyclophosphamide

F. Sacrifice and patholog

1. Terminal body weights and organ weight

There were no changes in terminal body weights at any dose. At 1800 ppm, there were no treatmentrelated changes in absolute and relative spleen and thymus weights. Mean absolute and relative spleen weights appeared numerically lower when compared to the controls (not statistically significant). Howeve this apparent change was related to lower values observed in three out of ten animals only. In isolation and in the absence of any associated similar tendency in the thymus weight, this change was



considered not to be relevant. Statistically significant markedly lower absolute and relative spleen and thymus weights were noted in the cyclophosphamide group, when compared to controls.

Atrophic/small spleen was noted in a few animals at 1800 and 600 ppm (2/10 and 1/10, respectively) compared to none in the control group. In isolation, this change was considered not to be relevant. In the positive control group (CPS), atrophic/small spleen and thymus were in a majority of the treated animals, 5/10 and 7/10, respectively, compared to 0/10 and 2/10 in the control group.

Organ weight changes in the 28-day immunotoxicity study with AE Co50948 mean Table 5.8.2-4 SD) añ

SD)				
Description		AE C656948 D	osoge level (ppp	9 ⁵ 2	Q CPS Q
Parameter	0	200 🚄	🔪 600 🧳	°1800√	(3.5 mg@g/day)
		Fema	iles 🕎		
Spleen weight Absolute (g)	0.741 ± 0.089	0.731 ± 0.109 (-1%)	@.742 ±0.117 × © (0%)	0.674 0.090 (-9%)	$0.584 \pm 0.079**$ (-21%)
Bodyweight-relative	0.2862 ±0.0290	0.2894 ± 0.038	0.28 1 ± 0.0 1	0.2696 ± 0.0446	Ø.2321 #0.0291** (-19%)
Thymus weight Absolute (g)	0.572 ± 0.113	0 ,598 0 .134	©0.52Ž¥0.108	0.582 ± 0.415	0,434 ±0,103* ∅ (2,4%)
	-	0.2352 ± 0.0419		0.2326 0.0448	0.173¥ ± 0.0452* (-22%)
*: p≤0.05; **: p≤0.01; Fig	ures in parentheses	are % differences	from control		<u></u>

G. Deficiencies

G. Deficiencies

III. Conclusions

III. Conclusions of the properties were detected for fluopyram in female Wistar rats as demonstrated by the same IgM response after injection of the antigen SRBC as compared to the control group. The dese level of 1800 ppm (corresponding to approximately 156 mg/kg body weight/day) is considered to be a NOAEL for the immunotoxicological parameters investigated.

Assessment and conclusion by applicant:

The study is acceptable as it provides immunofoxicological information on the effects of AE C656948 Co. C" in the rat. ñ

In conclusion, no immunosuppressive properties were detected for fluopyram in female Wistar rats as demonstrated by the same IgM response after injection of the antigen SRBC as compared to the control group. The dose level of 1800 for the immunotoxicological parameters investigated



CA 5.8.3 Endocrine disrupting properties

a. <u>Human estrogen i</u>	receptor (ERalpha) binding assay
Data Point:	KCA 5.8.3/01
Report Author:	
Report Year:	2018
Report Title:	In vitro Pharmacology Study of Fluopyram Technical BAYER SAS Study Number: TXGM0123 (Estogen ERalpha) mding and functional assays)
Report No:	100044628 TXGM0123
Document No:	<u>M-632695-01-1</u>
Guideline(s) followed in study:	No specific guideline but based on OECD TG 493 and 457
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~
GLP/Officially	Yes, conducted under GLR Officially recognised desting to ilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes Q X Q Q Q Q Q Q

Executive Summary

The purpose of this study was to test Fluop am Technical Estregen ERalpha and functional assays.

(The functional assays are described under point d. Enrogen receptor transactivation (OECD TG 457) - ERalpha - agolist and antagonist functional assay, (KCA 3.8.3/04))

Fluopyram Technical was tested at 1.0E-05 M and 1.0E-04 M (Equivalent to 10µM and 100 µM).

Compound binding was calculated as a % inhibition of the binding of a radioactively labeled ligand specific for the alpha strogen receptor (ERalpha)

Under the conditions of the test used, Fluopyram Technical shows no indication of binding to the alpha estrogen receptor Ralpha).

I. Matestals and methods

A. Materials

1. Test material:
Description
Lot / Batch #;
Purity:
CAS #
Stability of test compounds
2. Vehicle:
Fluoryram Technical
Powder
Mix-batch 08528/0002
94.5%
658066-35-4
Stable until 20 April 2020 (when stored at +10 to +30°C)
Dimethylsulfoxide



R	Thopytam
3. Reference substance:	Estrogen ER alpha (h) (agonist radioligand) Diethylstilbestrol If applicable, the respective reference compound was tested concurrently with Fluopyram Technical, and the data compared with historical values determined at Eurofines. The experiment is accepted in accordance with Eurofine valuation Standard Operating Procedure
4. Experimental conditions: Binding Assay	
Receptor:	Estrogen ER alpha (h)
Source: Ligand: Concentration: Kd: Non Specific: Incubation: Detection method: Assay volume and format: Compound addition:	with historical values determined at Eurofine. The experiment is accepted in accordance with Eurofine valuation Standard Operating Procedure. Estrogen ER alpha (h) (agonist radioligand) Human reconting [3H] Estradiol 0.5 nM 0.20 nM Dietar/stillbestrol (buM) 120 min RT Somtillation counting 200 µbm 96 well plates [100x] solution in solvent I protocol may have occurred during the testing, they have no impact d
Minor variations to the experimenta on the quality of the results obtaine	l protocol may have occurred during the testing, they have no impact
5. Test concentrations: Test com	pound was tested at 100μM and toμM for duplicate.
B. STUDY DESIGN AND METH	LODS 20 June 2018
B1 Binding Assey - Estrogen &R a	
1. Purpose: Evaluation of the af	inity of compounds for the humanestrogen receptor (ER alpha) letermined in a radialigand binding assay.
or presence of the test compound in DTT and 0.1% BSA.	ated for 120 min at 22 °C with 0.5 nM [3H] Estradiol in the absence a buffer containing 10 rom Tris/HCl (pH 7.4), 10 % glycerol, 1mM
Nonspecific binding is determined	n the presence of tot M diethylstilbestrol.
Packard presoaked with 0.3% PEL	and filtred rapidly under vacuum through glass fiber filters (GFB, and rinsed several times with ice-cold 50 mM tris/HCl using a 96- ackard). The filters are dried then counted for radioactivity in a Packard).
The results are expressed as experce	winhibition of the control radioligand specific binding.

The standard reference compound is disthylstilbestrol, which is tested in each experiment at several concentrations to obtain a competition curve from which its IC₅₀ is calculated.

Reference: Obourn H) et al (1993), Hormone- and DNA-binding mechanisms of the recombinant human estrogen receptor. Biochemistry 32(24): 6229 - 6236.

3. Analosis and expression of results:

The results are expressed as a percent of control specific binding



$\frac{measured\ specific\ binding}{control\ specific\ binding} \times 100$

and as a percent inhibition of control specific binding

$$100 - (\frac{measured specific binding}{control specific binding} \times 100)$$

obtained in the presence of Fluopyram Technical.

The IC50 values (concentration causing a half-maximal mhibition of control specific binding) and H coefficients (nH) were determined by non-linear regression analysis of the competition curves generate with mean replicate values using Hill equation curve atting

$$Y = D + \left[\frac{2^{OA-D}}{1 + (C/65^{\circ})^{nH}}\right]$$

where Y = specific binding, A = left asymptote of the curve, D \mathcal{F} right asymptote of the curve, $C \neq$ compound concentration, $C_{50} = IC_{50}$, and $p_{H} \neq slope factor. This analysis was performed using software$ developed at Cerep (Hill software) and wildated by comparison with data generated by the compercial software SigmaPlot[®] 4.0 for Windows[®] (© 1997 by SPSS line.).

The inhibition constants (Ki) were calculated using the Cheng Prasoff Aquation

$$Ki \stackrel{\mathcal{D}}{=} \frac{I_{C50}}{(1 + L/K_D^{\circ})} \stackrel{\mathcal{D}}{=} \stackrel{\mathcal{D}}{=}$$

where $L = \text{concentration of radioligand in the asset, and <math>RD = \text{affinity}$ of the radioligand for the receptor. A scatchard plot is used to determine the KD

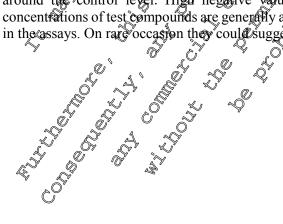
4. Results interpretation guide: «

Results showing an inhibition (of stimulation for assays run in basal conditions) higher than 50% are considered to represent significant effects of the test compounds. 50% is the most common cut-off value for further investigation (determination of 1050 or EC 50 values from concentration-response curves) that Ľ the laboratory recommends m

Results showing an inhibition (or stimulation) between 25% and 56% are indicative of weak to moderate effects for most assay they mould be confirmed by further testing as they are within a range where more inter-experimental vatiability can occur). L,

Results showing in inhibition (or stimulation) lower than 25% are not considered significant and mostly attributable to variability of the signal around the control devel.

Low to moderate negative values have no real meaning and are attributable to variability of the signal around the control level. High negative values (2 50%) that are sometimes obtained with high concentrations of test compounds are generally attributable to non-specific effects of the test compounds in the assays. On rare occasion they could suggest an allosteric effect of the test compound.





Results and discussion II.

Binding Assays

Binding Assays					o s
Table 5.8.3-1	Test compound	results		~	
Substance/Conce	entration		% Inhibitio	on of Control Spec	ific Binding
Compound	Client	Test	1st	2nd	Mean
I.D.	Compound	Concentration		A	
	Ī.D.		Ča	L.	
Estrogen ER alp	ha (h) (agonist ra	dioligand		Û (9, 10, 10, 10, 2
100044628-1	Fluopyram Technical	1.0E-05 M	-14.9	° -36.3 √	
100044628-1	Fluopyram Technical	1.0E-04 M	-11.0 ~~	° 37.1	-24.1 Q
A. Deficiencies		, Å			
None		4		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ô ô s
	_	$\langle \mathcal{I} \rangle \sim$		AS.	Š Š V
III. Concl Under the conditi alpha estrogen rec	usions			o indication of bi	
	ons of the test us		schnicar snows n		ngung to the
alpha estrogen rec	ceptor (ERalpha)			D D S	ž. "Ka
	ć				~~
Assessment and	l conclusion by	applicants		Ô.	0 V
Study meets the	current gaidance	Pand the requiren	nents in 283/2012		
Fluonvram Tech	mical shows and i	ndigation of bind	ing to the alphae	strogen receptor	(FR alpha)
Thuopyrain Teer				subgen receptor	(Litaipiia).
	Ũ,		ð a	0 49	
	S. On M		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		
b. <u>Estrogen</u>	PRbeta) Bindin	g Assay		J.	
	O			<u>A</u>	
Data Point:	0 ³ K CA 5	8.3/02		ý	
Report Author:	.«J		··· _0 _0	P	
Report Cear:					
Report Vitle:	5 Invitro	Effect of Compour	nd Fluopyrani Fech	nical on Estrogen	ERbeta Binding
	Assay				
Report No:	/) ⊭LSuuws	lo: FR095-0005801			
<i>Q</i> ,	Client A	Activity Id: YXGM	M23 🔊		
Document NO		<u>\$99-01-1</u> >			
Guideline(s) follo	wed in ONo spec	ific guideline but b	ased on OECD TC	i 493	
study:	r a a a a a a a a a a a a a a a a a a a	<u> </u>	K.		
Deviations from c	urrent None	\sim \mathcal{L} $\dot{\sim}$	¥		
test guideline:		<u>6' 0' ``Y'</u>			
Previous evaluation	on: Not pre	viously submitted			
		<u> </u>			
GLP/Officiall	A No, not	Conducted under G	LP/Officially reco	gnised testing facil	ities
recognised testing					
facilities:	ý de Se				
Acceptability/Rel	lability: Ye				
\$° 67					
Executive Summ	awy s				

Executive Summary

The objective of this study was to investigate the effect of test compound Fluopyram Technical on Estrogen ERbeta Binding Assay.

Fluopyram Technical was tested in duplicate at 10 and 100 μ M.



4^yto +36^yC) 2^yt^y</sup>

when stored at +10 to

Compound binding was calculated as a % inhibition of the binding of a radioactively labeled estradiol, ligand specific for the beta estrogen receptor (ERbeta).

Fluopyram Technica

Mix-batch 08528/0002

Stable unfil 20 April 2020

Haman recombiliant insec

10% Glycerol & mM DT

ðН

 \bigcirc

Dimethysulfoxide

DiethAstilbestrol

1.00%DMS00

2 hours @ 25°C 10 mM Tre-HCL

Powder

94.5%

658066-35-4

Under the conditions of the test used, Fluopyram Technical shows no ind	lication	of binding to	o the be	ta 🖉
estrogen receptor (ERbeta).	~		67	- Contraction of the second se

- I. Materials and methods
 - A. Materials
- 1. Test material: Description Lot / Batch #: **Purity:** CAS# **Stability of test compound:** 2. Vehicle:
- 3. Reference substance:
- 4. Experimental conditions: Source: Vehicle:

Incubation Time/Temp

Incubation Buffer

0.1% BSA Kd 0130M* 0.50 nM [H] Estradiol Ligand Non Specific Ligand: 20 uMDieth@stilbestrol Specific Binding: 90%@ Quantitation Method. Radioligand Binding 50% of max inhibition Significance Criteria: ~ 3000 pmole mg Protein * **Bmax:**

- B. Study design and methods,
- €12 June 2018 21 June 2018 1. Experiemental dates:

2. Purpose: The objective of this study is to investigate the effect of compound Fluopyram Technical on Estrogen ERbeta Binding

3. Experimental protocol

The effect of Fluopyram Technical was be evaluated by 3H]Estradiol binding assay on estrogen ERbeta receptor @

Test compound was dissolved in PMSOcto make a stock solution of 10 mmol/L, then diluted to the indicated concentrations

The assay procedure was performed based on Eurofins Panlabs Protocols.

Human recombinant Estrogen b (ERb) receptors expressed in insect Sf9 cells are used in modified Tris-HCl buffer pH 7.4 An adjuot is incubated with 0.5 nM [3H]Estradiol for 2 hours at 25°C. Non-specific binding is estimated in the presence of 1 uM diethylstibestrol.

Receptor proteins are filtered and washed, the filters are then counted to determine [3H]Estradiol specifically bound.

Test compound at 100 uM and 10 uM was performed in duplicate.



Reference: Obourn JD et al (1993), Hormone- and DNA-binding mechanisms of the recombinant human estrogen receptor. Biochemistry 32(24): 6229 - 6236.

4. Analysis and expression of results:

Binding assay results of test substance are expressed as a percent inhibition. The percent inhibition of the test substance at test concentration were calculated with the formula as follows: Inhibition (%)= $\{1-(c-a)/(b-a)\}\times 100$

- a: average cpm of non-specific binding
- b: average cpm of total binding
- c: cpm in the presence of test or reference substances

Results showing an inhibition of between 25% and 50% are indicative of a weak to moderate effect

Results showing an inhibition below 25% are considered to be mostly attributable to variability of the signal around the control value.

II. **Results and discussion**

Table 5.8.	3-2	Results of	Estrogen	ERbéta B	Binding As	say n			<u></u>	Š
Cat#	Assay	Batch*	Spec	Rep.	Conc.	/ %~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	IG90*	<i>к</i> кі 🕺	р инс	R
	Name		~~		b Ò	Inh.		d d	°~	
Compound: Fluopyram, PT # 12/19310 0 2 0 2 0 2 4										
226050	Estrogen	423211 %	hum	20	100µM	[~] 14 (ľ <u></u> -Ø	~-	0'-	-
	ERβ	Řa	hum	J.	<i>@</i> Υ0μΜ @	· 10 [•]	Ž	× - 0) -	-
		. 9	0	Re (۵	1 . A			

No significant responses (2 50% inhibition or stimulation for Biochemical Assays) were noted.

A. Deficiencies

None

Conchisions III.

Under the condition of the test used, Fluopyran Techpical shows no indication of binding to the beta estrogen receptor (ERbeta). S. estrogen receptor (ERbeta).

Study meets the surrent guidance and the requirements in 283/2013. Fluopyram Technical shows no indication of binding to the beta estrogen receptor (ERbera).

A

estregen receptor ren estregen receptor ren of the control of the



Androgen receptor (AR) Binding Assay c.

Data Point:	KCA 5.8.3/03	
Report Author:		
Report Year:		
Report Title:	In vitro Pharmacology: Human AR (h) (agonist radioligand) Receptor Bunding	
	Assay	
	Study of Fluopyram Technical	
	BAYER SAS Study Number: TXGM0123	
Report No:	100044626_TXGM0123	
Document No:	<u>M-632697-01-1</u>	
Guideline(s) followed in	No specific guideline but based on OECD 75 493	
study:		
Deviations from current	None Q A A A A	
test guideline:		
Previous evaluation:	Not previously submitted of the transformed of the	
GLP/Officially	Yes, conducted under CAP/Officially recognised testing facilities	
recognised testing		
facilities:		
Acceptability/Reliability:	$Yes \qquad \qquad$	
Executive Summary		
The purpose of this study was to test Fluogyram Jechnical in the AR (h) (agonist radioligand) assay.		
Fluopyram Technical wassiested at 1.0E-05 Mand 1 SE-04 M (equivalent to 10 MM and 100 µM).		
Compound binding was calculated as a % inhibition of the binding of a radioactively labeled ligand		
specific for the and agen receptor (AR), a sign of the and the second se		
Under the conditions of vest used, Fluopyram Technical shows no ardication of binding to the androgen		
receptor.	est using, i reopyrume compour snews no reducing of officing to the and ogen	

1. Test material 🖓 👋 🔗	Fltropyrapi, Technical
1. Test material Description	Powder C
Lot / Batch #:	Mix-batch 08528/0062
Purity: 🖓 🗘 🖉	94.5% ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
CAS #	658066-35-4
Stability of test compound:	Stable ontil 20 April 2020 (when stored at +10 to +30°C)
2. Vehicle:	Dimethylsuffoxide
 Vehicle: Reference substance: Experimental conditions: Receptors: Source: Ligand 	Tegosterepe
4. Experimental conditions	
Receptors A V	AR (hR(agonist radioligand)
Source:	LN@aP cells (cytosol)
	[3]]methyltrienolone
Concentration:	1 nM
Kal Gr A Z	0.8 nM
Non Specific:	Testosterone (1 μ M)
Incubation:	24 hr 4°C
Detection method:	Scintillation counting
Assay volume and format:	200 µl in 96-well plate
Compound addition:	[100x] solution in solvent



Minor variations to the experimental protocol may have occurred during the testing, they have no impact on the quality of the results obtained.

- 5. Test concentrations: Test compound was tested at 100µM and 10µM in duplicate.
- B. Study design and methods
- 1. Experiemental dates:
 - 04 June 2018- 05 June 2018

2. Purpose: Evaluation of the affinity of compounds for the human androgen receptor. (AR) in a NCaP cells determined in a radioligand binding assay.

3. Experimental protocol:

Fractions of cell cytosol (106 cell/point) are incubated for 24 h at 45°C with 1 nM [3H]methyltrenolor in the absence or presence of the test compound in a buffer containing 25 mM Hepes Tris (pH 7.4) 1 mM EDTA, 10 mM Na2MoO4, 2 mM DTT, 5 µM triamcinolor acetonide and 10% glycerol.

Nonspecific binding is determined in the presence of μ M testosterone \mathcal{D}

Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GP/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microssint 0, Packard).

The results are expressed as a percent inhibition of the control radiologiand specific binding.

The standard reference compound is testosterone, which is tested in each experiment at several concentrations to obtain a competition curve from which its IC564s calculated

Reference: Zava DT et dt (1979), Androgen receptor assav with [3H] Methyltrien lone (R1881) in the presence of progesterone receptor, Endocrinology 404: 1007.

The results are expressed as a percent of control specific bioding

and as a percent inhibition of control specific binding

$$\begin{array}{c} & & \\$$

obtained in the preserve of Ruopyram Technical.

The IC50 values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (nH) were determined by fon-linear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting

where $Y = specific binding A = teft asymptote of the curve, D = right asymptote of the curve, C = compound concentration <math>S_{50} = 4G_{50}$, an OnH = slope factor. This analysis was performed using software develope that Cerep (Hill software) and validated by comparison with data generated by the commercial software Signap lot 4,0 for Windows (© 1997 by SPSS Inc.).

The whibition constants (Ri) were calculated using the Cheng Prusoff equation



 \bigcirc

$$Ki = \frac{IC_{50}}{(1 + L/K_D)}$$

where L = concentration of radioligand in the assay, and KD = affinity of the radioligand for the receptor $\sqrt[3]{2}$ A scatchard plot is used to determine the KD.

5. Results interpretation guide:

Results showing an inhibition (or stimulation for assays run in basal conditions) higher than 30% are considered to represent significant effects of the test compounds. 50% is the most common cut off value for further investigation (determination of IC₅₀ or EC₅₀ values from concentration-response surves) that we would recommend.

Results showing an inhibition (or stimulation) between 25% and 50% are indicative of weak to moderate effects (in most assays, they should be confirmed by further testing as they are within a range where more inter-experimental variability can occur) &

Results showing an inhibition (or stimulation) lower than 25% are not considered significant and mostly attributable to variability of the signal around the control week.

Low to moderate negative values have no real meaning and are attributable to variability of the signal around the control level. High negative values $\geq 50\%$ that are sometimes obtained with high concentrations of test compounds are generally attributable to non-specific effects of the test compounds in the assays. On rare occasion they could suggest an allosteric effect of the test compound.

II. Results and discussion

Table 5.8.3-3 Results of Human AR(h) (agonist radioligand) Receptor Binding Assay; Pluopyram

100			
Compound	Client	S Test & S Inhibition of Control	Specific Binding
I.D.	Compound	Concentration 1st 0 2mg	Mean
	I.D.		
AR (h) (agonist radioligand)			
100044626-1	Floopyram	▲.0E-05 M ↓ ~ .2.0 ↓ -2.8	-2.4
ð	Technical	1.0E=04 M @ -2.2 0 -2.2	-1.4
O*	rechnical	<u>1.0E=04 M @ 2.2</u>	-1.4

Compound InDia				1111
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	L.Y	AR@h) (ago)	nist rædioligand)	
Testosteron	4	@4.6E-09M 0	© 20E-09 M	1.0
Q ,			~ ~	

#### A. Deficiencies

None

### III. Conclusions

Under the conditions of test used, Fluopyram Technical shows no indication of binding to the androgen receptor.

### Assessment and conclusion by applicant:

Study meets the ourrent guidance and the requirements in 283/2013. Fluopyram Technical shows no indication of binding to the androgen receptor.



#### Estrogen ERalpha agonist and antagonist functional assay d.

Data Point:	KCA 5.8.3/04
Report Author:	
Report Year:	2018
Report Title:	In vitro Pharmacology
_	Study of Fluopyram Technical
	BAYER SAS Study Number: TXGM0123 (Estrogen ERalpha binding and )
	functional assays)
Report No:	100044628_TXGM0123
Document No:	<u>M-632695-01-1</u>
Guideline(s) followed in	No specific guideline but based on OECD T& 493 and 457 V
study:	
Deviations from current	None Q ,
test guideline:	
Previous evaluation:	Not previously submitted 0 5 5 5 5
GLP/Officially	Yes, conducted ander GLP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	Yes Of y y y y y y y y

#### **Executive Summary**

The purpose of this study was to test Flugpyram Technical Estrogen PRalpha binding and functional Ô assays. Ò

assays. (The Estrogen ERalpha binding as any is described under pointy a. Estrogen or androgen receptor binding affinity (OE(P) TG 493) - Human estrogen receptor (Eralpha) binding assay (KCA 5.8.3/01)

Fluopyram Technical wastested at 1.0E-05 M and 1.0E-04 & (equivalent & 10 µM and 100 µM).

Cellular agonis Oeffect was calculated as a % of control response to a known reference agonist for the specified target and cellular antagonist effect was calculated as a g inhibition of control reference agonist response for the specified target  $\widehat{}$ n

Under the conditions of the testused, the functional assays showed that Fluopyram Technical had neither an agonist or antagonist effect on the alpha estragen receptor.

#### Materials and methods I.

A. Materials

1. Test material:	
1. Test material:	F Faopyram Technical
Description	
1. Test material: Description Lot / Batch #:	⁷ Mix@atch 08528/0002
Purity: 🔗	** 94.3% ()
CAS # @	<b>§</b> 5806 <b>§</b> 35-4
Stability of test compound	: Stable until 20 April 2020 (when stored at +10 to +30°C)
2. Vehicles 2 8	Dimethylsulfoxide
2. Vehicles 3. Reference sobstance:	ERalpha (h) (agonist effect) - Estriol
A A A	ERalpha (h) (antagonist effect) - 4-OH tamoxifen
	In each experiment and if applicable, the respective reference
	compound was tested concurrently with Fluopyram Technical,
× sôv	and the data was compared with historical values determined at
$\bigcirc$	Eurofins. The experiment is accepted in accordance with
	Eurofins validation Standard Operating Procedure.



4. Experimental conditions: Cellular and Nuclear Receptor F	unational Assaus
Agonist effect	
Receptors:	ERalpha (h) (agonist effect)
Source:	Human recombinant
Stimulus:	none (1 $\mu$ M Estriol for control)
Incubation:	RT
Measured component:	Coactivator recruitment
Detection method:	AlphaScreen & A A A A
Antagonist effect:	
Receptors:	ERalpha (h) (antagonist effect) $\bigcirc$ $\bigcirc$ $\bigcirc$ $\bigcirc$ $\bigcirc$ $\bigcirc$
Source:	Human recombinant
Stimulus:	Estradiol (30 $\text{M}$ ) $\sim$ $0^{\circ}$ $^{\circ}$ $^{\circ}$ $^{\circ}$
Incubation:	RT ( ) ( ) ( ) ( ) ( ) ( )
<b>Measured Component:</b>	Coactivator recruitment
<b>Detection Method:</b>	ERalpha (h) (antagonist effect) Human recombinant Estradiol (30 ffM) RT Coactivator recruitment AlphaScreen
Minor variations to the experimental on the quality of the results obtained	unctional Assays ERalpha (h) (agonist effect) Human recombinant none (1μM Estriol for control) RT Coactivator recruitment AlphaScreen ERalpha (h) (antegonist effect) Human recombinant Estradiol (30 μM) RT Coactivator recruitment AlphaScreen protocol may have occurred during the testing they have no impact und was tested at 100μM and 10 cM in duplicate une 2018- 20 June 2018
5. Test concentrations: Test comp	Sund was tested at 100 µM and 10 cM in doplicate
B. Study design and methods	
Experiemental dates:	ume 2018- 20 June 2018 0 0
B2 Cellular and Nuclear Receptor	Functional Assays - ERalpha (b) (agoilist effect)
alpha nuclear receptor, determin AlphaScreen technology 2. Experimental protocol: GST-tagged-ERalpha (Ligand Bindi SRC1-4 coactivator and 0.4 µg thuc containing 20 mM Hepes/NaCH (pl DTT. The mixture is then incubated for 3 buffer (basal control), the reference concentrations (ECs) determination)	
μg. 🖉 🕺 🖉	strepavidin coupled-beads) is added at a final concentration of 0.4 hal isomeasured at $\lambda ex=680$ nm and $\lambda em=520$ and 620 nm using a Elimer).
	b of the control response to 1 $\mu$ M estriol.
The standard reference agonist is est	riol which is tested in each experiment at several concentrations to urve from which its EC ₅₀ value is calculated.
Coactivator Decruitment. Mol.Endo	<i>Homogeneous in vitro Functional Assay for Estrogen Receptors:</i> <i>crinol., 17:346.</i>
3. General information:	

Assay volume and format: 20 µl in 384-well plate



Compound addition: [100x] solution in solvent then [10x] solution in incubation buffer

#### 4. Analysis and expression of results:

The results are expressed as a percent of control agonist response or inverse agonist response 

$$\frac{measured\ response}{control\ response} \times 100$$

and as a percent inhibition of control agonist response

$$100 - (\frac{measured response}{control response} \times 10$$

obtained in the presence of Fluopyram Technical.

The EC50 values (concentration producing a half-maximal response) and 1,50 values (concentration causing a half-maximal inhibition of the control agonist response) were determined by non-linear regression analysis of the concentration-response curves generated with mean replicate salues using Hill equation curve fitting

$$Y = D + \left[ \begin{array}{c} Y \\ 1 + \left( \begin{array}{c} A \\ C \\ 5 \end{array} \right) \\ \end{array} \right]$$

where Y = response, A = left asymptote of the curve,  $D = right asymptote of the curve, C \leq compound$ concentration, and C50= EC50 or IC50, and  $nH^2$  = slope factor?

This analysis was performed using software developed at Corep (Hot software) and validated by comparison with data generated by the commercial software SigmaPlot ® 4.0 for Windows ® (© 1997 by SPSS Inc.).

For the antagonists, the apparent dissociation constants (KB were calculated using the modified Cheng Prusoff equation

where A = concentration of reference agonist in the assay, and  $EC_50A = EC50$  value of the reference agonist.

L .

st A st

### 5. Results interpretation guide: «

Results showing an inhibition ( stimulation for assays rup, in basal conditions) higher than 50% are considered to represent organificant effects of the test compounds. 50% is the most common cut-off value for further investigation (determination of JC50 or EC50 values from concentration-response curves) that the laboratory recommends.

Results stowing an inhibition or stimulation between 25% and 50% are indicative of weak to moderate effects (in most assays, they should be confirmed by further testing as they are within a range where more inter-experimental variability can occur)

Results showing an inhibition for stip and a significant and mostly attributable to variability of the signal around the control level).

Low to moderate begative values have no real meaning and are attributable to variability of the signal around the control level. High negative values ( $\geq 50\%$ ) that are sometimes obtained with high conceptrations of test compounds are generally attributable to non-specific effects of the test compounds in the assay On rate occasion they could suggest an allosteric effect of the test compound.



# B3 Cellular and Nuclear Receptor Functional Assays - ERalpha (h) (antagonist effect)

1. Purpose: Evaluation of the antagonist activity of compounds at the human recombinant ER alpha nuclear receptor, determined by measuring their effects on light emission using the AlphaScreen technology.

#### 2. Experimental protocol:

GST-tagged-ERalpha (Ligand Binding Domain, 7.52 ng proteins) are preincubated for 50min af in an incubation buffer containing 20 mM Hepes/NaOH/(pH 7.4), 80 mM Nacl, 0.08% Tween 20 0.08% BSA and 0.8 mM DTT in the presence of one of the following: incubation Guffer basal and stimulated control), the reference antagonist (IC50 determination), or test compounds.

Thereafter, 30 nM estriol, 200 nM biotin-tagged-SRC1-4 coactivator and 0.4 µg/Iluorescence acceptor (anti-GST antibody coupled-beads) are added ano the mixture is incubated for 30 min at 22 °C

For control basal measurements, estriol is oppitted from the reaction mixture. Fluorescence donor (streptavidin coupled-beads) is then added at a finak concentration of 0.4 j.g.

Following 120 min at 22° C, the signal is measured at hex=680 nm and henry 520 and 620 nm using a microplate reader (EnVision, Perkin Elmer). ()Õ

The results are expressed as a percent of the control response to 30 nM estric

The standard reference agonist is 4-OH Tamexifem which is tested in each experiment at several concentrations to generate a concentration-response darve from which its 10 50 value is calculated.

Rereference: Liu J et al (2003), & Homogeneous, in vitro Functional A Estrogen Receptors: Coactivator Recruitment, Mol.Endocrinol., 17

#### 3. General information:

Assay volume and format: 20 µl in \$84-well plate

Compound addition: [100x] solution in solvent then [10x] solution in incubation buffer 4. Analysis and average is a solution of the solution o

# 4. Analysis and expression of results:

As described for B2 above

5. Results interpretation guide

As described for Baboves

#### П. Results and discussion

Cellular and Suclear Receptor Functional Assays

# Table 5.8.3-5 Agonist Effect: Fluepyram

Compound	, Glient Q Test	% of (	Control Agonist R	esponse
I.D.	Compound Concentrati	on 1st	2nd	Mean
ERalpha (h) (ag		¢.		
100044628-1	Fluopyram ~~1.0E-05 M	-0.5	-0.2	-0.3
Ő	Technical 1.05-04 M	3.1	-0.5	1.3

## Table 5.8.3-6 Agonist Effect: Estriol

Compound D.	IC50 (M)	nH
E Ralpha (4) (agonist effcet)		
Estriol	3.4E-09 M	n/a
Cĩ		



Compound	Client	Test	% Inhibitio	on of Control Agon		
I.D.	Compound	Concentration	1st	2nd	Mean 🖉	
	I.D.					
ERalpha (h) (an					Ô`	
100044628-1	Fluopyram	1.0E-05 M	6.3	-2.9	1.1 D	
	Technical	1.0E-04 M	-4.0	-7.9	-5.7 🔊	
				4	Ô, X.	
			Ra	st in the second		
able 5.8.3-7 Anta	agonist Effect: 4-0	<b>OH tamoxifen</b>	- Contraction of the second se	Û (	, , , , , , , , , , , , , , , , , , ,	
Compound I.D.		IC50				
ERalpha (h) (an	tagonist effect)			Ý Ő		
ERalpha (h) (antagonist effect)         4-OH tamoxifen       1.2E 49 M         A. Deficiencies         None         III. Conclusions         Under the conditions of the test used, the functional assays showed that Fluippyrang Technical had not be obtained on the other of the state of the s						
D.C		Q0		. O* ~ \(		
A. Deficiencies	5	Ű, Ť		x y p		
None		Ň	, v × ×	Y NY NY		
		4		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	à à k	
III. Conc	lusions			A S .	ĨĘĨĮ	
Inder the condit	ions of the test us	sed the functional	agays showed	trat Fluopyrant Te	chivical has	
either an agonic	t or antagonist ef	fection the sinha	strogen Pecenter	r a		
enner an agoins	t of antagoinst CI				, Ø	
•						
	<u>d conclusion by</u>				**	
Study meets the	e current guidance	e and the requiren	10nts in 283/201	3. Fluopyrant I ec.	hnical had	
neither an agon	ist or antagonist e	effect on the alpha	estrogen recept	or ô	0	
	<u> </u>					
	N.		Å S.			
e. <u>Aromatas</u>	<u>e assay (US KPA</u>	A OC SPP 890.12	<u>, 0' %</u>			
	S O			O 4		
Data Point:	KCA 5	\$3/05		· ·		
Report Author:	Brock,	C. , , , , , , , , , , , , , , , , , , ,				
Report Year:	$\approx 12018$	× 10 ×	Y 🔊 🔗	<i>w</i>		
Report Title: O	Jur vitro	Pharmacology: Qu	man Aromatase A	ssey_Bayer SAS As	ssay. Study of	
, Ø	Fluopy	am Teahnical BAY	YER SAS Study N	umber: TXGM012.	3	
Report No	<u>کې 1006</u> 44	627 9XGM0123				
Document No:	. © <u>M</u> @326	<u>596-01-1</u> , C	) ^v ^v ^v ^v ^v			
Guideline(s) follo	wed in No sper	affic gui@line but b	ased on US-EPA	OCSPP 890.1200		
study:			O' 💫			
Deviations from	currento Note					
test guideline;	o ^v o ^x	, <u></u> 0 , 0	° O ^r			
Previous evaluati	on: Not prê	viouslysubmitted	ð			
2			Į. V			
GLP/Officially	NeQ.co	nducted upder GLP	Mofficially recogn	ised testing facilitie	S	
recognised testing		~ ~ ~	, <u> </u>	0		
facilities:						
Acceptability/Rel	liability: Ves. C					
	liability: Yes O					
Executive Summ		N [×] a. [×]				
The purpose of t	fais study was to	test Fluopyram	Fechnical in Hu	man Aromatase A	ssay Bayer SAS	

Table 5.8.3-6 Antagonist Effect: Fluopyram

parts study was to test Fluopyram Technical in Human Aromatase Assay_Bayer SAS I ne purpos Assay.~ R

Flugg ram fechnical was tested at 1.0E-05 M and 1.0E-04 M. Compound enzyme inhibition effect was calculated as a % inhibition of control enzyme activity.

Under the conditions of the test, the results show that Fluopyram Technical does not cause enzyme inhibition in this Human Aromatase Assay.



tored at +10 to

- Materials and methods L
- Materials A.

1.	Test material:
	Description
	Lot / Batch #:
	Purity:
	CAS#
	Stability of test compound:

- 2. Vehicle:
- 3. Control:
- 4. Reference substance:
- 5. Experimental conditions: Source: Substrate/Stimulus/Tracer: **Incubation: Measured Component**^{*} Detection Method: O Assay volume and formats Compound addition:

Water Letrozole In each experiment and if applicable, the respective reference compound is tested concurrently with bluopy am Technical, and the data B compared with historical values determined at Eurofins. The experiment is accepted in accordance with Eurofins validation Standard Operating Procedure

Human recombinant (insect ce Testosterone (10 mM) °≨, min; 37°°C ⊘

Fluopyram Technical

Mix-batch 08528/0002

Dimethylsulfoxide

Stable until 20 April 2020 (when

Powder

94.5% 658066-35-4

Estraciol

HTRF

10 µl in \$84-well plates P100x] Solution in solvent then [5x] solution in water

Minor variations to the experimental protocol may have occurred during the testing, they have no impact on the quality of the results obtained.

- 6. Test concentrations: Test compound was tested at 100µM and 10µM in duplicate.
- Study design and methods B.

Si 1 June 20↓ 20 June 20↓ Experiemental dates:

2. Purpose: Evaluation of the effects of compounds on the activity of the aromatase (CYP19+P450 reductase) quantified by measuring the formation of estradiol from testosterone, using human recombinant enzymes perified from insect cells and the HTRF® detection method.

# 3. Experimental protocol

The test compound, reference compound or water (control) are mixed with 0.2 nM of aromatase and 10 nM of testosterone in a buffer containing 50 mM obtassium phosphate (pH 7.4), 0.5 mM EDTA and 5 mM_&MgCl2.

Thereafter, the reaction is initiated by the addition 75 nM of NADPH (co-substrate) and the mixture is incubated for X min at 37°C

For basal control measurements, the encyme is omitted from the reaction medium.

The fluorescence acceptor (Estradiol-XL665) and the fluorescence donor (Anti-Estradiol-Cryptate) are then added.

After 120 min, the fluorescence transfer is measured at  $\lambda ex=337$  nm,  $\lambda em=620$  nm and  $\lambda em=665$  nm using a microplate reader (Envision, Perkin).

The results are expressed as a percent inhibition of the control activity.



The standard inhibitory reference compound is letrozole, which is tested in each experiment at several concentrations to obtain an inhibition curve from which its IC50 value is calculated.

Reference: Ji J-Z et al (2014), Discovery of novel aromatase inhibitors using a homogeneous time- resolved fluorescence assay. APS, 35: 1082-1092.

#### 4. Analysis and expression of results:

The results are expressed as a percent of control specific activity

measured specific activity  $\times 100$ control specific activity

and as a percent inhibition of control specific activity

 $100 - (\frac{measured specific activity}{control specific activity})$ 

obtained in the presence of Fluopyram Technical.  $\sqrt{2}$ 

stime. The IC50 values (concentration causing a half-maximal/inhibition of control specific activity), EC50 values (concentration producing a half-maximal increase in control basal activity) and Hill coefficients (nH) were determined by non-linear regression analysis of the inhibition/conceptration response curves generated with mean replicate values using Hill equation eurve stiting

$$Y = D + \begin{bmatrix} A - D \\ 1 + (C/C_5 O^{HH}) \end{bmatrix}$$

where Y = specific activity, A = test asymptote of the surve, D = right asymptote of the curve, C =compound concentration, @50 = I@50 or EC50 and nH = slope factor.

This analysis was performed using software developed at Cerep (Hill software) and validated by comparison with data generated by the commercial software SigmaPlot® 4.0 for Windows® (© 1997 by SPSS Inc.).

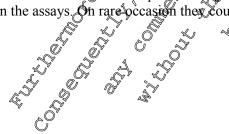
## 5. Results interpretation guide:

Results showing an whibition (or stimulation for assays run in basal conditions) higher than 50% are considered to represent significant effects of the test compounds. 50% is the most common cut-off value for further investigation (determination of IC50 or EC50 values from concentration-response curves) Ô that we would recommend. × Q

Results showing an inhibition (or stimulation), between 25% and 50% are indicative of weak to moderate effects (in most assays they should be confirmed by further testing as they are within a range where more inter-experimental variability can occur). 🐆

Results showing an inhibition (opstimulation) ower than 25% are not considered significant and mostly attributable to variability of the signal around the control level.

Low to moderate negative values have no real meaning and are attributable to variability of the signal around the control lever High negative values ( $\geq 50\%$ ) that are sometimes obtained with high concentrations of test compounds are generally attributable to non-specific effects of the test compounds in the assays. On rare occasion they could suggest an allosteric effect of the test compound.





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#### II. **Results and discussion**

Table 5.8.3-8 Resu	ults of Human Arc	omatase Assay: Fl	uopyram			a s.
Compound	Client	Test	% Inh	ibition of C	Control Spec	rific Binding 🖉 🖉
I.D.	Compound	Concentration	1st		2nd	Mean O
	I.D.				ð	, ČÍ (S)
Aromatase Assay Bayer SAS						
100044627-1	Fluopyram	1.0E-05 M	4.6		_∢ 4.7	~ -0. L~
	Technical			*	<i>A</i>	
100044627-1	Fluopyram	1.0E-04 M	<b>2</b> 4.6	Å	2.8	K 43?7 S
	Technical		- The second sec			
Table 5.8.3-9 Res	ults of Humon Ar	matasa Assaul L	tratala	08	Ň	
Table 5.8.3-9 Res	uits of Human Ar	omatase Assay: Le	eurozoie		0	
Compound I.D.		IC5	HM)	N B	° 🖄 n	K S

#### A. Deficiencies

Letrozole

None

#### III. Conclusions

Aromatase Assay Bayer SAS

Under the conditions of the test, the results show that Huopgram Technical does pot cause enzyme n P °, inhibition in this Human Aromatase Assay.

# Assessment and conclusion by applicants

Assessment and conclusion by applicate. Study meets the current goidancoand the requirements in 283/2013 Fluopyram Technical does not S cause enzyme inhibition in this Human Aromatase Assay

#### f. Thyroperox inhibition

	VQA59906 V C C
Data Point:	KCA 5.8.3/06 2 2 0 0
Report Autoor:	
Report Year:	
Report Vitle:	APC656948 (Fluopyram In vitro studies on the potential interactions with
	thyroid peroxidase-catalyzed reactions
Report No:	ÁT042681 2 P
Document No:	<u>M-299276-001-1</u> ~ A A A
Guideline(s) followed m	No specific guidenne
study:	No specific guideline
Deviations from current	Non of the second
Previous evaluation:	Les, evaluated and accepted in the DAR (2011)
GLROfficially	Yes, conducted under CLP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability Reliability:	jes q
	$\tilde{D}^{*}$ $\tilde{\omega}$ $\sqrt{2}$
O	

# Executive Summary

To intestigate a potential effect of AE C656948 on thyroid hormone synthesis at the level of thyroid perexidase (TPO), interactions of this compound with TPO-catalyzed reactions were studied in vitro using solution by the solution of the solution served as positive control substances.



Amitrole, a potent inhibitor of thyroid peroxidase, strongly inhibited TPO-catalyzed oxidation of guaiacol and formation of iodine. About 50% inhibition was observed in the presence of 1 µM Amitrole for guaiacol oxidation and in the presence of 0.1 µM Amitrole for iodine formation. Ethylenethiourea, which is not a TPO inhibitor, but a trap of the iodinating intermediate generated by TPO from iodide temporarily suppressed iodine formation.

In contrast, AE C656948 did not affect TPO-catalyzed guaiacol oxidation up to 300  $\mu$ M, the highest concentration tested. Similarly, TPO-catalyzed iodine formation was not affected by 300 μM C656948.

Costration
These findings strongly suggest that AE C656948 does not affect thyroid hormone synthesis at the level of TPO.
I. Materials and methods
A. Materials
1. Test material: AE C656948
bescription Light Deige powder
Light Deige powder
Light Deige powder
Light Deige powder
CAS # 658066 35-4
Stability of test compound: Stable for a period covering the study advantation
2. Vehicle and /or positive

- 2. Vehicle and /or positive
- control:

- Dimethylsulfoxide
- 3. Positive control substances: Amitrole (3 amino4,2,4-trazole) from Sigma Lot number 088K0649 and ETU (ethylenethioures, 2-initiazolidinethione) from Riedel-de Haën O ot number 3223X)
- 4. Bilogical raw material Hog thyroid glands from donestic pigs were obtained from Bayer CropScience, Mortheim. They were trimmed free of

excess fat and connective tissue. They were stored at -80°C until

- B. Study design
- was conducted on October 4, 2007 1. Experiemental date

we

2. Microsome preparation?

Interactions of AFC656948 with TPG-catalyzed reactions were studied in vitro using solubilized hog thyroid microsomes as an endowne source Amitrone and Ethylenethiourea (ETU) served as positive control substances.

prepared according to a standard procedure as described in Solubilized hog thyroid micro nes were Neary et al., 1984.

## C. Methods

# 1. Determination of TPO-catatyzed guaiaco oxidation :

Guaiacol oxidation was used as a measure for peroxidative activity. Incubations were carried out at room temperature n 0. M potassium phosphate buffer, pH 7.4 in a total volume of 1.0 mL. Guaiacol (125 µL of 40 mM solution in water, final concentration 5 mM), TPO (approximately 0.1 [E/min, corresponds to 5.5 µL of microsomal preparation) and test compound were preincubated for 1 minute, then the reaction was initiated by addition of hydrogen peroxide (20 µL of 12.5 mM solution in water, final concentration 250 µM). Test compounds were added in 20 µL DMSO, likewise control incubations lacking test compounds contained the same amount of solvent.



The following final concentrations were used:

AE C656948: 3.0 - 30 - 300 µM

Amitrole: 1.0 µM

The initial linear increase ( $\Delta E/min$ ) of the absorption at 470 nm was used to calculate the particulate the activity.

Amitrole: 0.1 µM

ETU: 5 µM

calculate the enzymatic The initial linear increase ( $\Delta E/min$ ) of the absorption activity.

II. **Results and discussion** 

**II.** Results and discussion of the results of the TPO-catalyzed oxidation of guaiacol are summarized below. Smitrole, the positive oxidation of the model substrate graiacol by more than 50%. AB/C656948, af concentrations up to 300 µM did not affect this reaction.

Table 5.8.3-10	Effect of	AE C656948	on TPO	-catalyz	edyguai	acol reaction	°¢ C
----------------	-----------	------------	--------	----------	---------	---------------	---------

	Ň					
Compound		Concentr	ation (µM)	$\Delta E/\partial a = SE$		% of control
Vehicle	S, Q	) 		$\sim 0 \sqrt{2}1 \pm 0.000$		100
AE C656948	<u>`</u> ``	· * &	\$ _ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~	0.122 * 0.002	2 🖉	100.8
- De la companya de l	L.		30 🖌 🌀	0.123 0.000	D)	101.6
Ô	O ^v		300 2	$0.124 \pm 0.004$		102.5
Amitrole	Č,		Ð,	$\sim -0.954 \pm 0.003$	3	44.6
	<i>.</i>	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~				

The results of the TPO-catalyzed iodine formation are summarized below. Up to 300 µM AE C656948 did not affect TPO patalyzed iodine formation Neither the initial rate of the reaction was affected, or a temporary suppression efficience formation was observed. In contrast, Ethylenethiourea (ETU), a trap of the iodinating intermediate, temporarly suppressed iodin formation, whilst Amitrole at a concentration of 0.1 µM infibited the initial rate of this reaction by 50%.

I able 5.8.9 Pl	Flieet	of AF 056948 on FPO-ca	sayzed lodine formation	
Compound		Concentration (MM)	$\Delta E/\min \pm SD$	% of control
Vehicle			$0.259 \pm 0.012$	100
AE @656948	, q		$0.269 \pm 0.012$	103.9
	> >	~~ <u>80</u> ~~	$0.246 \pm 0.005$	95.0
4	A	<i>₹</i> 300 <i>×</i>	$0.260 \pm 0.012$	100.4
Amitrole	$\sum_{i=1}^{n}$	0.10	$0.131 \pm 0.013$	50.6
· • • • •	$\lor$			

# Table 5 9 2 1

A. Deficiencies

# None

Fluopyram Technical (AE C656948), at concentrations up to 300 µM (the highest concentration tested) neither affected TPO-catalyzed guaiacol oxidation nor iodine formation. These findings strongly suggest that AE C656948 does not affect thyroid hormone synthesis at the level of TPO.



Assessment and conclu	sion hy annlicant.
	guidance and the requirements in 283/2013.
-	
AE C656948 does not a	ffect thyroid hormone synthesis at the level of TPO.
Data Point:	KCA 5.8.3/07
Report Author:	
Report Year:	
Report Title:	In vitro CYP and UGT induction in human app Wistar rat hebatocytes by
	Fluopyram
Report No:	KLC-BA20-06 $\mathcal{O}^{\mathcal{V}}$ $\mathcal{O}^{\mathcal{V}}$ $\mathcal{O}^{\mathcal{V}}$ $\mathcal{O}^{\mathcal{V}}$ $\mathcal{O}^{\mathcal{V}}$ $\mathcal{O}^{\mathcal{V}}$
Document No:	<u>M-759019-01-1</u>
Guideline(s) followed in	No specific guideline a the specific guideline a sp
study:	
Deviations from current test guideline:	Current guideline none & C & C & C & C & C & C & C & C & C &
Previous evaluation:	No, not previ@asly sitemitted
GLP/Officially	No, not conducted under CheP/Officially recognised testing facilities
recognised testing	
facilities:	
Acceptability/Reliability:	$\operatorname{Yes}_{\mathcal{O}} \qquad $

#### **Executive Summary**

Primary cultures of cryopreserved plateable male human and Wistar rat hepatocodes were used to assess the potential of Fluopyram to induce CYP and UGT expression and to increase thyroxine (T4) glucuronidation, after 3 or 7 days of daily treatment.

Induction study was performed with 3 male human and 3 male Wistar, at hepatocyte cultures treated daily for 3 days and 7 days with Fluopyran at 10 30, 60 and 100  $\mu$ M or with positive control inducers beta-Naphthoflavore (BNF), rifampicin (RIF) and phenobarbital (PB) for human hepatocytes and BNF, 5-pregnen 36-ol-20-one 46a-carbonitribe (PCN) and PB for at hepatocytes. RNA quantity established that Fluopyram at 10, 50 and 00  $\mu$ N were suitable concentrations for mRNA expression and activity analysis.

In Wistar rat hepatocytes, reference inducers BNF (5  $\mu$ M) PB (1000  $\mu$ M) and PCN (6  $\mu$ M) strongly induced CYP1A2, CYP2B1 and CYP3A1 expression, respectively and increased the related activities. PB and PCN also induced LGT2B1 and to a lesser extent UGT1A1 expression and BNF, PB and PCN increased UGT-T4 activity.

In human hepatocytes, reference inducers BNF (5  $\mu$ M), PB (1000  $\mu$ M) and RIF (15  $\mu$ M) induced CYP1A2, CYP2B6 and CYP3A4 expression, respectively, and increased the related activities. PB and RIF induced UGT1A1 expression, BNF and RW increased UGT-T4 activity.

#### In conclusion

The present results show that Fluopyrant at 10  $\mu$ M, 30  $\mu$ M and 100  $\mu$ M is a strong CYP3A and to a lesser extent a CYP1A2 inducer in Wistar rat hepatocytes. Fluopyram at all tested concentrations also consistently induces UGT2B and increases UGT-T4 activity in Wistar rat hepatocytes. Fluopyram at 10  $\mu$ M, 30  $\mu$ M and 100  $\mu$ M is a CYP1A2, CYP2B6, CYP3A4 and UGT1A1 inducer in human hepatocytes but does not increase UGT-T4 activity in human hepatocytes.

#### A detailed summary of this study report is provided under data point KCA 5.5/22



#### Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it assesses the potentiat of Fluopyram to induce human and rat inducible cytochrome P450 isoenzymes CYP1A, CYP2B and CYP3A as well as UDP-glucuronosyltransferases UGT1A and UGT2B and increase therefore the control of the control (T4)-glucuronidation in the liver

The present results show that Fluopyram at 10 µM, 30 µM and 100 µM is a strong CYPOA and to a lesser extent a CYP1A2 inducer in Wistar rat hepatocytes. Fluopyram at all tested concentrations also consistently induces UGT2B1 and increases UGT-T4 activity in Wistar fat hepatocytes. Fluopyram at 10 µM, 30 µM and 100 µM is a CYP1A2, CYP2B6, CYP3A4 and CGT1A1 inducer but does not increase UGT-T4 activity in human hepatocytes

#### **Endocrine disrupting (ED) properties**

Endocrine disrupting (ED) properties In addition to the evaluation of the endocrine disrupting potential activities of fluopyram in the in vitro assays described above, the potential of fluopyram to interact with endocrine systems in humans (and non-target organisms) was assessed in line with the EFSA/ECNA (2018) guidance for the identification of endocrine disruptors in the context of Regulations (EU), No 528 (2012 and (EC) No 1107/2009. The detailed assessment is reported in the Appendix I in accordance with Commission Regulation (EU) 2018/605.

Data Point:	KCA&8.3.08
Report Author:	
Report Year:	
Report Title:	Appendix I - Assessment of the endoerine distripting properties of the active
A	substance fluopyram in accordance with Commission Regulation (EU) 2018/605
Report No:	<u>M@6402201-1</u>
Document No:	M-76462-010 ~ ~ ~
Guideline(s) followed in	None V V V V V V V V V V V V V V V V V V V
study:	
Deviations from current	Not applicable to a for
test guideline:	
Previous evaluation:	TNO ADD DIE Y DUSTY SUDIFILIER AND A ADD ADD ADD ADD ADD ADD ADD ADD A
GLP/Officially	Not pplicable $\mathcal{O}^{\vee}$ $\mathcal{O}^{\vee}$ $\mathcal{O}^{\vee}$
recognised testing	
facilities:	
Acceptability/Relfability	Yes in the interview of the second

Standard toxicology and ecotoxicology studies conducted to meet the data requirements under Regulation (EU) 283/2013 have been submitted in support of the fluopyram application for approval under Regulation EU) No. 1109/2009. A literature search did not find any relevant studies conducted in the last 10 years published in the open diterative. At the time of preparation, there was no in vitro mechanistic data available on the US ERA CompTox Chemicals Dashboard. However, in vitro and in vivo mechanistic studies have been conducted to investigate EATS-mediated endocrine activity.

Ŵ Data from relevant studies were added to the Excel template provided as Appendix E1 to the EFSA/ECFA guidance for the identification of endocrine disruptors (2018). Each study was given a unique identification number Study ID matrix) for its identification in the data matrix and the Lines of Evidence (Lop) spreadsheets of the Excel template. Appendix E1 is provided as a supplement to this dogninent.

A summary of all the studies considered relevant for the mammalian toxicology review is outlined in Table 5.8.3-12.



Type of toxicity	Species	Duration/Type	Used for T/EAS modality	Reference	Matrix ID ()
Repeat	Rat	28 days	T & EAS	<u>M@85510-01-1</u>	
Dose Studies		28 days (dermal)	T & EAS	Ø <u>1-293833-01-1</u>	13,5
in mammals		90 days	T & EAS	<u>M-250946-001</u>	
		90 days (neurotoxicity)	T & EAS	<u>M-299118-01-1</u>	14
		104 weeks chronic/carcinogenicity	Т & Буля	<u>M-298939-01-0</u>	807 40
		2-generation reproduction	T & EAS	<u>Mr299334-01-1</u>	
		1-generation reproduction dose range	<b>₩</b> & ĘA <b>®</b>	<u>Mr299539-01-1</u>	
		Prenatal developmental tox offy	T & EAS	<u>Mag99438-01-2</u>	9 。
	Rabbit	Prenatal developmental foxicity	T& EAS	M-279779-01-1	10
	Mouse	28 days	T & AS	<u>M-088486-04-1</u>	O
		90 days & & & &	To EAS	M25113(0)1-1	5
		78 weeks carcinogenicity	T&EAS	CM-295688-01-1	7
	Dog	2 Solays S S	T & EAS	<u>M-276047-01-1</u> M-276047-01-1	3
	, bj		T& EAS	M-294279-01-1	6 15
					15
Table 5.			Ö ^Y ^K Y	L. C.	
Type of toxicity	Species	Discation/Rype	Used for THAS	Reference	Matri x ID
UNICITY	Species	Discation/Rype	priodality		
<i>In vivo</i> mechanisty	Rat		T	<u>M-299274-01-1</u>	21
ic studies		Rose 7 days	Î, Î	<u>M-408029-01-1</u>	22
K Y		728 day 5 24 4	Л	M-427431-01-1	23

Table 5.8.3-12:	Outline of dataset considered for mammalian toxicology ED assessment
-----------------	----------------------------------------------------------------------

<b>T A</b>				D (	35.1
Type of	Species	Discation/Expe	Used for	Reference	Matri
toxicity	Č al		TRAS T		x ID
	Species	Direction/Rype	TREAS		
In vivo	Rat O	7 days A S	ŤT ≪J	<u>M-299274-01-1</u>	21
mechanist 🗞			<u> </u>		
ic studies	Î Ô	Active 7 days	17	<u>M-408029-01-1</u>	22
mechanists ic studies			N N	M 407421 01 1	22
v		y28 day 67 27 4	_%I	<u>M-427431-01-1</u>	23
	Mouse		Т	M-426994-01-1	30
	Mouse		1	11-+2077+-01-1	50
		3 days 2 7 7 0 0 0	Т	M-308073-01-1	32
	v "O ^v				
		3 days	Т	M-408352-01-1	33
		3 days	Т	<u>M-426994-01-1</u>	34
, and a second s			T	M 220 ((2, 01, 1	21
		4 days	Т	<u>M-328662-01-1</u>	31
		3 & 4 days	Т	<u>M-299522-01-1</u>	24
"¥	1 Alianti al		1	11-277522-01-1	24
	<i>a</i> . [\]	28 days V	Т	M-428031-02-1	25
			-		
		28 days	Т	M-428303-01-1	26
Å		28 days	Т	<u>M-449821-03-1</u>	27
, Q	l .¢ ^v		-	N. 440000 01 1	20
1 and a	A A A	98 days (wild type v Pxr KO/Car KO)	Т	<u>M-449890-01-1</u>	28
L.	ON CN K	28 days (liver microme samples were	Т	M 451629 01 1	20
, St			1	<u>M-451628-01-1</u>	29
& s		provided from 28 day study M428031-			
<u> </u>		02-1)			
<u> </u>					



<i>In vitro</i> mechanist ic studies	Rat	Phase I enzyme activity assessment hepatocyte cultures and cellular proliferation (96 hours)	Т	<u>M-450157-01-1</u>	36 ©° ~
	Human	Phase I enzyme activity assessment hepatocyte cultures and cellular proliferation (96 hours)	Т	<u>M-450156-01-1</u>	
	Rat & Human	Phase I & II gene transcript & enzyme activity assessment hepatocyte cultures (3 & 7 days)	Т	<u>M-759019-014</u>	
	Domestic pig	inhibition of thyroperoxidase (TPØ)	T	<u>M-299</u> 46-01 40	200
	Human	Stably transfected Human ERα Transcriptional Activation assay	T L C	<u>M-532695-6Q-1</u>	
	Human	AR binding assay	EAS	<u>M-632697-01-1</u> ?	18
	Human	ER binding assays	EAS	M-6326950171 & M6632859-01-1	°16, 17
	Human	Aromatase assay (OCSR) 890.4200)	PAS C	<u>M-632696-01-4</u>	190

#### **Overall conclusion for humans:**

EAS and T modalities have been sufficiently investigated to above the ED assessment of fluopyram with respect to humans.

In guideline studies with floopyram, the liver was identified as a primary target organ in the rodent and dog, the thyroid was a target organ in the rodent, whilst the former was a target organ in the rat only.

EAS adversity and indocrine activity have been sufficiently investigated. Investigation of EASmediated endocrine activity *in vitro* indicated fluopy am had no endocrine activity *via* the E, A or S modality and there was no evidence of adversity related to an EAS MoA in any of the apical studies conducted.

No reproductive effects were observed in either the F0 or the F1 animals, nor was there any treatmentrelated effect on sexual maturation in females in the 2-generation reproduction study in F1 offspring. The slight delay in preputial separation of F1 males, which was well within the HCD range of the conducting laboratory, was considered to be secondar to reduced body weight. The mean body weight at attainment of preputial separation was comparable across all groups including the controls. In addition, there was no effect on the more sensitive parameter of anogenital distance in the F2 males or on sperm parameters, mate reproductive organ weight parameters, mating or fertility capacity in either generation. There were no morphological or functional changes observed in any of the organs sensitive to the E A, or S modalities, and no adverse effects were observed in any of these organs in any study conducted with fluopyram. The F, A, and S modalities can be considered to have been sufficiently investigated, and it can be concluded that the FD criteria are not met for these modalities.

T-mediated adversity and endocrine activity have been sufficiently investigated. Fluopyram caused microscopic changes in the thyroid and in thyroid hormones in the rat and mouse. However, the MoA analysis provided sufficient evidence to demonstrate the most plausible MoA was a secondary effect on the thyroid *via* changed hepatic clearance of thyroid hormones, mediated by enhanced hepatic phase II enzyme activity. There are known quantitative species-specific differences which make this MoA less relevant to humans. For example, there are species differences in the half-life of T4 of approximately 12 hours in the ratversus 5-9 days in humans (IARC 1999). The rodent also exhibits enhanced thyroid hormone elimination with less efficient enterohepatic recirculation than humans therefore the rodent is likely to be more sensitive to test substance enzyme induction leading to increased metabolism of T4.



Consequently, this MoA is of limited relevance to humans. In addition, a direct MoA could be excluded. As the effects on the thyroid are secondary to the effects of fluopyram on the liver, it can be concluded that fluopyram shows no adversity with regard to the T modality. Furthermore, based on the absence of  $\bigcirc$ UGT-T4 activity in vitro in the human hepatocytes, the data provided corroborate the MoA as being not relevant to humans.

In the absence of any effects on E, A, or S modalities, and in the absence of any adversity T modality, the ED criteria are not met for fluopyram with respect to buman heakly

CA 5.9 **Medical data** 

#### CA 5.9.1 Medical surveillance of manufac plant personnel and monitoring Ũ 0 studies M Ô

Information provided on medical sufveillance on manufacturing is confidential and included in (M-763420-01-1, M-763418-001 and M-76942 and document IC

#### CA 5.9.2 Data collected on humans

There are no publications on human poisoning

#### CA 5.9.3 rectobseroations

4 cases with son irrutation in one case with ever irritation from splashes or wind drift have come to the attention of Bayer. In all cases the irritation subsided quickly. In animal tests the formulations were not skin intitants

A child eating a soybean treated with Fluopyran only remained asymptomatic.

#### CA 5.9.4 pidemiological studies

No epidemiological studies have been published oncerning Fluopyram.

CA 5,9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

There are no reports on human poisoning cases in humans. In high dose animal experiments unspecific symptoms only have been seen.

**Proposed treatment: first aid measures, antidotes, medical treatment** First Add:

Remove patient from exposure/terminate exposure



- Thorough skin decontamination with copious amounts of water and soap, if available with polyethylenglykol 300 followed by water. .1. G
  - Note: Most formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polyethyleneglykol 300 is not required.
- Flushing of the eyes with lukewarm water for 15 minutes
- Induction of vomiting does not seem to be required in regard of the low to sic

- i low topic of the automation The application of activated charcoal and sodium supplate for other carchartics might be considered in significant ingestions. the provide and solar the transfer of the other the othe

A 5.9.7 Expected effects of poisoning



#### Summary tables for harmonised classification and labelling **Appendix 1:** (fluopyram)

#### 1. Acute oral toxicity

#### a) Animal studies on acute oral toxicity

#### Summary table of animal studies on acute gral toxicity Table 1:

(muopyram)					2 D			
1. Acu	Acute oral toxicity							
a) Ani	nimal studies on acute oral toxicity							
<ul> <li>a) Animal studies on acute oral toxicity</li> <li>One animal study of acute oral toxicity is available and is summarised in Table 1, below.</li> <li>Table 1: Summary table of animal studies on acute oral toxicity</li> </ul>								
Table 1: Sur	nmary table of a	nimal studies on	acute oral toxic	ity y				
Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels, duration of exposure	Quiue LD ₅₀	Reference to the second			
Acute oral toxicity (rat) OECD 423 (2001); EEC B.1; EPA OPPTS 870.1100 (1998); JMAFF (2000) No deviations		Fluopyram (94.7% purity) Batch No.: O 08528/0002	2000 mg/kg bw Single doso	2000 mg/kg bw				

#### b) Human data on acute oral toxicit

No human data are availables

#### Other studies relevant for acute or al toxicity c)

Studies of acute oral neurotoxicity in the rate erformed at dose levels of up to 2000 mg/kg bw did not report any mortality.

#### Summary of acute or al texicity and conclusion on classification d)

A GLP- and godeline compliant (QECD, 423) study of acute and toxicity in the rat performed with fluopyram reports an LD50 value of >2000 mg/kg bw. Studie of acute oral neurotoxicity also show a lack of mortality at dose levels of up to and including 2000 mg/kg bw. No additional studies using a single oral dose are available. No human data are available As the acute oral LD50 exceeds 2000 mg/kg bw, classification for acute and toxicity in any category is not required according to the CLP criteria.

Fluopyram was discussed at the 31st Meeting of the Committee for risk Assessment (RAC-31) on 4th December 2014. The GTH optimion acopted at RAG-31 concluded that no classification for acute oral toxicity was required.

#### 2. Acute dermal toxicit

#### Animal studies on acute dermal toocity a)

One animal study of acute dermaty toxicity is available and is summarised in Table 2, below.

#### Summary table of animal studies on acute dermal toxicity Table 2:

deviations if any	Species, serain, sex, norgroup	TestSubstance	Dose levels, duration of exposure	Value LD50	Reference
OECD 402 (1987); EEØ B.3; EPÅ OPFTS 870.1200 (1998). No deviations	Rat (Wistar): ∂,⊋ 57groups	Fluopyram (94.7% purity) Batch No.: 08528/0002	2000 mg/kg bw Single dose	>2000 mg/kg bw	(2005) <u>M-259275-01-1</u>



#### b) Human data on acute dermal toxicity

No human data are available.

#### c) Other studies relevant for acute dermal toxicity

No other relevant studies are available.

#### d) Summary of acute dermal toxicity and conclusion on classification

A GLP- and guideline-compliant (OECD 402) study of acute dermal toxicity in the rat performed with fluopyram reports an LD₅₀ value of >2000 mg/kg bw. No human data are available and no additional relevant studies are available. As the acute dermal LD₅₀ exceeds 2000 mg/kg bw, classification for acute dermal toxicity (in any category) is not required according to the CLP criteria.

Fluopyram was discussed at the 31st Meeting of the Committee for risk Assessment (RAC 31) or 4th December 2014. The CLH opinion adopted at RAC-31 concluded that to classification for acute dermal toxicity was required.

## 3) Acute inhalation toxicity

## a) Animal studies on acute whalation toxicity

One animal study of acute inhalation Poxicity is available and is summatised in Tables, below.

Table 3:	Summary table	of ann	mal <i>o</i> studi	es>on aç@te	e inbralat	ionToxic	iQĭ
	•	-					~

Method, guideline,	Species, strain,	Test substance,	Dose levels	Value	Beference
deviations if any	sex, no/group	formand	doration of	LC50 C	<b>)</b>
		particle size (MMAD)	exposure		
OECD 403 (1981);	Rat (Wispar): 🔬	Fluopyram	5,1)25 mg D	5.1125 mg/L	(2006)
EEC (1992); EPA OPPTS 870.1300 (1998); JMAFF	5/g@un	(xa.y /o purkey)	A-hour nose		M-283420-01-1
OPPTS 870.1300	5/group	Batch Noy.			
(1998); JMAFF 🖉		JU852840002 🔗		K, A	
(2000)		Solidaeroso	conly) sy iv	e,	
No de viacionas		(dtust)			
Ę,		MMAD 5.6 µm			
<u> </u>				1	

- b) Human data on acute inharation toxicito
- No human data are available 5

# c) Other studies relevant for acute inhalation toxicity

No other relevant studies are available

d) Summary of acute in ralation toxicity and conclusion on classification

A GEP- and guideline-compliant (OECD 400) study of acute inhalation toxicity in the rat performed with fluopyram reports an L $_{50}$  value (4-hour, nose-only) of 5.1125 mg/L. Although the MMAD attained in this study (5.6 km) is slightly high (CLP Guidance states that results from studies with MMAD >4 µm can 'generally not be used for classification'), attempts to generate atmospheres containing smaller particles were not successful. Furthermore, no mortality was seen at the exposure concentration (stated to be maximum technically achievable under the conditions of the study). No human data are available and no additional relevant studies are available. As the acute inhalation LC₅₀ exceeds 5 (hg/L (dust/mist), classification for acute inhalation toxicity (in any category) is not required according to the CLP criteria.



Fluopyram was discussed at the 31st Meeting of the Committee for risk Assessment (RAC-31) on 4th December 2014. The CLH opinion adopted at RAC-31 concluded that no classification for acute inhalation toxicity was required.

#### 4) Skin corrosion/irritation

#### Animal studies on skin corrosion/irritation a)

December 2014. The CLH opinion adopted at RAC-31 concluded that no classification for acute inhalation toxicity was required.							
initialition toxicity wa	s required.						
4) Skin cor	rosion/irritation						
a) Animal s	studies on skin co	orrosion/irrita	tion ble and is summarized in Table & below?				
One animal study of s	kin corrosion/irrit	ation is availab	ble and is summarized in Table & below.				
Table 4: Summary	y table of animal	studies on ski	ble and is summarized in Table & below.				
Method, Specie guideline, strain,		Dose levels, duration of					
deviations if no/gro		× I	- Observations and time point				
any		O ^v d	- Mean scoves/anipar				
			-Reversibility				
OECD 404 Rabbit	Fluopyram	∮0.5 g	No signs of irritation in any and (2005)				
(2002); EEC (NZW)	); 3♀   (94.7% _Q	(4 hours)	No signs of irritation in any animat 26005) Mean (24-72 hour) scores for erythema 0.00, 0.00 Mean 24-72 hour) scores for				
B.4. (1967); EPAOPPTS	purity) O'	ON N	Mean (24-72 hour) scores for				
870.2500	Batch@o.: 08528/0002		erythema 0.00, 0.00, 0.00				
(1998);	08328/0002		Mean 724-72 hour) scores for oedena: 0.00, 0.00, 0.00				
JMAFF (2000)	w .		Dewarsibility: NA				
No deviations							

Human data on skin corrosion orritation b)

No human data are available.

#### Other studies relevant for skin corrosion/irrutation c)

A 28-day demail repeated dose toxicity study in the rat is available (see Section 9), and is summarised in Table 5 below in Table 5, below.

# Table 5: Summary table of other studies relevant for skin corrosion/irritation

Method,	Fest substance	Realts X & A	Reference
guideline,	route of exposure,	z NOAFATLOARL	
deviations if any,	dose Tevels,	rarget tissue organ	
species, strain		- critical effects at the LOAEL	
sex, no/group	exposure		
28-day dermal	Fluopyram (24.6%	<b>WOAEL</b> (local effects): $3/2$ 1000 mg/kg bw/d	
OECD 410 (1981)	pursity)	LOASL (local effects): ∂/♀	(2007)
No deviations	Batch 082528/0002	Notevidence of local effects at the application site	<u>M-293833-01-1</u>
Rat (Wistar)	0, 100, 300, 4000		
10/sex/group	mg/kg bw/d		
	44 Weeks of the second		
	applications/week)		

#### Summary of skin corrosion/irritation d)

A GLP- and guideline-compliant (OECD 404) study of skin corrosion/irritation in the rabbit performed with fluory ram shows no local dermal reactions in any rabbit at any time point. No local dermal effects were reported in a 28-day repeated dose dermal toxicity study in the rat. No additional relevant studies are available, although the lack of skin effects in the OECD 402 study is supportive. No human data are available. The mean and individual scores for erythema and oedema seen in the rabbit study are not



sufficient to trigger classification for skin corrosion (Category 1) or skin irritation (Category 2) according to the CLP criteria.

Fluopyram was discussed at the 31st Meeting of the Committee for risk Assessment (RAC-31) of 4th December 2014. The CLH opinion adopted at RAC-31 concluded that no classification for skin corrosion / irritation was required.

#### 5. Serious eye damage/serious eye irritation

#### Animal studies on serious eye damage/serious eye irritation a)

One animal study of serious eye damage/serious eye irritation is available and is suppharised below.

Table 6:	Summany table of animal studios	of serious eye damage serious eye fritation	
Table 0:	Summary table of ammar studies	auserious eye damage/serious eye uritation	

			· · · · · · · · · · · · · · · · · · ·	
Method, guideline,	Species, strain, sex,	Test substance	Dose levels duration of	Results & Reference Reference
deviations if any	no/group		exposure	of waset ~ ~ ~ ~ ~ ~ ~
		Ő		Mean scores/animal - Reversibility Signs of infration timited to conjunctival erythema (Grade I) in two pabbits at 24 hours onlo
OECD 405	Rabbit	Fluopyram	0 <del>,</del> ,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	Signs of intration funited to (2005)
(2002); EEC	(NZW); 3♀	(94.7%) (94.7%)		Signs of intration united to (2005) conjunctoral erverema (Grade b) <u>M-263277-01-1</u> in two cabbits at 24 hours onlo
B.5. (1967);		purity) 🔬		in two abbits at 24 hours onlo
EPAOPPTS		Batch No.		Merry (24-72) Your) scores for
870.2400		08528,0002	O A	conneal on acity (1) (10 (1) (1) (1) (1) (1) (1) (1) (1) (1) (1)
(1998);				Mean (24-72 bour) scores for
JMAFF (2000)				Mean (24-72 hour) soores for iritis 9.00, 0.00, 0.00
No deviations				Mean (24-02 hours scores for
	Ö,		Ç Ş	conjunctival erythema: 0.66, 0.00,
	\$ . Ó			
		A' &		9.00 Mean (24-72 hour) scores for
2		$\circ \circ$	KU S	chemosisz 0.00, 0.00, 0.00
la la		L. Ø.		All signs reversible by 48 hours
\$\$\ \$	Ŵ		<del>y ()*</del>	········

🔊 Human data on seriouse ve damage/serious eye irritation b) Š,

# No human data are available

#### Other studies relevant for serious eye damage/serious eye irritation c) Ô

No other relevant studies are available.

#### A Summary of serious eye damage eye if itation and conclusion on classification d)

A GLP and guideline compliant (OECD 405) study of eye irritation in the rabbit performed with fluopyram shows a minimal and transient ocular reaction in two rabbits at 24 hours only. No additional relevant studies are available, and no human data are available. The mean and individual scores for corneal opacity pritis, conjunctival effthem and oedema seen in the rabbit study are not sufficient to trigger classification for serious eye damage (Category 1) or serious eye irritation (Category 2) according to the CLP criteria

Fluopyran was discussed at the 31st Meeting of the Committee for risk Assessment (RAC-31) on 4th December 2015. The CLH opinion adopted at RAC-31 concluded that no classification for eye damage / irritation was required.



#### 6. Respiratory sensitisation

#### a) Animal studies of respiratory sensitisation

No studies are available.

#### b) Human data on respiratory sensitisation

No human data are available.

#### c) Other studies relevant for respiratory sensitisation

No other relevant studies are available.

# d) Summary of respiratory sensitisation and conclusion on classification

In the absence of any relevant data, classification of huppyram for respiratory so sitism on (Category 1) is not required according to the CLP criteria.

#### 7. Skin sensitisation

# a) Animal studies on skin sensitisation

One animal study of skin sensitisation potential is available and is summarised in Table 7, below Table 7: Summary table of animal studies on skin sensitisation of the sensitis of the sensitisation of the sensitis of the sensitisation of the sensitis of the sensitis

Method, guideline, deviations if any	Species, strain, sex, no/group	OT ST	duration of s		Reference
OECD 429 (2002)	Mouse (CBAC):	Fluopyram 94 7‰purity)	0%, 0, 0%, 1.0% 2.5%, 7 5.0% (25, pL in	SI values: 1.0 (0%) 9.9 (05%), 1.9 (1.0%) 1.1	(2006)
No significant deviations	(CBAT): 5 ⁺ /group	li . A	5.6% (25.0°L in DMF); three & consecutive ~	(05%), 140(1.0%) 1.1 (2.5%), 0.9 (5.0%) EC ₃ not calculated	<u>M-281845-01-1</u>

b) Human data on skin sensitisation

No human data are available

# c) Other studies relevant for skin sensitisation

No other relevant studies are available.

# d) Summary of skin sensitisation and conclusion on classification

A GLP-and guideline-compliant (OECD 429) study in the mouse performed with fluopyram shows a clear lack of skin separation potential. No human data are available and no additional relevant studies are available. The stimulation index (SI) values in the mouse LLNA are not sufficient to trigger classification for skin sensitisation (Category I) according to the CLP criteria.

Fluopyram was discussed at the 34³⁴ Meeting of the Committee for risk Assessment (RAC-31) on 4th December 2014. The CFH opinion adopted at RAC-31 concluded that no classification for skin sensitisation was required.

# 8. STOT SE (specific target organ toxicity-single exposure)

# a) 🗳 🖉 Animal studies on STOT SE

Studies of acute oral toxicity, acute dermal toxicity and acute inhalation toxicity are available for fluopyram, and are summarised above in Sections 1-3. An oral neurotoxicity study in the rat and a



follow-up acute oral neurotoxicity study in female rats are additionally available, and are summarised in Table 8, below.

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference of the second
OECD 424 (1997); EPA OPPTS 870.6200 (1998); JMAFF (2000) No deviations Rat (Wistar) ♂,♀ 12/group	Fluopyram (94.7% purity) Batch 08528/0002 Oral (gavage); single dose 0, 125, 500, 2000 mg/kg bw	NOAEL ♂/♀: 125/ 425 mg/kg bw LOAEL ♂/♀: 500/25 mg/kg bw/Q ↓motor activity (≥500 mg/kg bw); ♀ (≥125 mg/kg bw) ↓locomotoractivity (≥500 mg/kg bw); ↔ (≥125 mg/kg mg/kg bw) ↓body temperature ♀ (≥500 mg/kg bw) No evidence of neuropathology	(2007) <del>)-289973-01-2</del> <del>)</del> <del>)</del> <del>)</del> <del>)</del> <del>)</del> <del>)</del> <del>)</del> <del>)</del>
OECD 424 (1997); EPA OPPTS 870.6200 (1998); JMAFF (2000) No deviations Rat (Wistar) ♀ 12/group	Fluopyram (94.7% purito) Batch 08528/0002 Oral (gavage); single dose 0, 25, 50, 100 52/kg by an data on STO	Jlocomotor activity c (-38%; 100 mg/kg bac)	(2007) M-289073-01-2

Tabla 8. Summary table of animal studies on STOT SE

No human data are available.

# c) Other studies retevant for STOT SE (Structure) d) Summary of STOC (Structure)

# Summary of STOF SE and conclusion on classification

Fluopyram is flown of be of low toxicity following single exposure by all routes investigated. In the acute oral toxicity study in the rat (Section 1), to sign of systemic toxicity observed at the limit dose of 2000 mg/kg bw. In the acute termat foxicity study in the rat (Section 2), no signs of systemic toxicity observed at the limit dose of 2000 mg/kg by In the acute inhalation toxicity study in the rat (Section 3), reversible signs of toxicity seen following exposure to 5.1125 mg/L included bradypnoea/dyspnoea, reduced motility, piloerection, upgroomed han coat and limpness. Measurements of reflexes made on the first day postexposure day were pormal invall males; one female showed a reduced tonus and vertical grip strength together with an impaired righting response. Rectal temperature was reduced in treated rats of both sexes with the reatment. All signs were fully reversible within five days. In the initial acute oral neurofoxicity study reduced motor activity and reduced locomotor activity at the time of peak effect were reported for both at dose levels of 500 and 2000 mg/kg bw, and additionally for females at 125 mg/kg bw. A feduction in body temperature was also noted for females at 500 and 1000 mg/kg bw. In the follow op study in female rats, reduced motor activity and reduced locomotor activity were noted at 100 mg/kg bw.

Classification for STOT SE in Category 1 or 2 is assigned on the basis of findings of 'significant' or 'severe' toxicity at relevant dose levels ( $\leq$ 300 mg/kg bw and  $\geq$ 300-2000 mg/kg bw, respectively).



Although the dose levels at which effects are reported in rats in the acute neurotoxicity studies are relevant in terms of classification, the subtle and transient behavioural effects reported in these studies do not represent either 'significant' or 'severe' toxicity. It is notable that neither this study nor the acute oral toxicity study reported any overt signs of toxicity. Furthermore, there was no evidence of neuropathology from the acute oral neurotoxicity studies at dose levels of up to and including the limit dose of 2000 mg/kg bw. The available data do not therefore trigger classification for STOT SE (Category 1 or 2) according to the CLP criteria.

Classification for STOT SE in Category 3 is assigned on the basis of narcotic effects or respiratory tract irritation. There is no evidence for narcotic effects from only study. The acute inhalation to verify study (Section 3) reports some findings including bradypnoea/dyspnoea and reduced rectal temperature that may be consistent with the response to a respiratory initiant. However, the clinical signs were transient and not marked and may reflect a general aversion to exposure. Reduced body temperature was also reported for rats administered a single gavage dose of fluopyram in the acute neurotoxicity studies; consequently, this finding may not reflect a response to respiratory irritant. Additionally, fluopyram was shown not to cause any skin irritation (Section 4) and to cause only the most minimal and transient eye irritation in rabbit studies (Section 5). The available data do not therefore trigger classification for STOT SE (Category 3) according to the CLP criteria.

Fluopyram was discussed at the 31st Meeting of the Committee for risk Assessment (FAC-31) on 4th December 2014. The CLH opinion adopted at RAC-31 concluded that no classification for STOT SE was required.

# 9. STOT RE (specific target organ toxicity-repeated@xposore)

# a) Animal studies for STOT RE

A number of repeated dose animal studies are available. Oral toxicity studies were performed for durations of 28 days (rat, more and dog), 90 days (rat, mouse, dog) and 12 months (dog). A 12-month oral toxicity study is also available for the dog. A 28-day repeated dose dermal toxicity study in the rat is also available. The studies are summarised in Table 9, befow.

W

Method, guideline deviations if any, species, strain, sex, no/group	Test substances route of exposure, dose levels? ouration of exposure	Results NOAEL/LOTEL - target tissue/organ - crutical effects at the LOAEL	Reference
28-day oral (dietary) Non-guideline Rat (Wistor) 5/sex/group	Fluggýram 198.6% pority)	NOAEL (400 ppm): $72:31.0/36.1 \text{ mg/kg bw/d}$ LOAEL (3200 ppm): $3/2:254/263 \text{ mg/kg bw/d}$ target organs: liver, kidney, thyroid 2 body weight gain (m/f) $\uparrow$ platelet court ( $3$ ) $\uparrow$ prothroutin time ( $3$ ) $\uparrow$ cholesterol, triglycerides ( $3,2$ ) $\downarrow$ ast, ap activity ( $3,2$ ) $\uparrow$ liver weight (m,f) $\uparrow$ thyroid weight ( $3$ ) hepatocyte hypertrophy (m/f) thyroid follicular cell hypertrophy ( $3$ )	(2004) <u>M-085510-01-1</u>
		kidney hyaline droplet nephropathy ( $\Im$ )	

# Table 9: Summary table of animal studies for STOE RE



	1		
Method,	Test substance,	Results	Reference
guideline,	route of exposure,		
deviations if any,	dose levels, duration of	- target tissue/organ - critical effects at the LOAEL	
species, strain, sex, no/group	exposure	- critical effects at the LOALL	ST P
28-day oral	Fluopyram (99.4%	NOAEL (1000 ppm) ♂/♀: 24.7/31.1 mg/kg.bw/d	42004
(dietary)	purity)	LOAEL (1000 ppm) ♂/♀: 24.//31.1 mg/kg bw/d	(2004) M (2004)
Non-guideline	Batch No.: FLH	Target organ: liver	
Mouse (C57BL/6J)	1046	Mortality (5000 ppm (€∂,♀)	
5/sex/group	0, 150, 1000,	$\downarrow$ body weight gain (M $\swarrow$	
J/SCA/group	5000 ppm	↑ALT activity ( )	
	28 days	$\uparrow$ liver weight $(M/F)$	
		Henatocyte antrilohular hypertrophy single cell	
		and focal necrosis eosinophilia and bile duct/oval	
		↑liver weight (M/F) Hepatocyte centrilobular hypertrophy, single cell and focat necrosis, éosinophilia and bile cuct/oya cell hyperplasia(M/F)	X 200,44,5 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,647-1 M-05,8486,747-1 M-05,8486,747-1 M-05,8486,747-1 M-05,8486,747-1 M-05,8486,747-1 M-05,8486,747-1 M-05,8486,747-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1 M-05,847-1
28-day oral	Fluopyram (99.0%		
(gavage)	purity)	LQAEL & : 7504750 mg/kg bw/d 0 4	<u>M-242097-01-1</u>
Non-guideline	Batch No.: PFI	NOALL 3/20150/190 mg/kg-bw/d LQAEL 22: 750/750 mg/kg bw/d Garget organs: liver, bone marrow	
Dog (Beagle)	0304	Verythrocyte count, harmoglopin concentration,	
2/sex/group	0, 30, 150, 750	haematocrit (8),	
	mg/kg bw/d	$\mathcal{A}ALP(\mathcal{A}Q)$	
	28 days	₩GGT (♀) 𝔅 𝔅 𝔅 𝔅	0 [×]
		↓alb@min (AA) @ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~	×
		↑mgglycentes ( )	
		fliver reight (199,F) 0 [°] ( , , , , , , , , , , , , , , , , , ,	
		NOAEL $3/40150/190 \text{ mg/kg/bw/d}$ LQAEL $4/2$ : 750/750 mg/kg bw/d Verythrocyte count, haemoglobin concentration, haematocrit ( $3$ ), 4LP(3/2) GGT ( $4$ ) 4Bbunin (3/2) 1000  mg/kg 1000	
90-day oral	Eluopytam (990%	NOAEL: 72 (200 ppm) 212.5/14.6 mg/kg bw/d	(2005)
(dietary)			<u>M-250946-01-1</u>
OECD 408; 0	Batch No PFI	Targeoorgans; liver thyroid ridneys	
Directive O	<b>\$304</b>	↓body weight, food consumption (9,2)	
2001/59/EC Annex V Method	0, 50, 200, 1009,	maemogobin concentration (JPP), haematocrit	
B.26: US-EPA.	3200ppm		
OPPTS 870.3100;	990 days and a	L'chonésterony	
JMAFF (2000)	P s Q		
No deviations	\$ \$ E	615H, 193, 14	
Rat (Wistar)		$\uparrow$ lix $\bigcirc$ weigh C centri (bular hypertrophy, periportal/	
10/sex/group	Õ _Ž _S O _Ž	The provide the function $(0, \pm)$	
Q,		$\uparrow$ kidre weight billing dronlet nenbronathy ( $\langle , \pm \rangle$ )	
	4304 0, 50, 200, 1008, 3200 ppm 5 90 days 5 90		
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		Q A	
		Q X	
Å	A	<pre>icholesterol icholesterol icholesterol</pre>	
		\sim	
je and and a start and a start		•	
S R	A S		
	O X		
	L.		
Ô			



	-		D
Method,	Test substance,	Results	Reference
guideline, deviations if any,	route of exposure, dose levels,	- NOAEL/LOAEL - target tissue/organ	n° 🔊
species, strain,	duration of	- critical effects at the LOAEL	
sex, no/group			
90-day oral	Fluopyram (99.0%	NOAEL : $\frac{2}{\sqrt{2}}$ (150 ppm): 25 6/32 0 mg/kg. $\frac{1}{\sqrt{2}}$	2011
(dietary)	purity)	LOAEL: $3/2$ (1000 ppm): 23:0/52:0 mg/kg/gw/d	M 201126 AV 1
OECD 408; EC	Batch No.: PFI	Target organs: liver adrenals	
B.26; EPA	0304	$\uparrow AIT(3^\circ) \qquad (3^\circ)$	
870.3100; JMAFF	0, 30, 150, 1000	$ \operatorname{albumin}(\mathcal{Z} \circ) = 0$	
No deviations	ppm	\downarrow cholesterol (A)	
Mouse (C57BL/6J)	90 days	\uparrow ALP (\checkmark)	
10/sex/group	-		
		Advance/ two is to the first of	
		adrenarsweight (2) 87-92%	
		Liver: centrilophar nypertrophy, iocal necrosis	
		A translad arreid immental to the	
		Automation ()	K S
00 days1	Electrony (04 cold	NOAEL: ∂/φ (150 ppm): 25.6/32.0 mg/kg bw/d LOAEL: ∂/φ (1000 ppm): 188/216 mg/kg bw/d Target organs: liver, adrenals \uparrow ALT (∂, φ) \downarrow albumin (∂, φ) \downarrow cholesterol (∂) \uparrow ALP (∂) \uparrow liver weight (∂) 87-90% Liver: centrilobilar hybertrophy, focal hecrosto (∂, φ) Adrenals ceroid pigment (∂), \uparrow corrical vacuolation (φ)	
90-day oral (dietary)	purity)	100 32.2 100 32.3 100 10	Q(2000)
OECD 409 (1998);	Batch 082528/0002	LOAEL: 3/2 (5000 ppm) 171/189 mg/k&bw/d Target organ: liver	
EEC B.27 (2001);	0, 800, 5000	Target organ: liver ↓ bodg weight gain ↓ food consumption	&y
EPA OPPTS	20000/10000 ppm	Vbody weight gain V O O	0
	90 days	↓food consumption	
JMAFF (2000)	JU days 4	$\uparrow \mathcal{A}LP\left(\mathcal{A}^{\mathcal{A}}\right) \qquad \qquad$	
No deviations	90 days	Target organ: liver \downarrow body weight gain \downarrow food consumption \uparrow ALP ($\langle \xi + \rangle$) \uparrow ALG ($\partial_{\tau}, \xi + \rangle$) \downarrow \downarrow \downarrow \downarrow \downarrow \downarrow \downarrow \downarrow \downarrow \downarrow	
Dog (Beagle)			
4/sex/group		$\begin{array}{c} 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 $	
Ŭ		↓albumin (♂,2)	
ð de ser al s		\downarrow biktrubin ($\xi, \hat{\varphi}$) \Diamond	
Ô		tiver werght (∂,⊋)	
		Diver: hepatocyte hyperfrophy intracytoplasmic	
<u> </u>		$(\mathcal{O}, \mathcal{V})$	
12-month oral	Ouopyram (94,6%)	$ \begin{array}{c} \downarrow \text{bikmbin}\left(\zeta, \varphi\right) \\ \uparrow \text{liver weight}\left(\zeta, \varphi\right) \\ \hline Diver: hepatocyte hyperbrophy, intracytoplasmic cosinophilic dioplets, single Cll necrosis (σ, φ) \\ \hline \text{NOAEL: $$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$	(2007)
(dietary)	purity	LOAEL: %/\$\(\overline\$ (2000 ppm)): 67.6/66.1 mg/kg bw/d	<u>M-294279-01-1</u>
OECD 452; EEC	Batch 082528/0002	Target organ hiver	
B.30 (1992); EPA	00100, 400, 2000	↓bôdy weight (3,8)	
OPPTS 870.; JMAFF (2000)	ppm	41000d consumption ($3, 2$)	
No deviations	12 months	$(ALP(\mathcal{O}, \mathbb{Q}))$	
	S A 2	Liver centriobular hypertrophy $(\mathcal{J}, \mathcal{Q})$	
Dog (Beagle)			
4/sex/group			
28-day dermal	Fluopyran@94.6%		(2007)
OECD 410 (981)	(purity)	L@AEL: ♂/♀ 1000 mg/kg bw/d	<u>M-293833-01-1</u>
No deviations	Batch 082528 0002		
Rat (Wistar)	0, 100, 30001000	↑cholesterol ($\stackrel{\bigcirc}{+}$)	
10/sex/group	ntgykg bwyd Of weeks (5	\uparrow prothrombin time (\circlearrowleft)	
10/sex group	applications/week)	\uparrow liver weight (\eth, \bigcirc)	
Ű "Ô ^ÿ		Hepatocyte hypertrophy $(\mathcal{A}, \mathcal{Q})$	
\cup			



b) Summary of human data on STOT RE

No human data are available.

c) Summary of other studies relevant for STOT RE

A sub-chronic neurotoxicity study in the rat is also available, and is summarised in Table 10, Below

Table 10: Summary table of other studies relevant for STOT RE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	- target tissue/organ - critical effects at the LOAEL
Sub-chronic neurotoxicity (dietary) OECD 424 (1997); US EPA OPPTS 870.6200 (1998); JMAFF (2000)	0, 100, 300, 2300	NOAEL: \mathcal{R}_{2} (2500 ppm); 164.2/4 \mathcal{R}_{1} mg/kg bw/d LOAEL \mathcal{R}_{2} (2608) No evidence of neurotoxicity of neuropathology
No deviations Rat (Wistar) 11-12/sex/group		

d) Summary of STOT KE and Conclusion on classification

STOT-RE is assigned on the basis of findings of 'significant or 'severe' texicity, where effects are seen below guidance values of ≤ 10 mg/kg/bw/d (for classification in Category 1) of $\geq 10-100$ mg/kg bw/d (for classification in Category 2). The guidance values refer to effect seen in a 90-day rat study, and can be used as a basis to extrapolate equivalent guidance values for toxicite studies of greater or lesser duration.

For fluopyran, none of the relevant repeated dose toxicity studies opport LOAEL values below the Guidance Values (GVs) for classification in STOT RECategory 1 (i.e. \leq 30 mg/kg bw/d for 28-day oral toxicity studies; \leq 10 mg/kg bw/d for 28-day oral toxicity studies; \leq 2.5 mg/kg bw/d for 12-month oral toxicity studies; \leq 60 mg/kg bw/d for 28-day derma toxicity studies). Consequently, classification for STOT RE (Category 1) is not considered further.

The 28-day oral foxicity studies in the rat and mouse report LOAELs below the Guidance Value (GV) for classification in SOOT RE Category 2 2300 mg/kg 6w/d). The relevance of the findings in these studies for STOT RE classification is considered below

The 28-day oral toxicity study in the dog reports a LOAEL above the Guidance Value (GV) for classification in STOT RE Category 2 (\leq 300 mg/kg bw/d). This study is therefore not of relevance for STOT RE classification and is not considered further.

The 90-day oral toxicity study in the rat teports a LOAEL below the Guidance Value (GV) for classification is STOT RE Category 2 (≤ 100 mg/kg bw/d). The relevance of the findings in this study for STOT RE classification is considered below.

The LOARL's reported for the 90-day oral toxicity studies in the mouse and dog are above the GV of 100 mg/kg bw/d. These studies are therefore not of relevance for STOT RE classification and are not considered for ther

The 12-month oral toxicity study in the dog reports a LOAEL above the extrapolated GV of 25 mg/kg bw/d. This study is therefore not of relevance for STOT RE classification and is not considered further.

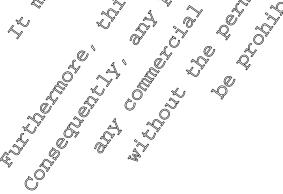


ð

The LOAEL reported for the 28-day dermal toxicity study in the rat is above the GV of 600 mg/kg bw/d. This study is therefore not of relevance for STOT RE classification and is not considered further.

Study	LOAEL	Guidance Value (GV)	Referen
Rat 28-day oral (dietary)	3200 ppm:	Cat 1 ≤30 mg/kg bw/d	(2004)
	3° 254 mg/kg bw/d	Cat $2 \leq 300 \text{ mg/kg bw/d}$	M-085510-02-1
	$\stackrel{\circ}{\downarrow}$ 263 mg/kg bw/d		
Mouse 28-day oral (dietary)	3200 ppm	Cat V≤30 mg/kg bw/d	2004) Ø
wouse 20 day of al (dietaly)	3200 ppm 3162 mg/kg bw/d	$\frac{\text{Cat} 2 \leq 300 \text{ mg/kg bw/d}}{\text{Cat} 2 \leq 300 \text{ mg/kg bw/d}}$	<u>M-058486-09-1</u>
	\bigcirc 102 mg/kg bw/d \bigcirc 197 mg/kg bw/d	[Rat GVs] Q Q	
D 0 0 1 1 ()	0		
Dog 28-day oral (gavage)	♂ 750 mg/kg bw/d Q	Cat 1 ≤30 mg/kg bw/d	(2004) (2004)
	\bigcirc 750 mg/kg bw/d	Cat $2 \le 306$ mg/kg 5 w/d (Cat $2 \le 306$ mg/kg 5 w/d (Rat Gxs)	<u>M-242097-61-1</u>
		(Rat Gys)	
Rat 90-day oral (dietary)	1000 ppm:	Cat $f \leq 10 \text{ mg/kg bw/d}$	2005)
	60.5 mg/g bw/d	$Cat/2 \leq 100 \text{ mg/kg/bw/d}$	<u>M-250946-01×1</u>
	\bigcirc 70.1 mg/kg bw/d		
Mouse 90-day oral (dietary)	1000,ppm; 🖓 📎	Cat 1≤10 mg/kg hw/d Cat 2≤100 mg/kg bw/d Cat 2<100 mg/kg bw/d Cat 2<10	(2011)
	් 188 mg/kg bw/ ඇ	Car2≤100 mg/kg bw/d O	<u>M-251136-01-1</u>
	₽216 mg/kg bw/d	Rat GWs]	\$
Dog 90-day oral (dietary)	ö 171 mg/kg w/d "	Cat 1 ≤10 mg/kg bw/d ू ⊘	(2006)
_ · · g / · · · · · / · · · · · (· · · · · / g / · · · · / g / · · · · / g / · · · ·	♀ 1 ®4 mg/kg bw/d	Cat $2 \leq 100 \text{ mg/kg/w/d}$	M-276047-01-1
		ØRat GX	
Dog 12-month oral (dictary)	2000 spm	Cat _k 1, ≤2.5 mg/kg bw/d	(2007)
	් 66 6 mg	$Cat 2 \leq 2.5$ mg/kg bw/d $Cat 2 \leq 2.5$ mg/kg bw/d	M-294279-01-1
\$. O	% 0 mg kg bw/d	AP at CH avtrage at at	<u>IVI-294279-01-1</u>
Rat 28-day deschal	$ \ge 1000 \text{ mg/kgebw/d} $	Cat = 60 mg/kg bw/d	(2007)
	\downarrow 5000 mg/kg bw/q.	Cats2 ≤600 mg/kg bw/d	<u>M-293833-01-1</u>
Rat 90-day neurotoxicity foral	>164 20mg/kg bw/d	Cat 1 ≤10 mg/kg bw/d	2008)
dietary v	₽₽ >197.1 mg/kg bw/d	$Cat 2 \leq 100 \text{ mg/kg bw/d}$	<u>M-299110-01-1</u>
) Summary of hu	man data on STOT R	RO ~	
No numan data are avariable			
c) Summary of of	ner studies pelevant fo	or SOFOT RE	
A sub-chronic neurotoxicity	story in the rat is also	vailable and is summarised in T	able 12 below
		7	,
W Or			
La AN			
	, v i		
	N Y		
		Cato, ≤2.3 mg/kg bw/d Cato, ≤2.3 mg/kg bw/d Rat GV, extrapolated Cato, ≤60 mg/kg bw/d Cato, ≥ ≤600 mg/kg bw/d Cato,	

Table 11•	Comparison of re	nested dose toxicit	v study LOAELs an	d CLP Guidance Values
Table II.	Comparison of re	μεαίευ μυχε ιυλιτιί	y sluuy LOAELS all	u CLI Guiuance values





Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference O
Sub-chronic neurotoxicity (dietary) OECD 424 (1997); US EPA OPPTS 870.6200 (1998); JMAFF (2000) No deviations Rat (Wistar) 11-12/sex/group	Fluopyram (94.7% purity) Batch 08525/0002 0, 100, 500, 2500 ppm 90 days	NOAEL: $3/2$ (2500 ppm):164.2/197.1 $\operatorname{ang/kg}$ bw/d LOAEL: $3/2$ (-) No evidence of neuroposicity or neuropathology	M-2995-10-01-6

Table 12:	Summary table of other studies relevant for STOT RE
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The 28-day oral toxicity study in the rat identifies effects on the liver (increased organ weight, hepatocyte hypertrophy) and thyroid (acreased organ weight, follieular hypertrophy) in both sexes, and additionally on the kidney (hyaline dropler nephropathy) in male rats at the LOAGL (equivalent to 254 and 263 mg/kg bw/d in male, and females, respectively). These effects are not regarded as of sufficient severity for classification. Furthermore, the renal effects (seen in male, rats only) are rat-specific and not relevant to humans. For the liver and thyroid effects, a rodent-specific mechanism can also be assumed based of evidence provided by the available comprehensive battery of mechanistic studies.

The 28-day oral toxicity study in the mouse identifies effects on the liver tincreased organ weight, hepatocyte centrilobular hypertrophy, single cell and focal necrosis, comophilia and bile duct/oval cell hyperplasia) at the OAEL (equivalent to 162 and 197 mg/kg bw/d in male and females, respectively). These effects are not regarded as of sufficient severity for classification and represent a rodent-specific mechanism (Section 19).

The 90-day oral toxicity study in the tat reports a COAFL of 1000 ppm (equivalent to 60.5 and 70.1 mg/kg bw/d in mates and remales respectively. Effects reported at the LOAEL of 1000 ppm in the 90-day rat study include a marginal, transient and non-significant reduction in body weight gain of 15% over the first study week. Mean food consumption by females in this group was also marginally (4-9%) and not significantly lower than controls. Findings indicate the red blood cell, liver, thyroid and kidney as target organs of fluopyram oxicity? Reductions in haemoglobin concentration (by 4% in both sexes) and hapmatocer (by do in males) were also seen; other erythrocyte parameters were not affected by treatment at this dose vel Findings at 6000 ppm are not of sufficient magnitude (<10%) to be considered as 'significant' of 'severe' toxicity, and are therefore not relevant for STOT RE classification. Bilitubin concentration was significantly reduced in both sexes (by 30% and 38% in males and females, respectively; however, the direction of change does not indicate an adverse effect of treatment. Serum cholester of concentrations were significantly increased in males (by 45%) and females (by 65%). Absolut Niver weight Overe significantly higher in males (+25%), as were liver weights relative to body weight (+20%) and relative to brain weight (+27%). Absolute (+24%), relative to body weight (+27%) and relative to brain weight (+25%) liver weights were also significantly higher in female? Necepsy revealed enlarged dark livers in both sexes, with prominent lobulation also noted for some males." Histopathologically, diffuse centrilobular hepatocyte hypertrophy (graded as minimal or slight) was observed in rats of both sexes; minimal periportal to mid-zonal hepatocyte vacuolation was also observed in females. Effects on the liver at 1000 ppm are consistent with adaptive change; adverse Ristopathological findings were not observed and changes in clinical chemistry parameters consistent with toxicity (significantly elevated GGT in females) were seen only at 3200 ppm. Findings at 1000 ppm do not represent 'significant' or 'severe' toxicity. Furthermore, liver effects in the rat are



shown to be due to an MoA not of relevance to humans and are therefore not relevant for STOT RE classification. Findings in the thyroid at 1000 ppm were limited to a non-significant increase in TSH at one time point, and an increased incidence of diffuse follicular in both sexes. These effects denot represent 'significant' or 'severe' toxicity, are shown to be due to an MoA not of relevance to manan (see Section 11), and are therefore not relevant for STOT RE classification. Kidney toxicity in this study was seen in males and was characterised by increased absolute and relative organ weight (25-30%), enlarged and/or pale kidneys. Histopathology showed basophilic tubules, hyaline droplets in the proximal tubules, granular casts in the medulla, and hyaline casts. Changes in clinical chemistry parameters (e.g. significantly increased creatinine (+11%) slightly increased urea (+6%), significantly increased inorganic phosphate (+9%), significantly increased calcium@+4%) and significantly reduced chloride (-3%) seen in males in this group are likely to reflect the kidney toxicity. Similarly the presence of cellular casts in the urine of males is also secondary to the kidney toxicity. The renal effects (increased organ weights, gross pathology, histopethology, changes in Africal Chemistry and urinalesis parameters) are attributable to a male rat-specific toxicity (2 µ-globulin pephropathy), and are not relevant to STOT RE classification.

In conclusion, therefore, although there are affects of treatment in some studies at dos Devels Below the relevant Guidance Values for STOT RE classification in Category 2, the effects are not relevant to humans and/or are not of sufficient sexerity to trigger classification Effects seen at higher door levels exceed the Guidance Values and are not there fore relevant for STOT RE classification

Fluopyram does not therefore require classification for STOT RE in any Category, according to the CLP criteria

Fluopyram was discussed at the 31st Meeting of the Committee for risk Assessment (RAC-31) on 4th December 2014. The CLH opinion adopted at RAC-31 concluded that no classification for STOT RE was required.

10. Gernocell mutagenicity

Genotoxieity tests in vitro a)

The results of in vitro genotoxicito studies with fluopy am are reported in Table 13, below.

Method,	Ost substance	Relevant information	Observations /Results	Reference
guideline, deviations if any		about the study including rationate for dose selection (as applicable)		
Ames test OECD 471 (1997); EEC B13/14 (2000); EPA OPPTS 870.5100 (1995); JMAFF (2000) No deviations	Fluopyram (94.7% purity) Batch 08528/0002	S. $tophimu(tam TA98, TA100, TA1535)$ FA153C TA162 All strains treated up to 5000 μ g/plate (+/-S9)	No cytotoxicity at any concentration (+/-S9) Precipitation: 1581 and 5000 µg/plate (+/-S9) Negative result in all strains tested (+/-S9)	(2006) <u>M-269978-01-1</u>
Amester OECD 471 (1997); ECC B13/14 (2000); EPA OPPTS 870.5100 (1998);	Fltopyram 95.7% purity) Batch 2007- 010986	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA102 All strains treated up to 1581 µg/plate (+/-S9); limited by cytotoxicity	Cytotoxicity at ≥500 µg/plate (+/-S9) No precipitation (+/-S9) Negative result in all strains tested (+/-S9)	(2008) M-298529-01-1

Table 13 Summary table of genotoxicity tests in vitro?



Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations /Results	Reference
No deviations				~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Chromosome aberration test OECD 473 (1997); EEC B.10 (2000); EPA OPPTS 870.5375 200 metaphases scored (current guideline states 300)	Fluopyram (94.7% purity) Batch 08528/0002	Chinese hamster V79 cells Concentrations limited by solubility and cytotoxicity 30, 60, 120, 180, 240 μ g/mL (4/18h) $+$ 89 120, 180, 240 μ g/mL (4/30h) +-/S9 60, 20, 180 μ g/mL (18/18 h) -89	Cytotoxicity at $\geq 120 \ \mu g/mL$ (-S9) Cytotoxicity at $\geq 180 \ \mu g/mL$ (+S9) Precipitation at $\geq 120 \ \mu g/mL$ (+/-S9) Negative resul(+/-S9)	(2005) <u>M-266906-01-1</u> Q Q D T D D D D D D D D D D D D D
Mammalian cell mutation test OECD 476 (1997); EEC B.17 (2000); EPA OPPTS 870.5300 (1998); JMAFF (2000) No deviations	Fluopyram (94.7% purity) Batch 08528/0002	Chinge hansser V79 cell (HPRT) Concentrations limited by solebility 4, 8, 46, 32, 64, 128 256 µg/mL (+/-S9)	Cytotoxicio at 256 µg/mL (\$9) No cytotoxicio (+S9) Precipitation at ≥128 µg/mU Negative tesult (+/-S9)	©1-268775-01-1 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

b) Genotoracity/mutagenicity tests in mammalian somation germ cells in vivo

One study of genetoxicit, mutagenicity in mammalian somatic cells *in vivo* is available and is reported in Table 14, below. No studies of genotoxicity mutagenicity in mammalian germ cells *in vivo* are available

Table 14: Summary table of genotoxicity/mutagenicity/tests in vivo

Method, guideline, deviations if any	Test substance	Relevant Anformation about the study (as applicable)	Observations/Results	Reference
Bone marrow micronucleus assay OECD 474 (1997); EEC B.12 (2000); US EPA OPPTS 870.5395 (1998); JMAEF (2000) 2000 PCEs scored (current guidefine states 4060)		250, 500, 1000 mg/kg bw 2 x ip mjection	No mortality Signs of toxicity (250, 500, 1000 mg/kg bw) NCE:PCE ratio was reduced in all treated groups (significantly at 1000 mg/kg bw) No increase in the proportion of MnPCEs in any group Bone marrow exposure demonstrated by signs of systemic toxicity, altered NCE:PCE ratio (also assumed due to the use of ip dosing)	(2005) <u>M-263710-02-1</u>



c) Human data relevant for germ cell mutagenicity

No human data are available.

d) Other studies relevant for germ cell mutagenicity

No other relevant studies are available.

e) Summary of germ cell mutagenicity and conclusion on classification

Fluopyram was shown to be negative in two Ames tests, a study of chromosomal aberration *invitro* and a study of mammalian cell mutation in vitro. A negative result is also shown *in vivo* in a mouse bone marrow micronucleus assay. No additional data are available. In the absence of any indication of genotoxicity/mutagenicity *in vitro* or somatic cell genotoxicity/mutagenicity *in vivo*, fluopyram does not required classification for germ cell mutagenicity according to the CLP criteria.

Fluopyram was discussed at the 31st Meeting of the Committee for fisk Assessment (RAC-31) of 4th December 2014. The CLH opinion adopted at RAC A concluded that no classification for germ cell mutagenicity was required.

11. Carcinogenicity

a) Animal studies on long-term toxicity and carcidogenicity

A combined chronic toxicity/catcinogenicity study in the rat and ocarcinogenicity study in the mouse are available for fluopyram, and are summarised in Table 11, below.

Table 15: Summary table of animal studies on long-term toxicity and carcinogenicity

	<u>`% { </u>		
	Test substance,	Results of the second s	Reference
deviations if any,	dose fevels	NOAEE/LOAEL	
species, strain, sex	durcation of	Z target tissue organ	
no/group	vexposure ² ~ ~	- critical effects at the LOAEL	
Combined chround	Fluopyram O	General toxicity	(2008)
toxicity/carcinogenicity	(94.5% purity)	NOAEL: 3/ \$0/30 ppm (1.20/1.68 mg/kg	M-298339-01-1
study (dietary)	Batch 🖉 👼	hw/d	<u>111 2) 0000 01 1</u>
OECD 403 (1981); EEC		LOAEL: 82 150 750 ppm (6.0/8.6 mg/kg	
	0 3°0×150 √750/	by (d) w	
OPPTS 870.4300 🗇	375 (from Week	Targe organs liver (372) , kidney (3) , thyroid	
(1998); JMAFF (2000)	\$5) ppp())	(3, 9), eye (9)	
No deviations	0, 30, 950, 1500	mortalio () 790 ppm	
$\mathbf{D} = \mathbf{I} \left(\mathbf{W}^{\dagger} = \mathbf{I} = \mathbf{W} \right) \otimes \mathbf{O}^{\dagger} \left(\mathbf{U}^{\dagger} = \mathbf{I} = \mathbf{U}^{\dagger} \right)$			
60/sex/group (24	° S' A	Liver toxicity $(3, 2)$ fliver weight, gross	
months)		changes, histopathology	
10/sex/group (12		Sephrapathy ()	
months) 🗸		Thyroid follicular hypertrophy $(3,2)$	
		Cateinogenicity	
L A		$\frac{Catefinogenerry}{\sqrt{2}}$	
		NOAEL: $3/2$ 750/150 ppm (29/8.6 mg/kg bw/d)	
	jo [®] _v ~9	LOAEL: (3/9 -/1500 ppm (-/89 mg/kg bw/d)	
		Increased incidences of hepatocyte adenoma and	
J & A		carcinoma in high dose females only	
	×2		
Kat (wistar) 60/sex/group (24 months) 10/sex/group (12 months) Cácinogeneity study (dietary) OECD 451 (1981): EEC	Fuopyram (94.5-	General toxicity	(2007)
(dietary)	94.7% purity)	NOAEL: ♂/♀ 30/30 ppm (4.2/5.3 mg/kg bw/d)	M-295688-01-1
OECD 451 (1981); EEC	Batch	LOAEL: (1/2 150/150 ppm (20.9/26.8 mg/kg	
B.32 (1987); EPA	08528/0002	bw/d)	



Method, guideline,	Test substance,	Results	Reference
deviations if any,	dose levels	- NOAEL/LOAEL	
species, strain, sex,	duration of	- target tissue/organ	
no/group	exposure	- critical effects at the LOAEL	
OPPTS 870.4200 (1998); JMAFF (2000) No deviations Mouse (C57BL/6J) 50/sex/group (78 weeks)	0, 30, 150, 750 ppm (♂,♀)	Target organs: liver, thyroid $(\mathcal{J}, \mathcal{Q})$, kidne $\mathfrak{H}(\mathcal{Q})$ Nephropathy (\mathcal{Q}) 750 ppm Thyroid follicular cell hyperplasia $(\mathcal{J}, \mathcal{Q})$ Centrilobular to panlobular hepatoeyte hypertrophy, single cell necrosis $(\mathcal{J}, \mathcal{Q})$ Thyroid gland follicular cell <u>Carcinogenieity</u> NOAEL: \mathcal{H} 150/750 ppm (20.9/129 mg/kg bw/d) \mathcal{H} LOAEL: \mathcal{J}/\mathcal{Q} 750/- ppm (105/- mg/kg/bw/d) The orcidence of thyroid follicular cell adenoma was increased in males only, at 750 ppm	

b) Human data on long-term toxicity and carcinogenicity

No human data are available.

c) Other studies relevant for long-term toxicity and care mogenteity

A number of mechanistic studies have been performed to characterise the mode of action (MoA) for both the increased incidence of liver tumours seen in high dose females in the rat study; and for the increased incidence of thyroid follocular tumours seen in high dose males in the modes study. Data are described in detail in a Position Paper (Waser, 2013; <u>M-465168-01-2</u>) and Expert Summary Report (Geter *et al*, 2013; <u>M-454439 (2-1</u>); the key points are summarised in Table 16 Delow.

Table 16:	Summary table of	f other studies	relevant to lo	ng-term toxi	city and carcinogenicity
	_ v v			V/92	_ / / / /

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08)
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Method, guideline,	Test substance,	Results	Reference
deviations if any, species, strain, sex, no/group	dose levels duration of exposure		
Non-guideline	Fluopyram	30 ppm: no effects	@2011)
mechanistic study	(94.7% purity)	75 ppm: $\uparrow cyp3a3$ expression (marginal)	<u>M-408029-01-01</u>
Rat (Wistar) ♀ 15/group	Batch 08528/0002	150 ppm: ^hepatocyte proliferation <i>cyp2B1</i>	
15/gloup	0, 30, 75, 150,	expression, $\uparrow cyp3a3$ expression, $\uparrow cyp1a1$ expression	
	600, 1500 ppm	600 ppm: ↑liver weight, ↑heparocyte	
	3 and 7 days	proliferation@lepatocyte hypertrophy,	Q Q A
		↑BROD/PROD activity, ↑CDPG5 activity,	
		$\uparrow cyp2b\mathcal{R}$ expression, $\uparrow cyp3a3$ expression,	
		$\uparrow cypt al expression, fidpgttQ/gstm4/Pphx1 expression.$	
		1500 ppm live@weight@hepatocyte	
		proliferation (marked) hepatoeste hyportrophy,	$(2011)_{C}$ M-408029-01C1 $(57)^{0}$
		TEROD/BROD/PROD activity, TUPPGT	
	LO ^Y	expression, $\uparrow cypHa1$ expression, $\uparrow cypHa5$	
		1udpgur2/gstin4/ephx4 expression.	°~γ
Non-guideline	Fluopyram	30 ppm: App 3a3 expression	(2012)
mechanistic study	(94.7% purity)	The man balanced liver the networks	<u>M-427431-01-1</u>
Rat (Wistar) \bigcirc	Batch 0* 08528/0002	proliferation	
15/group	0, 30, 49, 150, 9	150 ppm: The weight (slight), enterged over, The patocyce prolific ratio $f cyp 2bl$ expression,	
S.	600, 1500 ppm	\mathcal{G}_{cyp1a} \mathcal{G}	
	28 days	expression, PRODactivity, UDPOT activity	
	(with 14month	600 ppm: Diver weight, enlarged liver,	
ð S	recovery)	sepatocyte hypethrophy hepatocyte proliteration, f_{yp2b1} expression, f_{cyp1a1}	
, Q		expression <i>dupp dupp dupp</i>	
E.S.		expression EROD/BROD/PROD activity,	
		OUDPOT activity.	
	A	1500 ppm: 1 veight, enlarged liver,	
Ø,Č	R' S . C	hepatocyte hypertophy, ↑hepatocyte peoliferation (marked), ↑ <i>cyp2b1</i> expression,	
, Â, Ô		<i>cypl@l</i> expression,	
		1udpgtr2/gstm4/gsta2/ephx1 expression,	
		↑EROD/BROD/PROD activity, ↑UDPGT	
		1-month recovery	
Ű Ø »		1560 ppm, hepatic changes showed signs of	
A		reversibility	
Non-guideline	Flaopyram	Slight cytotoxicity at 100 μ M; more marked	(2013)
mechanistic study Cultured primaty	(98.7% purity) Batch	cytotoxicity at 300 μ M	<u>M-450156-01-1</u>
Lultured primaty A humati hepataeytes	EDFL03235	No increase in replicative DNA synthesis \uparrow PROD activity (1-100 μ M)	
	43, 10, 30, 100,	\uparrow BROD activity (3-300 μ M)	
× Č×	300 µM	\uparrow BQ activity (3-30 μ M)	
\bigcirc	96 hours		



Method, guideline,	Test substance,	Results	Reference
deviations if any,	dose levels		
species, strain, sex, no/group	duration of exposure		
Non-guideline	Fluopyram	Cytotoxicity at 300 µM	(2013)
mechanistic study	(98.7% purity)	\uparrow replicative DNA synthesis (1-100 μ M)	<u>M-450¥57-01 x</u>
Cultured primary rat hepatocytes	Batch EDFL03235	↑PROD activity (1-300 μM) ↑BROD activity (4-100 μM)	
	1, 3, 10, 30, 100, 300 μM	\uparrow BQ activity (1400 μ M)	
	96 hours	<u> </u>	<u>Q</u> <u>Ö</u>
Non-guideline mechanistic study	Fluopyram (94.7% purity)	No inhibition of TPO activity by the opyrain at any concentration	
Porcine thyroid	Batch	& g J L L	<u>M-299276-01-1</u>
microsomes	08528/0002 0, 3, 30, 300 μM	No inhibition of TPO activity by theory and at any concentration	
Non-guideline	Fluopyram	3 day 14 day	(2008)
mechanistic study	(94.7% purity)	111 ver weights (~1) %/~22%	<u>xx29952Q-01-1</u>
Mouse (C57BL/6J) 👌	Batch ^O	diffuse ceptrilobalar/pantobular hepatocyte	
15/group	08528/0002	hypertrophy a a a a a a a a a a a a a a a a a a a	
	2000 ppm	1 mitoses fr a fr	4
	3 and days s	Single cell necrosis	O`
		F14 (-30%/-27%)	
	× A	↑TS(\$*(+18%)+7%)	
*		<pre></pre>	
Ĩ		(CEROD activity (+116%/+165%)	
J. J		TBROD activity (+2890%/+2163%)	
		↑ PROD activity (+8717%+9061%)	
Non-guideline	Phenobarbital	3 days 24 days	(2008)
mechanisticostudy	(99.0 % pearty) 🛁		<u>M-299521-01-1</u>
Mouse (C\$7/BL/6J) ♀	Batch and 100228	henatocyte Wyperfilipphy	
15/group	80 mg/kg bw/day	AT4 (-f0%/-19%)	
	3 and 14 days	↑T3 @10%/-Ŵ)	
Q	3 and 14 days	↑tiver P45@rconte@r(+146%/+36%)	
		EROD activity (+297%/+375%)	
.1		BRSD activity (+4930%/+2844%)	
, and the second		↑₽₿ŎD activity (+1381%/+1345%)	
		BDPGT (no changes)	
Nonguideline	Etwopyramy &	↑clearance of ¹²⁵ I-thyroxine from the blood	(2008)
mechanistic study N	(94.7% purity)		M-308369-01-1
Mouse (C57BL(6J)	Batcle		
5/group	08528/0002	¢	
	2000 ppm	1	
	3 days		
Mouse (C57BL(bJ) 6 5/group	Batcher 08538/0002 2900 ppm 3 day		
* ^o o ^y *			
\bigcirc			



Method, guideline,	Test substance,	Results	Reference
deviations if any, species, strain, sex, no/group	dose levels duration of exposure		
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 10/group	Fluopyram (94.7% purity) Batch 08528/0002 2000 ppm 3 days	$\uparrow cyp1b1$ transcription $\uparrow cyp2b9$ transcription $\uparrow cyp3a11$ transcription $\uparrow sulfotransferase transcription\uparrow UDPGT transcription$	2008) M-308973-0457
Non-guideline mechanistic study Mouse (C57BL/6J) 8/group	Fluopyram (94.7% purity) Batch 08528/0002 2000 ppm 4 days	↑ clearance @ ¹²⁵ I-thyroxing from the blood	2008) 4 M-328662-01 4 ************************************
Non-guideline mechanistic study Mouse (C57BL/6J) 15/group	Fluopyram (94.7% purity) Batch 08528/0002 100, 300 mg/kg bw/d 3 days	$\downarrow T 4_{A}-26\% \downarrow$ $\uparrow T 4_{A}-26\% \downarrow$ $\uparrow T 4_{A}-26\% \downarrow$ $\uparrow T 4_{A}-26\% \downarrow$ $\downarrow T 4_{A}-26\% \downarrow$	(2011) 57408359-01-1 57 57 57 57 57 57 57 57 57 57
Non-guideline mechanistic study Mouse (C57BL/6J) 8 15/group	(04.70/60 urity)	$\downarrow T \bigoplus (-26\%)$	(2012) M- 426994-01-1



Method, guideline, deviations if any,	Test substance, dose levels	Results 30 ppm: \downarrow T4 (-28%) 75 ppm: \downarrow T4 (-31%) 1liver weight (+0%) 150 ppm: \downarrow T4 (-25%) \uparrow T4 (-35%) \uparrow T4 (-38%) \downarrow T4 (-38%)	Reference
species, strain, sex, no/group	duration of exposure		
Non-guideline	Fluopyram	30 ppm:	2012
mechanistic study Mouse (C57BL/6J) ♂	(94.7% purity) Batch	\downarrow T4 (-28%)	<u>M-428031-02-1</u>
15/group	08528/0002	75 ppm:	
15/group	0, 30, 75, 150,		
	300, 750 ppm	Thiver weight (+6%)	
	28 days	150 ppm:	
		↓T4 (-25%)	
		$ \begin{array}{c} \uparrow \text{liver weight (+11\%)} \\ \hline \\ \downarrow \\ \downarrow$	
		Three UDPG5 activity & &	
		300 ppm: 2 0 0 0	
		4 (-37%) γ γ γ γ γ γ	
	Ó	Tliver weight 427%	
	, Ó¥	The UDRGT activity	
		fortuitary tsh transcription of or or	
		750 ppm: 5 0 5 0 0	%
		T4 (-38%) & O	O [¥]
		Priver weight (166%)	
	N A	Thive UDPGC activity	
*		Tpututary and transer priors	
Non-guideline mechanistic study Mouse (C57BL/60)	Fluopyram	Thive ODP CT activity	(2012)
Mouse (C57BL/60)	Batch	the the labeling	<u>M-428303-01-1</u>
15/group	08528/0002		
	750 ppm		
<u></u>	🔊 days 🗸 🖉	↑p@uitary@h transcription Cnlarged/liver ↑ thycoid BrdQ labelong Fularged/liver Fularged/liver 9 thyroid BrdQ labelling at 150 ppm (1.21x),	
Non-guideline	Fluopyram	Enfarged Over *	(2013)
mechanistic study	(9407% purity)	Ithyroud Brdl Habelling at 150 ppm (1.21x),	<u>M-449821-03-1</u>
Mouse (C57BL/6L)	Hatch \mathcal{Q} \mathcal{Q}	600, mm (1.40x), 759 ppm (1.61x), 1500 ppm (2.33x), mmm (2.33x	
	0.3075.150	(2.35X). Percising and 28 days (1500 ppm)	
	600,750,300		
<u> </u>	ppm N OF		
	28 daýs		
J. A		Enlarged liver Thyroid BrdU labelting Finlarged Over Thyroid BrdU labelling at 150 ppm (1.21x), 600 ppm (1.40x), 759 ppm (1.61x), 1500 ppm (2:33x). reversible after 28 days (1500 ppm)	
le da	Ĩ.		
Č ^{O°}			
-			



Method, guideline, deviations if any, species, strain, sex,	Test substance, dose levels duration of	Results	Reference
no/group	exposure		
Non-guideline	Fluopyram	<u>WT mice / 750 ppm:</u>	<u>M-449890-0151</u>
mechanistic study	(94.7% purity)	Enlarged livers	<u>M-449890-0151</u>
Mouse (C57BL/6J) 👌	Batch	↑liver weight (+39-42%)	
15/group	08528/0002	Hepatocyte hypertrophy, single cell hecrosis	
Mouse CAR/PXR KO	0, 750, 1500 ppm	↑thyroid BrdU herelling (1.8x)	
	28 days	↑liver P450 content (3.6x)	
		↑PROD (79%), ↑BQ (5.5x), ↑T4-GT (1.8x)	
		$ \begin{array}{c} \uparrow \text{PROD} (70\%), \uparrow \text{BQ} (5.5x) \uparrow \text{T4-GT} (1.8x) \\ \uparrow \text{bilirubineCT} (1.8x) \\ \hline \end{array} $	
		↑pituitary tsh transcription (1,6x)	
		↑PROD (70%), ↑BQ (5.5x) ↑T4-GT (1.8x) ↑bilirubineGT (1.8x) ↑pituitary tsh transcription (1,6x) WTOnice / 1500 ppnr. Enlarged/pivers	
		Enlarged divers & Q	
		Enlarged/prvers Q Pliver weight (+39-4208)	$\begin{array}{c} (2013) \\ \underline{M-449890-0151} \\ \hline \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0$
		Hepatocyte hypertrophy, single celk necrosis	
	, Ô¥	ftbyroid BrdU labelling (@.6x) 5	
		Pliver weight (\Rightarrow 39-42 \otimes) Hepatocyte hypertrophy, single celk necrosis (hyroid BrdU labelling (2.6x) (1.9x) (1	
	Q X	↑PRØD (15%), ↑BØ (7.9x5 ↑T4-©r (1.9x9,	<u>k</u>
		Leilirubin-GT (2.0x)	O [×]
		The function of the function	
		Ibilirubin-GT (2.0x) Image: Comparison of the second sec	
*		1 liver weight by by the same	
Ĩ		buirubin-GT (2.0x) pituitary tsh transcription (1.7x) <u>CARPEXR KO mice 750 ppm</u> \uparrow liver weight \clubsuit PROED(1.4x) \clubsuit BQ (J.5x)	
J. J.		CARPXR KO mice 1500 pm	
		forer weight ~ _	
O S	~~~~	$\uparrow PROD(1.4x) \Rightarrow BQ (\overline{P.7}x), \neq 4-GT (1.3x),$	
No No	K K K	\downarrow pitultary tsh transception (\emptyset .2x)	
Non-gradeline	Fluor	$\Delta D = O $	(2013)
	(9407% putity)	BO activity up to 622.6% (30-750 ppm)	<u>M-451628-01-1</u>
Mouse (C57BL/6L)	Batch V		
15/group	0852840002 0	N 68 68	
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	0,, 30, 150, 600		
4	750 ppm	BQ activity up to 622.6% (30-750 ppm)	
Non guilta			(2020)
Non-guideline mechanistic study	Fluopyram (9453% put@ty)	Kat hepatocytes (10 - 100 μM) ↑ CYP 3A, ↑ CYP 1A,	(2020) M-759019-01-1
Primary cultures of	Batch 🖉 🚬	↑ UGT 2B1, ↑UGT-T4	<u>1v1-757017-01-1</u>
cryopreserved male	08528,0002	Human hepatocytes (10 - 100 $\mu$ M)	
human and Wistar rat		$\square$ CYP 1A2, $\uparrow$ CYP 2B6, $\uparrow$ CYP 3A4	
hepatocytes	HOF L ~C	↑ UGT 1A1	
	HOF ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	No activation of UGT-T4	
	A A A A A A A A A A A A A A A A A A A		
human and Westar rat hepatocytes			



#### d) Summary of carcinogenicity and proposal for classification

Chronic administration of fluopyram caused liver tumours in high dose females in the rat oral chronic toxicity/carcinogenicity study. Chronic administration of fluopyram caused thyroid tumours in high dose males in the mouse oral carcinogenicity study.

The 28-day rat study showed the induction of cytochrome P450 (increased total hepatic cytochrome P450 content, increased BROD and PROD activities) consistent with a phenobarbital-like MoA. Further. mechanistic studies performed in female rats for durations of 3, 7 or 28 days, demonstrate that fluopyram? at the dose levels tested in the carcinogenicity study recolled in the induction of synochrome P450 content; increased cytochrome P450 (principally PROD and BROD) and UDPGT isoenzyme activities and corresponding changes in gene expression, hepatocyto proliferation and associated histopathological change. Findings were reversible and were similar to those induced by phenobarbital A study performed in CAR/PXR wild-type (WT) and knock-out (KO) mice howed significant liver enlargement, hepatocyte hypertrophy, and liver enzyme induction in MT but pot in KO mice, indicating that activation of CAR/PXR is the initiating event in the rodent for liver tumours of the rat (and thyroid tumours in the mouse). An in vitro comparative study examining the proliferative response in rat and human primary hepatocytes showed a clear concentration-related increase in proliferation in rat but hot in human cells in response to fluopyrap and phenobarbital . Overall, the mechanistic studies together with the standard repeated dose toxicity studies clearly demonstrate that the MoA for the rat liver tumours following chronic exposure to fluoryramys via activation of the CARPXR ouclear receptors. Furthermore, the lack of proliferation in primary human hepatocytes (compared to tat hepatocytes), an essential key step in the formation of liver minours via the postalated MoA provides convincing evidence that the liver tumours seen in the rat are non-relevant to a man and the man are non-relevant to a man and the man are non-relevant to a man and the man are non-relevant to a man are non-re been excluded. Consequently, the MoA demonstrated to be responsible for fiver tumour formation in the female rat following chronic exposure to high dose levels of fluepyram in the fewant to humans.

Thyroid effects seen in male mice, were associated with marked liver effects and can therefore be assumed to be secondary to the induction of liver enzymes, specifically UDPGT. This CAR/PXR initiated MoA is further supported by animber of mechanistic studies. Administration of fluopyram to male mice resulted in cytochrome P450 induction (increased BRCD/PROD activities), reduced T4 and increased TSH evels, and a more rapid coarance of T4. The invotal CAR/PXR wild-type (WT) and knock-out (KO) mice study showed significant liver enlargement, hepatocyte hypertrophy, and liver enzyme induction in WK but new in KO mice, this study prevides compelling evidence for CAR/PXR induction being the initiating event for the MoA that eventually results in the formation of a low incidence of thyroid adenomas in the male mouse at the high dose in the cancer bioassay. Further supportive evidence is provided in an *in vitro* comparative study examining CYP and UGT induction in human and Wistar rat hepatocytes with fluopyram Phased and II liver enzymes were induced in both species, but critically OGT-T4 was induced in rat hepatocytes but not in human hepatocytes. This MoA (increased TSH secondary of liver enzyme induction causing increased T4 clearance, resulting in thyroid follicular cell hyperplasia and carcinogenesis) is not of relevance to humans. Fluopyram was shown not to be anothibitor of thyroid peroxidase (TPO) activity, thereby discounting an alternative direct MoA.

Comprehensive mechanistic data are available, which elucidate the MoA and demonstrate that neither tumour type is of relevance to lumans. Classification of fluopyram for carcinogenicity is therefore not triggered on the basis of the liver tumours seen in female rats. Similarly, classification of fluopyram for carcinogenicity is not triggered on the basis of the thyroid tumours seen in male mice. In the absence of any carcinogenicity of relevance to humans, fluopyram does not require classification for carcinogenicity in any category, according to the CLP criteria.

Fluopyram was discussed at the 31st Meeting of the Committee for risk Assessment (RAC-31) on 4th December 2014. The CLH opinion adopted at RAC-31 concluded that no classification for carcinogenicity in any category was required.



#### 12. **Reproductive toxicity (sexual function and fertility)**

#### a) Animal studies on adverse effects on sexual function and fertility

One study of reproductive toxicity is available and is summarised in Table 17, below.

# Table 17: Summary table of animal studies on adverse effects on sexual function and fertilit

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure		Reference
2-generation reproductive toxicity study (oral) OECD 416 (2001) EPA OPPTS 870.3800 (1998) JMAFF (2000) No deviations Rat (Wistar) 30/sex/group		↓body weight gain ( ) during pre-mating, gestation ↓hæmoglöbin, haematocrit ( ) Kreney toxicity and clinical chemistry changes ( ) Niver weights, centrilobular hypertraphy ( ) ( ) Offspring toxicity NØAEL (220 ppm): 14.5/mg/kg/bw/d LOAEU (1200 ppm): 82.8 mg/kg bw/d ↓weight gato Fb Delayed sexual maturation, secondary to body weight ( ) Reproductive toxicity NOAEL / ( 1200/1200 ppm): 82.8/96 9 mg/kg bw/d	

## b) Human data on adverse effects on sexual function and fertility

No human data are available

# c) Other studies refevant for toxicity of sexual function and fertility

Studies of repeated dose toxicity with floopyrate (sumparised in Section 9, above) do not indicate any effects of potential relevance to sexual function and tertility.

# d) Summary of sexual function and for tility and conclusion on classification

There was no evidence of period vertice toxicity in the two-generation oral reproductive toxicity study in the rat. Studies of repeated dose toxicity dy not indicate any effects of potential relevance to sexual function and fertility. No human data are available. Fluopyram does not therefore require classification for reproductive toxicity (effects on sexual function and fertility) in any category according to the CLP criteria.

Fluopytam was discussed at the 31st Meeting of the Committee for risk Assessment (RAC-31) on 4th December 2014. The CLF opinion adopted at RAC-31 concluded that no classification for reproductive toxicity (offects on sexual function and fertility) was required.

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## 13. Reproductive toxicity (adverse effects on development)

#### a) Animal studies on adverse effects on development

Studies of prenatal developmental toxicity in the rat and rabbit are available, and are summarised in Table 18, below.

# Table 18: Summary table of animal studies on adverse effects on development

		4	
Method, guideline, deviations if any, species, strain, sex, no/group	•	Results - NOAEL/LOACL (for parent and for developmental effects) - target tissuc/organ - critical effects at the LOAEL Materna foxicity	Reference 2
Pre-natal developmental toxicity study (oral) OECD 414 (2001); EPA OPPTS 870.3700 (1998); JMAFF (2000) No deviations Rat (Sprague- Dawley) 23♀/group		NOAEL: 30 ang/kg bw/d LOAEL: 100 mg/kg bw/d body weight gain food consumption hiver weight Centrilobular hepatocyte hypertrophy Developmental toxicity: NOAEL: 150 mg/kg bw/d KoAEL: 450 mg/kg bw/d foetar weight (25%) visceral variations (thymus remnant, covoluted dilated ureter skeletar variations (bipartite thoracic centrum)	(2008) M-299438-01
Pre-natal developmental toxicity study (pal) OECD 414 (2001); EPA OPRTS 870.3700 (1998); JMAFF (2000) No deviations Rabbit (NZW) 23 \2/group	Fluopfram (94.6% 2) purity) Barch Ng 0852800002 0, 10, 25, 75 mg/kg bw/dc GD 6-28	Maternal toxocity NOAEL:23 mg/kg bw/d UOAEL:75 mg/kg bw/d Ubody/weight/gain tood consumption Developmental toxicity	(2006) M-299773-01

## b) Aluman data on accerse effects on development toxicity

No human data are available.

# c) V Other studies relevant for developmental toxicity

No other relevant studies are available

# d) Simmary of developmental toxicity and proposal for classification

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Studies of developmental toxicity in the rat and rabbit do not indicate any specific developmental toxicity or fluopyram. In the at study, slightly reduced foetal weight and slightly increased incidences of visceral variations (theracic centrum split/split cartilage (only one thoracic centrum affected per animal, would not affected long-term functionality), thoracic centrum: dumbbell and/or bipartite thoracic centrum / normal cartilage). Findings were associated with maternal These findings are not of sufficient severity to be of relevance to classification. In the rabbit study, developmental effects were limited to slightly reduced



foetal weight observed at a maternally toxic dose level. This finding is not of relevance to classification. No human data are available. Fluopyram does not therefore require classification for reproductive toxicity (adverse effects on development) in any category according to the CLP criteria.

#### 14. **Reproductive toxicity (effects on or via lactation)**

a) Animal studies on effects on or via lactation
No specific animal studies are available. The oral 2-generation reproductive toxicity study in rate (summarised above) assessed effects on or via lactation.
b) Human data on effects on or via lactation
b) Human data on effects on or via lactation
No human data are available.
c) Other studies relevant for effects on or via lactation
No other relevant studies are available.
d) Summary of effects on or via lactation and conclusion on classification
There is no evidence for any effects of fluopytan on or via lactation. Summary of effects on or via lactation and conclusion on classification evidence for any effects of fluopyram on or via lactation. Reduced vo-generation oral reproductive toxicity study in the to) maternal toxicity. Fluopyram d) Summary of effects on or vial actation and conclusion on classification is the two-generation oral reproductive toxicity study in the advasses or classification reproductive toxicity (effects on or vial actation according to the CLP criteria.