Document Title

1st amendment of

Summary of the toxicological and metabolism studies for fluopyram

Data Requirement(s)

Regulation (EC) No 1107/2009 & Regulation (EU) No 283/2013

Document MCA

Section 5: Toxicological and metabolism studies – Part 2

According to the Guidance Document SANCO/10181/2013 for applicants on preparing dossiers for the approval of a chemical active substance

Date

2021-07-14

Author(s)

[redacted]

Bayer AG,
Crop Science Division

OWNERSHIP STATEMENT

This document, the data contained in it and copyright therein are owned by Bayer AG and/or affiliated entities. No part of the document or any information contained therein may be disclosed to any third party without the prior written authorisation of Bayer AG and/or affiliated entities.
The summaries and evaluations contained in this document are based on unpublished proprietary data submitted for the purpose of the assessment undertaken by the regulatory authority. Other registration authorities should not grant, amend, or renew a registration on the basis of the summaries and evaluation of unpublished proprietary data contained in this document unless they have received the data on which the summaries and evaluation are based, either:

- from Bayer AG or respective affiliate; or
- from other applicants once the period of data protection has expired.

Version history

Date [yyyy-mm-dd]	Data points containing amendments or additions[1] and brief description	Document identifier and version number
2021-04-20	Original document as submitted by the applicant	M-766370-02-1
2021-07-14	Update of section CA 5.8.1 to include toxicological data on the metabolite TFA	M-766370-03-1

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4, 'How to revise an Assessment Report'.

Table of Contents

CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

This document provides detailed summaries of metabolism and toxicological studies.

This document supports the application for regulatory approval of fluopyram in Europe under Regulation (EC) No 1107/2009.

Substance coding

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

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

Studies are summarized in Fluopyram Active Substance, MCA Section 5, Part 1, Toxicology and metabolism studies

CA 5.2 Acute toxicity

Table 5.2-1 Summary of Fluopyram (AE C656948) acute toxicity data, with classification endpoints according to Regulation (EC) No 1272/2008

Study	Result	Reference	Classification according to Reg. (EC) No 1272/2008
Acute oral rat	No mortalities observed at 2000 mg/kg bw	[redacted] 2005; M-259398-01-1	Unclassified
Acute dermal rat	Dermal LD_{50} > 2000 mg/kg bw	[redacted] 2005; M-259275-01-1	Unclassified
Acute inhalation rat	Inhalation LC_{50} at 4 hours > 5112 mg/m^3	[redacted] 2006; M-283420-01-1	Unclassified
Skin irritation, rabbit	Non-irritating	[redacted] 2005; M-263302-01-1	Unclassified
Eye irritation, rabbit	Non-irritating	[redacted] 2005; M-263277-01-1	Unclassified
Skin sensitization (LLNA)	Non-sensitizing	[redacted] 2006; M-281845-01-1	Unclassified

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 in accordance with prevailing OECD, EU, USEPA or Japanese MAFF testing guidelines.

The acute toxicity of fluopyram (AE C656948) was low for all routes evaluated (oral, dermal and inhalational). The oral LD_{50} was > 2000 mg/kg body weight as no mortality, clinical signs or abnormalities at necropsy were reported. The rat acute dermal LD_{50} was > 2000 mg/kg body weight, with no mortality or clinical signs observed. The rat acute inhalation LC_{50} (4-hour) was > 5112 mg/m^3, which was the highest achievable concentration and did not cause mortality. Clinical signs (reversible) included bradypnea, laboured 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:	[redacted]
Report Year:	2005
Report Title:	AE C656948 - Acute toxicity in the rat after oral administration
Report No:	AT02530
Document No:	M-259398-01-1
Guideline(s) followed in study:	OECD 423 (2001); EEC 67/548 Annex V - Method B.1. tris; EPA 40 CFR part 160: OPPTS 870.1100; MAFF 12 Nousan n° 8628 (Dec. 06, 2000); EPA 712-C 98-190 (1998)
Deviations from current test guideline:	current guideline: Current Guideline: OECD 423, 2001 Deviations: None
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an acute oral toxicity study using a stepwise procedure, two groups of three fasted, young adult female Wistar rats (HsdCpb:Wu) were given successively a single oral dose of AE C656948 (batch number Mix-batch:08528/0002, 94.7% purity) in 2% Cremophor EL of 2000 mg/kg bw and were observed for 14 days.

The dose of 2000 mg/kg bw was tolerated without mortalities, clinical signs, effects on weight gain or gross pathological findings.

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

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Beige powder
Lot / Batch #:	Mix-batch:08528/0002
Purity:	94.7 %
CAS #	658066-35-4
Stability of test compound:	Stable at 1 and 200 mg/mL at room temperature for at least 2 hours
2. Vehicle and / or positive control:	2% Cremophor EL in demineralized water
3. Test animals:	
Species:	Rat
Strain:	HsdCpb:Wu
Age:	10 - 12 weeks approximately
Sex:	Female
Weight at dosing:	170 - 195g
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Provimi Kliba 3883.0.15 Maus/Ratte Haltung, Kaiseraugst Switzerland, *ad libitum*
Water:	Tap water, *ad libitum*

Housing:	Animals were group caged conventionally in polycarbonate cages on low dust wood granulate bedding
Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	55 ± 5%
Air changes:	Approximately 10 changes per hour
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 rats. The animals were assigned to their groups by randomization. The random list was based on evenly distributed chance numbers by a software application. Following an overnight fast (16 to 24 hours), each group received a single dose of 2000 mg/kg of AE C656948 (94.7% purity) by gavage. The test substance was administered in demineralized water with 2% Cremophor EL at a volume of 10 mL/kg bw. Clinical 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 15. On day 15, surviving animals were sacrificed and all animals were necropsied and examined for gross pathological changes.

3. Statistics

The data did not warrant statistical analysis.

II. Results and discussion

A. Mortality

Details are provided in Table 5.2.1-1. No mortalities occurred at 2000 mg/kg bw, the only dose tested.

Table 5.2.1-1 Doses, mortality / animals treated

Dose (mg/kg bw)	Females
2000 (1st)	0/3
2000 (2nd)	0/3

B. Clinical observations

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

C. Body weight

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

D. Necropsy

No abnormalities were observed at gross necropsy.

E. Deficiencies

No deficiencies are noted.

III. Conclusions

Under the conditions of this study, the acute oral LD_{50} was > 2000 mg/kg bw (Unclassified 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 oral route is low in the rat. The LD_{50} > 2000 mg/kg bw does not trigger classification.

CA 5.2.2 Dermal

Data Point:	KCA 5.2.2/01
Report Author:	[redacted]
Report Year:	2005
Report Title:	AE C656948 - Acute toxicity in the rat after dermal application
Report No:	AT02500
Document No:	M-259275-01-1
Guideline(s) followed in study:	OECD 402 (1987); EEC 67/548 Annex V- Method B.3; EPA OPPTS 870.1200, EPA 712-C-98-192 (1998)
Deviations from current test guideline:	current guideline: OECD 402, 2017 Old procedure: 5 animals/sex/dose treated once, rather than testing 3 animals of single sex in a stepwise approach described in the with the use of up to 3 animals of a single sex per step. 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).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an acute dermal toxicity study, groups of young adult Wistar rats, 5/sex were exposed by the dermal route to AE C656948 (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.

The dermal LD50 for the males was > 2000 mg/kg bw
for the females was > 2000 mg/kg bw
for the combined sexes was > 2000 mg/kg bw

AE C656948 was of very low 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 C656948 does not warrant classification for acute dermal toxicity according to Regulation (EC) No. 1272/2008.

I. Materials and methods

A. Materials

1. **Test material:** AE C656948
   Description Beige powder
   Lot / Batch #: Mix-batch:08528/0002
   Purity: 94.7%
   CAS # 658066-35-4
   Stability of test compound: Stable at room temperature for the duration of the study.

2. Vehicle and / or positive control:	Pure solid test material was transferred to wet gauze-layer
3. Test animals:	
Species:	Rat
Strain:	HsdCpb:Wu
Sex	Male and females
Age:	9-13 weeks approximately
Weight at dosing:	Males: 228-259g Females: 212-222g
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Provimi Kliba 3883.0.15 maus/Ratte Haltung, Kaiseraugst Switzerland, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were group caged conventionally in polycarbonate cages on low dust wood granulate bedding
Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	55 ± 5%
Air changes:	10 per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. Study design and methods

1. In life dates: 14 July 2005 -28 July 2005

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 back and 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 gauze-layer (6.0 cm x 5.0 cm) of a "Cutiplast® steril" coated with air-tight "Leukoflex®. The gauze strip was placed on the rat's back and secured in place using "Peha® -haft" cohesive stretch tape (8 cm x 23 cm) and additionally covered with "Lomir biomedical Inc rat jacket". After 24 hours the dressing was removed and the area rinsed 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 sacrificed by carbon dioxide at the end of the study and examined macroscopically.

3. Statistics

The data did not warrant statistical analysis.

II. Results and discussion

A. Mortality

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

Table 5.2.2-1 Doses, mortality / animals treated

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

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.

III. Conclusions

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 being toxic or harmful on the basis of its acute dermal 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 dermal route is low in the rats. The LD_{50} > 2000 mg/kg bw does not trigger classification

CA 5.2.3 Inhalation

Data Point:	KCA 5.2.3/01
Report Author:	[redacted]
Report Year:	2006
Report Title:	AE C656948 - Acute inhalation toxicity in rats
Report No:	AT03464
Document No:	M-283420-01-1
Guideline(s) followed in study:	OECD 403; Directive 92/69/EEC, Annex V, Method B.2.(1992);, US-EPA OPPTS 870.1300 (1998); Japan MAFF Notification no. 12 Nousan-8147 (2000)
Deviations from current test guideline:	current guideline: OECD 403 (2009) Deviation: Traditional LC 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).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an acute inhalation 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/m^3. 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 mg/m^3

The inhalation LC50 for the females was > 5112.5 mg/m^3

The inhalation LC50 for the combined sexes was > 5112.5 mg/m^3

AE C656948 (solid aerosol) proved to be non-toxic via the inhalation route to rats. No mortality occurred 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 sexes 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 harmful or toxic by inhalation according to Regulation (EC) No. 1272/2008.

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Beige powder
Lot / Batch #:	Mix-batch:08528/0002
Purity:	94.7 %
CAS #	658066-35-4
Stability of test compound:	Stable until 5 April 2007 at room temperature
2. Vehicle and / or positive control:	The test substance was aerosolized as dry powder
3. Test animals:	
Species:	Rat
Strain:	Hsd:Cp:Wu
Age:	Approximately 2 months
Weight at dosing:	170-196g (male) or 169-191g (female)
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Provimi Kliba 3883 = NAFAG 9441 pellets maintenance diet for rats and mice, Kaiseraugst Switzerland, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were individually caged in conventional Makrolon® Type III cages.
Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	50 ± 10%
Air changes:	At least 10 air exchanges per hour
Photoperiod:	12 hours of light, 12 hours of dark

B. Study design and methods

1. In life dates: 08 May -20 May 2006

2. Animal 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 (finger 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

N Group /sex	Target Concentration (mg/m³)	Toxicological Result	Onset and Duration of Signs	Onset of Mortality	Rectal Temperature (°C)
1/m	0	0 / 0 / 5	--	--	38.3
2/m	5000	0 / 5 / 5	0d - 1d	--	36.1
1/f	0	0 / 0 / 5	--	--	38.4
2/f	5000	0 / 5 / 5	0d - 5d	--	34.3

N = group assignment, m = males, f = females, 0d= exposure day
Values given in the 'Toxicological results' column are:
1st = number of dead animals.
2nd = number of animals with signs after cessation of exposure.
3rd = number of animals exposed.

3. Generation of the test atmosphere / chamber description

Directed-flow nose-only inhalation chambers (TSE, 61348 Bad Homburg) 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 cascade impactor.

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

4. Statistics

A one-way ANOVA (vide infra) was used to analyze body weight gain data and rectal temperature measurements.

II. Results and discussion

A. Test atmosphere concentration and particle size analysis

The real-time aerosol monitoring (filter analyses) 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 inhalable. Internationally recognized recommendations such as of SOT (1992) were not fulfilled in regard to the requirement MMAD < 4 µm. 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. Humidity values were slightly lower due to dry air aerosolization. This deviation from the guideline had no apparent negative impact on the outcome of study.

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 (% < 4 µm) for each treatment group

Parameter	Control group	Tested group
Target conc., mg/m³	0	5000
Gravimetric conc., mg/m³	-	5112.5
Inlet air flow (L/min)	15	28
Exhaust air flow (L/min)	13	24
Temperature (mean, °C)	22.8	21.8
Relative humidity (mean, %)	6.2	32.1
MMAD, µm	-	5.60
GSD	-	2.02
Aerosol mass <3 µm (%)	-	19.0
Mass recovered (mg/m3)		4062.5

B. Mortality

No mortality occurred up to 5112.5 mg/m³, the maximum technically achievable concentration. The 4 hour inhalation LC_{50} for the males was > 5112.5 mg/m³, for the females was > 5112.5 mg/m³, and for the combined sexes was > 5112.5 mg/m³.

C. Clinical observations

All rats tolerated the exposure with some evidence of reversible signs. Clinical signs in both sexes exposed to AE C656948 included: bradypnea, labored breathing patterns, reduced motility, pilo-erection, ungroomed hair coat and limpness.

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 tonus and vertical grip strength together with an impaired righting response. Rectal temperature was lowered in both sexes with the treatment.

All clinical signs were fully reversible within 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 necropsy.

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 low in the rat. The LC_{50} > 5112.5 mg/m³ does not trigger classification.

CA 5.2.4 Skin irritation

Data Point:	KCA 5.2.4/01
Report Author:	[redacted]
Report Year:	2005
Report Title:	Acute skin irritation/corrosion on rabbits
Report No:	AT02737
Document No:	M-263302-01-1
Guideline(s) followed in study:	OECD 404 (2002); EEC Directive 67/548 Annex V - Method B.4 (1967); EPA 40 CFR part 160; 712-C-98-196 (1998); OPPTS 870.2500; MAFF 12 Nousan No 8628 (December 06, 2000)
Deviations from current test guideline:	current guideline: Current Guideline: OECD 404, 2015 Deviations: None. Conduct of study predates the in vitro screening recommendations.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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 pulverized 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 removed after three minutes. As no serious skin reactions were observed, the second patch was removed after one hour and then the third patch applied 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, AE C656948 was not a dermal irritant and does not warrant classification as being irritating to the skin according to Regulation (EC) No. 1272/2008.

I. Materials and methods

A. Materials

1. **Test material:** AE C656948
   Description Beige powder
   Lot / Batch #: mix-Batch:08528/0002
   Purity: 94.7%
   CAS # 658066-35-4
   Stability of test compound: Until 04 May 2007
2. **Vehicle and / or positive control:** Aqua p.i to ensure good contact with skin
3. **Test animals:**
   Species: Rabbit
   Strain: HsdIf:NZW - females
   Age: Young adult animals
   Weight at dosing: 2.2 – 2.6 kg
   Source: [redacted]
   Acclimation period: At least 5 days

Diet:	Ssniff K-Z" 4mm (manufacturer: Ssniff Spezialdiaten GmbH, 59494 Soest, Germany), *ad libitum*
Water:	Tap water *ad libitum*
Housing:	Individually in cage units Metall/Noryl by EBECO
Environmental conditions:	
Temperature:	20 ± 3°C
Humidity:	50 ± 25%
Air changes:	not provided in the report
Photoperiod:	12 hours daily

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 C656948, Batch No. mix-Batch:08528/0002; 94.7% 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 2.5 cm. The patch was held in place with non-irritating tape for the duration of exposure period.

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 observation of reactions after one time-point before moving to a longer duration of exposure. Then, the test was completed with 2 additional animals.

Dermal irritation was scored approximately at 1, 24, 48 and 72 hours after patch removal. In the case of an irritation reaction, animals were further monitored for reversibility of the effect on day 7 and day 14 (maximum) after patch removal. The degree of erythema/eschar formation and oedema formation was recorded and scored according to the Draize scheme. Any serious lesions or toxic effects other than dermal irritation were also recorded and described.

3. Statistics

Statistical analysis of the results was not required.

II. Results and discussion

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.

Table 5.2.4-1 Individual values for skin irritation in three rabbits administered AE C656948 for four hours

Animal	Observation	24h	48h	72h	Mean scores	Response	Reversible (days)
1	Erythema and eschar	0	0	0	0.0	-	N/A
	Edema	0	0	0	0.0	-	N/A
2	Erythema and eschar	0	0	0	0.0	-	N/A
	Edema	0	0	0	0.0	-	N/A
3	Erythema and eschar	0	0	0	0.0	-	N/A
	Edema	0	0	0	0.0	-	N/A

N/A: 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.

Assessment and conclusion by applicant:
Study meets the current guidance and the requirements in 283/2013. Based on those results, it was concluded that fluopyram was not an irritant to the skin.

CA 5.2.5 Eye irritation

Data Point:	KCA 5.2.5/01
Report Author:	[redacted]
Report Year:	2005
Report Title:	AE C656948 - Acute eye irritation on rabbits
Report No:	AT02738
Document No:	M-263277-01-1
Guideline(s) followed in study:	OECD 405 (2002); EEC Directive 67/548 Annex V - Method B.5. (1967); EPA 40 CFR part 160, 712 C-98-195 (1998); OPPTS 870.2400; MAFF 12 Nousan No 8628 (December 06, 2000)
Deviations from current test guideline:	current guideline: Current Guideline: OECD 405, 2020 Deviations: None, Conduct of study predates the *in vitro* screening recommendations.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a primary eye irritation study, 0.1 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 1, 24, 48 and 72 hours after application. As no irritation indices were observed after 72 hours, the study was finished.

The degree of ocular lesions 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-treatment). According to the classification criteria AE C656948 was not irritating to the eye and there were no systemic intolerance reactions.

I. Materials and methods

A. Materials

1. **Test material:** AE C656948
   Description Beige powder

Lot / Batch #:	Mix-Batch:08528/0002
Purity:	94.7%
CAS #	658066-35-4
Stability of test compound:	Until 04 May 2005
2. Vehicle and / or positive control:	none
3. Test animals:	
Species:	Rabbit
Strain:	Crl:KBL(NZW)BR (female)
Age:	Young adult animals
Weight at dosing:	2.2 -2.8 kg
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Standard diet "Ssniff K-Z 4mm (manufacturer: Ssniff Spezialdiaten GmbH, 59494 Soest, Germany) *ad libitum*
Water:	Municipal tap water *ad libitum*
Housing:	Individually in cage units Metall/Noryl by EBECO
Environmental conditions:	
Temperature:	30 ± 3°C
Humidity:	30± 25%
Air changes:	Not reported
Photoperiod:	12 hours rhythm

B. Study design and methods

1. In life dates: 10 -13 November 2005

2. Animal assignment and treatment

On the day of treatment, 0.1 g of fluopyram (AE C656948, Batch No. Mix-Batch:08528/0002; 94.7% purity) was placed into the conjunctival sac of one eye of the first animal. The lids were then gently held together for about 1 second in order to prevent 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 recorded 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 hours. The degree of ocular lesions was recorded according to Draize. In addition any serious lesions or toxic effects other than ocular lesions were also recorded and described.

3. Statistics

Statistical analysis of the results was not required.

II. Results and discussion

A. Findings

There was no systemic intolerance reaction.

A summary of the irritant effects is given in table 5.2.5-1.

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

Animal	Effects	24h	48h	72h	Mean score	Response	Reversible (days)
1	Corneal opacity	0	0	0	0.0	-	N/A
	Iritis	0	0	0	0.0	-	N/A
	Redness conjunctivae	1	0	0	0.3	-	2
	Chemosis conjunctivae	0	0	0	0.0	-	N/A
2	Corneal opacity	0	0	0	0.0	-	N/A
	Iritis	0	0	0	0.0	-	N/A
	Redness conjunctivae	0	0	0	0.0		1*
	Chemosis conjunctivae	0	0	0	0.0	-	N/A
3	Corneal opacity	0	0	0	0.0	-	N/A
	Iritis	0	0	0	0.0	-	N/A
	Redness conjunctivae	1	0	0	0.3		2
	Chemosis conjunctivae	0	0	0	0.0	-	N/A

Corneal opacity: "-" if mean score <2
Iritis: "-" if mean score <1
Redness conjunctivae: "-" if mean score <2.5
Chemosis conjunctivae: "-" if mean score <2
N/A: not applicable
1*: considering the results 1 hour post application

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 classification according to Regulation (EC) No. 1272/2008.

Assessment and conclusion by applicant:
Study meets the current 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:	[redacted]
Report Year:	2006
Report Title:	AE C656948 - Evaluation of potential dermal sensitization in the local lymph node assay in the mouse
Report No:	SA 06320
Document No:	M-281845-01-1
Guideline(s) followed in study:	OECD guideline 429 (2002); Equivalent to US EPA OPPTS Guideline No 870.2600
Deviations from current test guideline:	Current guideline: OECD 429, 2010 Deviation: None.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a dermal sensitisation study AE C656948 (Batch-No.: Mix-Batch:08528/0002, 94.7% 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 µL of test substance solution at the adequate concentration. On day 5, mice were injected intravenously with 3H-methyl thymidine. Auricular lymph nodes were then removed 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, AE C656948 did not show any sensitization potential in this assay and does not warrant classification as being a dermal sensitizer according to Regulation (EC) No. 1272/2008.

I. Materials and methods

A. Materials

1. **Test material:** AE C656948
   Description Beige powder
   Lot / Batch #: Mix-Batch: 08528/0002
   Purity: 94.7%
   CAS # Not stated in this report
   Stability of test compound: Until 04 May 2007
2. **Vehicle:** Dimethylformamide (DMF)
   Positive control: Alpha-Hexylcinnamaldehyde (HCA)
3. **Test animals:**
   Species: Mice (females)
   Strain: CBA/J
   Age: Approximately 8 weeks old
   Weight at dosing: Not stated
   Source: R Janvier, 53940 Le Genest-St-Isle, France
   Acclimation period: At least 5 days
   Diet: Certified rodent pellet diet: AO4C-10, S.A.F.E. (Scientific Animal Food and Engineering, Route de Saint Bris, Augy, France)

Water:	Municipal tap water, *ad libitum*
Housing:	Individually in suspended, stainless steel, wire-mesh cages
Environmental conditions:	
Temperature:	20 -24°C
Humidity:	40-70%
Air changes:	10-15 exchanges per hour
Photoperiod:	12 hours daily

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:08328/0002; 94.7% 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 of 0.5, 1.0, 2.5 and 5% in DMF. These dose levels were chosen on the basis of preliminary 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 daily during the study. In particular, the site of application was examined for signs of local irritation. Individual bodyweight were also measured at study start and end (scheduled sacrifice).

On day 5, each mouse was placed in a retention box, intravenously injected via the tail vein with 250 µL of NaCl (0.9%) containing 20 µCi of ^{3}H methyl thymidine and then placed in a plastic cage for 5 hours. Then mice were sacrificed and the 2 auricular lymph 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 crushing them with a plastic piston.

Cell suspensions were washed with 5 ml of physiological saline, centrifuged and then resuspended in 2 ml of 5% trichloroacetic acid (TCA) and stored overnight at 4°C. After a final centrifugation, the cell pellets were resuspended in 1 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.

Table 5.2.6-1 Summary of animal assignment and treatment:

Group	Test substance	Concentration % (days 0, 1, 2)	Number of animals groups	Animal identity
1	Vehicle (DMF)	0	5	QT1F4121 to 4125
2	AE C656948	0.5	5	QT2F4126 to 4130
3		1	5	QT3F4131 to 4135
4		2.5	5	QT4F4136 to 4140
5		5	5	OT5F4141 to 4145
6	HCA	25	5	QT6F4146 to 4150

Evaluation criteria

A test substance is regarded as a skin sensitizer when one concentration results in a 3-fold or more increase in ^{3}H 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.

The results of proliferation assay are presented in the table below.

Table 5.2.6-2 Lymph node DPM values and Stimulation Indices

Test group	DPM/node	Stimulation index	Response
Control (DMF)	581.4	1.0	-
Fluopyram 0.5% in DMF	543.4	0.9	-
Fluopyram 0.5% in DMF	591.6	1.0	-
Fluopyram 0.5% in DMF	647.5	1.1	-
Fluopyram 0.5% in DMF	519.0	0.9	-
HCA 25% in DMF	3330.3	5.7	+

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

Negative responses were observed at all dose levels of fluopyram.

B. Deficiencies

No deficiencies were noted.

III. Conclusions

Under the conditions of the study, fluopyram did not show any skin sensitization potential in the local lymph node assay and does not 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 results do not trigger classification.

CA 5.2.7 Phototoxicity

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 (ε) of the UV-VIS absorption maxima (calculated according to OECD TG 101) is above 1000 L x 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 less than 1 000 L× mol^{-1} × cm^{-1}, phototoxicity testing is not required.

CA 5.2.8 Potentiation/interactions of multiple active substances or products

Not applicable

CA 5.3 Short-term toxicity

The short-term toxicity studies with fluopyram (AE C656948) were conducted between 2003 and 2008. All subchronic 90-day studies were in accordance with OECD, EU, USEPA and Japanese MAFF 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 in compliance with GLP as they were not subjected to QA inspections although the same standardized routine operating procedures applied to GLP studies were used. A summary of these results is presented in Table 5.3-1.

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 16-29% 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 examination, males had a 30% increase in platelet count and a 32% increased prothrombin time compared with controls. At the clinical chemistry evaluation, higher mean total cholesterol and triglyceride concentrations were found in both sexes, when compared to the controls, together with slightly lower aspartate aminotransferase and alkaline phosphatase activities. No effects on body weights, hematology or clinical chemistry parameters were observed at 400 ppm or below. The liver was a target organ with increased weights in both sexes associated with centrilobular hepatocellular 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 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 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 hepatotoxicity parameters, AE C656948 caused a dose-related increase in total cytochrome P-450, BROD and PROD activities at 3200 and 400 ppm. AE C656948 was therefore considered to be a moderate Phenobarbital-like cytochrome P-450 inducer.

There were no treatment-related effects noted at 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 kidneys (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 sexes, and food consumption was reduced in females for the last two months of treatment. Mean prothrombin 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, γ-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 sexes, 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 liver weights and thyroid gland weights were increased in both sexes, whilst mean kidney weights were increased in males only. Microscopically, in the liver, minimal to moderate centrilobular hepatocellular hypertrophy was observed in both sexes, together with minimal to moderate periportal to midzonal hepatocellular macrovacuolation in females. In the thyroid gland, minimal to slight diffuse hypertrophy of follicular 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 α2µ-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 α2µ-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 droplet nephropathy and hyaline casts persisted in some animals. All other affected parameters were found to be reversible after 1 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 females, compared to controls. Lower mean hemoglobin concentrations and mean hematocrit were noted in males. Mean total cholesterol concentration was increased in both sexes, whilst creatinine, inorganic phosphorus and calcium were increased in males, and total bilirubin decreased in both sexes and chloride decreased in males. Urinalysis revealed an increased incidence and severity of cellular casts in males. Mean TSH levels were increased in males at Week 13 only. Mean liver weights were increased in both sexes and mean kidney weights 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 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 nephropathy and the incidence of hyaline 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 droplet 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 14.6 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, hepatocellular eosinophilia, bile duct/oval cell hyperplasia, focal necrosis, single cell hepatocellular necrosis and centrilobular degeneration/necrosis. At 1000 ppm, body weight gain was slightly decreased 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 glands in 3/5 females. In the liver, changes were seen in both sexes and included hypertrophy of centrilobular hepatocytes, single hepatocellular necrosis, focal necrosis, hepatocellular eosinophilia and bile duct/oval cell hyperplasia. At 150 ppm, only adaptive changes were observed in the liver; liver weights were increased by between 15% and 21% in both sexes and microscopically, minimal to slight hypertrophy of centrilobular 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 150 ppm (equating to 24.7 mg/kg body weight/day in males and 34.1 mg/kg body weight/day in females).

In the 90-day mouse study, treatment-related findings observed at the high dose of 1000 ppm included an increase in food consumption (up to 12%) in males. Higher mean alanine aminotransferase activity, lower mean albumin concentration in both sexes together with lower mean total cholesterol concentration, higher mean alkaline phosphatase activity and a tendency towards higher mean aspartate aminotransferase 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 hypertrophy 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 minimal to slight focal necrosis in 6/10 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. At 150 ppm, mean liver weights were increased by 9% to 16% in males and 25% to 28% in females, in correlation with a minimal to slight 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 lower mean total cholesterol concentration was seen in males, however, as the change 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 were observed.

The NOAEL in both sexes was established 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 exposures of AE C656948. 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, liquid feces 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 least 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, erythrocyte counts and hemoglobin concentration) and some clinical chemistry parameters (mainly alkaline phosphatase and δ-glutamyl transferase activities and bilirubin, albumin and protein concentrations).

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

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

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

At 800 ppm, there was no treatment-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 at least 52 weeks. This study confirmed the liver as the main target organ in dogs. As in the 90-day study, the poor palatability 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 sexes 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 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. 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 group), 100, 300, and 1000 mg/kg/day. The dose was based on each animal’s body weight on days 0, 7, 14, 21, 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.

Table 5.3-1 Fluopyram - Summary of short-term toxicity studies

Study Doses tested: ppm or *mg/kg bw/d* GLP	NOEL/ NOAEL ppm	NOEL/ NOAEL mg/kg bw/d	LOAEL ppm	LOAEL mg/kg bw/d	Main findings	Reference
28-day rat study, 0, 50, 400, 3200 ppm ***0, 4.0, 31.0, 254 M*** ***0, 4.6, 36.1, 263 F***	400* (M/F)	31.0/36.1* (M/F)	3200 (M/F)	254/263 (M/F)	↓ body weight gain (M/F), ↑ platelet count and prothrombin time (M), ↑ cholesterol and triglycerides conc. (M/F), ↓ aspartate aminotransferase and alkaline phosphatase ↑ liver weight with hepatocellular hypertrophy (M/F) ↑ thyroid weight with follicular cell hypertrophy (M) ↑ kidney : hyaline droplet nephropathy	[redacted] 2004 M-085510-01-1
28-day mouse study, 0, 150, 1000, 5000 ppm ***0, 24.7, 162, 747 M*** ***0, 31.1, 197, 954 F***	150 (M/F)	24.7/31.1 (M/F)	1000 (M/F)	162/197 (M/F)	Mortality at 5000 ppm (M/F), ↓ body weight gain (M), ↑ ALT (M), ↑ liver weights, 27-49% (M/F) associated with : centrilobular hypertrophy, single cell and focal necrosis, hepatocellular eosinophilia and bile duct/oval cell hyperplasia (M/F)., Adrenal gland: hypertrophy of zona fasciculata (F)	[redacted] 2004 M-088486-01-1
28-day dog study, 0, 30, 150, 750 mg/kg/day	n.a	150 (M/F)	n.a	750 (M/F)	↓ erythrocyte count, hemoglobin, 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).	[redacted] 2004 M-242097-01-1

Study Doses tested: ppm or *mg/kg bw/d* GLP	NOEL/ NOAEL ppm	NOEL/ NOAEL mg/kg bw/d	LOAEL ppm	LOAEL mg/kg bw/d	Main findings	Reference
90-day rat study, 0, 50, 200, 1000, 3200 ppm ***0, 3.06, 12.5, 60.5, 204 M*** ***0, 3.63, 14.6, 70.1, 230 F***	**200*** (M/F)	**12.5/14.6*** (M/F)	**1000** (M/F)	**60.5/70.1** (M/F)	↓ body weight & food consumption, hematology parameters affected. ↓ cholesterol conc & δ-glutamyltransferase activity. ↑ TSH, T3 & T4. Target organs were liver and thyroid gland (M/F) & kidney (M). ↑ liver, thyroid gland & kidney weights. Liver: centrilobular hypertrophy & periportal to midzonal hepatocellular macrovacuolation. Thyroid gland: follicular cell hypertrophy. Kidney: hyaline droplet nephropathy.	[redacted] 2005 M-250946-01-1
90-day mouse study, 0, 30, 150, 1000 ppm ***0, 5.4, 26.6, 188 M*** ***0, 6.8, 32.0, 216 F***	150 (M/F)	26.6/32.0 (M/F)	1000 (M/F)	188/216 (M/F)	↑ ALT & ↓ albumin conc. (M/F), ↓ cholesterol conc & ↑ alkaline phosphatase (M), ↑ liver weights , 34-45% (M/F), ↑ adrenal gland weights, 87-92% (M) Liver: centrilobular hypertrophy and focal necrosis (M/F) Adrenal gland: ↓ ceroid pigment (M), ↑ cortical vacuolation (F)	[redacted] 2011 M-251136-01-1

Study Doses tested: ppm or *mg/kg bw/d* GLP	NOEL/ NOAEL ppm	NOEL/ NOAEL mg/kg bw/d	LOAEL ppm	LOAEL mg/kg bw/d	Main findings	Reference
90-day dog study, 0, 800, 5000, 20000/10000 ppm ***0, 28.5, 171, 332 M*** ***0, 32.9, 184, 337 /F***	800 (M/F)	28.5/32.9 (M/F)	5000 (M/F)	171/184 (M/F)	↓ body weight & food consumption, hematology parameters affected at high dose only. ↑ alkaline phosphatase δ-glutamyltransferase activity, ALT & AST, ↓ albumin conc and bilirubin, ↑liver weights associated with hepatocellular hypertrophy, intracytoplasmic eosinophilic droplets and single cell necrosis. Both sexes affected.	[redacted] 2006 M-276047-01-1
1 year dog study, 0, 100, 400, 2000 ppm ***0, 3.0, 13.2, 67.6 M*** ***0, 3.8, 14.4, 66.1 F***	400 (M/F)	13.2/14.4 (M/F)	2000 (M/F)	67.6/66.1 (M/F)	↓ body weight & food consumption (M/F), ↑ alkaline phosphatase (M/F), Liver: centrilobular hypertrophy	[redacted] 2007 M-294279-01-1
Percutaneous 28-day rodent (rat) study, 0, 100, 300, 1000 mg/kg/day	n.a.	300 (M/F)	n.a.	1000 (M/F)	↑ cholesterol (F), ↑ prothrombin time (M) ↑ liver weights associated with hepatocellular hypertrophy	[redacted] A.; 2007 M-293833-01-1

* relevant values when considering human risk assessment which do not take into account male rat specific effects observed in these studies as they are known not to be relevant for humans
n.a.: not applicable

Short-term studies were conducted prior to the requirement for toxicokinetic data (i.e., measurement of blood concentration of the parent and / or its metabolites). However, as extensive ADME data show plasma concentrations of fluopyram, the conduct of additional in vivo studies to generate toxicokinetic data is considered not to be necessary.

CA 5.3.1 Oral 28-day study

Data Point:	KCA 5.3.1/01
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE C656948 - Exploratory 28-day toxicity study in the rat by dietary administration
Report No:	SA 03332
Document No:	M-085510-01-1
Guideline(s) followed in study:	Not applicable
Deviations from current test guideline:	Current guideline: not applicable
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

AE C656948 (batch No: FLH 999; 98.6% purity) was administered to Wistar rats at dietary concentrations of 0, 50, 400, or 3200 ppm for 28 days, corresponding to 0, 4.0, 31.0, 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% in females (weeks 1, 2 and 4). 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 examination, males had a 30% increase in platelet count and a 32% increased prothrombin time compared with controls. In clinical chemistry, higher mean total cholesterol and triglyceride concentrations were found in both sexes, when compared to the controls together with slightly lower aspartate aminotransferase and alkaline phosphatase activities. The liver was a target organ with increased weights in both sexes associated with centrilobular hepatocellular 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 hepatotoxicity parameters, AE 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 Phenobarbital-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). However, changes at 400 ppm were limited 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 NOAEL for human risk assessment.

I. Materials and methods

A. Materials

1. **Test material:** AE C656948
   Description Pale yellow powder
   Lot / Batch #: FLH 999

Purity: 98.6%
CAS # 658066-35-4
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:

Species: Rat
Strain: Wistar Rj: WI (IOPS HAN)
Age: 7 weeks approximately at start of dosing
Weight at dosing: 255 -283 g (males); 171-193g (females)
Source: [redacted]
Acclimation period: 7-8 days
Diet: Certified rodent powdered and irradiated diet A04C-10P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water: Tap water, *ad libitum*
Housing: Rats were housed individually in suspended stainless-steel 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: 10 December 2003 - 07 January 2003

2. Animal assignment and treatment

There were 5 animals of each sex per dose group. Animals were assigned to dose groups randomly by body weight. AE C656948 was administered in the diet for 28 days to Wistar rats at the following doses – 0, 50, 400 and 3200 ppm (equating to 4.0, 31.0 and 254 mg/kg/day in males and 4.6, 36.1 and 263 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 "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 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%.

Table 5.3.1-1 Study design and group sizes

Test group	Concentration in diet (ppm)	Dose per animal (study averages)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	5	5
2	50	4.0	4.6	5	5
3	400	31.0	36.1	5	5
4	3200	254	263	5	5

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 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 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

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 periods. Diet-fasted animals were weighed 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 1 to 4 was calculated for each sex.

4. Ophthalmoscopic examination

Ophthalmoscopic examination was not conducted.

5. Clinical chemistry

On study day 29, prior to necropsy, 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 lithium heparin for plasma and clot activator for serum for clinical chemistry and on sodium citrate for coagulation parameters.

The following hematology parameters were assayed using a Advia 120 (Bayer Diagnostics, Puteaux, 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 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, glucose, urea, creatinine, total cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase activities, total protein and albumin concentrations were assayed on serum samples using an Hitachi 911 (Roche Diagnostics, Meylan, France).

6. Urinalysis

Urinalysis 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-fasted prior to scheduled sacrifice. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically. Adrenal gland, brain, kidney, liver, ovary, spleen, testis, thyroid gland (with parathyroid gland) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

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

The following tissues were sampled: adrenal gland, brain, liver, kidney, lung, ovary, pituitary, spleen, 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 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. 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 clinical signs evident in any group.

2. Mortality

There was no mortality in any group.

B. Body weight and body weight gain

Body weight gain was reduced at 3200 ppm in both sexes, by 12-28% 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% lower in females. Body weight was unaffected at 400 or 50 ppm in either sex.

C. Food consumption and compound intake

At 3200 ppm, food intake was reduced by 4 to 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, 4.6, 36.1, and 263 mg/kg/day for females, and 0, 4.0, 31.0, and 254 mg/kg/day 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.

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

Dosage level (ppm)	0	50	400	3200
Males				
Platelet count (10^9 / l)	1183 ± 193	1331 ± 105	1341 ± 150	1539 ± 126**
Prothrombin time (s)	13.4 ± 1.5	14.0 ± 0.7	15.0 ± 0.6	18.0 ± 2.9*

*: p≤0.05; **: p≤0.01

2. Clinical Chemistry

Clinical chemistry findings included higher total cholesterol and triglyceride concentrations together with slightly lower aspartate aminotransferase and alkaline phosphatase activities in both sexes.

No relevant changes were observed at 400 or 50 ppm in either sex.

Table 5.3.1-3 Changes in clinical chemistry parameters in the 28-day rat study with AE C656948 (mean ± SD)

Dosage level (ppm)	0	50	400	3200
Males				
Total cholesterol (mmol/L)	1.45 ± 0.17	1.56 ± 0.28	1.52 ± 0.28	2.64 ± 0.52**
Triglycerides (mmol/L)	0.81 ± 0.24	0.83 ± 0.25	0.76 ± 0.23	2.01 ± 0.38**
Aspartate aminotransferase (IU/L)	86 ± 23	80 ± 14	67 ± 5	63 ± 7*
Alkaline phosphatase (IU/L)	127 ± 36	113 ± 13	124 ± 11	84 ± 10
Females				
Total cholesterol (mmol/L)	1.90 ± 0.19	2.09 ± 0.38	2.21 ± 0.32	3.50 ± 0.47**
Triglycerides (mmol/L)	0.40 ± 0.05	0.47 ± 0.10	0.40 ± 0.05	1.01 ± 0.41**
Aspartate aminotransferase (IU/L)	76 ± 7	70 ± 4	74 ± 16	63 ± 5
Alkaline phosphatase (IU/L)	90 ± 25	86 ± 21	95 ± 24	54 ± 8*

*: p≤0.05; **: p≤0.01

3. Urinalysis

Urinalysis was not conducted in this study.

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 ppm dose 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 enlarged 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, hypertrophy of the follicular cells was observed in 3/5 males. Thyroid gland weight was not affected in females although 2/5 animals showed a minimal follicular cell hypertrophy.

In males, increased kidney 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 cast in the medulla). This typical nephropathy is a well know lesion occurring in male rat kidney and which is linked to accumulation of α2µ-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 α2μ-globulin specifically in the rat. As humans secrete only trace amounts of this globulin, this mechanism is generally accepted as being non-relevant for humans. Therefore, although this effect was considered treatment-related in the rat, it is considered not to be relevant to man.

Table 5.3.1-4 Organ weight changes in the 28-day rat study with AE C656948 (mean ± SD)

Dosage level (ppm)	0	50	400	3200
Males				
Liver weight Absolute (g)	11.13 ± 0.93	11.18 ± 0.93 (+0.4%)	12.45 ± 1.15 (+12%)	17.11 ± 1.76** (+54%)
Bodyweight-relative	2.864 ± 0.210	2.811 ± 0.137 (-2%)	3.055 ± 0.176 (+7%)	4.401 ± 0.239** (+54%)
Brain-relative	567.042 ± 57.354	565.078 ± 48.426 (0%)	607.382 ± 50.971 (+7%)	878.091 ± 86.733 (+55%)
Kidney weight Absolute (g)	2.63 ± 0.09	2.49 ± 0.18 (-5%)	3.16 ± 0.24** (+20%)	3.13 ± 0.20** (+19%)
Bodyweight-relative	0.676 ± 0.046	0.627 ± 0.053 (-7%)	0.777 ± 0.052 (+15%)	0.801 ± 0.094 (+18%)
Brain-relative	133.726 ± 6.952	125.616 ± 7.214 (-6%)	154.282 ± 9.524** (+15%)	161.284 ± 9.292** (+21%)
Thyroid weight Absolute (g)	0.0176 ± 0.0040	0.0197 ± 0.0023 (+12%)	0.0222 ± 0.0026 (+26%)	0.0252 ± 0.0033** (+43%)
Bodyweight-relative	0.00456 ± 0.00118	0.00499 ± 0.00085 (+9%)	0.00548 ± 0.00086 (+20%)	0.00653 ± 0.00109** (+41%)
Brain-relative	0.89067 ± 0.18060	0.99508 ± 0.11592 (+12%)	1.08461 ± 0.13174 (+22%)	1.29779 ± 0.20244** (+46%)
Females				
Liver weight Absolute (g)	5.84 ± 0.66	6.24 ± 0.32 (+7%)	6.76 ± 0.46 (+16%)	9.64 ± 1.00** (+65%)
Bodyweight-relative	2.665 ± 0.209	2.773 ± 0.064 (+4%)	3.072 ± 0.151 (+15%)	4.599 ± 0.349** (+73%)
Brain-relative	315.044 ± 21.373	330.241 ± 27.788 (+4%)	384.924 ± 27.400 (+21%)	550.595 ± 63.589** (+74%)

*: p≤0.05; **: p≤0.01; Figures in parentheses are % differences from control

No other treatment-related effects were observed in other organs.

2. Liver enzyme induction

Changes that were observed are described in Table 5.3.1-5. A dose-related slight increase in total cytochrome P-450, BROD and PROD activities 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)

Dosage level (ppm)	0	50	400	3200
Males				
Total P-450 (nmol/mg prot.)	1.19 ± 0.19	1.24 ± 0.25 (-)	1.43 ± 0.11 (+20%)	1.63 ± 0.25 (+37%)
BROD (pmol/min/mg prot.)	8.90 ± 1.00	9.86 ± 2.89 (-)	71 ± 20.66 (+698%)	171.82 ± 29.02 (+1831%)
EROD (pmol/min prot.)	59.24 ± 6.94	46.80 ± 4.14 (-)	66.78 ± 2.26 (-)	79.23 ± 8.22 (-)
PROD (pmol/min/mg prot.)	6.56 ± 1.30	4.68 ± 1.00 (-)	29.54 ± 11.80 (+350%)	68.31 ± 6.05 (+941%)
Females				
Total P-450 (nmol/mg prot.)	0.85 ± 0.07	0.89 ± 0.01 (+5%)	0.98 ± 0.23 (+15%)	1.20 ± 0.13 (+41%)
BROD (pmol/min/mg prot)	2.79 ± 0.54	4.32 ± 0.63 (-)	26.27 ± 10.94 (+842%)	86.57 ± 37.92 (+3002%)
EROD (pmol/min prot.)	52.16 ± 3.31	54.4 ± 3.92 (-)	68.57 ± 8.61 (-)	87.90 ± 5.33 (-)
PROD (pmol/min/mg prot.)	2.81 ± 0.52	3.49 ± 0.64 (-)	12.92 ± 4.87 (+360%)	45.68 ± 24.43 (+1526%)

No statistical analysis performed.
(%) as compared to control
(-) no relevant change

G. Deficiencies

No specific deficiencies were noted in the study.

III. Conclusions

In conclusion, the NOAEL in this study was 50 ppm (4.0 and 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 (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.

Assessment and conclusion by applicant:

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

In conclusion, the NOAEL in this study was 50 ppm (4.0 and 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 (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

Data Point:	KCA 5.3.1/02
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE C656948 - Preliminary 28-day toxicity study in the mouse by dietary administration
Report No:	SA 04013
Document No:	M-088486-01-1
Guideline(s) followed in study:	Not applicable
Deviations from current test guideline:	Current guideline: not applicable
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

AE C656948, (batch number FLH 1046, 99.4% purity) was administered via the diet to separate groups of C57BL/6J mice (5/sex/group) at dose levels of 0, 150, 1000 and 5000 ppm (equivalent to 0, 24.7, 162 and 747 mg/kg body weight/day in males and 0, 31.1, 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 sacrificed for humane reasons between study Days 17 and 27. Clinical signs in these decedent animals included reduced motor activity, hunched posture, piloerection, wasted appearance, coldness to touch, abnormal respiration and distended abdomen, together with a loss in body weight and reduced food consumption. Red liquid was observed in the thoracic cavity of all males. Microscopically, hypertrophy, vacuolation and degeneration/necrosis of the zona fasciculata were seen in the adrenal glands in all animals, together with perivascular and intra-alveolar hemorrhage and degeneration/inflammation of pulmonary veins in the lungs and erythroid extramedullary hematopoiesis in the spleen. Focal hemorrhage was seen in the thyroid gland in 3/5 males and decreased cellularity of the cortex and focal hemorrhage were seen in the thymus in all animals where examination was possible. In the liver, hypertrophy of hepatocytes, hepatocellular eosinophilia, bile duct/oval cell hyperplasia, focal necrosis and single cell hepatocellular necrosis were seen in all animals, and centrilobular degeneration/necrosis in 1/5 males. In the two surviving females, distended abdomen was noted between study Days 8 and 10 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 relative liver weights were increased by between 132% and 147%. Microscopically, changes were seen in the adrenal gland and liver in both animals which were consistent with those observed in decedent animals.

At 1000 ppm, there were no mortalities or clinical signs. There was a slight decrease in the body weight gain during study Week 2 in males. Mean absolute and relative liver weights were increased by between 27% and 49% in 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. 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 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.

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 (MTD) due to the overt toxicity noted, whilst a dose level of 150 ppm represented the NOAEL of AE 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
Description	Beige powder
Lot / Batch #:	FLH 1046
Purity:	99.4%
CAS #	658066-35-4
Stability of test compound:	Stable in rodent diet at 20 and 10000 ppm after 95 days frozen storage and 10 days at room temperature.
2. Vehicle and / or positive control:	none
3. Test animals:	
Species:	Mouse
Strain:	C57BL/6J
Age:	Approximately 6 weeks at start of treatment
Weight at dosing:	18.7 -21.6 g (males), 15.1-18.3 g (females)
Source:	[redacted]
Acclimation period:	13 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	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: 25 February - 07 April 2004

2. Animal assignment and treatment

There were 5 animals per group. Animals were randomly assigned to treatment groups by body weight, and were given AE C656948 in the diet at concentrations of 0, 150, 1000, or 5000 ppm for 28 days. 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 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°C when not in use. AE C656948 concentrations were shown in a previous study (M-085510-01-1) to be stable at 20 and 10000 ppm for 95 days frozen followed by 10 days at room temperature.

Table 5.3.1-6 Study design and achieved doses

Test group	Achieved dose (mg/kg/day)	Animals assigned
Males		
Control	0	5
150	24.7	5
1000	162	5
5000	747*	5
Females		
Control	0	5
150	31.1	5
1000	197	5
5000	954	5

* Calculated for Weeks 1 to 3 only due to mortality or early sacrifice in this group.

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'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) 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).

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. Cages and cage trays were inspected daily for signs of adverse effects, such as blood or loose 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 necropsy.

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, albumin, total cholesterol, AST, ALT, and alkaline phosphatase on a Hitachi 911 (Roche Diagnostics, Meylan, France).

6. Urinalysis

Urinalysis 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. All animals were fasted prior to scheduled sacrifice. Macroscopic abnormalities were recorded, sampled and examined microscopically. Adrenal gland, brain, kidney, liver, spleen, testis and ovary were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

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

II. Results and discussion

A. Clinical signs and mortality

1. Clinical signs of toxicity

Clinical signs in the decedent animals comprised reduced motor activity, hunched posture, piloerection, wasted appearance and/or coldness to touch in both sexes together with labored respiration in 3/5 males and distended abdomen in 2/3 females. 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 ppm or 150 ppm dose groups.

2. Mortality

All males and 3/5 females at 5000 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 (0.03 g/day vs. 0.10 g/day in the control group, not statistically significant).

No treatment-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 total cholesterol (+118%) and total protein (+16%) concentrations, and alanine aminotransferase activities (+384%).

At 1000 ppm, mean alanine aminotransferase activity was higher in males (+259%, not statistically significant) and mean albumin concentration was lower in females (-12%, $p \leq 0.05$). Lower mean total bilirubin and albumin concentrations were noted in males (-56%, $p \leq 0.05$ and -10%, $p \leq 0.01$, 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 bilirubin and albumin concentrations were noted in males (-50 and -5%, respectively, $p \leq 0.05$ compared to controls). These changes were considered not to be toxicologically relevant as the individual values were within historical control ranges.

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.

Table 5.3.1-7 Terminal body weight and absolute and relative liver weights (mean ± SD)

Sex	**Males**			
Dietary level (ppm)	**0**	**150**	**1000**	**5000**
Terminal body wt, g	19.8±0.9	19.2±0.9	19.2±0.8	a
Liver wt, g	0.80±0.10	0.94*±0.05	1.13+±0.08	a
Liver to body wt, %	4.052±0.495	4.896±0.131	5.905+±0.223	a
Liver to brain wt, %	175.232±18.266	211.332+±12.975	261.966+±15.650	a
Sex	**Females**			
Dietary level (ppm)	**0**	**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 ± 0.12**	1.66 ± 0.12#
Liver to body wt, %	4.502 ± 0.163	5.279 ± 0.294*	5.712 ± 0.594**	10.435 ± 0.43#
Liver to brain wt, %	161.379 ± 11.017	185.388 ± 14.792	214.903 ± 35.597**	398.576 ± 8.592#

*: $p \leq 0.05$; **: $p \leq 0.01$
a = no surviving animals
#: 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 adrenal 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 intra-alveolar hemorrhage and degeneration/inflammation of pulmonary veins in the lungs and erythroid extramedullary hematopoiesis in the spleen. Focal hemorrhage was seen in the thyroid gland in 3/5 males and decreased cellularity of the cortex and focal hemorrhage were seen in the thymus in all animals where examination was possible. In the liver, hypertrophy of hepatocytes (mainly 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 males and in 1/3 females dosed at 5000 ppm was associated with intrathoracic hemorrhage. The majority of decedent animals 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 most moribund animals. Hence, the increase in the incidence and severity of extramedullary hematopoiesis 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 treatment 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 glands 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 150 ppm, with evidence of a dose-response. Single cell hepatocellular 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. Hepatocellular eosinophilia and bile duct/oval cell hyperplasia were noted in the two surviving females at 5000 ppm and in 1/5 females at 1000 ppm.

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

Sex	Males				Females			
Dietary level (ppm)	**0**	**150**	**1000**	**5000**	**0**	**150**	**1000**	**5000**
Liver, N examined	5	5	5	0*	5	5	5	2*
Hypertrophy, hepatocellular, centrilobular - Minimal	0	3	0	-	0	2	4	1
Hypertrophy, hepatocellular, centrilobular - Slight	0	2	0	-	0	0	4	0
Hypertrophy, hepatocellular, centrilobular - Moderate	0	0	5	-	0	0	0	2
Total	**0**	**5**	**5**	**-**	**0**	**2**	**5**	**2**
Necrosis, hepatocellular, single cell - Minimal	0	0	5	-	0	0	0	1
Necrosis, focal - Minimal	0	0	3	-	0	0	2	0
Necrosis, focal - Slight	0	0	0	-	0	0	0	2
Total	**0**	**0**	**3**	**-**	**0**	**0**	**2**	**2**
Eosinophilia, hepatocellular - Minimal	0	0	0	-	0	0	1	2
Hyperplasia, bile ducts / oval cells - Minimal	0	0	0	-	0	0		

* 0/2 survivals in males/females, respectively

Hypertrophy of centrilobular hepatocytes is associated with the proliferation of sub-cellular organelles, although their identity cannot be confirmed in hematoxylin and eosin sections. The change is predominantly centrilobular but appears to be more diffuse in a few animals dosed 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 necropsy.

Necrosis and eosinophilia of hepatocytes and focal 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 noted in this study.

III. Conclusions

A dose level of 5000 ppm clearly exceeded the MTD due to the overt toxicity noted, whilst a dose level of 150 ppm represented the NOAEL of AE 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).

Assessment and conclusion by applicant:

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

A dose level of 5000 ppm clearly exceeded the MTD due to the overt toxicity noted, whilst a dose level of 150 ppm represented the NOAEL of AE 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).

Data Point:	KCA 5.3.1/03
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE C656948 - Preliminary 28-day toxicity study in the dog by gavage
Report No:	SA 04049
Document No:	M-242097-01-1
Guideline(s) followed in study:	Not applicable
Deviations from current test guideline:	Current guideline: not applicable
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Technical grade AE C656948 was administered by gavage to Beagle dogs (2/sex/dose) at dose levels of 0, 30, 150, and 750 mg/kg/day for at least 28 days. 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 just prior to necropsy. Clinical chemistry, hematology, and urinalysis 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 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 showed a high alkaline phosphatase activity and a low albumin concentration (as a consequence a low albumin/globulin ratio). In addition, the same female also showed a high δ-glutamyltransferase activity and triglyceride concentration.

The liver was the only identified target organ in both 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 material:** AE C656948
   Description Beige powder
   Lot / Batch #: PT0304
   Purity: 99.0%
   CAS # 658066-35-4
   Stability of test compound: Stable at 25 ± 5°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:	[redacted]
Acclimation period:	At least 21 days
Diet:	Certified canine meal 153C3 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France). Each animal received approximately 300 g of diet daily approximately 2.5 hours after gavage administration for 1.5 hours
Water:	Municipal tap water, *ad libitum*
Housing:	Animals were housed individually in stainless steel kennels with a floor surface area of 1.2 m^2. 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. At the end of working day, dogs were pair-housed overnight by opening the partition between 2 animals of the same sex and dose group.
Environmental conditions:	
Temperature:	18-21°C
Humidity:	40-70%
Air changes:	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: 02 June - 30 June 2004

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 ophthalmologic examination, hematology and clinical chemistry investigations and urinalysis once during the acclimatization phase. Animals were assigned to dosage groups using a computerized randomization procedure in order to ensure a similar body weight distribution among groups of each sex. Test groups were as described in the table 5.3.1-9.

Table 5.3.1-9 Test groups and dose levels

Test Group (Group number)	Sex : Number of animals	Dose levels (mg/kgbw/day)
Control (1)	Male: 2 Female:2	0
Low (2)	Male: 2 Female:2	30
Mid (3)	Male: 2 Female:2	150
High (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 demonstrated 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 therefore 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). Daily examination of the kennels was also carried out for vomitus, diarrhea or blood.

b. Clinical examinations

Detailed clinical observations for clinical signs of toxicity 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 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 treatment, ophthalmic examinations were conducted on all animals by means of an indirect ophthalmoscope after instillation of an atropinic agent (mydriaticum).

5. Clinical pathology: Hematology and clinical chemistry

Clinical chemistry and complete blood count, including differentials, were performed on all animals once prior to administration of the test substance (day -9) and on day 27. Animals were diet fasted overnight prior 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, Glucose, Total 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 collected overnight. The following parameters were measured:

Appearance, Volume, Specific gravity/ osmolality/ Refractive index, pH, Sediment (microscopic), Protein, Glucose, Ketones, Bilirubin, Blood/ red blood cells, Urobilinogen.

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 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:

	Digestive system		Cardiovasc. / Hemat.		Neurologic
X	Tongue	X	Aorta	XX	Brain (with cerebellum)
X	Submaxillary (salivary) gland	XX	Heart	X	Sciatic nerve
		X	Bone marrow, sternum	X	Spinal cord (cervical, thoracic, lumbar)
X	Esophagus	X	Lymph node, mesenteric	X	Eyes
X	Stomach			X	Optic nerves
X	Duodenum	X	Lymph node, retropharyngeal	XX	Pituitary gland*
X	Jejunum				
X	Ileum	XX	Spleen		
X	Cecum	XX	Thymus		**Glandular**
X	Colon			XX	Adrenal gland
X	Rectum		**Urogenital**		
XX	Liver	XX	Kidney	X	Parathyroid gland
X	Pancreas	X	Urinary bladder	XX	Thyroid gland (weighed with parathyroid gland)
X	Gallbladder	XX	Testis		
	Respiratory	XX	Epididymis		**Other**
X	Trachea	XX	Prostate gland	X	Bone (sternum)
X	Lung	XX	Ovary	X	Skeletal muscle
X	Pharynx	XX	Uterus (with cervix)	X	Skin
X	Larynx	X	Mammary gland	X	All gross lesions and masses
		X	Vagina	X	Articular surface (femorotibial joint)

(X) Tissues were collected for histological examination.
(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% formalin with the exception of the eye, 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 groups.

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 feces that were observed on a few occasions in 2 animals at the high dose. On 1 or 2 occasions only, liquid feces was also observed in 2/4 animals at 150 mg/kg/day and in 1/4 animals at 30 mg/kg/day. However as liquid feces 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-276047-01-1), this finding at 150 and 30 mg/kg/day was considered to be incidental.

No abnormal findings were noted at the detailed physical examinations throughout the study.

Rectal temperature was within the normal range.

2. Mortality

There were no mortalities throughout the study.

B. Body weight and body weight gain

There was no treatment-related effect on body weights or body weight gains at any dose level in either sex.

C. Food consumption and compound intake

Mean food consumption was not affected throughout the study.

D. Hematology, clinical chemistry, and urinalysis

1. Hematology

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

No change was observed in any other group.

2. Clinical Chemistry

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

No relevant changes were observed at 150 or 30 mg/kg/day.

3. Urinalysis

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

E. Sacrifice and pathology

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 were also slightly 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.

Table 5.3.1-10 Histopathological changes in the 28-day dog study with AE C656948

Sex	Males				Females			
AE C656948, dietary concentration in mg/kg bw/day	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Liver, N examined	2	2	2	2	2	2	2	2
Centrilobular to panlobular hepatocellular hypertrophy: diffuse - Minimal	0	0	0	1	0	0	0	0
Centrilobular to panlobular hepatocellular hypertrophy: diffuse - Slight	0	0	0	1	0	0	0	2
Total	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**2**
Eosinophilic inclusion bodies: focal/ multifocal - Minimal	0	0	0	1	0	0	0	1
Eosinophilic inclusion bodies: focal/ multifocal - Slight	0	0	0	0	0	0	0	1
Total	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**2**

F. Deficiencies

No specific deficiencies were noted in this study.

III. Conclusions

The NOEL was found to be 150 mg/kg/day in both sexes.

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 sexes.

CA 5.3.2 Oral 90-day study

Data Point:	KCA 5.3.2/01
Report Author:	[redacted]
Report Year:	2005
Report Title:	AE C656948 - 90-day toxicity study in the rat by dietary administration
Report No:	SA 04048
Document No:	M-250946-01-1
Guideline(s) followed in study:	OECD 408; Directive 2001/59/EC, Annex V, Method B.26; US-EPA, OPPTS 870.3100; JMAFF notification 12 Nousan No. 8147
Deviations from current test guideline:	Current guideline: OECD 408, 2018 Deviations: no measurements of HDL, LDL, but the study acceptability was not affected because total cholesterol were measured. These deviations have no impact the outcome of the study and interpretation of the results.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

AE C656948 (batch number PFL 0304, 99.0% purity) was administered continuously via the diet to separate groups of Wistar rats (10/sex/group) at dose levels of 0, 50, 200, 1000 and 3200 ppm equivalent to 0, 3.06, 12.5, 60.5 and 204 mg/kg/day in males and 0, 3.63, 14.6, 70.1 and 230 mg/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 females, compared to controls. Mean food consumption was slightly decreased by between 5% and 12% from Days 29 to 90 in females, compared to controls. Mean prothrombin time was increased in males, whilst mean platelet and reticulocyte (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 corpuscular hemoglobin in females. An increase in mean total cholesterol, γ-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 sexes, glucose in males, and alkaline phosphatase 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 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, 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 kidney in males. In the liver, minimal to moderate centrilobular hepatocellular hypertrophy was observed in both sexes, together with minimal to moderate periportal to midzonal hepatocellular macrovacuolation in females. In the thyroid gland, minimal to slight diffuse hypertrophy of follicular 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 α2μ-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 α2µ-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 in males, compared to the controls. Microscopic examination showed that hyaline droplet nephropathy and hyaline casts 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 hemoglobin concentrations and lower mean hematocrit were noted 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, urinalysis 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 gland in both sexes and in the kidney in males. 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 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 nephropathy, considered not to be toxicologically relevant to man, and the incidence of hyaline casts were 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 liver 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 hyaline droplet nephropathy at the microscopic examination of the kidney, but these changes are considered not to be toxicologically relevant to man.

At 50 ppm, treatment-related changes consisted only of a slight increase in incidence and severity of cellular casts in the urine in males, compared to controls. These changes were considered not to be toxicologically relevant to man.

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 a dose level of 200 ppm represented the NOEL 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).

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Beige powder
Lot / Batch #:	PFI 0304
Purity	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
Strain:	Wistar Rj:WI (IOPS HAN)
Age:	6 weeks approximately
Weight at dosing:	182-221 g (males), 159-190 g (females)
Source:	[redacted]
Acclimation period:	7 days
Diet:	Certified rodent powdered and irradiated diet A04C-10-P1 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually, suspended stainless steel and wire mesh cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	15 per hour
Photoperiod:	12 hours dark, 12 hours light (7 am - 7 pm)

B. Study design

1. In life dates: 05 May 2004 – 08 September 2004

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 Wistar rats at the following doses – 0, 50, 200, 1000, 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, 70.1, and 230 mg/kg/day in females). An additional 10 males and 10 females fed either 0 or 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. 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".

Table 5.3.2-1 Details of group sizes and treatment

Group	Test substance	Diet concentration (ppm)	No. Of animals per group	Animal identity	
				Dosing phase	Recovery phase
Males					
1	Control	0	10 +10*	OT1M1773 to 1782	OT1M1783 to 1792
2	AE C656948	50	10	OT2M1813 to 1822	-
3		200	10	OT3M1833 to 1842	-
4		1000	10	OT4M1853 to 1862	-
5		3200	10 + 10*	OT5M1873 to 1882	OT5M1883 to 1892
Females					
1	Control	0	10 +10*	OTlF1793to 1802	OTlF1803to 1812
2	AE C656948	50	10	OT2F1823to 1832	-
3		200	10	OT3F1843to 1852	-
4		1000	10	OT4F1863to 1872	-
5		3200	10 + 10*	OT5F1893to 1902	OT5F1903to 1912

*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 dietary 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 females, respectively). However, changes at 400 ppm were limited 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 NOAEL for human risk assessment.

4. 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 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 nominal concentrations

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 homogenous, 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 Dunn test (2-sided) 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 urine 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 PathTox 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 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 periods and before necropsy.

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 mg/kg/day for each week and for Weeks 1 to 13 was calculated for each sex using the formula:

$$\text{Test substance intake (mg/kg/day)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day)}}{\text{Group mean body weight (g) at the end of the food consumption period}}$$

4. Ophthalmoscopic 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 13 of the treatment period, all surviving animals from control and high dose group were re examined

5. Neurological examination

During study Weeks 11 to 12, a neurotoxicity assessment was performed for all surviving animals (except for animals of the reversibility groups) 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 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 was collected on EDTA for hematology, on lithium heparin for plasma and clot activator for serum for clinical chemistry and on sodium citrate for coagulation parameters.

Hematology parameters investigated:

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 and stained with Wright stain. 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, calcium and inorganic phosphorus concentrations and aspartate 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 recovery phase, blood samples were collected from the retro-orbital venous plexus of all surviving 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 radioimmunoassay 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 urine collection.

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 (Bayer Diagnostics, Puteaux, France). Urinary refractive index was measured using a RFM320 refractometer (Bioblock Scientific, Illkirch, France).

The following semi-quantitative parameters were assayed using a Clinitek 200+ and Ames Multistix dipsticks: glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen. 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.

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 exsanguinated before necropsy. 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, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically. Adrenal gland, brain, epididymides, heart, kidney, liver, ovary, pituitary gland, prostate gland, 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 tissues were sampled: adrenal 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, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, 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, 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 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 α2μ-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 humane reasons on Days 37 and 57. One male (1000 ppm) was sacrificed on Day 37 having had a distended abdomen between Days 22 and 37 and general 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 sacrificed on Day 57 having been noted to have labored and noisy respiration, a wasted appearance, piloerection 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 macroscopic examination revealed soiled fur around both eyes and a fracture of the nasal cavity. These two premature sacrifices were considered not to be treatment-related.

There were no mortalities during the recovery phase of the study.

3. Neurological examinations

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 treatment-related effect.

Grip strength - At 3200 ppm, fore- and hindlimb 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 females was considered most likely to be due to a decrease in body weight, rather than to be a specific treatment-related neurotoxic effect. At 1000, 200 and 50 ppm in both sexes, fore- and hindlimb grip strength were essentially comparable to control values. The few changes noted in females at 1000 ppm were considered to reflect inter-individual variations rather than any treatment-related effect.

B. Body weight and body weight gain

At 3200 ppm, mean body weight was decreased by between 4% and 6% in males ($p \leq 0.01$ or $p \leq 0.05$) and 4% and 8% in females ($p \leq 0.01$ or $p \leq 0.05$ on 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 treatment in males and females (-26 and -29%, respectively, $p \leq 0.01$), 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 ($p \leq 0.05$) and 17% in females ($p \leq 0.01$). 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 ppm, 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 either 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 ($p \leq 0.01$ or $p \leq 0.05$ on most occasions), compared to the controls. No treatment-related effect on mean food consumption was noted 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 slightly 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 sex.

The mean achieved dosage intake per group was as follows:

Table 5.3.2-2 Mean achieved dose levels of AE C656948, in mg/kg bw/day

Sex	Males				Females			
Dietary level (ppm)	**50**	**200**	**1000**	**3200**	**50**	**200**	**1000**	**3200**
Weeks 1-13 (mg/kg/day)	3.06	12.5	60.5	204	3.63	14.8	70.1	230

D. Ophthalmoscopic examination

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

E. Hematology, clinical chemistry, and urinalysis

1. Hematology

a) Dosing Phase

Higher mean prothrombin time was noted at 3200 ppm in males only (+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 findings (Main study (dosing) phase) (mean ± SD)

Main study (dosing) phase					
Dose levels (ppm)	**0**	**50**	**200**	**1000**	**3200**
Males					
Hemoglobin (g/dL)	15.5 ± 0.3	15.4 ± 0.3	15.2 ± 0.6	14.9 ± 0.5 (-4%)	14.6 ± 0.7** (-6%)
Hematocrit (%)	47.6 ± 0.9	47.6 ± 0.9	47.0 ± 1.9	45.7 ± 1.2*	45.4 ± 1.9**
Females					
Hemoglobin (g/dL)	15.0 ± 0.5	15.2 ± 0.5	15.4 ± 0.3	15.1 ± 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 ± 0.5	17.8 ± 0.4	17.9 ± 0.7	17.5 ± 0.5	16.3 ± 0.4** (-8%)

* = p≤0.05. **= p≤0.01

Additionally, higher mean platelet (+24%, p≤0.01) and reticulocyte (+50% for absolute count and +42% for percentage, p≤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 tendancy towards reversibility although mean hemoglobin concentration was still lower after the recovery period in males and females (-4%, p≤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, no 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:

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

Main study (dosing) phase										
Sex	**Males**					**Females**				
Dietary level (ppm)	**0**	**50**	**200**	**1000**	**3200**	**0**	**50**	**200**	**1000**	**3200**
Total bilirubin, µmol/L	2.0 ± 0.4	1.7 ± 0.2	1.8 ± 0.5	1.4 ± 0.2** (-30%)	1.3 ± 0.3** (-35%)	2.6 ± 0.4	2.1 ± 0.3**	1.9 ± 0.5**	1.6 ± 0.3** (38%)	1.7 ± 0.04** (-35%)
Triglycerides (mmol/L)	NC	NC	NC	NC	NC	0.44 ± 0.12	0.48 ± 0.12	0.45 ± 0.04	0.50 ± 0.08	0.85 ± 0.33** (+102%)
Total cholesterol, mmol/L	1.87 ± 0.5	1.73 ± 0.25	2.23 ± 0.57	2.72 ± 0.84* (+45%)	2.95 ± 0.47** (+58%)	1.88 ± 0.17	2.13 ± 0.39	2.11 ± 0.43	2.79 ± 0.65** (-48%)	3.78 ± 0.78** (-101%)
γ-glutamyltransferase (IU/L)	0 ± 1	0 ± 0	0 ± 0	0 ± 1	2 ± 1** (+625%)	0 ± 0	0 ± 0	1 ± 1	1 ± 1	9 ± 4** (+4300%)
Alkaline Phosphatase (IU/L)	NC	NC	NC	NC	NC	47 ± 10	45 ± 9	44 ± 10	42 ± 9	31 ± 6** (-34%)
Total protein (g/L)	72 ± 3	73 ± 3	72 ± 3	73 ± 4	78 ± 4** (+8%)	72 ± 3	70 ± 4	72 ± 5	75 ± 4	78 ± 7* (+8%)
Globulin (g/L)	28 ± 2	28 ± 1	29 ± 2	29 ± 2	31 ± 2	24 ± 2	25 ± 2	27 ± 2	27 ± 2	30 ± 3** (+25%)
Albumin/Globulin ratio	NC	NC	NC	NC	NC	1.96 ± 0.26	1.82 ± 0.15	1.74 ± 0.06	1.78 ± 0.10	1.64 ± 0.10** (-16%)
Creatinine mmol/L	35 ± 3	35 ± 4	33 ± 2	39 ± 5* (+11%)	40 ± 3** (+14%)	NC	NC	NC	NC	NC

Main study (dosing) phase										
Sex	**Males**					**Females**				
Urea (mmol/L)	5.14 ± 0.64	4.96 ± 0.39	4.86 ± 0.44	5.45 ± 0.79	6.01 ± 0.57** (+17%)	NC	NC	NC	NC	NC
Glucose (mmol/L)	6.64 ± 1.04	7.05 ± 1.38	6.87 ± 1.15	5.85 ± 0.49	4.88 ± 0.32** (-27%)	NC	NC	NC	NC	NC
Inorganic phosphorus (mmol/L)	1.79 ± 0.09	1.86 ± 0.15	1.86 ± 0.18	1.96 ± 0.14* (+9%)	2.11 ± 0.15** (+18%)	1.46 ± 0.13	1.45 ± 0.13	1.48 ± 0.11	1.58 ± 0.18	1.68 ± 0.23* (+15%)
Calcium mmol/L	2.71 ± 0.07	2.71 ± 0.05	2.73 ± 0.06	2.81 ± 0.07* (+4%)	2.86 ± 0.08** (+6%)	2.67 ± 0.07	2.65 ± 0.06	2.70 ± 0.05	2.74 ± 0.07	2.80 ± 0.12** (+5%)
Chloride (mmol/L)	103 ± 2	103 ± 1	102 ± 1	100 ± 1** (-3%)	100 ± 1** (-3%)	104 ± 1	103 ± 1	103 ± 2	103 ± 1	101 ± 1** (-3%)

Significant at * p ≤ 0.05; ** p ≤ 0.01; NC = Not relevant change

b) Recovery phase

After 1 month of recovery in the high dose group, the general tendancy was towards reversibilty. Nevertheless, statistically significant differences were still noted in mean total cholesterol, globulin concentrations and albumin/globulin ratio in females (+19%, +14% and -14% 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, no significant differences were noted between the high dose and the control groups. The slightly higher mean alkaline phosphatase activity seen in females was considered not to be biologically or toxicologically relevant.

Thyroid hormones

a) Dosing phase

At 3200 ppm, an increase in mean TSH level was noted at Week 3 in both sexes (+63%, p≤0.05 and +71%, p≤0.01, respectively), together with an increase in mean T3 and T4 levels in females (+24%, p≤0.05 and +54%, p≤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≤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 biologically or toxicologically relevant changes at 200 or 50 ppm in either sex.

b) Recovery phase

All changes observed at the end of the dosing phase were considered to be reversible, as after the 1-month 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 toxicologically relevant.

3. Urinalysis

a) Dosing phase

The incidence and severity of cellular casts in urine were increased in all male treated groups in a dose-related 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

Main study (dosing) phase					
Sex	**Males**				
Dietary level (ppm)	**0**	**50**	**200**	**1000**	**3200**
Number of animals examined	10	9	10	9	10
Slight	1	4	2	0	1
Moderate	0	2	1	3	2
Severe	0	0	2	5	7
Total	1	6	5	8	10

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

b) Recovery phase

After 1 month of recovery, cellular casts in urine were still observed in male high dose group. However, 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	**Males**	
Dietary level (ppm)	**0**	**3200**
Number of animals examined	10	10
Slight	0	5
Moderate	0	3
Severe	0	0
Total	0	8

F. Sacrifice and pathology

1. Terminal body weight and organ weight

a) Dosing phase

Mean terminal body weight at 3200 ppm was lower than in controls in males and females (-5%, not statistically significant and -9%, $p \leq 0.05$, respectively).

Mean absolute and relative liver weights were statistically significantly higher at 3200 and 1000 ppm in both sexes, when compared to controls. A tendency towards higher liver weights was also noted at 200 ppm in both sexes (not statistically significant).

Mean absolute and relative kidney weights were statistically significantly higher at 3200 and 1000 ppm in males, when compared to controls. A tendency towards higher kidney weights was also observed at 200 ppm in males (not statistically significant). At 3200 ppm in females, mean kidney to body weight ratio was statistically significantly higher than controls, but this change was attributable to lower terminal body weight and was thus considered not to be toxicologically relevant.

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

Table 5.3.2-7 Mean terminal body weight and absolute and relative weights of liver, kidney and thyroid (mean ± SD)

Main study (dosing) phase					
Dietary level (ppm)	**0**	**50**	**200**	**1000**	**3200**
Males					
Terminal body wt, g	479.2±25.9	476.1±35.7	500.7±38.8	498.2±19.6	457.5±42.7
Liver wt, g	10.94 ± 0.80	10.82 ± 0.63 (-1%)	12.02 ± 1.19 (+10%)	13.63 ± 1.13** (+25%)	16.77 ± 1.70** (+53%)
Liver wt, % body wt	2.282 ± 0.096	2.277 ± 0.101 (0%)	2.400 ± 0.151 (+5%)	2.736 ± 0.188** (+20%)	3.669 ± 0.223** (+61%)
Liver wt, % brain wt	518.50 ± 51.102	516.57 ± 41.111 (0%)	569.50 ± 75.647 (+10%)	657.050 ± 57.55** (+27%)	839.82 ± 83.091** (+62%)
Kidney wt, g	2.81 ± 0.21	2.89 ± 0.20 (+3%)	3.10 ± 0.30 (+10%)	3.65 ± 0.32** (+30%)	3.60 ± 0.38** (+28%)
Kidney wt, % body wt	0.587 ± 0.045	0.608 ± 0.029 (+4%)	0.620 ± 0.049 (+6%)	0.732 ± 0.043** (+25%)	0.788 ± 0.069** (+34%)

Main study (dosing) phase					
Dietary level (ppm)	**0**	**50**	**200**	**1000**	**3200**
Males					
Kidney wt, % brain wt	133.04 ± 12.503	137.98 ± 10.830 (+4%)	146.70 ± 17.950 (+10%)	175.96 ± 15.175** (+29%)	180.51 ± 21.128** (+62%)
Thyroid gland, g	0.0272 ± 0.0052	0.0224 ± 0.0058 (-18%)	0.0269 ± 0.0048 (-1%)	0.0282 ± 0.0060 (+4%)	0.0299±0.0051 (+10%)
Thyroid wt, % body wt	0.00568 ± 0.00107	0.00471 ± 0.00123 (-17%)	0.00496 ± 0.00081 (-13%)	0.00565 ± 0.00106 (-1%)	0.00657 ± 0.00121 (+16%)
Thyroid wt, % brain wt	1.287 ± 0.25164	1.068 ± 0.27200 (-17%)	1.178 ± 0.23204 (-8%)	1.352 ± 0.23465 (+5%)	1.500±0.26417 (+17%)
Females					
Terminal body wt, g	270.1±23.0	269.3±16.8	274.5±22.5	263.7±23.3	245.3*±19.6
Liver wt, g	5.74 ± 0.58	5.94 ± 0.32 (+3%)	6.40 ± 0.43 (+11%)	7.09 ± 0.58** (+24%)	9.09 ± 1.55** (+58%)
Liver wt, % body wt	2.123 ±0.096	2.235 ± 0.105 (+5%)	2.366 ± 0.071 (+11%)	2.697 ± 0.172** (+27%)	3.699 ± 0.503** (+74%)
Liver wt, % brain wt	292.99 ± 24.236	306.43 ± 18.640 (+5%)	323.45 ± 15.254 (+10%)	367.12 ± 24.632** (+25%)	501.61 ± 70.554** (+71%)
Kidney wt, g	1.65 ± 0.14	1.74 ± 0.13 (+5%)	1.73 ± 0.15 (+5%)	1.73 ± 0.18 (+5%)	1.70 ± 0.17 (+3%)
Kidney wt, % body wt	0.614 ± 0.044	0.655 ± 0.041 (+7%)	0.640 ± 0.050 (+4%)	0.659 ± 0.062 (+7%)	0.693 ± 0.069** (+13%)
Kidney wt, % brain wt	84.540 ± 6.618	89.904 ± 7.905 (+6%)	87.468 ± 5.139 (+3%)	89.817 ± 9.611 (+6%)	94.027 ± 10.455 (+11%)
Thyroid gland, g	0.0185 ± 0.0021	0.0203 ± 0.0040 (+10%)	0.0164 ± 0.0042 (-11%)	0.0184 ± 0.0038 (-1%)	0.0206 ± 0.0026 (+11%)
Thyroid wt, % body wt	0.00689 ± 0.00093	0.00767 ± 0.00163 (+11%)	0.00610 ± 0.00156 (-11%)	0.00696 ± 0.00116 (0%)	0.00841 ± 0.00097* (+22%)
Thyroid wt, % brain wt	0.948 ± 0.1218	1.050 ± 0.2168 (+11%)	0.829 ± 0.1925 (-13%)	0.954 ± 0.2046 (0%)	1.140 ± 0.1423 (+20%)

Significant at * p ≤0.05; ** p ≤0.01

b) Recovery phase

After 1 month of recovery in the high dose groups, mean terminal body weight was still lower than in controls in males and females (-9%, p≤0.01and -7%, not statistically significant, respectively).

Mean liver 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.

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 treatment-related as it was not associated with any microscopic finding.

Table 5.3.2-7 continued

Recovery phase		
Dietary level (ppm)	**0**	**3200**
	Males	
Terminal body wt, g	522.7±31.5	478.2±32.4**
Kidney wt, g	2.89 ± 0.16	3.08 ± 0.22* (+7%)
Kidney wt, % body wt	0.554 ± 0.024	0.645 ± 0.044** (+16%)
Kidney wt, % brain wt	133.05 ± 9.466	147.31 ± 12.287** (+11%)

* = p<0.05. **= p<0.01

2. Gross pathology

a) Dosing phase

Terminal sacrifice: Enlarged and dark liver and/or prominent lobulation 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 5.3.2-8 Incidence macroscopic changes in the liver and kidneys terminal sacrifice

Main study (dosing) phase										
Sex	**Males**					**Females**				
Dose level (ppm)	**0**	**50**	**200**	**1000**	**3200**	**0**	**50**	**200**	**1000**	**3200**
Liver: Obviously large	0/10	0/9	2/10	6/9	10/10	0/10	0/10	1/10	7/10	10/10
Liver: Dark	0/10	0/9	0/10	2/9	9/10	0/10	0/10	0/10	0/10	9/10
Liver: Prominent lobulation	0/10	0/9	0/10	4/9	6/10	0/10	0/10	0/10	1/10	0/10
Kidneys: Obviously large	0/10	1/9	3/10	4/9	6/10	0/10	0/10	1/10	0/10	0/10
Kidney: Pale	1/10	0/9	1/10	7/9	9/10	0/10	1/10	0/10	0/10	0/10

b) Recovery phase

Terminal sacrifice: After 1 month of recovery in the high dose group, enlarged kidneys were noted in 2/10 males.

3. Microscopic pathology

a) Dosing phase

Terminal sacrifice: 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 dose-related increase in incidence and severity at 3200 and 1000 ppm in both sexes and minimal centrilobular hepatocellular hypertrophy was observed at 200 ppm in males. In addition, minimal to moderate periportal to midzonal hepatocellular macrovacuolation was observed in females at 3200 and 1000 ppm.

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

Main study (dosing) phase										
Sex	**Males**					**Females**				
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	10	10
Centrilobular hepatocellular hypertrophy: diffuse										
Minimal	0	0	2	5	0	0	0	0	5	3
Slight	0	0	0	4	2	0	0	0	2	5
Moderate	0	0	0	0	8	0	0	0	0	1
Total	**0**	**0**	**2**	**9**	**10**	**0**	**0**	**0**	**7**	**10**
Periportal to midzonal hepatocellular macrovacuolation : focal/multifocal										
Minimal	0	0	0	0	0	0	0	3	6	3
Slight	0	0	0	0	0	0	0	0	0	1
Moderate	0	0	0	0	0	0	0	0	0	1
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**3**	**6**	**5**

In the kidney, hyaline droplet nephropathy (characterized by basophilic tubules, hyaline droplets in proximal tubules and granular casts in the medulla) 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 α2μ-globulin in the proximal tubules of affected animals. Accumulation of α2μ-globulin in the male kidney was confirmed with a protein-specific immunohistochemical determination, which showed a higher storage of α2μ-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.

α2μ-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 α2μ-globulin and is male rat-specific (G.C. HARD et al., Susceptibility of the kidney to toxic substances, in MOHR U. et al., Pathobiology of the aging rat, vol. 1, p. 252, ILSI Press, 1992, KIIA 5.3.2 /03; Hard, G. C., Alden, C.L., 1992, M-344988-01-1). As humans secrete α2μ-globulin only in trace amounts, this mechanism 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.

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

Main study (dosing) phase										
Sex	**Males**					**Females**				
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	10	10
Hyaline droplets : proximal tubules										
Minimal	1	3	7	0	0	0	0	0	0	0
Slight	0	0	3	0	0	0	0	0	0	0
Moderate	0	0	0	9	1	0	0	0	0	0
Marked	0	0	0	0	9	0	0	0	0	0
Total	**1**	**3**	**10**	**9**	**10**	**0**	**0**	**0**	**0**	**0**
Basophilic tubules : focal/multifocal										
Minimal	1	1	3	3	1	0	0	0	0	0
Slight	0	1	0	6	8	0	0	0	0	0
Moderate	0	1	0	0	1	0	0	0	0	0
Total	**2**	**3**	**3**	**9**	**10**	**0**	**0**	**0**	**0**	**0**
Granular casts: medulla										
Minimal	0	0	1	2	6	0	0	0	0	0
Slight	0	0	0	6	2	0	0	0	0	0
Moderate	0	0	0	0	1	0	0	0	0	0
Total	**0**	**0**	**1**	**8**	**9**	**0**	**0**	**0**	**0**	**0**
Hyaline casts: focal/multifocal										
Minimal	2	0	0	4	6	0	1	1	0	2
Slight	1	0	0	0	0	0	0	0	0	0
Total	**2**	**0**	**0**	**4**	**6**	**0**	**1**	**1**	**0**	**2**

In the thyroid gland, higher incidences of minimal to slight diffuse hypertrophy of follicular cells was seen at 3200 and 1000 ppm 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 of treatment

Main study (dosing) phase										
Sex	**Males**					**Females**				
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	10	10
Follicular cell hypertrophy: diffuse										
Minimal	0	0	1	3	5	0	0	0	2	1
Slight	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 tubules, granular casts in the medulla and hyaline casts were persistent in the kidney of some males.

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

Main study (dosing) phase				
Sex	**Males**		**Females**	
Dietary level (ppm)	**0**	**3200**	**0**	**3200**
Number of animals examined	10	10	10	10
Hyaline droplets : proximal tubules				
Minimal	0	2	0	0
Total	**0**	**2**	**0**	**0**
Basophilic tubules : focal/multifocal				
Minimal	0	3	0	0
Slight	0	7	0	0
Total	**0**	**10**	**0**	**0**
Granular casts : medulla				
Minimal	0	5	0	0
Slight	0	2	0	0
Moderate	0	1	0	0
Total	**0**	**8**	**0**	**0**
Hyaline casts: focal/multifocal				
Minimal	1	9	0	1
Total	**1**	**9**	**0**	**1**

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

No specific deficiencies are noted.

III. Conclusions

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 a dose 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 related to hyaline droplet nephropathy (known not to be relevant for humans) and minimal adaptive liver changes, the appropriate NOAEL for human risk assessment purpose is considered to be 200 ppm (equivalent to 12.5 mg/kg bw/day).

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 a dose 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 related to hyaline droplet nephropathy (known not to be relevant for humans) and minimal adaptive liver changes, the appropriate NOAEL for human risk assessment purpose is considered to be 200 ppm (equivalent to 12.5 mg/kg bw/day).

Data Point:	KCA 5.3.2/02
Report Author:	[redacted]
Report Year:	2005
Report Title:	AE C656948 - 90-day toxicity study in the mouse by dietary administration
Report No:	SA 04052
Document No:	M-251136-01-1
Guideline(s) followed in study:	OECD 408; Directive 2001/59/EC Method B.26; US-EPA series 870, No 870.3100; JMAFF 12 Nohsan no 8147
Deviations from current test guideline:	Current guideline: OECD 408, 2018 Deviations: no measurement of thyroid hormone, no measurements 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 (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

AE C656948 (batch number PFI 0304, 99.0 % purity), was administered continuously via the diet to groups of C57BL/6J mice (10/sex/group) at concentrations of 0, 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 mg/kg/day in females).

At 1000 ppm, mean food consumption was increased by up to 12% on a few occasions in males, compared to controls. Clinical 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 adrenal 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 animals together with a greater incidence of minimal focal necrosis in 3/10 males and minimal to slight focal necrosis in 6/10 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 150 ppm, at necropsy, mean liver weights were increased by 9% to 16% in males and 25% to 28% in females, 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

A. Materials

1. Test material:	AE C656948
Description	Beige powder
Lot / Batch #:	PFI 0304
Purity:	99.0%
CAS #	658066-35-4
Stability of test compound:	Stable at 20 to 10000 ppm at room temperature for 105 days
2. Vehicle and / or positive control:	None
3. Test animals:	
Species:	Mouse
Strain:	C57BL/6J
Age:	Approximately 6 weeks old
Weight at dosing:	Males: 18 – 22.3 g; Females: 14.5 – 17.9 g
Source:	[redacted]
Acclimation period:	7 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually in suspended, stainless steel and wire mesh cages
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	10-15 per hour
Photoperiod:	12 hours dark, 12 hours light (7 am – 7 pm)

B. Study design

1. In life dates: 30 June 2004 - 07 October 2004

2. Animal assignment and treatment

There were 10 animals of each sex per dose group. 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 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".

Table 5.3.2-13 Details of group sizes and treatment,

Group	Test substance	Diet concentration (ppm)	No. Of animals per group	Animal identity
Males				
1	Control	0	10	OTlM2792to2801
2	AE C656948	30	10	OT2M2812to2821
3		150	10	OT3M2832to2841
4		1000	10	OT4M2852to2861
Females				
1	Control	0	10	OTlF2802to2811
2	AE 1170437	30	10	OT2F2822to2831
3		150	10	OT3F2842to2851
4		1000	10	OT4F2862to2871

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 were two preparations of each concentration during the study. The stability had been demonstrated in a previous study where 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. 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 was checked for all dose levels for each preparation and was within 92-98% of the nominal concentration. Hence all values were within the target range of 85-115% of the nominal concentration.

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 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 or more group variances) 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).

C. Methods

1. Observations

The animals were observed twice daily for morbundity 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 1 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:

$$\text{Mean achieved dosage (mg/kg body weight/day)} = \frac{\text{Mean weekly food consumption (g/day)} \times \text{Dose level (ppm)}}{\text{Mean body weight at the end of the week (g)}}$$

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 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 was collected on lithium heparin for plasma chemistry determinations. Any significant change in the appearance of the plasma was recorded and the following clinical chemistry parameters were measured on a Hitachi 911 (Roche Diagnostics, Meylan, France) for: total bilirubin, total protein, albumin, total cholesterol and urea concentrations, and aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities.

6. Urinalysis

Urinalysis was not conducted in this study.

7. Sacrifice and pathology

On study days 91, 92 or 93, a complete necropsy was performed on all animals. Animals were deeply anesthetized by Isoflurane inhalation, then exsanguinated before necropsy. All animals were fasted prior to scheduled sacrifice.

The following organs or tissues were sampled:

Adrenal gland	Eye
Aorta	Exorbital (lachrymal) gland
Articular surface (femoro-tibial)	Gallbladder
Bone (sternum)	Harderian gland
Bone marrow (sternum)	Heart
Brain	Intestine (ileum, jejunum)
Cecum	Kidney
Colon	Larynx/pharynx
Duodenum	Liver
Epididymis	Lung
Oesophagus	Lymph nodes (submaxillary)
Lymph nodes (mesenteric)	Spinal cord (cervical, thoracic, lumbar)
Mammary gland	Spleen
Nasal cavities	Stomach
Optic nerve	Submaxillary (salivary) gland
Ovary	Testis
Pancreas	Thymus
Pituitary gland	Thyroid gland (with parathyroid gland)
Prostate	Tongue
Rectum	Trachea
Sciatic nerve	Urinary bladder
Seminal vesicle	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 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 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 level.

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 a body weight loss of 6.9 g between Days 15 and 29 and a reduced food consumption between Days 9 and 29. Clinical signs recorded prior to death for this animal were reduced motor activity on Days 22 and 23, together with wasted 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 attributable 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 C656948 per group is presented in the following table:

Table 5.3.2-14 Mean achieved dose levels of AE C656948, in mg/kg bw/day,

Sex	Males			Females		
Dietary level (ppm)	**30**	**150**	**1000**	**30**	**150**	**1000**
Weeks 1-13	5.4	26.6	188	6.8	32.0	216

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 an outlier in the female control values, respectively, p≤0.01), compared to the control groups. In 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≤0.01) and a tendency 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≤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 aminotransferase and cholesterol (mean ± SD)

Sec	Males				Females			
Dietary level (ppm)	**0**	**30**	**150**	**1000**	**0**	**30**	**150**	**1000**
Alamine aminotransferase IU/L	40±13	88±79	55±16	122±35*	83±118	56±20	64±24	96±32*
Albumin, g/L	41±1	39±2	38±2*	36±2*	43±3	43±3	42±2	39±2*
Aspartate aminotransferase , IU/L	108±40	139±50	92±21	158±51**	218±320	118±29	152±43	166±71
Cholesterol mmol/l	1.35±0.17	0.94±0.21*	0.79±0.14*	0.81±0.16*	1.22±0.33	1.08±0.15	0.93±0.15*	1.33±0.12

Significant at * p < 0.01; ** p <0.05;

E. Sacrifice and pathology

1. Organ weight

There was no relevant change in terminal body weight in either sex. Mean absolute and/or relative liver weights were statistically significantly higher 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 Liver weight changes at terminal sacrifice (% change when compared to controls) (mean ± SD)

Sex	Males			
Dietary level (ppm)	**0**	**30**	**150**	**1000**
Terminal body wt, g	23.0±1.2	23.2±0.8	22.5±0.9	22.6±0.6
Liver wt, g	0.91±0.08	0.94±0.15	1.04±0.07	1.24±0.14*
Liver wt, % body wt	3.973±0.312	4.064±0.640	4.590±0.147*	5.497±0.688*
Liver wt, % brain wt	210.400±21.305	212.555±36.690	230.188±14.668	282.470±33.447*
	Females			
	0	**30**	**150**	**1000**
Terminal body wt, g	18.3±0.7	18.5±0.5	18.8±0.6	19.1±0.5*
Liver wt, g	0.75±0.09	0.84±0.06**	0.96±0.08*	1.09±0.07*
Liver wt, % body wt	4.102±0.452	4.566±0.294**	5.117±0.302*	5.681±0.298*
Liver wt, % brain wt	165.237±22.807	181.546±12.771	212.017±19.438*	239.379±13.339*

Statistically significant at * p < 0.01; ** p <0.05;

Mean absolute and relative adrenal gland weights were increased by between 87% and 92% at 1000 ppm in males compared to controls, the difference being statistically significant for mean adrenal gland to body weight ratio only (p≤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 livers were observed in 5/10 males and 10/10 females. Other gross pathology changes were considered as incidental and not treatment-related.

Microscopic pathology: effects of treatment with AE C656948 were seen in the liver and adrenal gland in both sexes.

In the liver, there was a minimal to moderate hypertrophy of centrilobular hepatocytes in both sexes 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 3/10 males and 6/10 females given 1000 ppm, compared to one female in the control group.

Table 5.3.2-17 Incidence of microscopic changes in the liver and adrenal gland

Sex	Males				Females			
Dietary level (ppm)	**0**	**30**	**150**	**1000**	**0**	**30**	**150**	**1000**
Liver, N examined	9	9	10	10	10	10	10	10
Centrilobular hepatocellular hypertrophy:								
Minimal	0	0	3	0	0	0	5	1
Slight	0	0	7	0	0	0	0	8
Moderate	0	0	0	10	0	0	0	1
Total	**0**	**0**	**10**	**10**	**0**	**0**	**5**	**10**
Focal necrosis:								
Minimal	0	0	0	3	1	1	0	3
Slight	0	0	0	0	0	0	0	3
Total	**0**	**0**	**0**	**3**	**1**	**1**	**0**	**6**
Adrenal, N examined	9	9	10	10	10	10	10	10
Cortical ceroid pigment:								
Minimal	5	3	4	0	0	0	0	0
Slight	1	1	0	0	0	0	0	0
Total	**6**	**4**	**4**	**0**	**0**	**0**	**0**	**0**
Cortical vacuolation:								
Minimal	0	0	0	0	3	1	2	9
Slight	0	0	0	0	0	0	0	1
Total	**0**	**0**	**0**	**0**	**3**	**1**	**2**	**10**

In the adrenal glands at 1000 ppm, there was a lower incidence of cortical ceroid pigment in males and a greater incidence of minimal to slight cortical vacuolation in females, compared to controls. The change noted in males at 1000 ppm was considered to be slight compared to the magnitude of increase in adrenal gland weights seen 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 treatment-related. In females, the incidence and severity of this finding were comparable between controls and treated animals.

F. Deficiencies

No deficiencies were noted

III. Conclusions

Under the conditions of this study, a dose level of 150 ppm AE C656948 by dietary administration to the C57BL/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).

Assessment and conclusion by applicant:
Study meets the current guidance and the requirements in 283/2013.
Under the conditions of this study, a dose level of 150 ppm AE C656948 by dietary administration to the C57BL/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).

Data Point:	KCA 5.3.2/03
Report Author:	[redacted]
Report Year:	2006
Report Title:	AE C656948 - 90-day toxicity study in the dog by dietary administration
Report No:	SA05046
Document No:	M-276047-01-1
Guideline(s) followed in study:	O.E.C.D. guideline 409 (1998); E.E.C. Directive 2001/59/EC, Method B.27 (August 21, 2001); U.S. E.P.A., OPPTS Series 870, Health Effects Testing Guidelines, No 870.3150 (August, 1998); M.A.F.F. in Japan, notification 12 Nousan n°8147 (November 24, 2000)
Deviations from current test guideline:	Current guideline: OECD 409, 1998 Deviation: None
Previous evaluation:	Yes, evaluated and submitted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary
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 least 13 weeks (equivalent to 0, 28.5, 171, 332 mg/kg bw/day in males and 0, 32.9, 184, 337 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 just prior to necropsy. 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 processed for histopathological examination.

At 20000/10000 ppm:

Due to a lack of palatability of AE C656948 in the diet, a marked reduction in food consumption was observed in both sexes with an associated effect on body weight gains and body weights. Despite the reduction of the dietary level of the test compounds 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, erythrocyte counts, hemoglobin concentration and hematology) and some clinical chemistry parameters (mainly alkaline phosphatase and δ-glutamyl transferase activities and bilirubin, 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 thymus was found to be generally atrophic and associated with a higher severity in thymic 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.

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

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Beige powder
Lot / Batch #:	Mix-Batch 08528/0002
Purity:	Min. 94.6%
CAS #	658066-35-4
Stability of test compound:	Stable at 25 ± 5°C / room temperature
2. Vehicle and / or positive control:	None
3. Test animals:	
Species:	Dog
Strain:	Beagle
Age:	9-month old
Weight at dosing:	Males: 6.3 – 9.0 kg & females: 4.5 - 7.1 kg
Source:	[redacted]
Acclimation period:	20 days
Diet:	Certified canine meal 125C3-P1 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France). Three hundred grams of diet moistened with 450 ml of water at the time of distribution was given daily to each animal for approximately 1.5 hours each morning, except on study Day 337 when animals were fed 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®) was spread over the normal dietary ration of 2 animals (PT4M1291 and PT4F1295) to stimulate their appetite as described below:

Animal	Study days	Beef pâté added to the diet
PT4M1291	16	200 g
	17 to 19	100 g
PT4F1295	16	200g
	17 to 21	100g
	66 to 69	100g
	90	400g

Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*
Housing:	Animals were housed individually in stainless steel kennels with 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 acclimatization 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 once per week for dogs of the same sex and treatment group.
Environmental conditions:	
Temperature:	18-21°C
Humidity:	40-70%
Air changes:	Target of 15 per hour during the dosing period
Photoperiod:	12 hours dark, 12 hours light (7 am – 7 pm)

B. Study design

1. In life dates: 06 April 2005 – 07 July 2005

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 ophthalmological examination, hematology and clinical chemistry 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 diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 5.3.2-18 Test groups, dose levels and achieved dosages

Test Group (Group number)	Sex : Number of animals	Concentration of AE C656948 in Diet (ppm)	Achieved dose to animal (Weeks 1-13) (mg/kg/day)
Control (1)	Male: 4 Female: 4	0	0
Low (2)	Male: 4 Female: 4	800	Male: 28.5 Female:32.9
Mid (3)	Male: 4 Female: 4	5000	Male: 171 Female: 184
High (4)	Male: 4 Female: 4	20000 (Day 1 to 14) 10000 (Day 15 to sacrifice)	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 at 87-115% of nominal concentration confirming that the formulations were homogeneously distributed.

AE C656948 was found to be stable in the diet at 800 and 20000 ppm over 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 89-109% of the nominal levels; 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 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 (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 Dunn test (2-sided), if the Kruskal-Wallis test 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% 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 ill-health, moribundity, mortality and clinical signs of toxicity (except once daily on weekends and holidays). 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 for clinical signs of toxicity 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 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 recorded 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 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). Ophthalmic 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 diet fasted overnight prior to blood collection, which was drawn via jugular vein puncture.

The following parameters were evaluated:

Hematology:

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 were assayed using an Advia 120 (Bayer Diagnostics, Puteaux, France). A blood smear was prepared and stained with Wright stain. It was examined when the results of Advia 120 determinations were abnormal. Prothrombin time and activated partial thromboplastin time were assayed on an ACL 3000 (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-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). Globulin and albumin/globulin ratio values were calculated.

6. Urinalysis

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

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

Urine samples were weighed to determine urinary volume, pH was assayed using Clinitek 200+ and Ames Multistix dipsticks (Bayer Diagnostics, Puteaux, France). Urinary refractive index was measured using a RFM 320 refractometer (Bioblock Scientific, Illkirch, 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 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.

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 acepromazine (50 µl/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:

	DIGESTIVE SYSTEM		CARDIOVASC./HEMAT.		NEUROLOGIC
X	Tongue	X	Aorta	XX	Brain (with cerebellum)
X	Submaxillary (salivary) gland	XX	Heart	X	Sciatic nerve
X	Esophagus	X	Bone marrow, sternum	X	Spinal cord (cervical, thoracic, lumbar)
X	Stomach	X	Lymph node, mesenteric	X	Eyes
X	Duodenum	X	Lymph node, retropharyngeal	X	Optic nerves
X	Jejunum	XX	Spleen	XX	Pituitary gland
X	Ileum	XX	Thymus		
X	Cecum				GLANDULAR
X	Colon		UROGENITAL	XX	Adrenal gland
X	Rectum	XX	Kidney	X	Parathyroid gland
XX	Liver	X	Urinary bladder	XX	Thyroid gland (weighed with parathyroid gland)
X	Pancreas	XX	Testis		
X	Gallbladder	XX	Epididymis		OTHER
		XX	Prostate gland	X	Bone (sternum)
	RESPIRATORY	XX	Ovary	X	Skeletal muscle
X	Trachea	XX	Uterus (with cervix)	X	Skin
X	Lung	X	Mammary gland	X	All gross lesions and masses
X	Pharynx	X	Vagina	X	Articular surface (femorotibial joint)
X	Larynx				

(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% formalin with the exception of the eye, 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 groups. After the examination by the study pathologist, an independent review by a second pathologist was conducted. The results presented in the report are a consensus view of the 2 pathologists.

II. Results and discussion

A. Clinical signs and mortality

1. Clinical signs of toxicity and physical examination

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

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

There was no treatment-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 18% 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 females, 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. At 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 of palatability of the test substance in the diet.

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

Dosage level (ppm)	0	800	5000	20000/10000
Sex		**Males**		
Initial BW (%C)	7.4 ± 0.8	7.4 ± 0.4 (100%)	7.2 ± 0.7 (97%)	7.7 ± 0.3 (104%)
BWG Week 1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	-0.3 ± 0.1**
BWG Weeks 1-4	0.6 ± 0.1	0.6 ± 0.2	0.4 ± 0.3	-0.7 ± 0.4**
BWG Weeks 1-8	0.9 ± 0.2	0.8 ± 0.4	0.4 ± 0.6	-0.8 ± 0.4**
BWG Weeks 1-13	1.0 ± 0.3	1.0 ± 0.7	0.6 ± 0.8	- 0.8 ± 0.5**
Final BW (%C)	8.4 ± 0.8	8.4 ± 0.7 (100%)	7.8 ± 1.1 (93%)	6.9 ± 1.0 (82%)
Sex		**Females**		
Initial BW (%C)	5.5 ± 0.6	5.6 ± 1.1 (102%)	5.6 ± 0.6 (102%)	5.7 ± 0.7 (104%)
BWG Week 1	0.3 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	-0.4 ± 0.2**
BWG Weeks 1-4	0.7± 0.1	0.5 ± 0.3	0.1 ± 0.4	-0.8 ± 0.3**
BWG Weeks 1-8	0.8 ± 0.2	0.4 ± 0.4	0.0 ± 0.5	-1.1 ± 0.6**
BWG Weeks 1-13	1.0 ± 0.3	0.4 ± 0.5	0.2 ± 0.5	-1.1 ± 0.5**
Final BW (%C)	6.5 ± 0.6	6.0 ± 1.2 (92%)	5.8 ± 0.8 (89%)	4.6 ± 0.7 (71%)

*: p≤0.05; **: p≤0.01; BWG: body weight gain; (%C) = % versus control

C. Food consumption 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 C656948 was reduced to 10000 ppm, the food consumption was still reduced by 9-38% in males and 28-53% 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.

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

Dose level (ppm)	0	800	5000	20000/10000	0	800	5000	20000/10000
Sex	Males				Females			
Week 1 (% C)	641	671 (105%)	548 (85%)	478* (75%)	638	524 (82%)	490 (77%)	299** (47%)
Weeks 1-13 (% C)	701	722 (103%)	653 (93%)	528 (75%)	676	612 (91%)	527 (78%)	368 (54%)

*: p≤0.05; **: p≤0.01; (%) = % versus control

D. Ophthalmic examination

No treatment-related ocular abnormalities were observed at ophthalmic examination.

E. Blood analyses

1. Hematology

Changes in hematology parameters were limited to the high dose group where higher mean platelet counts were noted throughout the study, although the effect was less pronounced at the end of the study (Week 13) than at week 8. In addition, lower erythrocyte counts, hemoglobin concentrations and hematocrit values were observed in females.

Table 5.3.2-21 Hematology results in the 90-day dog study with AE C656948 (mean ± SD)

Dosage level (ppm)	0	800	5000	20000/10000	0	800	5000	20000/10000
Sex	Males							
Week	Week 8				Week 13			
Platelet count (10^9/L)	361 ± 77 -	381 ± 46 (+6%)	396 ± 71 (+10%)	548 ± 151 (+52%)	379 ± 78 -	406 ± 32 (+7%)	390 ± 84 (+3%)	491 ± 103 (+30%)
Sex	Females							
Week	Week 8				Week 13			
Platelet count (10^9/L)	370 ± 70 -	435 ± 47 (+18%)	415 ± 164 (+12%)	577 ± 233 (+56%)	411 ± 82 -	470 ± 57 (+14%)	492 ± 100 (+20%)	570 ± 183 (+39%)
Red blood cell count (10^{12}/L)	7.16 ± 0.57 -	7.06 ± 0.35 (-1%)	6.93 ± 0.53 (-3%)	6.45 ± 0.65 (-10%)	7.27 ± 0.73 -	6.89 ± 0.50 (-5%)	6.70 ± 0.47 (-8%)	6.49 ± 0.82 (-11%)
Hemoglobin concentration (g/dL)	16.9 ± 0.9 -	16.6 ± 1.0 (-2%)	15.8 ± 0.9 (-7%)	14.8 ± 1.3 (-12%)	16.5 ± 1.4 -	15.8 ± 1.2 (-4%)	15.0 ± 0.8 (-9%.)	14.7 ± 2.0 (-11%)
Hematocrit (L/L)	0.480 ± 0.028 -	0.476 ± 0.031 (-1%)	0.456 ± 0.020 (-5%)	0.429 ± 0.038 (-11%)	0.479 ± 0.035 -	0.457 ± 0.041 (-5%)	0.436 ± 0.023 (-9%)	0.431 ± 0.061 (-10%)

(%) = % variation versus control

2. Clinical Chemistry

At 20000/10000 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 ± SD)

Dosage level (ppm)	0	800	5000	20000/ 10000	0	800	5000	20000/ 10000
Sex	**Males**							
Week	**Week 8**				**Week 13**			
Alkaline phosphatase (IU/l)	97 ± 16 -	129 ± 15 (+33%)	325 ± 150* (+235%)	461 ± 309** (+375%)	93 ± 22 -	139 ± 15 (+49%)	372 ± 172* (+300%)	555 ± 420** (+497%)
γ-glutamyl transferase (IU/l)	1 ± 1 -	2 ± 0 (+100%)	3 ± 1 (+200%)	10 ± 7** (+900%)	2 ± 1 -	3 ± 1 (+50%)	3 ± 1 (+50%)	13 ± 8** (+550%)
Aspartate aminotransferase (IU/l)	28 ± 5 -	25 ± 8 (-11%)	25 ± 4 (-11%)	49 ± 23 (+75%)	28 ± 4 -	31 ± 7 (+11%)	26 ± 5 (-7%)	43 ± 20 (+54%)
Alanine aminotransferase (IU/l)	32 ± 12 -	27 ± 4 (-16%)	34 ± 12 (+6%)	115 ± 98 (+259%)	31 ± 8 -	28 ± 6 (-10%)	38 ± 15 (+23%)	215 ± 189 (+594%)
Total bilirubin (µmol/l)	4.4 ± 1.0 -	2.7 ± 0.4** (-39%)	2.0 ± 0.3** (-55%)	1.9 ± 0.5** (-57%)	4.5 ± 1.0 -	3.1 ± 0.6 (-31%)	2.2 ± 0.9* (-51%)	2.8 ± 1.4 (-38%)
Albumin (g/l)	36 ± 2 -	34 ± 1 (-6%)	30 ± 3* (-17%)	29 ± 3** (-19%)	36 ± 1 -	33 ± 1 (-8%)	28 ± 3** (-22%)	27 ± 4** (-25%)
Total protein (g/l)	57 ± 4 -	55 ± 1 (-4%)	54 ± 3 (-5%)	52 ± 3 (-9%)	59 ± 4 -	55 ± 2 (-7%)	53 ± 4 (-10%)	52 ± 1* (-12%)
Albumin/globulin ratio	1.72 ± 0.16 -	1.61 ± 0.08 (-6%)	1.20 ± 0.16 ** (-30%)	1.23 ± 0.22 ** (-28%)	1.64 ± 0.16 -	1.45 ± 0.04 (-12%)	1.09 ± 0.12** (-34%)	1.11 ± 0.25** (-32%)
Sex	**Females**							
Week	**Week 8**				**Week 13**			
Alkaline phosphatase (IU/l)	111 ± 21 -	190 ± 43 (+71%)	299 ± 161 (+169%)	375 ± 73** (+238%)	111 ± 28 -	190 ± 72 (+71%)	355 ± 208 (+220%)	383 ± 85* (+245%)
γ-glutamyl transferase (IU/l)	2 ± 1 -	2 ± 1 (0%)	4 ± 3 (+100%)	10 ± 5* (+400%)	2 ± 2 -	3 ± 1 (+50%)	5 ± 3 (+150%)	14 ± 12** (+600%)
Total bilirubin (µmol/l)	3.4 ± 0.7 -	2.8 ± 0.7 (-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 (g/l)	36 ± 2 -	36 ± 1 (0%)	30 ± 1** (-17%)	30 ± 2** (-17%)	36 ±1 -	37 ± 1 (+3%)	29 ± 1** (-19%)	28 ± 2** (-22%)
Total protein (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 (+2%)	1.32 ± 0.10 (-21%)	1.41 ± 0.14 (-16%)	1.61± 0.17 -	1.63 ± 0.27 (+1%)	1.24 ± 0.04 * (-23%)	1.28 ± 0.20 (-20%)

*: p≤0.05; **: p≤0.01; (%) = % versus control

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 thymus 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 and absolute and relative weights of liver and thymus (mean ± SD)

Dosage level (ppm)	0	800	5000	20000/10000
Sex	**Males**			
Terminal body wt, kg	8.49 ± 0.83	8.47 ± 0.81	8.00 ± 1.20	6.95 ± 1.01
Liver Absolute wt, g	280.9 ± 31.6	351.9 ± 20.4 (+25%)	448.0 ± 67.4** (+59%)	410.3 ± 60.9** (+46%)
Liver wt, % body wt	3.32 ± 0.38	4.18 ± 0.50* (+26%)	5.61 ± 0.37** (+69%)	5.91 ± 0.11** (+78%)
Liver wt, % brain wt	378.2 ± 47.9	472.4 ± 47.0 (+25%)	620.7 ± 86.6** (+64%)	580.6 ± 49.0** (+54%)
Sex	**Females**			
Terminal body wt, kg	6.47 ± 0.63	6.09 ± 1.14	5.90 ± 0.72	4.68 ±0.46*
Liver Absolute wt, g	228.2 ± 15.8	295.8 ±26.7 (+29%)	308.2 ±41.2* (+35%)	307.8 ±55.1* (+35%)
Liver wt, % body wt	3.55 ± 0.24	4.95 ± 0.69 (+39%)	5.30 ± 1.11* (+49%)	6.57 ± 0.90** (+85%)
Liver wt, % brain wt	315.5 ± 29.6	448.7 ± 40.52 (+42%)	478.6 ± 68.7* (+52%)	487.1 ± 115.7* (+54%)
Thymus Absolute wt, g	8.32 ± 2.40	6.13 ± 1.31 (-26%)	6.97 ± 2.69 (-16%)	2.38 ± 0.59** (-71%)
Thymus wt, % body wt	0.128 ± 0.034	0.102 ± 0.018 (-20%)	0.115 ± 0.034 (-10%)	0.050 ± 0.008** (-61%)
Thymus wt, % brain wt	11.55 ± 3.77	9.39 ± 2.48 (-19%)	10.75 ± 3.96 (-7%)	3.71 ± 0.86** (-68%)

*: p≤0.05; **: p≤0.01

2. Gross and histopathology

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

At the microscopic 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

Dose level (ppm)	**0**	**800**	**5000**	**20000/ 10000**	**0**	**800**	**5000**	**20000/10000**
Sex	**Males**				**Females**			
Liver, N examined	4	4	4	4	4	4	4	4
Hepatocellular hypertrophy: diffuse:								
Minimal	0	0	3	1	0	0	3	4
Slight	0	0	1	3	0	0	1	0
Total	**0**	**0**	**4**	**4**	**0**	**0**	**4**	**4**
Intracytoplasmic eosinophilic droplets: multifocal								
Minimal	0	1	1	3	0	1	1	2
Slight	0	0	2	1	0	0	2	0
Moderate	0	0	0	0	0	0	1	0
Total	**0**	**1**	**3**	**4**	**0**	**1**	**4**	**2**
Hepatocellular single cell necrosis: focal/multifocal								
Minimal	0	0	2	3	0	0	0	0
Moderate	0	0	0	0	0	0	1	0
Total	**0**	**0**	**2**	**3**	**0**	**0**	**1**	**0**
Thymus, N examined	4	4	4	4	4	4	4	3
Decreased size of cortex: involution								
Minimal	4	1	1	0	4	3	2	0
Slight	0	3	3	2	0	0	2	1
Moderate	0	0	0	1	0	0	0	2
Marked	0	1	0	1	0	0	0	0
Total	**4**	**4**	**4**	**4**	**4**	**3**	**4**	**3**
Estrus phases in the uterus:								
Proestrus					1	1	4	0
Early metestrus					1	2	0	0
Midmetestrus					1	0	0	0
Anestrus					1	1	0	4

H. Deficiencies

None

III. Conclusions

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

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013.
The NOAEL for this study was considered to be 800 ppm for both sexes (equivalent to 28.5 and 32.9 mg/kg bw/day for males and females, respectively).

Data Point:	MCA 5.3.2/04
Report Author:	[redacted]
Report Year:	2007
Report Title:	AE C656948 - Chronic toxicity study in the dog by dietary administration
Report No:	SA 05047
Document No:	M-294279-01-1
Guideline(s) followed in study:	OECD test guideline 452; EEC Directive 88/302, Method B.30 (1992); OPPTS 870.; MAFF in Japan, 12 Nousan No 8147
Deviations from current test guideline:	Current guideline: OECD 452, 2018 Deviation: None
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/officially recognised testing facilities
Acceptability/Reliability:	Yes

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 (equivalent to 0, 3.0, 13.2 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 just 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 tissues were examined microscopically.

At 2000 ppm:

There was an initial body weight loss during the 1st week of treatment in both sexes concomitant to lower food consumption most likely due to a lack of palatability 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 resulted 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 centrilobular 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 (13.2/14.4 mg/kg/day for males/females).

I. Materials and methods

A. Materials

1. **Test material:** AE C656948
   Description Beige powder
   Lot / Batch #: Mix-Batch 08528/0002
   Purity: Min 94.6%
   CAS # 658066-35-4
   Stability of test compound: Stable at 25 ± 5°C / room temperature

2. Vehicle and / or positive control:	none
3. Test animals:	
Species:	Dog
Strain:	Beagle
Age:	Approximately 8 months old
Weight at dosing:	7.0 – 9.4 kg, males; 5.2 - 7.7 kg, females
Source:	[redacted]
Acclimation period:	33 days
Diet:	Certified canine meal 125C3-P1 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France). Three hundred grams of diet moistened with 450 ml of water at the time of distribution was given daily to each animal for approximately 1.5 hours each morning, except on study Day 337 when animals were fed in the afternoon due to a change in planning. Supplementary food ration: due to a decrease in body weight of between 0.8 to 1.3 kg during the study for animals PT1M6617 (control group), PT3M6632 (mid dose group), PT1F6620 and PT1F6621 (control group), despite the fact these animals ate their entire daily food ration, a supplementary untreated pelleted diet ration (153C3 from S.A.F.E.) was given to these animals starting on Day 149, 130, 199 and 109, respectively, until study termination. Approximately 100 g per day of untreated pelleted diet was distributed to each dog after the measurement of its empty feeder of the dietary 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 ration was left overnight where necessary on weekends and holidays). Data were kept in the study file.
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*.
Housing:	Animals were housed individually in stainless steel kennels with 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 acclimatization 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 once per week for dogs of the same sex and treatment group.
Environmental conditions:	
Temperature:	18-21°C
Humidity:	40-70%
Air changes:	Target of 15 per hour
Photoperiod:	12 hours dark, 12 hours light (7 am – 7 pm)

B. Study design

1. In life dates: 31 January 2006 to 01 February 2007

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 urinalysis 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, body weight, food 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 sex, 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.

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

Dose Group (ppm)	No. Animals/ Dose/Sex	Males (mg/kg/day)	Females (mg/kg/day)
0 (control)	4	0	0
100	4	3.0	3.8
400	4	13.2	14.4
2000	4	67.6	66.1

3. Diet preparation and analysis

The test substance formulations were prepared to cover the dietary requirements over approximately 6-week 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 homogeneity 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.

Concentration analysis: 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-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 (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 Dunn test (2-sided), if the Kruskal-Wallis test 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% levels of significance. Statistical analyses were carried 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 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.

b. Clinical examinations

Detailed clinical observations for clinical signs of toxicity 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 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 recorded 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 minimum 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

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

X	Hematocrit (HCT)*	X	Leukocyte differential count*
X	Hemoglobin (HGB)*	X	Mean corpuscular hemoglobin (MCH)*
X	Leukocyte count (WBC)*	X	Mean corpuscular hemoglobin conc. (MCHC)*
X	Erythrocyte count (RBC)*	X	Mean corpuscular volume (MCV)*
X	Platelet count*	X	Reticulocyte count and % reticulocytes
	Blood clotting measurements:		
X	Activated partial thromboplastin time	X	Prothrombin time

*Recommended for chronic studies based on OPPTS Guideline 870.4100

b. Clinical Chemistry

	ELECTROLYTES		**OTHER**
XX	Calcium (calc)*	X	Albumin (ALB)*
XX	Chloride (Cl)*	XX	Creatinine (Creat)*
	Magnesium (Mg)*	XX	Urea*
XX	Phosphorous (Phos)*	XX	Total Cholesterol (Chol)*
XX	Potassium (K)*		Globulins (Glob)
			A/G ratio (A/G)
XX	Sodium (Na)*	XX	Glucose (gluc)*
	ENZYMES (more than 2 hepatic enzymes)*	XX	Total bilirubin (T-Bili)
XX	Alkaline phosphatase (ALK)*	X	Total protein (TP)*
XX	Alanine aminotransferase (ALT/SGPT)*	XX	Triglycerides (Trig)
XX	Aspartate aminotransferase (AST/SGOT)*		
XX	Gamma Glutamyl transferase (GGT)*		

*Recommended for chronic studies based on OPPTS Guideline 870.4100
(X) Parameters examined on serum samples.
(XX) Parameters examined on plasma samples.
Globulins and A/G ratio by calculation of TP - ALB

6. Urinalysis

Urinalysis (including parameters mentioned in the table below) was performed on all animals once prior to administration of the test substance and on all animals during study weeks 14, 24 and 52. Urine volume was collected overnight.

X	Appearance*	X	Glucose (Glu)*
X	Volume (UVol)*	X	Ketones (Ket)
X	Specific gravity / osmolality (Sp.Gr.)*	X	Bilirubin (Bil)
X	pH (pH)*	X	Blood (Bld)*
X	Sediment (microscopic)		
X	Protein (Pro)*	X	Urobilinogen (Uro)

*Recommended for chronic studies based on OPPTS Guideline 870.4100

7. Sacrifice and pathology

On study Days 365 to 368, 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 (60 mg/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:

	DIGESTIVE SYSTEM		CARDIOVASC. / HEMAT.		NEUROLOGIC
X	Tongue	X	Aorta*	XX	Brain with cerebellum*
X	Submandibular (salivary) gland*	XX	Heart*	X	Sciatic nerve*
X	Esophagus*	X	Bone marrow, sternum*	X	Spinal cord (cervical, thoracic, lumbar) *
X	Stomach*	X	Lymph node, mesenteric*	X	Eyes*
X	Duodenum*	X	Lymph node, submaxillary*	X	Optic nerves*
X	Jejunum*	XX	Spleen*	XX	Pituitary gland*
X	Ileum*	XX	Thymus		
X	Cecum*				GLANDULAR
X	Colon*		UROGENITAL	XX	Adrenal gland*
X	Rectum*	XX	Kidney*	XX	Parathyroid gland*
XX	Liver*	X	Urinary bladder*	XX	Thyroid gland* (weighed with parathyroid gland)+
X	Pancreas*	XX	Testis*	X	Lacrymal exorbital gland*
		XX	Epididymis*	X	Harderian gland
	RESPIRATORY	XX	Prostate gland*		OTHER
X	Trachea*	X	Oviduct*	X	Bone (sternum)
X	Lung*	XX	Ovary*	X	Skeletal muscle
X	Pharynx*	XX	Uterus (with cervix)*	X	Skin*
X	Larynx*	X	Mammary gland*	X	All gross lesions and masses
X	Nasal cavities*	X	Vagina	X	Articular surface (femorotibial joint)
		X	Seminal vesicle*		
		X	Oviduct*		

* recommended for chronic studies based on US EPA guideline 870.4100
X tissues were collected for histological examinations
XX organs were weighed fresh at scheduled sacrifice only, with paired organs weighed together, and collected for histological examination

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 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 all dose groups.

II. Results and discussion

A. Clinical signs and mortality

1. Clinical signs of toxicity and physical examination

No treatment-related clinical signs were noted in either sex.

2. Mortality

There were no mortalities throughout the study

B. Body weight and body weight gain

At 2000 ppm 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.

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

Dosage level (ppm)	0	100	400	2000
Sex	**Males**			
Initial BW (%C)	**8.3 ± 0.6**	**8.1 ± 0.6 (98%)**	**8.2 ± 0.9 (99%)**	**8.2 ± 0.7 (99%)**
BWG Week 1	0.1 ± 0.2	0.1 ± 0.1	0.0 ± 0.1	-0.2 ± 0.2
BWG Weeks 1-52	0.4 ± 1.0	2.3 ± 1.3	1.3 ± 1.8	0.2 ± 1.1
Final BW (%C)	**8.7 ± 0.8**	**10.4 ± 1.7 (120%)**	**9.6 ± 1.3 (110%)**	**8.5 ± 0.8 (98%)**
Sex	**Females**			
Initial BW (%C)	**6.9 ± 0.6**	**6.6 ± 0.9 (96%)**	**6.8 ± 0.8 (99%)**	**6.7 ± 0.4 (97%)**
BWG Week 1	0.0 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	-0.1 ± 0.2
BWG Weeks 1-52	1.1 ± 0.6	1.4 ± 0.9	1.2 ± 0.6	1.0 ± 0.2
Final BW (%C)	**8.0 ± 1.0**	**8.0 ± 1.3 (100%)**	**8.1 ± 1.4 (101%)**	**7.6 ± 0.3 (95%)**

BWG: body weight gain; (%C) = % versus control

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 effect 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-276047-01-1, M-276047-01-1). Overall, food consumption was comparable to control in males whereas this parameter remained slightly lower in female throughout the study resulting in an overall 10% reduction.

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

Dose level (ppm)	0	100	400	2000	0	100	400	2000
Sex	**Males**				**Females**			
Week 1 (Days 1-8) (%C)	709	648 (91%)	656 (93%)	**498 (70%)**	645	636 (99%)	625 (97%)	**493 (76%)**
Weeks 1-52 (Days 1-364) (%C)	740	730 (99%)	745 (101%)	720 (97%)	688	703 (102%)	693 (101%)	**620 (90%)**

(%C) = % versus control

D. Ophthalmic examination

No ocular abnormalities were observed in males or females at any dietary level.

E. Blood analyses

1. Hematology

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

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-test values.

The slightly elevated ALP 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.

No effect was seen at 100 ppm.

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

Dosage level (ppm)	0	100	400	2000
Sex	**Males**			
Pre-test	111 ± 19	142 ± 35	119 ± 16	119 ± 16
Month 3	120 ± 51 (+8%)	107 ± 17 (-25%)	162 ± 41 (+36%)	256 ± 108* (+115%)
Month 6	154 ± 121 (+39%)	108 ± 31 (-24%)	215 ± 122 (+81%)	341 ± 135 (+187%)
Month 12	117 ± 46 (+5%)	82 ± 30 (-43%)	176 ± 67 (+48%)	299 ± 166 (+151%)
Sex	**Females**			
Pre-test	121 ± 18	134 ± 41	135 ± 34	145 ± 51
Month 3	119 ± 27 (-2%)	123 ± 30 (-8%)	129 ± 44 (-4%)	229 ± 162** (+58%)
Month 6	147 ± 49 (+21%)	134 ± 30 (0%)	134 ± 24 (-1%)	334 ± 153 (+130%)
Month 12	140 ± 43 (+16%)	135 ± 72 (+1%)	161 ± 61 (+19%)	285 ± 126 (+97%)

(%) change compared to pre-test value
*: p≤0.05; ** p≤0.01

3. Urinalysis

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

F. Sacrifice and pathology

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 histopathological effect.

2. Gross and histopathology

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

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

A minimal diffuse hypertrophy of the follicular 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 level (ppm)	0	100	400	2000	0	100	400	2000
Sex	**Males**				**Females**			
Liver Centrilobular hepatocellular hypertrophy (minimal, diffuse)	0/4	0/4	0/4	3/4	0/4	0/4	0/4	0/4
Thyroid gland 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 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).

CA 5.3.3 Other routes

Data Point:	KCA 5.3.3/01
Report Author:	[redacted]
Report Year:	2007
Report Title:	A subacute dermal toxicity study in rats with technical grade AE C656948
Report No:	201617
Document No:	M-293833-01-1
Guideline(s) followed in study:	US-EPA OPPTS 870.3200 (1998)
Deviations from current test guideline:	Current guideline: OECD 410, 1981 Deviation: none
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

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 group), 100, 300, and 1000 mg/kg/day. The dose was based on each animal's body weight on days 0, 7, 14, 21, and 28. During the study, the animals were evaluated for the effect of the test compound on body weight, food consumption, clinical signs, the eyes, clinical chemistry, and hematology. Gross necropsy 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 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 5.3.1) 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

1. Test material:	AE C656948 -- N-[2-[3-Chloro-5-(trifluoromethyl)-2-pyridinyl]ethyl]-2-(trifluoromethyl)benzamide
Description	Beige powder
Lot / Batch #:	Mix-batch:08528/0002
Purity:	94.7%
CAS #	658066-35-4
Stability of test compound:	Stable at room temperature
2. Vehicle and / or positive control:	For treated animals gauze pads were moistened with deionized water and the test substance was then applied to the gauze. The control animals were treated with gauze pads moistened with deionized water.
3. Test animals:	
Species:	Rat
Strain:	[redacted] (nulliparous and nonpregnant)
Age:	11 weeks
Weight at dosing:	281.3-351.0 g (males), 203.1-237.6 g (females)
Source:	Charles River Laboratories, Incorporated, Raleigh, North Carolina, U.S.A.
Acclimation period:	7 days prior to release for the study.
Diet:	PMI Certified Rodent Diet 5002 in "meal" form provided continuously for *ad libitum* consumption, except when fasted prior to bleeding.
Water:	Tap water provided continuously for *ad libitum* consumption. The water was sampled monthly by the KCMO Water Department and was periodically sampled and analyzed by a Continental Analytical Services, Inc., Salina, KS for a variety of potential impurities (e.g., aflatoxins, chlorinated hydrocarbons, heavy metals, etc.).
Housing:	Individually housed in stainless steel cages.
Environmental conditions:	
Temperature:	18-26°C
Humidity:	30-70%
Air changes:	Averaged at least 12.7 per hour
Photoperiod:	Approximately 12 hours of light alternating with 12 hours of darkness, except when lights were turned off for eye exams.

B. Study design

1. In life dates: 25 September 2006 to 30-31 October 2006

2. Animal assignment and treatment

The rats were randomly assigned to dose groups, based on weight, using INSTEM DATATOX®. Weight variation of animals used were targeted not to exceed ±20% of the mean weight for each sex.

Table 5.3.3-1 Study Design

Dose Group (mg/kg/day)	No. Animals/ Dose/Sex
0 (control)	10
100	10
300	10
1000	10

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 hair growth.

Individual doses of the test substance were weighed out for each animal and applied to a commercially-available adhesive bandage (2 in. x 4 in.; dose area 2 in. x 2 in.) that was moistened before dose 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 deionized water was applied to the dose site.

The test substance/bandage was held in 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 wiped with deionized water-dampened and dry gauze to remove as much test substance residue as feasible without damaging the skin.

4. Statistics

Statistical significance was determined at $p \leq 0.05$ for all tests with the exception of Bartlett's test, in which a probability value of $p \leq 0.001$ was used. All tests were two-tailed, except for gross and histopathologic lesion evaluations 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 Dunnett's t-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-test 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 animals were observed at least twice daily (AM and PM) for clinical signs of toxicity, except once daily on weekends. Findings were recorded when first observed and then at 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 for calculation of organ-to-body weight ratios.

3. Food consumption

Food consumption was measured weekly during the study.

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

X	Hematocrit (HCT)*	X	Leukocyte differential count*
X	Hemoglobin (HGB)*	X	Mean corpuscular hemoglobin (MCH)*
X	Leukocyte count (WBC)*	X	Mean corpuscular hemoglobin conc.(MCHC)*
X	Erythrocyte count (RBC)*	X	Mean corpuscular volume (MCV)*
X	Platelet count*	X	Reticulocyte count
	Blood clotting measurements*	X	Blood cell morphology
X	(Thromboplastin time)	X	Red Blood Cell Distribution Width (RDW)
	(Clotting time)	X	Hemoglobin Distribution Width (HDW)
X	(Prothrombin time)		

* Recommended for 28-day dermal toxicity studies based on Guideline 870.3200

b. Clinical Chemistry

	ELECTROLYTES		**OTHER**
X	Calcium (calc)		Glutamate dehydrogenase
X	Chloride (Cl)	X	Albumin (ALB)*
	Magnesium (Mg)	X	Creatinine (Creat)*
X	Phosphorous (Phos)	X	Urea nitrogen (Urea-N)*
X	Potassium (K)*	X	Total Cholesterol (Chol)*
X	Sodium (Na)*	X	Globulins (Glob)
	ENZYMES (more than 2 hepatic enzymes)*	X	Glucose (gluc)*
X	Alkaline phosphatase (AP)*	X	Total bilirubin (T-Bili)
	Cholinesterase (ChE)	X	Total protein (TP)*
X	Creatine phosphokinase (CK)	X	Triglycerides (Trig)
X	Lactic acid dehydrogenase (LDH)		Serum protein electrophoresis
X	Alanine aminotransferase (ALT/SGPT)*	X	Uric Acid (Uric-A)
X	Aspartate aminotransferase (AST/SGOT)*		Bile Acids
X	Gamma Glutamyl transferase (GGT)*	X	A/G ratio (A\G)

* Recommended for 28-day dermal toxicity studies based on Guideline 870.3200

6. Urinalysis

Urinalysis was not performed and is not required by the guideline

7. Sacrifice and pathology

Animals were euthanized 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 collected and weighed at necropsy is presented in the table below (marked with an x). All tissues were processed, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (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 low- and 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

	DIGESTIVE SYSTEM		CARDIOVASC./HEMAT		GLANDULAR
X	Cecum*	X	Aorta*	X	Adrenal gland*+#
X	Colon*	X	Bone marrow*	X	Exorbital/lacrimal gland@
X	Duodenum*	X	Heart*+#	X	Thyroid (with parathyroid)*
X	Esophagus*	X	Lymph node, mesenteric*		
X	Ileum*	X	Lymph node, cervical*		NEUROLOGIC
X	Jejunum*	X	Spleen*#	X	Brain*+#
X	Liver*+#	X	Thymus*+#	X	Cerebellum
X	Pancreas*			X	Cerebrum-Midbrain
X	Rectum*		UROGENITAL	X	Medulla/Pons
X	Salivary glands*	X	Harderian gland@	X	Eyes*
X	Stomach, glandular *	X	Cervix@	X	Nerve optic*
X	Stomach, non-	X	Clitoral gland@	X	Nerve, sciatic*
X	Tongue@	X	Epididymides*+#	X	Pituitary*
X	Tooth	X	Kidneys*+#	X	Spinal cord, cervical*
		X	Mammary gland*	X	Spinal cord, thoracic*
	RESPIRATORY	X	Ovary*+#	X	Spinal cord, lumbar*
X	Larynx*	X	Preputial gland@		
X	Lung*	X	Prostate*		OTHER
X	Nasal structure*	X	Testicle*+#	X	Bone, femur@
X	Nasopharynx*	X	Urinary bladder*	X	Bone, sternum
X	Oral structure	X	Uterus*+#	X	Gross lesions*
X	Trachea*	X	Vagina@	X	Muscle, protocol@
		X	Seminal vesicles*	X	Physical Identifier
		X	Zymbal's gland@	X	Skin, treated*
				X	Skin, untreated*

* Recommended for 28-day dermal toxicity studies based on Guideline 870.3200.
+Organ weight required in a 21/28-Day Dermal Study
#Organ weighed
@No histopathology performed

II. Results and discussion

A. Observations and mortality

1. Clinical signs of toxicity

There were no compound-related clinical observations for males and females at any dose level.

2. Mortality

No animals were found dead or sacrificed in-extremis during the study.

B. Body weight and body weight gain

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. However, this change was considered not adverse since the values were only marginally elevated relative to concurrent controls.

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

2. Clinical Chemistry

The only clinical chemistry parameter which was affected by compound administration was a statistically significant increase in total cholesterol 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 females as compared to controls. The significant decrease in potassium values was considered not to be treatment-related for the following reasons; they were not dose dependent and the decreased 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 in males and females at any dose level.

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

A statistically significant increase in mean kidney weights (relative) in 1000 mg/kg/day males and a statistically significant increase in mean liver 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 controls was 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, statistically significant increases in the incidence of hypertrophy (centrilobular and mid-zonal) 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 rat 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 283/2013.
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-EPA guidelines. The studies were performed in compliance with GLP 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 Ames test was conducted with a representative batch of the proposed specification for the technical AE 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-708691-01-1).

Table 5.4-1 Summary of genotoxicity studies with Fluopyram

Study	Concentrations of [Substance] tested	Result	Reference
***In vitro* assays**			
Ames Salmonella microsome test - Plate incorporation and preincubation method	16, 50, 158, 500, 1581 and 5000 µg/plate	Negative	[redacted] 2006; M-269978-01-1
Ames Salmonella /microsome test - Plate incorporation and preincubation method	16, 50, 158, 500, and 1581 µg/plate	Negative	[redacted] 2008; M-298529-01-1
Chromosome aberration test with Chinese hamster V79 cells	30, 60, 120, 180 and 240 µg/mL	Negative	[redacted] 2005; M-266066-01-1
V79 / HPRT mammalian mutagenicity study	1.95 to 256 µg/mL	Negative	[redacted] 2006 M-268775-01-1
***In vivo* assay**			
Mouse (male) micronucleus assay	250, 500 and 1000 mg/kg bw	Negative	[redacted] 2005; M-263710-02-1
Fluopyram 600 (600 g/L) solo formulation			
Mouse (male) micronucleus assay	500, 1000 and 2000 mg/kg bw	Negative	[redacted] 2016; M-560911-03-1

CA 5.4.1 *In vitro* studies - Bacterial reverse mutation studies

Data Point:	KCA 5.4.1/01
Report Author:	[redacted]
Report Year:	2006
Report Title:	AE C656948 - Salmonella/microsome test plate incorporation and preincubation method
Report No:	AT02911
Document No:	M-269978-01-1
Guideline(s) followed in study:	OECD 471 (1997) EEC Commission Directive 2000/32/EC Method B13/14 (2000) EPA 40 CFR part 160 OPPTS 870.5100 (August 1998) MAFF 12 Nousan No8628 (December 06, 2000)
Deviations from current test guideline:	current guideline: OECD 471, 2020 Deviation: None
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In this *in vitro* assessment of the mutagenic potential of AE C656948 (Batch-No.: Mix-Batch:08528/0002, 94.7% purity), histidine-dependent auxotrophic mutants of Salmonella typhimurium, strains TA 1535, TA 1537, TA 98, TA 100 and TA 102 were exposed to AE C656948 up to 5000 µg/plate, diluted in dimethyl sulfoxide (DMSO). For each bacterial strain and dose level, triplicate plates were used in both the presence and absence of an Aroclor 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°C, the numbers of revertant colonies were scored using an automated colony counter.

There was no indication of a bacteriotoxic effect of AE C656948 at any dose up to and including 5000 µg/plate. From 1581 µg/plate onwards 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 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 presented in documents Herbold, B.;2009; M-345002-01-1 (Check of S9 metabolizing capacity S9 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 C656948 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.

I. Materials and methods

A. Materials

1. Test material:	AE C656948
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 study
2. Control materials:	
Negative:	None (Culture medium was used as the negative control)
Solvent / final concentration:	DMSO
Positive:	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 µg/plate only in plate 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 S9 mix in all strains at 3 µg/plate.
3. Activation:	
Source	The S9 fraction was isolated from the livers of Aroclor 1254 induced male Sprague Dawley rats.
S9 mix composition:	per 70 ml MgCl2 · 6 H2O 162.6 mg KCl 246.0 mg Glucose-6-phosphate, disodium salt 179.1 mg NADP disodium salt 315.0 mg In 100 mM sodium-ortho-phosphate buffer at pH 7.4
4. Test organisms:	Salmonella typhimurium LT2 strains: TA 1535, TA 100, TA 1537, TA 98, and TA 102
5. Test concentrations:	
Preliminary cytotoxicity assay (+/-S9) and plate incorporation:	16, 50, 158, 500, 1581 and 5000 µg/plate
Mutation assay – pre-incubation:	For all strains with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 µg/plate

B. Test performance

1. In life dates: 30 November 2005 – 19 December 2005

2. Bacterial maintenance

The S. typhimurium bacteria used in this assay have been maintained at the test facility since 15 August 1997 and were originally received from Dr. B.N. Ames. Bacteria were cultured as described in the original papers (Ames et al., McCann et al.) and were stored frozen at -80°C. Stock cultures were generated as needed through culturing on nutrient agar followed by overnight incubation in nutrient broth at 37°C. Stock cultures were then checked for crystal-violet sensitivity and UV sensitivity, and were frozen at -80°C until use.

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 agar containing histidine 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. DMSO (0.1 mL) containing AE C656948 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 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 described mixture in a water bath at 37°C for 20 minutes. 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.

5. Statistics

According to the OECD guideline 471, statistical analysis of the data is not mandatory.

6. Evaluation criteria

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

a) The negative controls were within the range of either published data or the laboratory's own historical control data.

b) The positive control substances produced revertant counts within those previously seen in the laboratory and

c) Determination of titer showed sufficient bacterial density in the cultures used for the tests.

A positive test (revertant counts were increased with test 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 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 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 at least 100 colonies.

IC Results and discussion

A. Analytical determinations

AE C656948 is stable in the solvent at room temperatureat 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 last treatment.

B. Preliminary cytotoxicity assay

The preliminary cytotoxicity 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. There was no indication of a bacteriotoxic effect of AE C656948 technical at doses of up to and including 5000 µg per plate. From 1581 µg/plate onwards, the test substance precipitated nevertheless assessment was possible up to the highest dose of 5000 µg/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

The preliminary cytotoxicity 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 test a dose-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 number of revertant colonies compared to the controls demonstrating the sensitivity 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 without metabolic activation. The results from both the preliminary and the definitive studies are presented in the following tables.

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

Summary of Mean Values ±Standard Deviation *without S9 Mix*					
Test substance and concentration (µg/Plate)	**Strain**				
	TA 1535	**TA 100**	**TA 1537**	**TA 98**	**TA 102**
0 (DMSO)	12±2	91±1	6±1	27±2	190±5
16	10±2	86±14	6±3	25±5	144±20
50	7±2	84±6	5±1	22±5	167±8
158	9±3	93±17	4±1	20±5	198±21
500	8±3	91±13	4±2	22±4	161±19
1581	8±2 (P)	75±6 (P)	4±2 (P)	18±8 (P)	134±6 (P)
5000	10±2 (P)	77±14 (P)	6±2 (P)	23±5 (P)	127±22 (P)
Na-azide	314±32				
NF		239±22			
4-NPDA			71±12	166±18	
MMC					550±23
Summary of Mean Values ±Standard Deviation *with S9 Mix*					
0 (DMSO)	7±2	112±2	8±2	32±2	248±17
16	7±1	110±34	5±2	28±2	247±6
50	6±2	106±5	6±3	27±2	237±20
158	5±2	138±8	5±0	24±2	255±16
500	6±2	99±5	8±2	28±2	215±38
1581	6±2 (P)	95±5 (P)	6±2 (P)	19±8 (P)	219±23 (P)
5000	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 5.4.1-2 Results from the pre-incubation assay in Salmonella typhimurium strains

Test substance and concentration (µg/Plate)	Strain				
	TA 1535	TA 100	TA 1537	TA 98	TA 102
Summary of Mean Values ±Standard Deviation *without S9 Mix*					
0 (DMSO)	20±3	120±3	8±3	26±5	209±29
16	24±5	111±6	6±1	25±5	248±8
50	17±3	106±6	7±2	27±10	204±6
158	21±8	105±19	6±1	24±6	240±26
500	17±6	112±8	6±1	24±5	200±22
1581	16±3 (P)	108±10 (P)	6±1 (P)	22±10 (P)	174±8 (P)
5000	17±3 (P)	104±10 (P)	6±2 (P)	30±14 (P)	177±14 (P)
Na-azide	537±18				343±40
NF		309±26			
4-NPDA			82±17	164±7	
Cumene					343±40
Summary of Mean Values ±Standard Deviation *with S9 Mix*					
0 (DMSO)	10±4	130±11	7±3	45±12	249±18
16	7±1	120±12	7±1	38±4	236±16
50	8±3	119±7	5±2	33±10	247±25
158	9±4	121±6	7±2	34±5	252±53
500	10±2	118±11	7±4	27±4	241±42
1581	6±1 (P)	100±9 (P)	7±1 (P)	30±4 (P)	241±28 (P)
5000	8±2 (P)	106±10 (P)	6±2 (P)	29±6 (P)	205±12 (P)
2-AA	84±3	1384±49	141±8	816±98	436±22

P= Precipitate

D. Deficiencies

No deficiencies were identified.

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 to be non-mutagenic.

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

Data Point:	KCA 5.4.1/02
Report Author:	[redacted]
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 2000/32/EC Method B13/14 (2000); US EPA712-C-98-247, OPPTS 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 (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

This supplementary test was performed with a representative technical batch of the proposed specifications as there were a few changes in the impurity profile of the test material compared to the one used in the initial test. In this *in vitro* assessment of the mutagenic potential of AE C656948 (Batch-No.: 2007-010986, 95.7% purity), histidine dependent auxotrophic mutants of Salmonella typhimurium, strains TA 1535, TA 1537, TA 98, TA 100 and TA 102 were exposed to AE C656948 up to 5000 µg/plate, diluted in dimethyl sulfoxide (DMSO). For each bacterial strain and dose level, triplicate plates were used in both the presence and absence of an Aroclor 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°C, the numbers of revertant colonies were scored using an automated colony counter.

There was indication of a bacteriotoxic effect of AE C656948 starting at 500 µg/plate. Nevertheless assessment was possible up to 1581 µg/plate but not at 5000 µg/plate.

AE C656948 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 C65948 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.

I. Materials and methods

A. Materials

1. **Test material:** AE C656948
   Description Fine white powder
   Lot / Batch #: 2007-010986
   Purity: 95.7 %
   CAS # 658066-35-4
   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

Positive:	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 µg/plate only in plate 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 S9 mix in all strains at 3 µg/plate.

3. **Activation:**

Source	Rat liver S9, from rats induced with phenobarbital and Aroclor 1254 The S9 mix was freshly prepared, kept on ice and used only on the same day.
S9 mix composition:	per 70 ml $MgCl_2 \cdot 6\ H_2O$ 162.6 mg KCl 246.0 mg Glucose-6-phosphate, disodium salt 1791.1 mg NADP-disodium salt 315.0 mg In 100 mM sodium-ortho-phosphate buffer at pH 7.4

4. **Test organisms:** Salmonella typhimurium strains: TA 1535, TA 100, TA 1537, TA 98, and TA 102

5. **Test concentrations:**

Preliminary cytotoxicity assay (+/-S9):	For all strains with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 µg/plate
Mutation assays:	For all strains with or without S9 mix: 5, 16, 50, 158, 500 and 1581 µg/plate

B. Test performance

1. **In life dates:** 12 December 2007 – 14 January 2008

2. **Bacterial maintenance**

The S. typhimurium bacteria used in this assay have been maintained at the test facility since 15 August 1997 and were originally received from Dr. B.N. Ames. Bacteria were cultured as described in the original papers (Ames et al., McCann et al.) and were stored frozen at -80°C. Stock cultures were generated as needed through culturing on nutrient agar followed by overnight incubation in nutrient broth at 37°C. Stock cultures were then checked for crystal-violet sensitivity and UV sensitivity, and were frozen at -80°C until use.

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 sufficient 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 agar containing histidine 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 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 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 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 described mixture in a water bath at 37°C for 20 minutes. 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.

5. Statistics

According to the OECD guideline 471, statistical analysis of the data is not mandatory.

6. Evaluation criteria

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

a) The negative controls were within the range of either published data or the laboratory's own historical control data,

b) The positive control substances produced revertant counts within those previously seen in the laboratory, and

c) Determination of titer showed sufficient bacterial density in the cultures used for the tests.

A positive test (revertant counts were increased with test 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 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 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 at least 100 colonies.

II. Results and discussion

Concentrations of up to 158 µg/plate did not produce any indication of bacteriotoxicity. From 500 µg/plate onwards, AE C656948 produced strain specific toxicity. Nevertheless, concentrations up to 1581 µg/plate could be used 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 system.

Table 5.4.1-3 Results from the plate incorporation assay in Salmonella typhimurium strains

Summary of Mean Values ±Standard Deviation *Mix without S9 Mix*					
Test substance and concentration (µg/Plate)	**Strain**				
	TA 1535	**TA 100**	**TA 1537**	**TA 98**	**TA 102**
0	25±5	111±14	8±1	22±4	189±10
16	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 and concentration (µg/Plate)	**Strain**				
	TA 1535	**TA 100**	**TA 1537**	**TA 98**	**TA 102**
158	20±1	81±14	6±2	14±3	216±32
500	17±5	81±5	7±1	15±2	183±18
1581	16±1	47±20	8±1	11±2	163±6
5000	9±5	28±7	4±3	2±1	159±12
Na-azide	502±94	-	-	-	-
NF	-	346±67	-	-	-
4-NPDA	-	-	85±18	156±28	-
MMC	-	-	-	-	629±56
Summary of Mean Values ±Standard Deviation *with S9 Mix*					
0	14±3	113±5	9±5	35±12	217±22
16	13±4	93±11	7±2	23±7	221±20
50	9±3	64±18	10±2	21±6	203±35
158	6±1	88±28	7±3	29±4	197±17
500	8±2	83±10	6±3	37±11	229±18
1581	6±2	67±4	6±1	28±5	136±13
5000	3±2	27±7	4±2	10±5	114±11
2-AA	123±15	1253±28	181±12	1480±233	493±162

Table 5.4.1-4 Results from the pre-incubation assay in Salmonella typhimurium strains

Summary of Mean Values ±Standard Deviation *without S9 Mix*					
Test substance and concentration (µg/Plate)	**Strain**				
	TA 1535	**TA 100**	**TA 1537**	**TA 98**	**TA 102**
0	9±3	132±19	6±1	27±7	254±39
5	8±4	115±16	7±2	27±5	244±8
16	9±2	130±24	7±2	28±7	257±17
50	10±4	162±27	6±2	31±4	266±45
158	12±4	101±6	7±4	24±8	232±64
500	9±3	112±19	5±1	25±5	222±20
1581	12±3	90±10	4±2	20±5	208±23
Na-azide	757±44	-	-	-	-
NF	-	626±38	-	-	-
4-NPDA	-	-	103±12	181±29	-
Cumene hydroperoxide	-	-	-	-	474±11
Summary of Mean Values ±Standard Deviation *with S9 Mix*					
0	10±4	154±15	9±3	41±10	232±30
5	11±3	148±29	8±3	39±3	258±11
16	9±2	134±13	8±6	36±7	251±10
50	8±2	163±30	5±2	40±8	278±58
158	11±3	138±11	7±1	37±1	273±12
500	9±6	130±9	7±1	34±3	267±26
1581	9±2	108±16	4±1	40±7	209±20
2-AA	115±24	1958±176	158±27	1464±93	512±39

A. Deficiencies

No deficiencies were identified.

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 assay.

Data Point:	KCA 5.4.1/03
Report Author:	[redacted]
Report Year:	2005
Report Title:	AE C656948 (Project: AE C656948) *In vitro* chromosome aberration test with chinese hamster V79 cells
Report No:	AT02798
Document No:	M-266066-01-1
Guideline(s) followed in study:	OECD 473 (1997); EEC 2000/32/EC Method B.10 (2000); US EPA 712-C-98-223, OPPTS Series 870.5375 (August 1998)
Deviations from current test guideline:	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.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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 µg/mL, diluted in dimethyl sulfoxide (DMSO) in the absence and in presence of an Aroclor 1254-induced rat liver metabolic activation system (S9 mix). Cultures of all concentrations were harvested 18 hours after treatment start. In addition, cells treated at 120, 180 and 240 µg/mL were harvested 30 hours after treatment start. Without S9 mix, an additional experiment was performed using continuous treatment for 18 hours, harvest at the same time, and AE C656948 concentrations of 60, 120 and 180 µg/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 µg/mL and above after 4 and 18 hours treatment. With S9 mix, cytotoxic effects were observed at 180 µg/mL and above. Precipitation in the medium occurred at 120 µg/mL and above.

None of the cultures treated with AE C656948 in the presence and in the absence of S9 mix showed biologically relevant or statistically significant increased numbers of aberrant metaphases.

The positive controls mitomycin 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*.

I. Materials and methods

A. Materials

1. Test material:	AE C656948
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 study
2. Control materials:	
Negative:	Culture medium
Solvent:	DMSO for AE C656948 and Hanks's balanced salt solution for positive controls (Seromed)
Positive:	Mitomycin C (Fluka, batch 454188/2 449031 16) without S9 mix at 0.1 µg/mL for a treatment period of 4 hours and 0.03 µg/mL for a treatment period of 18 hours. Cyclophosphamide (Endoxan 100 mg injection vials of dry substance, Baxter Oncology GmbH) with S9 mix at 2 µg/mL.

3. Activation:

S9 derived from

X	induced	X	Aroclor 1254	X	Rat	X	Liver
	non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other		Other		

Composition of S9 mix composition (containing 40% S9 fraction)

Cofactor solution per 25 ml S9 mix:	
Sodium phosphate buffer (100 mM, pH 7.4)	15.0 ml
$MgCl_2 \times 6 H_2O$	40.7 mg
KCl	61.5 mg
Glucose-6-phosphate (disodium salt)	38.0 mg
NADP (disodium salt)	78.8 mg

4. Test cells:

V79 cells were obtained from Dr. Utesch, Merck AG, Darmstadt. The cells arrived at the Toxicology of Bayer HealthCare AG, Wuppertal, on November 8, 1993.

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

Prior to the start of the study Chinese hamster V79 cells from a frozen permanent, which was stored in liquid nitrogen, were normally grown in 20 ml medium and 75 cm^2 flasks or under comparable conditions. Incubation of the cells was always performed at 37°C in a $C0_2$-incubator (5% CO_2). 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)	PAA Ready Mix (2% FBS)
1% L-glutamine	1% L-glutamine
1% MEM-vitamins	1% MEM-vitamins
1 % MEM NEAA	1 % MEM NEAA
1 % Pen/Strep	1 % Pen/Strep
10%FBS(=FCS)	2%FBS(=FCS)

6. Test compound concentrations used:

Non activated conditions: 30, 60, 120, 180 and 240 µg/mL.
Activated conditions: 30, 60, 120, 180 and 240 µg/mL.

B. Test performance

1. In life dates: 14 September 2005 -02 December 2005

2. Preliminary cytotoxicity assay

250 µg/mL with or without S9 mix for 4 hours or 18 hours (without S9 mix). The mitotic 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 interphase were defined as mitotic.

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 µg/mL with a harvest time of 18 hours and at concentrations of 120 to 240 µg/mL with a harvest time of 30 hours. Additional cultures were exposed without S9 mix for 18 hours to concentrations of AE C656948 ranging from 60 to 180 µg/mL. These cultures were harvested at the end of the treatment period.

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

3. Cytogenetic assay

	Test Material	**Solvent Control**	**Positive Control**
a. Cell exposure time:			
Non-activated:	18.0 h	18.0 h	18.0 h
Activated:	4.0 h	4.0 h	4.0h

b. Spindle inhibition

Inhibition used/concentration: Colcemid, 40 µg/ml water
Administration time: 2 hours prior to harvest, respectively

	Test Material	Solvent Control	Positive Control
c. Cell harvest time:			
Non-activated:	4 or 18 h	4 or 18 h	4 or 18 h
Activated:	4 h	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 5 minutes at 700 rpm. The supernatant was removed and 1 to 2 mL of a hypotonic solution (0.4% KCl; 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 pelleted 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

4. Statistics

The statistical analysis was performed by pair-wise comparison of AE C656948-treated and positive 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 gaps) and of metaphases with exchanges were compared (provided that these data superseded the respective solvent control). The one-side Chi2-test was used for the statistical evaluation.

A difference was considered to be significant, if the probability of error was below 5%.

5. Evaluation criteria

An increased incidence of gaps of both types without concomitant increase of other aberration types was not considered as indication of a clastogenic effect.
A test was considered positive if

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

A test was considered negative if

- there was no such increase at any time interval.
- there were statistical significant values, which were, however, 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 biologically 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

II. Results and discussion

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 last 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 treatment.

Mitotic index without S9 mix:

In absence of S9 mix, the mitotic index was only significantly reduced at 180 µg/mL after 18 hours of treatment

Mitotic index with S9 mix:

With S9 mix, there was no reduction of the mitosis rate.

Survival Index

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

Survival index without S9 mix:

Survival indices were significantly reduced from 120 and 180 µg/mL onwards in the absence of S9 mix.

Survival index with S9 mix:

Survival indices were significantly reduced from 120 and 180 µg/mL onwards in the presence of S9 mix.

C. Cytogenetic assays

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

AE C656948 without 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 same was true for a treatment period and total culture time of 18 hours.

The treatment with the positive control mitomycin C resulted in a clear and statistically significant increase of metaphases with aberrations 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 S9 mix.

Table 5.4.1-5 Results of the chromosomal aberration study with (harvest time 18h)

Exposure, h	S9	Conc., µg/mL	Mitotic index, % control	Incl. gaps	Excl. gaps	Carrying exchanges
4	-	Solvent - DMSO	100.0	4.0	3.5	0.0
		Mitomycin C: 0.1	112.4	73.5	72	0.0
		60	111.3	2.0	2.0	0.0
		120	119.2	1.0	1.0	0.0
		180	133.9	3.5	3.5	0.0
4	+	Solvent -DMSO	100.0	5.0	5.0	2.0
		Cyclophosphamide: 2	77.0*	53.5	52.5	21.5
		60	120.5	3.0	3.5	1.5
		120	108.7	3.0	3.0	1.5
		180	96.9	2.5	2.0	0.0
18	-	Solvent - DMSO	100.0	1.5	1.5	0.0
		Mitomycin C: 0.03	89.5	46.5	45.0	12.0
		60	108.8	3.5	3.0	1.0
		120	118.4	1.5	1.5	0.0
		180	103.5	2.0	1.5	1.0

*p < 0.05

D. Deficiencies

None.

III. Conclusions

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

Assessment and conclusion by applicant:
Study meets the current guidance and the requirements in 283/2013.
Based on the results of this test, fluopyram is considered not to be clastogenic for mammalian cells *in vitro*.

Data Point:	KCA 5.4.1/04
Report Author:	[redacted]
Report Year:	2006
Report Title:	AE C656948 - V79/HPRT-test *in vitro* the detection of induced forward mutations
Report No:	AT02875
Document No:	M-268775-01-1
Guideline(s) followed in study:	OECD 476 (1997); EEC Commission Directive 2000/32/EC, Method B.17. (2000); OPPTS 870.5300; EPA 712-C-98-221 (August 1998); MAFF 12 Nousan No 8628 (December 06, 2000)
Deviations from current test guideline:	Current guideline: OECD 476, 2016 Deviation: None.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

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 (HPRT) 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 culture 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 mix.

Based on these results, AE C656948 was considered to be non-mutagenic in the V79/HPRT forward mutation assay, both with and without metabolic activation.

I. Materials and methods

A. Materials

1. **Test material:** AE C656948
   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 study
2. **Control materials:**

Negative:	Culture medium [Eagle's minimal essential medium supplemented with 1% L-glutamine, 1% MEM-vitamins, 1% MEM NEAA, 1% penicillin/streptomycin and 10% fetal calf serum (FCS)]
Solvent:	DMSO for AE C656948 and Dimethylbenzanthracene not exceeding 1% (v/v) in the culture medium. No solvent needed for ethyl methanesulfonate as it is a liquid.
Positive:	Ethyl methanesulfonate (EMS), a directly alkylating agent, used at 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 S9 mix.

3. **Activation:**

The S9 fraction was isolated from the livers of Aroclor 1254 induced male Sprague Dawley rats. The preparation dated from April 6, 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 study. Cofactors were freshly dissolved in sodium phosphate buffer (150 mM, pH 7.4). Three parts of the cofactor solution were mixed with two parts of the S9 fraction giving rise to the following final concentrations in the S9 mix:

8 mM	$MgCl_2 \times 6H_2O$
33 mM	KCl
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

5. Locus examined: Hypoxanthine-guanine phosphoribosyl transferase (HPRT)

6. Test compound concentrations used:

A preliminary cytotoxicity test was conducted without and with metabolic activation using concentrations of AE C656948 ranging from 1.95 ug/ml to 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 osmolality 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

1. In life dates: 04 November 2005 – 15 December 2005

2. Cell treatment

Determination of cytotoxicity

Exponentially growing V79 cells were plated in 20 mL culture medium in a 75 cm2 flask with a total volume of 275 mL (4x106 cells per flasks). For each concentration, one culture was available. After attachment (16 to 24 hours 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).

Treatment without Metabolic Activation

Exponentially growing V79 cells were plated in 20 mL culture medium in two 75 cm^2 flasks 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%). Thereafter, cell monolayers were washed with PBS, trypsinized and replated in 20 mL culture medium using 1.5x106 cells per 75 cm^2 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 cytotoxicity associated with each test substance directly after treatment (survival to treatment).

Cells in 75 cm^2 flasks were incubated to permit growth and expression of induced mutations. Cells were subcultured (= count 1, normally after 3 days) by reseeding 1.5x106 cells into 20 mL of medium in 75 cm^2 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 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 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 $\alpha = 0.05$ using the Dunnett 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 highest concentration was dropped and the analysis repeated. This procedure was repeated until $p > 0.05$. 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.

Mutant frequencies will only be used for assessment, if

- 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 will be considered positive if

- a concentration-related and in parallel cultures reproducible increase in mutant frequencies is 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 can be reproduced in a second trial, the test substance is considered to be mutagenic.

Despite these criteria, a positive result will only be considered relevant, if

- no significant change in osmolality compared to the vehicle control can be observed. Otherwise, unphysiological culture conditions may be the reason for the positive result (Scott et al, 1991).

A test substance will be judged as equivocal if

- 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

- no reproducible and relevant increases of mutant frequencies were observed.

II. Results and discussion

A. Preliminary cytotoxicity assay

In the absence and in the presence of S9 mix Chinese hamster V79 cells were exposed to AE C656948 at concentrations of up to and including 256 µg/mL. With or without S9 mix, fluopyram precipitation occurred in the medium at concentration of 128 µg/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.

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 a biologically relevant increase in mutant frequencies as compared to the corresponding controls.

There was no relevant increase in mutant frequencies after treatment with fluopyram at any concentration (up to the highest dose of 256 µg/mL) either with or without metabolic activation.

Table 5.4.1-6 Results of the HPRT locus mammalian gene mutation *in vitro* assay

Treatment	Conc., µg/mL	S9	Culture I			Culture II		
			CE II, %	Mutant colonies / 10^6 cells	Total Mutant colonies	CE II, %	Mutant colonies / 10^6 cells	Total Mutant colonies
DMSO		-	49.8±3.0	1.7	2	63.3±6.8	9.9	15
EMS	900.0		33.3±3.3	230.0	184	43.2±9.4	437.3	453
AE C656948	4		43.0±6.3	6.8	7	50.5±3.5	6.6	8
	8		40.8±6.8	10.2	10	56.7±5.4	1.5	2
	16		-	-	-	42.8±2.3	6.8	7
	32		47.7±0.6	7.0	8	45.7±0.3	3.6	4
	64		52.7±5.3	3.2	4	65.0±6.2	7.1	11
	128P		53.8±4.6	0.8	1	66.0±1.8	8.2	13
	256P		58.3±7.8	0.7	1	55.3±4.5	1.5	2
DMSO			51.2±2.9	4.1	5	75.3±3.3	7.2	13
DMBA	20		71.0±3.5	73.2	123	74.3±7.2	105.9	189
AE C656948	4	+	48.8±3.7	8.5	10	63.2±5.0	13.2	20
	8		61.2±3.2	6.1	9	71.5±5.8	4.1	7
	16		65.5±5.0	2.5	4	68.7±2.9	5.5	9
	32		55.8±4.6	4.5	6	60.7±5.4	8.2	12
	64		42.7±3.3	2.0	2	61.2±4.8	3.4	5
	128P		61.8±6.0	4.7	7	49.5±8.0	2.5	3
	256P		LC		11	53.8±4.0	10.1	13
DMSO			55.3±6.1	3.8	5	70.7±10.6	2.4	4
EMS	900.0		40.2±3.4	301.1	254	47.7±4.0	465.0	532
AE C656948	4		61.3±6.2	2.0	3	56.0±9.3	8.2	11
	8		53.2±2.8	3.9	5	62.0±9.0	7.4	11
	16		64.7±2.9	3.9	6	56.5±8.0	5.9	8
	32		42.0±3.6	6.0	6	50.2±7.8	5.8	7
	64		58.3±5.8	2.9	4	51.7±3.8	1.6	2
	128P		58.3±7.8	1.7	2	77.2±4.0	11.9	22
	256P		70.5±17.3	0.0	0	72.0±4.9	0.0	0
DMSO		+	48.8±4.3	2.6	3	54.2±0.8	4.6	6
DMBA	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
	8		69.3±6.3	3.0	5	54.7±4.2	8.4	11

Treatment	Conc., µg/mL	S9	Culture I			Culture II		
			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±4.0	4.5	6
	64		56.0±1.5	6.7	9	63.5±7.5	9.8	15
	128P		56.5±10.0	8.8	12	64.3±5.4	7.1	11
	256P		C	-	6	50.7±5.1	3.3	4

P= precipitation
C: Cells lost due to contamination

C. Deficiencies

No deficiencies were noted.

III. Conclusions

Under the experimental conditions described, fluopyram was considered non mutagenic in this V79/HPRT forward mutation assay.

Assessment and conclusion by applicant:
Study meets the current guidance and the requirements in 283/2013. There is no concern for induction of gene mutation in the mammalian cells.

CA 5.4.2 *In vivo* studies in somatic cells

Data Point:	KCA 5.4.2/01
Report Author:	[redacted]
Report Year:	2011
Report Title:	AE C656948 - Micronucleus-test on the male mouse - 1st amendment to Bayer report AT02753 of December 2, 2005
Report No:	AT02753A
Document No:	M-263710-02-1
Guideline(s) followed in study:	OECD 474 (1997) EEC 2000/32/EC Method B12 (2000) US EPA OPPTS Series 870.5395 (August 1998) MAAF 12 Nousan No 8628 (December 06, 2000)
Deviations from current test guideline:	current guideline: OECD 474, 2016 Deviation: intraperitoneal administration rather than oral. No measurement of test material in the blood, but signs of systemic toxicity noted at all doses tested. These deviations have no impact the outcome of the study and interpretation of the results
Previous evaluation:	yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

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 intraperitoneal 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. Slides of bone marrow cells were prepared and scored for the occurrence of micronucleated polychromatic erythrocytes (micronucleated PCEs), of micronucleated normochromatic erythrocytes and PCE/total erythrocytes ratios.

All animals survived until the end of the study but showed symptoms of toxicity after administration starting at 250 mg/kg bw, which included apathy, semi-anesthetized state, roughened fur, weight loss, 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 demonstrating a relevant systemic exposure to the test substance.

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

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

I. Materials and methods

A. Materials

1. **Test material:** AE C656948
   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 study
2. **Control materials:**
   Negative: none
   Solvent: 0.5% aqueous Cremophor emulsion
   Positive: cyclophosphamide used in form of Endoxan 100 mg injection vials of dry substance (Baxter Oncology GmbH)
3. **Test animals:**
   Species: mouse
   Strain: Hsd/Win :NMRI
   Age: 6-12 weeks approximately
   Weight at dosing: 36-43 g (males only)
   Source: [redacted]
   Acclimation period: At least 5 days
   Diet: Feed 3883 (10 mm cubes), produced according to specification by Provimi Kliba SA, CH-4303 Kaiseraugst, ad libitum
   Water: Tap water ad libitum
   Housing: Single housing type II cages with bedding of soft wood granules
   Environmental conditions:
   Temperature: 21-22°C
   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.

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 intraperitoneal injection, the positive control was sampled 24 hours after the single intraperitoneal injection.

3. Tissues and cells examined

Bone marrow; 2000 polychromatic erythrocytes (PCEs) examined per animal; the number of normochromatic erythrocytes (NCEs, more mature RBCs) per 2000 PCEs was noted.

4. Details of slide analysis

At 24 hours after the second intraperitoneal injection of AE C656948 or 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 intact 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 PCEs per 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

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

- Whether or not there were dose-dependent effects that were consistent with a treatment-induced response and

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

6. Statistical methods

The AE C656948 groups(s) with the highest mean (provided this superseded the negative control mean) and the positive control were checked by Wilcoxon's non-parametric rank sum test with respect to the number of micronucleated polychromatic erythrocytes 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 using the one-sided Chi^2-test.

II. Results and discussion

A. Dose Range-finding 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-related clinical 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 substantialdifferences 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 difficulty in breathing were observed at all dose levels. This demonstrated a relevant systemic exposure of the animals to the test substance.

2. PCE ratio

The positive control cyclophosphamide caused a significant increase in the number of micronucleated 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 normochromatic 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 Results of the Micronucleus Test

Experimental group	Number evaluated PCE	Number of NCE per 2000 PCE	Number of MNNCE per 2000 NCE	Number of MNPCE per 2000 PCE
Negative control	10000 (5 x 2000)	3775 ± 737	2.1 ± 2.0	4.0 ± 1.6
Fluopyram 250 mg/kg	10000 (5 x 2000)	5309 ± 1274	2.2 ± 1.5	4.4 ± 1.8
Fluopyram 500 mg/kg	10000 (5 x 2000)	6090 ± 852	1.5 ± 0.9	3.2 ± 2.3
Fluopyram 1000 mg/kg	10000 (5 x 2000)	7866* ± 2018	1.3 ± 0.7	4.2 ± 1.5
Positive control Cyclophosphamide	10000 (5 x 2000)	3243 ± 337	1.7 ± 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 convincing 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, 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 Fluopyram 600 (600 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 (13.2%) in the number of PCEs in the 48 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. This study provides further support for the exposure of the bone marrow to fluopyram.
- Additionally, two quantitative whole-body autoradiography (QWBA) studies in the rat (fluopyram is labelled with ^{14}C in the pyridyl ring (M-296485-01-1) in one study and in the phenyl ring in the other (M-296623-02-1) 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 marrow to fluopyram in the mouse micronucleus study

III. Conclusions

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered fluopyram in the micronucleus test on the male mouse, i.e. in a somatic test system *in vivo*.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013. There is no concern for clastogenic effect of intraperitoneally administered fluopyram 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:	[redacted]
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:	M-560911-03-1
Guideline(s) followed in study:	OECD 474 (2014); EEC 2000/32/EC Method B12 (2000); US EPA OPPTS Series 870.5395 (August 1998); MAAF 12 Nousan No 8628 (December 06, 2000)
Deviations from current test guideline:	OECD 474, 2014 ninth amendment Deviation: No measurement of test material in the blood, but signs of systemic 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
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a preliminary dose range finder study Fluopyram FS 600 (600 g/L) (formulated in 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 posture, squinted/closed eye, ruffled fur, spontaneous activity). All clinical signs of toxicity resolved 30 hours post dosing, with all animals 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 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 range-finder test, male only were used in the main experiment.

A single mid dose group animal was killed *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 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.

All animals treated with Fluopyram FS 600 (600 g/L) exhibited both group mean and individual MN PCE (micronucleated polychromatic erythrocyte) values which were comparable with both the concurrent vehicle control and the laboratory's historical 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 ($p \leq 0.05$) greater than the observed frequency in the concurrent vehicle control group, thereby demonstrating the sensitivity and specificity of the test system and the analysts ability to detect MN in the PCE population.

Animals dosed with Fluopyram 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 – 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 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 toxicity 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 bw (the maximum recommended dose in accordance with current regulatory test guidelines for the *in vivo* micronucleus assay). This methodology included a single dose of Fluopyram FS 600 (600 g/L) orally via gavage, with bone marrow sampling 24 and 48 hours post dosing.

I. Materials and methods

A. Materials

1. Test material:	Fluopyram FS 600 (600 g/L) (~alternative name: Fluopyram (AE C659648)
Description	Light-beige liquid
Lot / Batch #:	NK43FX395
Purity:	48.7 (w/w) (no correction for purity undertaken)
CAS #	*658066-35-4*
Stability of test compound:	Stable for the duration of the study
2. Control materials:	
Negative:	none
Solvent:	Sterile water
Positive:	cyclophosphamide (40 mg/kg bw)
3. Test animals:	
Species:	mouse
Strain:	NMRI
Age:	7-8 weeks approximately
Weight at dosing:	32.6-41.8 g (males only)
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Teklad Certified Global 18% Protein Rodent Diet, produced by Envigo, *ad libitum*
Water:	Tap water *ad libitum*
Housing:	Single housing type II cages with bedding of soft wood granules
Environmental conditions:	
Temperature:	20-22°C
Humidity:	Approximately 45-65%
Air changes:	Ten times per hours
Photoperiod:	12 hours light, 12 hours dark

4. Test compound concentrations used:

Range-finding test (2 animals/sex): a single dose by oral gavage at 2000 mg/kg bw.

Micronucleus assay (7 males/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 dosed, 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 tissue.

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 marrow smears were prepared from the intact femurs for each animal. Cell smears were prepared and stained with Giemsa, coverslipped and mounted.

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 frequency 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 concurrent 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. Poisson-based 95% control limits).

6. Statistical methods

Statistical significance of MN PCE frequency at ($p < 0.05$ was evaluated by means of the non-parametric Mann-Whitney test

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 animals 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 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 range-finder test, male only were used in the main experiment.

B. Micronucleus 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 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 – 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 474 (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 ($p \leq 0.05$) 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, 1000 and 2000 mg/kg bw at the 24 hour time point, the mean %MN 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 0.045 – 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 Fluopyram 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 ($p < 0.05$) greater than the observed frequency in the concurrent vehicle control group, thereby demonstrating the sensitivity and specificity of the test system and the analysts ability to detect MN in the PCE population.

Table 5.4.2-2 Results of the Micronucleus Test

Parameter	MN PCE/4000 PCE							% MN PCE							% PCE/NCE						
	24 hour time point																				
	Vehicle control: sterile water																				
Animal no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Individual score	7	9	0	4	12	1	3	0.18	0.23	0.00	0.10	0.30	0.03	0.08	64.9	63.5	66.6	59.7	67.2	64.2	65.4
Group mean (±SD)	5.1/4000							0.13 ±0.11							64.5 ±2.5						
Parameter	**500 mg/kg bw**																				
Animal no.	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Individual score	4	4	4	10	8	2	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)	5.1/4000							0.13 ±0.07							64.3 ±5.2						
Parameter	**1000 mg/kg bw**																				
Animal no.	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Individual score	10	3	7	-	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
Group mean (±SD)	5.3/4000							0.13 ±0.08							67.1 ±3.1						
Parameter	**2000 mg/kg bw**																				

Parameter	MN PCE/4000 PCE							% MN PCE							% 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**	**28**
Individual score	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
Group mean (±SD)	4.9/4000							0.12 ±0.06							65.1 ±5.1						
Parameter	**Positive control: CPA 40 mg/kg bw**																				
Animal no.	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**29**	**30**	**31**	**32**	**33**	**34**	**35**
Individual score	98	122	150	111	93	87	132	2.45	3.05	3.75	2.78	2.33	2.18	3.30	58.8	67.8	58.6	50.2	56.4	64.2	63.5
Group mean (±SD)	113.3/4000							2.83 ±0.57*							59.9 ±5.8						
Parameter	**48 hour time point**																				
	2000 mg/kg bw																				
Animal no.	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**36**	**37**	**38**	**39**	**40**	**41**	**42**
Individual score	4	6	8	7	3	3	1	0.10	0.15	0.20	0.18	0.08	0.08	0.03	56.2	53.6	57.9	57.3	53.8	58.3	54.9
Group mean (±SD)	4.6/4000							0.12 ±0.06							56.0 ±1.9						

Historical control data (/4000 PCE)		
	Vehicle control (25 studies; Sept 2014 – Dec 2015)	**Positive control (25 studies; Sept 2014 – Dec 2015)**
	% MN PCE	**% MN PCE**
Mean (%) ±S.D:	0.125 ±0.039	2.536 ±0.476
Min-Max (%):	0.046 – 0.205	1.650 – 3.495
95% RR	0.043 – 0.198	1.605 – 3.507
Individual animal MN range	0 - 12	37 - 204.7

* $p \leq 0.05$
CPA: cyclophosphamide
MN PCE: micronucleated polychromatic erythrocytes
RR: reference range [lower limit – upper limit]
NCE: normochromatic erythrocytes

C. Deficiencies

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 slight decrease (13.2%) in the number of PCEs in the 48 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.

III. Conclusions

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 bw (the maximum recommended dose in accordance with current regulatory test guidelines for the *in vivo* micronucleus assay). This methodology included a single dose of Fluopyram FS 600 (600 g/L) orally via gavage, with bone marrow sampling 24 and 48 hours post dosing.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013. There is no concern for clastogenic effect of orally administered Fluopyram FS 600 (600 g/L) in the micronucleus test on the male 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 OECD, 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 females 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 retinal fundus was observed in females at 1500 ppm. At the 24-month examination, this condition was observed in females at 1500 ppm and males at 750/375 ppm, together with small retinal vessels at these dose levels and in males at 150 ppm. In addition, hyperreflectivity in the retina was noted in females and corneal opacity, oedema of the cornea and nuclear opacity noted in males at these dose levels.

In females at 1500 ppm, higher mean cholesterol levels were observed throughout the study, higher mean triglyceride concentrations were noted up to month 6 and lower mean glucose concentrations at months 6, 12 and 18, orange to red colored urine 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, thyroid gland, kidney and eye. In females at 1500 ppm, at the 12-month sacrifice, liver and thyroid gland weights were increased. Histological changes which included a higher incidence of altered hepatocytes (eosinophilic foci) and hepatocellular brown pigments, focal/multifocal hepatocellular vacuolation, increased number of mitoses, centrilobular to panlobular hypertrophy and hepatocellular single cell necrosis in the liver. In the thyroid gland, follicular cell hypertrophy, in association with a higher incidence and severity of colloid alteration was observed. In the kidney a higher incidence of tubular golden brown pigments and of hyaline casts was observed. At the 24-month sacrifice, 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-neoplastic/preneoplastic changes observed at 12 months and marked hepatocellular toxicity after 24 months. In the kidney, marked degenerative changes resulting from exacerbation of the microscopic findings noted 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 (follicular 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 lens degeneration and peripheral bilateral retinal atrophy.

In males at 750/375 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 hyaline droplets and of bilateral basophilic tubules was observed. In the thyroid gland, follicular cell 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 liver, 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 were 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 Wistar rat, the NOAEL was 30 ppm in males (equivalent to 1.37 mg/kg body weight/day) and the NOEL in females 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 Wistar 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 tumors observed in female rats 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 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 resulted in the induction of cytochrome P450 content; increased cytochrome P450 and UDPGT isoenzyme activities and corresponding changes in gene expression, hepatocyte 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 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 in the rodent for liver tumours in 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 not in human cells in response to fluopyram 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 fluopyram is via activation of the CAR/PXR nuclear receptors. Furthermore, the lack of proliferation in primary human hepatocytes (compared to rat hepatocytes), provides convincing evidence that the liver tumours 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 depth discussion 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 males at 750 ppm. Liver weights were increased at both the 12-month and 18-month sacrifice in both sexes at 750 ppm and 150 ppm, and at 18 months only in males at 30 ppm. At the high dose level, kidney weights were decreased in both sexes at 12 months and females only at 18 months. The target organs were the liver, kidney and thyroid gland. Histologically, after 12 months, the pre-neoplastic change of follicular 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 cell adenoma of the thyroid gland in males at 750 ppm. Non-neoplastic changes were seen in the liver, kidney and thyroid gland. The principal change noted in the liver was centrilobular to panlobular hypertrophy, which was seen in both sexes at the two highest dose levels, together 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, hyaline casts(s) and interstitial mononuclear cell infiltrates, together with a higher incidence of glomerular congestion/hemorrhage(s), associated with higher severity of amyloid deposition was noted in females at 750 ppm. In the thyroid gland, a higher incidence of follicular cell hyperplasia was noted in both sexes at 750 ppm and males only at 150 ppm.

Dietary administration of AE C656948 over an 18-month period to the C57BL/6J mouse, at a dose level of 750 ppm in males (equivalent to 105 mg/kg/day) resulted in a higher incidence of follicular 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 follicular 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 mouse thyroid tumors

In summary, the 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 a number of mechanistic studies. Administration of fluopyram to male mice resulted in cytochrome P450 induction (increased BROD/PROD activities), reduced T4 and increased TSH levels and a more rapid 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 not in KO mice provides 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. Phase I and II liver enzymes were induced in both species, but critically UGT-T4 was induced in rat hepatocytes but not in human hepatocytes. This MoA (increased TSH secondary to 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 an inhibitor of thyroid peroxidase (TPO) activity, thereby discounting an alternative MoA.

Overall, comprehensive mechanistic data are available, which elucidate the MoA and demonstrate that neither tumour 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

Supporting data regarding male mouse thyroid tumors

Mechanistic studies are summarized under data points 5.5/09 – 5.5/22

Table 5.5-1 Summary of long-term toxicity/carcinogenicity with AE C656948

Study **Doses tested** ***mg/kg bw/d***	**NOAEL** **ppm** **mg/kg bw/d**	**LOAEL** **ppm** **mg/kg bw/d**	**Main findings**	**Reference**
Rat – 104-week Chronic Toxicity/ Oncogenicity 30, 150 & 750/375 (males) 1500 females ppm ***0, 1.20, 6.0, 29 M*** ***0, 1.68, 8.6, 89 /F,*** ***over 24 months***	1.20/1.68 (M/F)	6.0/8.6 (M/F)	Eye lesions in high dose females Treatment-related marked liver toxicity, nephropathy in the kidney and follicular cell hypertrophy in the thyroid gland Liver cell tumors (carcinoma and adenoma) in high dose females – Additional data support an indirect, non-genotoxic, phenobarbital-like mechanism of action	[redacted] M-298339-01-1
Mouse - 78 week-Chronic/ Oncogenicity 30, 150 & 750 ppm ***0, 4.2, 20.9, 105 M*** ***0, 5.3, 26.8, 129 /F,*** ***over 18 months***	4.2/5.3 (M/F)	20.9/26.8 (M/F)	Nephropathy in the kidney in high dose females. Treatment-related follicular cell hyperplasia in the thyroid gland, centrilobular to panlobular hypertrophy and hepatocellular single cell degeneration /necrosis in the liver. Thyroid gland follicular cell adenomas in high dose males – Additional data support an indirect, non-genotoxic threshold mechanism of action secondary to liver effect.	[redacted] M-295688-01-1

Data Point:	KCA 5.5/01
Report Author:	[redacted]
Report Year:	2008
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:	M-298339-01-1
Guideline(s) followed in study:	OECD 453 (1981); EEC Directive 88/302/EEC Method B33 (1987); EPA Health Effects Test Guideline (OPPTS 870.4300; 1998), M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines
Deviations from current test guideline:	Current guideline: OECD 453, 2018 Deviation: None
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

AE C656948, (Mix-Batch 08528/0002, a beige powder, ≥94.5% purity) was administered by continuous dietary treatment to groups of 60 male Wistar rats at 30, 150 and 750 ppm (due to the high mortality rate in the high dose group males, this dose level was reduced to 375 ppm from Week 85 onwards), corresponding to 0, 1.20, 6.0 and 29 mg/kg/day, respectively, and groups of 60 female Wistar rats at 30, 150 and 1500 ppm, corresponding to 1.68, 8.6 and 89 mg/kg/day, respectively, over a 24-month period. Additionally, groups of 10 male and 10 female rats 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 males and 1.88, 9.6 and 95 mg/kg/day in females, 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. Hematology and clinical chemistry determinations and urinalysis were performed during months 3, 6, 12, 18 and 24 on selected animals. At the scheduled chronic and carcinogenicity phase sacrifice, selected organs were weighed and designated tissues sampled and examined microscopically.

At 1500 ppm in females

The overall incidence and percentage of mortality was low with no evidence of a treatment-related increase throughout the study. There were no treatment-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 14 to 26 (-29%, $p \leq 0.01$), 26 to 54 (-15%, not statistically significant) and 54 to 79 (-59%, $p \leq 0.01$) in comparison to the controls, whilst mean body weight was lower by 3, 5, 14 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.

Ophthalmological 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,abnormal color (pale) of the retinal fundus and hyperreflectivity in retina after two years, in comparison 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). Urinalysis 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 hepatocytes (eosinophilic foci) and hepatocellular brown pigments, focal/multifocal hepatocellular vacuolation, increased number of mitoses, centrilobular to panlobular hypertrophy and hepatocellular single cell necrosis. In the thyroid gland, mean absolute and relative weights were 23 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 kidney was found in most animals, compared to no cases in the controls, and a higher incidence of tubular golden/brown pigments 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 incidence of tumors of any type in any organ.

At the end of the carcinogenicity 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 weights were 39 to 56% higher than the controls and were associated with a higher incidence of enlarged liver, dark liver, prominent lobulation, red and white foci on the liver at the macroscopic observation, when compared to the controls. In addition, liver nodules/masses (5/60 females, compared to no cases in the controls) were noted and correlated with liver cell carcinoma or adenoma noted at the microscopic examination. Histological changes attributed to the treatment and indicative of marked liver toxicity were also noted, including metabolic, degenerative or proliferative changes. They correspond to the exacerbation of the microscopic findings observed at the end of the chronic phase. In addition in the liver, minimal to slight extramedullary hematopoiesis was observed. In the kidney, 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, together with an increased incidence of tubular golden/brown pigments and collecting ducts hyperplasia. In the thyroid gland, exacerbation of the microscopic findings (follicular 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 lens degeneration and peripheral bilateral retinal atrophy. Retinal atrophy was characterized by degeneration of the outer plexiform layer, outer nuclear layer and rod/cones lamina.

Treatment-related neoplastic changes 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 hepatocellular toxicity.

<u>At 750/375 ppm in males:</u>

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 or 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 incidence of the usual signs associated with morbidity (prostration, general pallor and soiled anogenital 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% ($p \leq 0.01$ or $p \leq 0.05$) 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 retinal vessels and abnormal color (pale) of the retinal fundus was noted after two years, in comparison to the controls.

There were no treatment-related changes at the hematology and clinical chemistry evaluations throughout the study. Urinalysis revealed a higher incidence and severity of cellular casts at Months 3 and 6, when compared to the controls. This change was found to be reversible, as no 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 liver weights were higher by between 17 to 18% and mean absolute and relative kidney weights by 28%, in comparison to the controls. At the macroscopic observation, enlarged liver was found in 1/10 animals 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 no cases 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 (eosinophilic foci) was higher. In addition, centrilobular to panlobular hypertrophy was observed. In the kidney, chronic progressive nephropathy was noted. This change is a combination of thickened basement membranes (tubular and glomerular), basophilic tubules and hyaline casts with a variable inflammatory cell infiltrate. In addition, a higher incidence of hyaline droplets and of bilateral basophilic 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 liver weights were 5 to 12% higher, in comparison to the control group. At the macroscopic observation, a higher incidence of enlarged liver and white foci on 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 liver, kidney, thyroid gland, and secondary effects in the testis and stomach, in comparison to the controls. In the liver and thyroid 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 microscopic findings noted at the end of the chronic phase were observed, together with a higher incidence of tubular hypertrophy, collecting duct hyperplasia and hyaline droplets. In the testis, a higher incidence of arteritis and periarteritis was noted. This vascular change was isolated (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 in the testis was considered not to be a direct effect of the treatment. In the stomach, 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 morbidity. 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.

At 150 ppm

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 throughout the study. Urinalysis revealed a higher incidence and severity of cellular 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, 18 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 panlobular hypertrophy was observed. In the kidney, chronic progressive nephropathy was noted, 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 months), mean terminal body weights and mean organ weights were unaffected by treatment in either sex, with the exception of a slight increase 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, thyroid gland and testis, in comparison to the controls. In the liver, changes resulting from exacerbation of the findings noted at the end of the chronic phase were observed in males. In the 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 thyroid 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 neoplastic changes were observed at this dose level in either sex.

At 30 ppm:

No toxicologically relevant changes were 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-related 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

1. Test material:	AE C656948
Description	beige powder
Lot / Batch #:	Mix-Batch: 08528/0002
Purity:	≥94.5% a.i.

CAS #	658066-35-4
Stability of test compound:	Stable in rodent diet at 20 and 10000 ppm over a 105-day period at ambient temperature, checked in a previous study ()
2. Vehicle and / or positive control:	none
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj:WI (IOPS HAN)
Age:	6 weeks approximately (start of dosing)
Weight at dosing:	216 - 219 g mean group weight for the males - 155 - 157 g mean group weight for the females
Source:	[redacted]
Acclimation period:	13 days
Diet:	A04CP1-10 (formerly referenced as A04C-10 P1) from S.A.F.E. (Scientific Animal Food and Engineering, Augy, *ad libitum* except at designated time periods
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*
Housing:	By sex in groups of 5, unless reduced by mortality or isolation. The cages were suspended, stainless steel wire mesh.
Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	55 ± 15%
Air changes:	10-15 per hour
Photoperiod:	Alternating 12-hour light and dark cycles (7 am-7 pm)

B. Study design

1. In life dates: From 09 February 2005 to 06 March 2007

2. Dose level selection

The dose levels were selected based on the results from a previous 90-day dietary study in the rat (M-250946-01-1), where the NOAEL was established at 50 ppm for males and 200 ppm for females. The NOAEL was mainly 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 chemistry parameters. In addition in males, a higher incidence of cellular casts was observed at urinalysis and higher incidences/severity of hyaline droplet nephropathy (related to the accumulation of alpha 2u-globulin in the proximal tubules) 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 of randomization, 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 ±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 untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 5.5-2 Details of group sizes and treatment,

Test Group	Diet concentration (ppm)	Achieved dose to animal (weeks 1-104) (mg/kg/day)	Interim sacrifice 52 weeks		Main study 104 weeks	
			Male	Female	Male	Female
Control (1)	0	0	10	10	60	60
Animal identity			PT1M0677 to 0686	PT1F0747 to 0756	PT1M0687 to 0746	PT1F0757 to 0816
Low (2)	M: 30 F: 30	M: 1.20 F: 1.68	10	10	60	60
Animal identity			PT2M0817 to 0826	PT2F0887 to 0896	PT2M0827 to 0886	PT2F0897 to 0956
Mid (3)	M: 150 F: 150	M: 6.0 F: 8.6	10	10	60	60
Animal identity			PT3M0957 to 0966	PT3F1027 to 1036	PT3M0967 to 1026	PT3F1037 to 1096
High (4)	M: 750/375* F: 1500	M: 29 F: 89	10	10	60	60
Animal identity			PT4M1097 to 1106	PT4F1167 to 1176	PT4M1107 to 1166	PT4F1177 to 1236

* 750 ppm up to study Day 588 (study Week 84), 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 ppm. The test substance formulations were prepared to cover the dietary requirements over 6 to 8 weekly periods apart from the thirteenth formulation (F13) due to the decrease in dose level 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.

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 least for all loads at all dose levels for formulations F1, F2, F4, F7, F10, F13 and F16.

Homogeneity and concentration results of AE C656948 in the diet were within the in-house target range of 85 to 115% of nominal concentration, except for 14 out of 242 results, which however were considered acceptable for use on 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 ($p \leq 0.05$) (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-sided) if the Kruskal-Wallis test indicated significance.

- If the Bartlett test was significant ($p \leq 0.05$) (for body weight and average 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 ($p \leq 0.05$) 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 ($p \leq 0.05$) (for hematology 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 ($p \leq 0.05$) even after square root transformation, group means were compared using the non-parametric Kruskal-Wallis which was followed by the Dunn 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 test indicated significance.

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.

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 accidents (accidental trauma or died 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 dose-related trend in survival were assessed using Cox's and Tarone's tests on life table data. Probability values presented were two-sided for pairwise comparisons and trend test. Group mortality rates were compared at the 5% and 1% levels 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 treated 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 lesion-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 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 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 animal, incidental otherwise.

Incidental tumors and non-neoplastic lesions data were analyzed by logistic regression of tumor 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 dose-related trends were compared using the corrected score test. Fatal tumors were analyzed by the life-table 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 dose-related trends were investigated using Cox's 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 excluding the high dose group was also done. 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 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.

3. Food consumption and compound intake

Food consumption was recorded twice weekly during the first 6 weeks of treatment, then weekly up to Week 13, and once approximately every 4 weeks thereafter.

The weekly mean achieved dosage intake in mg/kg body weight/day for Weeks 1 to 13, then 1 week per month thereafter was calculated as follows:

$$\text{Test substance intake (mg/kg/day)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day)}}{\text{Group mean body weight (g) at the end of the food consumption period}}$$

The monthly and overall mean achieved dosage intake for the 24 months of treatment were derived from the weekly data.

4. Ophthalmoscopic examination

During the acclimatization phase, all animals were examined by indirect ophthalmoscopy. During the treatment period, funduscopic (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 atropinic agent (Mydriaticum, Merck Sharp and Dohme), each eye was re-examined by means of a slit lamp and an indirect ophthalmoscope.

5. Hematology and clinical chemistry

Blood was sampled from Isoflurane anesthetized animals by puncture of the retro orbital venous plexus 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 on Weeks 12 or 13, 25 or 26 and 51, and on the first ten suitable surviving rats of the terminal sacrifice groups on 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 Laboratory, Paris, France) for blood clotting measurements.

Hematocrit	Leukocyte differential count*
Hemoglobin concentration	Mean corpuscular HGB
Leukocyte count	Mean corpuscular HGB concentration
Erythrocyte count	Mean corpuscular volume
Platelet count	Reticulocyte count
Blood clotting measurements: Prothrombin time	

* Minimum required for carcinogenicity studies (Control and high dose groups unless effects are observed) based on Guideline 870.4300 & OECD 453

A blood smear was prepared and stained with a Wright (Months 3, 6, 12 and 18) or May-Grünwald-Giemsa (Month 24) stains. It was examined only when the results were abnormal.

For moribund and terminal sacrificed animals, the blood smears were stained with Wright stain (until October 05, 2006) or May-Grünwald-Giemsa stain (from October for possible differential white blood cell determination (but were not examined).

b. Clinical Chemistry

Parameters were measured using an Advia 1650 (Bayer Diagnostics, Puteaux, France).

ELECTROLYTES	OTHER
Calcium*	Albumin*
Chloride*	Creatinine*
Magnesium*	Urea *
Inorganic phosphorus*	Total Cholesterol*
Potassium*	Globulins*
Sodium*	Glucose (fasting)*
ENZYMES (more than 2 hepatic enzymes)*	Total bilirubin
Alkaline phosphatase*	Total protein*
Cholinesterase	Triglycerides
Creatine phosphokinase	Serum protein electrophoresis
Lactic acid dehydrogenase	
Alanine aminotransferase*	
Aspartate aminotransferase*	
Gamma glutamyltransferase*	
Sorbitol 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 sacrifice 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. Urinary refractive index was measured using a RFM320 refractometer.

The following semi-quantitative parameters were assayed using a Clinitek 200+ and Ames Multistix dipsticks:

Appearance*	Glucose*
Volume*	Ketones*
Specific gravity / osmolality / refractive index*	Bilirubin*
pH*	Blood / red blood cells*
Sediment (microscopic)	Urobilinogen
Protein*	Creatinine

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

a. Quantitative parameters

- Urine samples were weighed to determine urinary volume. pH was assayed using a Clinitek 200+ and Multistix dipsticks (previously referenced as Ames Multistick in the protocol) (Bayer Diagnostics, Puteaux, France).
- Urinary refractive index was measured using a RFM 320 refractometer (Bioblock Scientific, Illkirch, France).

b. Semi-quantitative parameters

- Glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen were assayed using a Clinitek 200+ and Multistix dipsticks (previously referenced as Ames Multistick in the protocol) (Bayer Diagnostics, Puteaux, France).

c. Microscopic examination of the sediment

- 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.

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 24-month carcinogenicity phase, all surviving animals dedicated to chronic phase and carcinogenicity phase groups, respectively, were sacrificed by exsanguination under 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 necropsied. The necropsy included the examination of external surfaces, all orifices, all major organs, tissues and body cavities. All significant 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	Tongue	X	Aorta, thoracic*	XX	Brain (3 sections)*+
X	Submandibular (salivary) gland	XX	Heart*+		
		X	Bone marrow*, sternum	X	Sciatic nerve*
X	Esophagus*	X	Lymph node*, mesenteric	X	Spinal cord (cervical, thoracic, lumbar)*
X	Stomach*				
X	Duodenum*	X	Lymph node, submaxillary	X	Eyes (retina)*

	Digestive system		Cardiovasc. / Hemat.		Neurologic
X	Jejunum*			X	Optic nerves*
X	Ileum*	XX	Spleen*+		
X	Cecum*	XX	Thymus		**Glandular**
X	Colon*			XX	Pituitary gland*
X	Rectum*		**Urogenital**	XX	Adrenal gland*+
XX	Liver*+	XX	Kidney*+	X	Parathyroid gland*
X	Pancreas*	X	Urinary bladder*		
		XX	Testis*+	XX	Thyroid gland* (weighed with parathyroid gland)
	Respiratory	XX	Epididymis*+		
X	Trachea*	XX	Prostate gland*	^	Lacrymal exorbital gland
X	Lung*	X	Seminal vesicle*	X	Harderian gland
^	Pharynx*	XX	Ovary*+		**Other**
^	Larynx*	XX	Uterus (with cervix)*+	X	Bone (sternum)
^	Nasal cavities*	X	Mammary gland*	X	Skeletal muscle
		X	Vagina	X	Skin*
				X	All gross lesions and masses (ncluding their lymph nodes if possible)*
				X	Articular surface (femorotibial joint)

* required for carcinogenicity studies based on US EPA guideline 870.4300
\+ organ weights required for rodent studies
X tissues were collected for histological examinations
XX 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 Grünwald Giemsa, but not examined as no relevant changes were observed in hematology or bone marrow histology. The second smear was stored unstained.

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

a. Histotechnology

- All the above samples listed in the above table (except exorbital lachrymal gland, larynx/pharynx and nasal cavities) were embedded in paraffin wax.
- Histological sections, stained with hematoxylin and eosin, were prepared from all organs and tissue samples listed in the above table from all animals.

b. Histopathology

- Histopathology examinations were performed as follows (interim sacrifice):
- all organs and tissue samples from animals sacrificed or dying during the treatment period,
- all organs and tissue samples from animals of control and high dose groups,
- liver, lung, kidney and target organs detected at microscopic examination (thyroid gland) from animals of the intermediate dose groups,
- Histopathology examinations were performed as follows (terminal sacrifice):
- all organs and tissue samples from all animals,
- gross abnormalities from all animals.

For all unscheduled sacrificed or dead animals on study, the cause of death was determined when possible. The diagnoses 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 hindlimbs, 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: Mortality rate in male and female rats – weeks 1-52

Sex	Males				Females			
Diet concentration of AE C656948 (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Group size	70	70	70	70	70	70	70	70
Mortality (% mortality)	6 (8.6)	3 (4.3)	2 (2.9)	11 (15.7)	1 (1.4)	2 (2.9)	5 (7.1)	3 (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 ($p \leq 0.05$). 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

Table 5.5-4: Mortality rate in male and female rats – weeks 1-104

Sex	Males				Females			
Dose level of AE C656948 (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of animals	60	60	60	60	60	60	60	60
Killed for humane reasons	17	15	6	20	12	24	19	23
Found dead	20	29	28	28	7	8	4	6
Dead during anesthesia	0	0	0	1	0	0	2	1
Total mortality (% mortality)	37 (61.7)	44 (73.3)	34 (56.7)	49 (81.7)	19 (31.7)	32 (53.3)	25 (41.7)	30 (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 survival rates at the end of the study after adjusting for censored animals
*: $p \leq 0.05$

B. Clinical observations

Chronic 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:

Table 5.5-5: Incidence of treatment-related clinical signs days 367-742

Sex	Males				Females			
Diet concentration (ppm)	0	30	150	750/375	0	30	150	1500
Number of animals examined	55	57	58	49	59	58	56	57
Hair loss	nc	nc	nc	nc	8 (13.6%) [371]	10 (17.2%) [371]	5 (8.9%) [371]	17 (29.8%) [371]
Prostration	0 (0%) -	1 (1.8%) [724]	0 (0%) -	3 (6.1%) [613]	nc	nc	nc	nc
General pallor	2 (3.6%) [543]	2 (3.5%) [624]	1 (1.7%) [445]	4 (8.2%) [445]	nc	nc	nc	nc
Wasted appearance	nc	nc	nc	nc	10 (16.9%) [464]	12 (20.7%) [553]	11 (19.6%) [483]	14 (24.6%) [504]
Soiled anogenital region	2 (3.6%) [704]	1 (1.8%) [654]	1 (1.7%) [641]	4 (8.2%) [368]	nc	nc	nc	nc

nc: not concerned or no relevant change; (%): incidence
[ ]: first day of appearance during the second year of treatment.

In the female high dose group (1500 ppm), a higher incidence of hair loss and wasted appearance was noted, in comparison to the controls.

In the male high dose group (750/375 ppm), a slightly higher incidence of the usual signs associated with morbidity (prostration, general pallor and soiled anogenital region) was noted, in comparison to the controls, reflecting the higher mortality observed in this group.

No treatment-related clinical signs were noted at the mid and low dose levels in either sex.

C. Body weight

In the female high dose 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%, $p \leq 0.01$), 26 to 54 (-15%, not statistically significant) and 54 to 79 (-59%, $p \leq 0.01$), whilst mean body weight was lower by 3, 5, 14 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				
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)	278±14 (100)	279±15 (101)
BW Week 14 (Day 92) (%C)	527±37	526±42 (100)	529±41 (100)	526±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 (101)	694±61 (100)
BW Week 78 (Day 547) (%C)	719±78	728±86 (101)	733±76 (102)	702±64 (98)
BW Week 102 (Day 708) (%C)	**672±89**	**639±86 (95)**	**686±68 (102)**	**627±64 (93)**
BWG Weeks 1-2 (Days 1 to 8) (%C)	**59±5**	**59±7 (100)**	**62±9 (105)**	**61*±6 (103)**
BWG Weeks 1-14 (Days 1 to 92) (%C)	308±34	307±36 (100)	313±40 (102)	308±37 (100)
BWG Weeks 14-26 (Days 92 to 176) (%C)	77±16	74±16 (96)	79±17 (103)	82±17 (106)
BWG Weeks 26-54 (Days 176 to 372) (%C)	88±32	97±35 (110)	93±31 (106)	81±34 (92)
BWG Weeks 54-78 (Days 372 to 547) (%C)	25±34	31±33 (124)	28±47 (112)	23±30 (92)
BWG Weeks 79-102 (Days 547 to 708) (%C)	-48±57	-67±49 (143)	-42±55 (89)	-60±45 (128)
Overall BWG (Days 1 to 708) (%C)	**453±91**	**421±86 (93)**	**470±69 (104)**	**410±65 (91)**
Females				
AE C656948 dosage level (ppm)	0	30	150	1500
Initial BW (Day 1) (%C)	**155±10**	**156±11 (101)**	**157±10 (101)**	**157±10 (101)**
BW Week 2 (Day 8) (%C)	183±13	186±14 (102)	186±13 (102)	183±13 (100)
BW Week 14 (Day 92) (%C)	280±22	287±21 (103)	283±23 (101)	278±21 (99)
BW Week 26 (Day 176) (%C)	310±25	316±25 (102)	314±26 (101)	300*±25 (97)
BW Week 54 (Day 372) (%C)	350±49	361±40 (103)	351±42 (100)	333±36 (95)
BW Week 79 (Day 540) (%C)	412±75	422±67 (102)	408±59 (99)	356**±45 (86)
Final BW (Day 708) (%C)	**425±78**	**452±92 (106)**	**425±68 (100)**	**374**±56 (88)**
BWG Weeks 1-2 (Days 1 to 8) (%C)	**27±7**	**30*±7 (111)**	**29±7 (107)**	**27±7 (100)**
BWG Weeks 1-14 (Days 1 to 92) (%C)	124±17	131*±16 (106)	126±18 (102)	122±16 (98)
BWG Weeks 14-26 (Days 92 to 176) (%C)	31±10	30±10 (97)	30±9 (97)	22**±9 (71)
BWG Weeks 26-54 (Days 176 to 372) (%C)	39±33	43±24 (110)	37±24 (95)	33±20 (85)
BWG Weeks 54-79 (Days 372 to 547) (%C)	61±42	60±42 (98)	56±32 (92)	25**±23 (41)
BWG Weeks 79-102 (Days 547 to 708) (%C)	21±44	22±52 (105)	25±34 (119)	20±39 (95)
Overall BWG Weeks 1-102 (Days 1 to 708) (%C)	**269±73**	**296±89 (110)**	**269±64 (100)**	**216**±53 (80)**

# 750 ppm up to study Day 588 (study Week 84), 375 ppm from study Day 589 onwards (study Week 85)
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, mean food consumption was similar to the controls throughout the study in both sexes and at all dose levels, with the only exception of a slight reduction by up to 7% (p≤0.01 or p≤0.05) 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 treatment-related effects.

Table 5.5-7 Group mean food consumption (g/animal/day)

Males					
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)	-
Week period 14 to 26 (% C)	25.3	25.2 (100)	25.8 (102)	25.7 (102)	-
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)	-
Females					
Week period 1 to 13 (%C)	19.0	19.5 (103)	19.6 (103)	-	18.7 (99)
Week period 14 to 26 (% C)	18.1	18.4 (102)	18.9 (104)	-	17.6 (97)
Week period 27 to 52 (% C)	18.2	18.8 (103)	18.6 (1002)	-	18.0 (99)
Week period 53 to 78 (% C)	19.3	19.6 (102)	19.3 (100)	-	18.6 (96)
Week period 79 to 104 (% C)	20.5	21.8 (106)	20.8 (101)	-	20.9 (102)

Percentage from control in parentheses.

E. Achieved intake

Compound intake is shown in Table 5.5.2-6 for various periods of the study.

The mean achieved dietary intakes of AE C656948 expressed in mg/kg body weight/day received by the animals during the study were as follows:

Table 5.5-8 Mean achieved dietary intake of AE C656948 (mg/kg/day)

Sex	**Males**			**Females**		
Dosage level (ppm)	**30**	**150**	**750/375***	**30**	**150**	**1500**
Week period 1 to 13	1.84	9.2	46	2.35	12	117
Week period 1 to 52	1.37	6.9	35	1.88	9.6	95
Week period 1 to 104	1.20	6.0	29	1.68	8.6	89

* 750 ppm up to study Day 588 (study Week 84), 375 ppm from study Day 589 onwards (study Week 85)

F. Ophthalmoscopic examinations

At the end of the first year of treatment, the following treatment-related ophthalmological findings were observed:

Table 5.5-9 Incidence of treatment-related ophthalmological findings noted at the first year examination (animals allocated to the chronic and carcinogenicity phases)

Sex	**Males**				**Females**			
Dosage level (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Number of animals examined	65	68	68	59	69	68	66	67
Retina fundus abnormal color: pale	0 (0%)	0 (0%)	1 (1.5%)	0 (0%)	0 (0%)	1 (1.5%)	0 (0%)	4 (6%)

(%): incidence

In the female high dose group, abnormal color (pale) of the retinal fundus was observed in 4/67 animals, compared to no case in the controls.

No treatment-related ophthalmological findings were noted at any dose level tested in males or at the mid and low dose levels 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:

Table 5.5-10 Incidence of treatment-related ophthalmological findings noted at the second year examination (animals allocated to the carcinogenicity phase)

Sex	Males				Females			
Dosage level (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of animals examined	25	21	31	14	43	31	37	32
Corneal opacity	2 (8.0%)	2 (9.5%)	7 (22.6%)	3 (21.4%)	2 (4.7%)	0 (0%)	1 (2.7%)	0 (0%)
Oedema of the cornea	1 (4.0%)	1 (4.8%)	4 (12.9%)	3 (21.4%)	2 (4.7%)	0 (0%)	1 (2.7%)	0 (0%)
Nuclear opacity of lens	12 (48%)	11 (52.4%)	27 (87.1%)	12 (85.7%)	35 (81.4%)	26 (83.9%)	31 (83.8%)	30 (93.8%)
Small retina vessels	1 (4.0%)	1 (4.8%)	3 (9.7%)	3 (21.4%)	2 (4.7%)	2 (6.5%)	2 (5.4%)	14 (43.8%)
Retina fundus abnormal color: pale	2 (8.0%)	1 (4.8%)	2 (6.5%)	6 (42.9%)	3 (7%)	3 (9.7%)	2 (5.4%)	15 (46.9%)
Hyperreflectivity in retina	1 (4.0%)	0 (0%)	0 (0%)	1 (7.1%)	1 (2.3%)	1 (3.2%)	1 (2.7%)	3 (9.4%)

(%): incidence

In the female high dose group, a higher incidence of small retinal vessels, abnormal color (pale) of the retinal fundus and hyperreflectivity in retina was noted, in comparison to the controls.

In the male high dose group, a higher incidence of corneal opacity, oedema of the cornea, nuclear opacity of lens, small retinal vessels and abnormal color (pale) of the retinal fundus was noted, in comparison to the controls.

In the male mid dose group, a higher incidence of corneal opacity, oedema 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 mid 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 urinalysis

1. Hematology

A tendency towards lower erythrocyte parameters (hemoglobin concentration, mean corpuscular volume, hematocrit and/or mean 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.

No treatment-related findings were noted at the hematology 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 cholesterol 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 mid dose group at Month 3, but as this change was observed in isolation and as its magnitude was low, 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 ± 0.28* (+15%)	2.38 ± 0.32 ** (+35%)
Month 6	2.07 ± 0.37 -	2.01 ± 0.34 (-3%)	2.32 ± 0.27 (+12%)	2.72 ± 0.56 ** (+31%)
Month 12	1.98 ± 0.33 -	2.01 ± 0.49 (+2%)	2.19 ± 0.27 (+11%)	2.74 ± 0.76 ** (+38%)
Month 18	2.22 ± 0.31 -	2.78 ± 1.94 (+25%)	2.21 ± 0.31 (0%)	2.92 ± 0.76 (+32%)
Month 24	2.44 ± 0.57 -	2.14 ± 0.68 (-12%)	2.32 ± 0.51 (-5%)	3.30 ± 0.46 ** (+35%)

*: p≤0.05, **: p≤0.01

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≤0.01) and slightly lower mean glucose concentrations were noted at Months 6, 12 and 18 (-9%, -13% and -16%, respectively, p≤0.01 or p≤0.05).

No treatment-related findings were noted at the clinical chemistry evaluation at any dose level tested in males or at the low dose level in females. Slightly lower mean total bilirubin 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 orange 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.

Table 5.5-12 Incidence and severity of the presence of cellular casts in urine in males

Males					
Dose level of AE C656948 (ppm)		**Control**	**30**	**150**	**750**
Month 3	Slight	1/19	2/19	4/20	0/19
	Moderate	0/19	0/19	4/20	6/19
	Marked	0/19	0/19	2/20	5/19
	Severe	0/19	0/19	0/20	6/19
	total	1/19	2/19	10/20	17/19
Month 6	Slight	0/20	0/19	5/20	6/20
	Moderate	0/20	0/19	1/20	9/20
	Marked	0/20	0/19	1/20	4/20
	Severe	0/20	0/19	0/20	0/20
	total	0/20	0/19	7/20	19/20

This effect was temporary (seen only after 3 and 6 months), since cellular casts (moderate severity) were observed in only 1/20 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.

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 (-10%, not statistically significant) but was unaffected at all dose levels tested in males and at the mid and low dose levels in females.

In the high dose groups, mean absolute and relative liver weights were higher by between 39 to 54% in females and by 17 to 18% in males, when compared to the controls.

Table 5.5-13 Liver weight changes (± SD) at scheduled sacrifice of the chronic phase (% change when compared to controls)

Sex	Males				Females			
Dose level of AE C656948 (ppm)	0	30	150	750	0	30	150	1500
Mean absolute liver weight (g)	12.21 ± 1.33	12.70 ± 1.50 (+4%)	12.43 ± 1.05 (+2%)	14.31 * ± 1.92 (+17%)	7.51 ± 0.99	7.37 ± 1.01 (-2%)	7.67 ± 1.43 (+2%)	10.44 ** ± 1.61 (+39%)
Mean liver to body weight ratio	1.86 ± 0.12	1.93 ± 0.18 (+4%)	1.88 ± 0.15 (+1%)	2.19 ** ± 0.18 (+17%)	2.17 ± 0.19	2.19 ± 0.22 (+1%)	2.26 ± 0.20 (+4%)	3.35 ** ± 0.33 (+54%)
Mean liver to brain weight ratio	540.35 ± 61.69	552.56 ± 59.71 (+2%)	569.79 ± 65.10 (+5%)	635.39 * ± 87.57 (+18%)	367.35 ± 44.71	354.69 ± 43.06 (-3%)	378.12 ± 62.80 (+3%)	517.51 ** ± 61.47 (+41%)

*: 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 5.5-14 Kidney weight changes (± SD) at scheduled sacrifice of the chronic phase (% change when compared to controls)

Sex	Males				Females			
Dose level of AE C656948 (ppm)	0	30	150	750	0	30	150	1500
Mean absolute kidney weight (g)	3.51 ± 0.32	3.64 ± 0.33 (+4%)	3.78 ± 0.23 (+8%)	4.48 ** ± 0.70 (+28%)	2.39 ± 0.24	2.40 ± 0.37 (0%)	2.48 ± 0.37 (+4%)	2.64 ± 0.36 (+10%)
Mean kidney to body weight ratio	0.54 ± 0.03	0.55 ± 0.04 (+2%)	0.58 ± 0.08 (+7%)	0.69 ** ± 0.13 (+28%)	0.70 ± 0.08	0.71 ± 0.09 (+1%)	0.73 ± 0.08 (+5%)	0.85 ** ± 0.08 (+22%)
Mean kidney to brain weight ratio	155.15 ± 13.34	158.65 ± 15.77 (+2%)	173.58 ± 18.90 (+12%)	198.22 ** ± 25.86 (+28%)	117.11 ± 11.71	115.46 ± 14.64 (-1%)	122.15 ± 16.97 (+4%)	131.11 ± 13.48 (+12%)

**: p≤0.01

In the female 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	Males				Females			
Dose level of AE C656948 (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Mean absolute thyroid gland weight (g)	0.0272 ± 0.0059	0.0280 ± 0.0036 (+3%)	0.0244 ± 0.0066 (-10%)	0.0295 ± 0.0059 (+8%)	0.0201 ± 0.0052	0.0196 ± 0.0063 (-2%)	0.0213 ± 0.0048 (+6%)	0.0248 ± 0.0058 (+23%)
Mean thyroid gland to body weight ratio	0. 0042 ± 0.0009	0.0043 ± 0.0006 (+3%)	0.0038 ± 0.0012 (-10%)	0.0045 ± 0.0008 (+9%)	0.0058 ± 0.0013	0.0057 ± 0.0015 (-1%)	0.0063 ± 0.0013 (+9%)	0.0080 ** ± 0.0019 (+38%)
Mean thyroid gland to brain weight ratio	1.2025 ± 0.2522	1.2226 ± 0.1797 (+2%)	1.1202 ± 0.3036 (-7%)	1.3064 ± 0.2478 (+9%)	0.9833 ± 0.2479	0.9423 ± 0.2958 (-4%)	1.0512 ± 0.2287 (+7%)	1.2358 ± 0.2813 (+26%)

**: p≤0.01

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 unaffected 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 mid dose groups, mean absolute and/or relative liver weights were also slightly higher by between 5 to 12%. This change 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 or microscopic examination.

Table 5.5-16 Liver weight changes (± SD) at scheduled sacrifice of the carcinogenicity phase (% change when compared to controls)

Sex	Male				Female			
Dose level of AE C656948 (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Mean absolute liver weight (g)	12.56 ± 1.71	11.09 * ± 1.12 (-12%)	14.02 * ± 2.06 (+12%)	13.24 ± 2.25 (+5%)	9.46 ± 2.19	9.69 ± 2.12 (+2%)	9.89 ± 2.12 (+5%)	13.16 ** ± 2.87 (+39%)
Mean liver to body weight ratio (%)	2.06 ± 0.33	1.94 ± 0.26 (-6%)	2.22 ± 0.39 (+8%)	2.32 ± 0.43 (+12%)	2.37 ± 0.37	2.31 ± 0.30 (-3%)	2.52 ± 0.35 (+6%)	3.70 ** ± 0.59 (+56%)
Mean liver to brain weight ratio (%)	536.35 ± 78.37	482.19 ± 55.17 (-10%)	590.42 ± 91.06 (+10%)	573.01 ± 90.66 (+7%)	447.20 ±100.55	458.65 ± 96.36 (+3%)	466.02 ± 95.77 (+4%)	656.49** ± 151.35 (+47%)

*: p≤0.05, **: p≤0.01

2. Macroscopic findings

Chronic phase

a. Unscheduled deaths

Two animals from the chronic phase were found dead before scheduled sacrifice.

- 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, small thymus, dark liver, white foci on spleen and a dilatation of uterine horns.

b. Terminal sacrifice:

Treatment-related findings were found in the liver 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 prominent lobulation on the liver in 3/10 animals, compared to no case in the controls.

Table 5.5-17 Incidence of macroscopic changes in the liver, scheduled sacrifice of the chronic phase

Sex	Males				Females			
Dose level of AE C656948 (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Obviously large	0/9	0/10	0/10	1/10	0/10	0/10	0/9	9/10
Dark	0/9	0/10	0/10	0/10	0/10	0/10	0/9	8/10
Prominent lobulation	0/9	0/10	0/10	3/10	0/10	0/10	0/9	4/10

In the female high dose group, dark kidneys were found in 8/10 animals, compared to no case in the controls.

In the male high dose group, pale kidneys, enlarged kidneys or irregular surface on the kidneys were found in some animals, compared to no case in the controls.

Table 5.5-18 Incidence of macroscopic changes in the kidney, scheduled sacrifice of the chronic phase

Sex	Males				Females			
Dose level of AE C656948 (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Dark	0/9	1/10	0/10	1/10	0/10	0/10	0/9	8/10
Pale	0/9	0/10	0/10	6/10	0/10	1/10	1/9	0/10
Obviously large	0/9	0/10	0/10	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 female high dose group, a higher incidence of enlarged liver, dark liver, white foci or red foci on the liver 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.

Table 5.5-19 Incidence of macroscopic changes in the liver-unscheduled sacrifices of the carcinogenicity phase

Sex	Males				Females			
Dose level of AE C656948 (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
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	1/32	1/25	0/30
Dark	2/37	0/44	0/34	1/49	0/19	1/32	1/25	02/30
Focus (i), white	2/37	6/44	4/34	9/49	3/19	3/32	6/25	9/30
Focus (i), red	6/37	9/44	9/34	9/49	5/19	6/32	7/25	14/30

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 enlarged kidneys was also observed.

Table 5.5-20 Incidence of macroscopic changes in the kidney, unscheduled sacrifices of the carcinogenicity phase

Sex	Males				Females			
Dose level of AE C656948 (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Dark	1/37	0/44	0/34	1/49	0/19	2/32	0/25	5/30
Obviously large	2/37	2/44	0/34	5/49	0/19	2/32	0/25	6/30
Irregular surface	3/37	2/44	4/34	8/49	1/19	2/32	1/25	7/30

b. Terminal sacrifice

In the female high dose group, a higher incidence of enlarged liver, 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 noted and correlated with liver carcinoma or adenoma noted at the microscopic examination. The overall incidence (unscheduled deaths plus terminal sacrifice) of liver nodules/masses was 5/60 high dose females, compared to no case in the controls.

Table 5.5-21 Incidence of macroscopic changes in the liver - scheduled sacrifices of the carcinogenicity phase

Sex	Males				Females			
Dose level of AE C656948 (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Nodules/masses	0/23	0/16	0/26	1/11	0/41	1/28	2/35	4/30
Obviously large	1/23	0/16	2/26	0/11	6/41	1/28	7/35	28/30
Dark	0/23	0/16	1/26	2/11	0/41	1/28	1/35	19/30
Focus (i), white	4/23	1/16	4/26	3/11	14/41	7/28	9/35	22/30
Prominent lobulation	1/23	0/16	2/26	0/11	2/41	3/28	3/35	13/30

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 rate of enlarged kidneys or irregular surface on the kidneys was noted.

Table 5.5-22 Incidence of macroscopic changes in the kidney - scheduled sacrifices of the carcinogenicity phase

Sex	Males				Females			
Dose level of AE C656948 (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
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	3/35	6/30

3. Microscopic findings

Chronic phase

a. Unscheduled deaths:

One control male was found dead on Day 246; meningeal hemorrhage 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.

b. Terminal sacrifice:

Treatment-related non-neoplastic changes of AE C656948 were found in the liver, kidney and thyroid gland.

Non-neoplastic findings

In the liver of high dose females, a higher incidence of altered hepatocytes (eosinophilic foci), focal/multifocal hepatocellular vacuolation, increased number of mitoses, hepatocellular single cell necrosis and hepatocellular brown pigments was noted, when compared to the controls. In addition in this group, centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were observed. Hepatocellular macrovacuolation or vacuolation was considered not to be an adverse effect, since it is a reversible 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 higher incidence of altered hepatocytes (eosinophilic foci) was observed. In addition, centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were observed. In the male mid dose group, centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were noted. In the male low dose group, centrilobular to midzonal hepatocellular macrovacuolation was observed. Hepatocellular vacuolation or macrovacuolation noted in males at the three dose levels was also considered not to be adverse, as it was not associated with any degenerative change in the liver.

No treatment-related changes were noted in the liver at the mid and low doses in females.

Table 5.5-23 Incidence and severity of microscopic changes in the liver, all animals of the chronic phase

Sex	Males				Females			
AE C656948 Dose level (ppm)	0	30	150	750	0	30	150	1500
Number of examined animals	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Focus(i) of hepatocellular alteration: eosinophilic: focal/multifocal								
Minimal	2	1	2	4	0	0	0	2
Slight	0	0	0	1	0	0	0	1
Total	**2**	**1**	**2**	**5**	**0**	**0**	**0**	**3**
Centrilobular to panlobular hepatocellular hypertrophy: diffuse								
Minimal	0	0	2	5	0	0	0	0
Slight	0	0	1	5	0	0	0	2
Moderate	0	0	0	0	0	0	0	8
Total	**0**	**0**	**3**	**10**	**0**	**0**	**0**	**10**
Hepatocellular macrovacuolation: centrilobular to midzonal: diffuse								
Minimal	0	4	4	5	0	0	0	2
Slight	0	1	2	3	0	0	0	2
Moderate	0	0	0	1	0	0	0	3
Total	**0**	**5**	**6**	**9**	**0**	**0**	**0**	**7**
Hepatocellular vacuolation: focal/multifocal								
Minimal	0	4	0	2	2	2	2	3
Slight	0	1	0	0	0	0	0	1
Total	**0**	**5**	**0**	**2**	**2**	**2**	**2**	**4**
Increased number of mitoses								
Present	0	0	0	0	2	0	1	6
Total	**0**	**0**	**0**	**0**	**2**	**0**	**1**	**6**
Hepatocellular brown pigment (s): focal/multifocal								
Minimal	0	0	0	0	0	0	0	1
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**
Hepatocellular single cell necrosis: focal/multifocal								
Minimal	0	0	0	0	0	0	0	1
Slight	0	0	0	0	0	0	0	1
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**

In the kidney of high dose females, a higher incidence of tubular golden/brown pigments and of hyaline casts was noted, when compared to the controls.

In the male high and mid dose groups, 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 glomerular), basophilic tubules and hyaline casts with a variable inflammatory cell infiltrate. In addition in the male high dose group, a higher incidence of bilateral basophilic tubules was noted.

No treatment-related changes were noted in the kidney at the mid dose in females or low dose in either sex.

Table 5.5-24 Incidence and severity of microscopic changes in the kidney, all animals of the chronic phase

Sex	Males				Females			
AE C656948 Dose level (ppm)	0	30	150	750	0	30	150	1500
Number of examined animals	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Chronic progressive nephropathy: focal/multifocal								
Minimal	0	0	3	1	0	0	0	0
Slight	0	0	0	1	0	0	0	0
Total	**0**	**0**	**3**	**2**	**0**	**0**	**0**	**0**
Intratubular golden/brown pigments: focal/multifocal								
Minimal	0	3	1	0	1	2	1	4
Slight	0	0	0	0	0	0	0	1
Total	**0**	**3**	**1**	**0**	**1**	**2**	**1**	**5**
Hyaline droplets: proximal tubules								
Minimal	0	1	3	1	0	0	0	0
Slight	0	0	1	5	0	0	0	0
Moderate	0	0	0	4	0	0	0	0
Total	**0**	**1**	**8**	**10**	**0**	**0**	**0**	**0**
Basophilic tubules: bilateral: focal/multifocal								
Minimal	3	4	0	3	1	0	0	2
Slight	1	0	0	3	1	0	0	0
Total	**3**	**4**	**0**	**6**	**2**	**0**	**0**	**2**
Hyaline cast(s): focal/multifocal								
Minimal	2	1	0	1	1	0	1	4
Total	**2**	**1**	**0**	**1**	**1**	**0**	**1**	**4**

In the thyroid gland of high dose females and males, follicular cell hypertrophy was noted together with a higher incidence and severity of colloid alteration, when compared to the controls.

In the male mid dose group, only follicular cell hypertrophy was observed.

No treatment-related changes were noted in the thyroid gland 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 phase

Sex	Males				Females			
AE C656948 Dose level (ppm)	0	30	150	750	0	30	150	1500
Number of examined animals	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Follicular cell hypertrophy: diffuse								
Minimal	0	0	2	2	0	0	0	4
Slight	0	0	0	2	0	0	0	1
Total	**0**	**0**	**2**	**4**	**0**	**0**	**0**	**5**
Colloid alteration								
Minimal	4	4	3	2	1	0	0	3
Slight	0	1	1	2	0	0	0	1
Moderate	0	0	0	3	0	0	0	0
Total	**4**	**5**	**4**	**7**	**1**	**0**	**0**	**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 animals found 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 liver, kidney, thyroid gland and eye.

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 exacerbation of microscopic findings observed after the chronic phase.

Proliferative changes like altered hepatocytes (eosinophilic foci), clear cell foci, multinucleated hepatocytes with anisocaryosis or increased number of mitoses were associated with metabolic morphological changes: centrilobular to panlobular hypertrophy, focal/multifocal hepatocellular vacuolation, centrilobular to midzonal hepatocellular macrovacuolation, brown pigments in Kupffer cells or hepatocellular brown pigments. 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 extramedullary hematopoiesis was observed in the liver in this group.

In the male high and mid dose groups, treatment-related effects (seen also in the female 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 incidence of altered hepatocytes (eosinophilic foci) was observed. In addition, centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation 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 change in the liver, centrilobular to midzonal hepatocellular macrovacuolation was considered not to be adverse in males.

No treatment-related changes were noted in the liver at the mid dose in females or at the low dose in either sex.

Table 5.5-26 Incidence and severity of microscopic changes in the liver, all animals of the carcinogenicity phase

Sex	Males				Females			
Dose level (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of examined animals	**60**	**60**	**60**	**58**	**60**	**60**	**60**	**59**
Centrilobular to panlobular hepatocellular hypertrophy: diffuse								
Minimal	1	1	14	15	0	0	0	6
Slight	0	0	0	15	0	0	0	21
Moderate	0	0	0	0	0	0	0	21
Total	**1**	**1**	**14****	**30****	**0**	**0**	**0**	**48****
Focus(i) of hepatocellular alteration: clear: focal/multifocal								
Minimal	8	6	6	11	0	3	3	8
Slight	2	2	1	5	1	1	1	3
Total	**10**	**8**	**7**	**16**	**1**	**4**	**4**	**11****
Focus(i) of hepatocellular alteration: eosinophilic: focal/multifocal								
Minimal	12	20	18	15	23	17	14	25
Slight	4	3	10	12	6	7	15	13
Moderate	0	1	2	1	0	2	1	7
Marked	0	0	1	0	0	0	0	3
Total	**16**	**24**	**31***	**28****	**29**	**26**	**30**	**48****
Hepatocellular vacuolation: focal/multifocal								
Minimal	10	4	13	5	5	10	7	13
Slight	0	2	3	0	1	4	2	9
Moderate	0	1	0	0	0	0	0	0
Total	**10**	**7**	**16**	**7**	**6**	**14****	**9**	**22****
Increased number of mitoses								
Present	0	0	0	0	6	1	5	33
Total	**0**	**0**	**0**	**0**	**6**	**1**	**5**	**33****
Multinucleated hepatocytes with anisocaryosis								
Present	1	0	1	0	4	2	6	38
Total	**1**	**0**	**1**	**0**	**4**	**2**	**6**	**38****
Hepatocellular single cell necrosis: focal/multifocal								
Minimal	2	0	1	1	0	3	1	25
Slight	0	0	1	0	0	1	0	12
Total	**2**	**0**	**2**	**1**	**0**	**4**	**1**	**37****
Hepatocellular brown pigment(s): focal/multifocal								
Minimal	0	0	0	0	1	1	2	22
Slight	0	0	0	0	0	0	0	2
Total	**0**	**0**	**0**	**0**	**1**	**1**	**2**	**24****
Accumulation of brown pigments in Kupffer cells: focal/multifocal								
Minimal	6	2	9	5	8	7	9	27
Slight	1	0	1	2	4	3	2	4
Moderate	0	0	0	0	0	0	0	1
Total	**7**	**2**	**10**	**7**	**12**	**10**	**11**	**32****
Hepatocellular macrovacuolation: centrilobular to midzonal: diffuse								
Minimal	0	0	0	9	0	0	0	5
Slight	0	0	2	1	0	0	0	4
Moderate	0	0	0	0	0	0	0	2
Total	**0**	**0**	**2**	**10****	**0**	**0**	**0**	**11****
Extramedullary hematopoiesis: multifocal								
Minimal	16	7	10	15	17	24	21	30
Slight	1	3	1	1	2	1	3	3
Moderate	1	0	0	0	0	0	0	0
Total	**18**	**10**	**11**	**16**	**19**	**25**	**24**	**33****

**: $p \leq 0.01$

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 high dose group, a higher incidence of tubular hypertrophy, collecting ducts hyperplasia and hyaline droplets was also noted.

In the male mid dose group, a higher incidence of tubular hypertrophy was also noted.

No treatment-related changes were noted in the kidney at the mid dose in females or at the low dose in either sex.

Table 5.5-27 Incidence and severity of microscopic changes in the kidney, all animals of the carcinogenicity phase

Sex	Male				Female			
Dose level (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of examined animals	**60**	**60**	**60**	**60**	**60**	**60**	**59**	**60**
Chronic progressive nephropathy: focal/multifocal								
Minimal	17	24	16	11	17	12	14	19
Slight	7	8	10	18	3	7	11	13
Moderate	5	2	11	15	0	3	1	7
Marked	1	1	4	8	0	2	0	3
Severe	1	2	1	0	0	0	0	0
Total	**31**	**37**	**42**	**52****	**20**	**24**	**23**	**42****
Tubular hyperplasia: focal/multifocal								
Minimal	5	1	5	8	2	5	3	4
Slight	0	1	3	2	0	0	1	1
Moderate	0	0	0	1	0	0	0	0
Total	**5**	**2**	**8**	**11***	**2**	**5**	**4**	**5**
Collecting ducts hyperplasia: unilateral: focal/multifocal								
Minimal	2	1	3	8	1	1	0	4
Slight	3	1	2	1	1	1	0	2
Moderate	0	0	0	0	0	0	0	2
Total	**5**	**2**	**5**	**9**	**2**	**2**	**0**	**8**
Tubular hypertrophy: focal/multifocal								
Minimal	5	8	18	17	7	13	11	8
Slight	4	3	6	2	2	3	2	4
Total	**9**	**11**	**24****	**19****	**9**	**16**	**13**	**12**
Intratubular golden/brown pigments: focal/multifocal								
Minimal	4	3	4	7	22	16	17	17
Slight	0	0	0	1	10	3	7	32
Moderate	0	0	0	0	0	0	0	8
Total	**4**	**3**	**4**	**8**	**32**	**19**	**24**	**57****
Hyaline droplets: proximal tubules								
Minimal	0	1	1	5	1	1	0	1
Slight	2	0	0	4	0	1	1	1
Moderate	1	0	0	1	1	0	0	0
Marked	0	1	0	0	0	0	0	0
Total	**3**	**2**	**1**	**10**	**2**	**2**	**1**	**2**
Cortical tubular dilatation: focal/multifocal								
Minimal	6	2	9	17	4	0	6	7
Slight	3	3	5	11	0	4	1	6
Moderate	1	0	1	0	0	1	0	3
Total	**10**	**5**	**15**	**28****	**4**	**5**	**7**	**16****
Medullary tubular dilatation: focal/multifocal								
Minimal	5	12	8	21	11	4	10	9
Slight	4	2	13	17	2	3	2	11
Moderate	1	1	1	2	0	1	0	2
Total	**10**	**15**	**22***	**40****	**13**	**8**	**12**	**22***
Renal cyst(s): focal/multifocal								
Present	7	3	6	13	2	2	3	3
Total	**7**	**3**	**6**	**13***	**2**	**2**	**3**	**3**

*: $p \leq 0.05$, **: $p \leq 0.01$

In the thyroid 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.

Table 5.5-28 Incidence and severity of microscopic changes in the thyroid gland, all animals of the carcinogenicity phase

Sex	Male				Female			
Dose level (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of examined animals	**58**	**59**	**57**	**54**	**60**	**60**	**60**	**58**
Follicular cell hyperplasia: focal/multifocal								
Minimal	0	0	0	2	1	1	0	2
Slight	0	0	0	1	1	1	1	2
Moderate	1	0	0	0	0	0	0	0
Marked	0	0	1	0	0	0	0	0
Total	**1**	**0**	**1**	**3**	**2**	**2**	**1**	**4**
Follicular cell hypertrophy: diffuse								
Minimal	1	0	3	5	0	0	1	3
Slight	0	0	1	2	0	0	0	4
Moderate	0	0	0	0	0	0	0	1
Total	**1**	**0**	**4**	**7****	**0**	**0**	**1**	**12****
Colloid alteration								
Minimal	18	5	12	10	5	6	10	17
Slight	8	9	16	20	2	1	5	15
Moderate	1	1	4	9	0	0	1	3
Marked	0	0	0	0	0	0	0	1
Total	**28**	**15**	**32**	**39****	**7**	**7**	**16***	**36****

*: p≤0.05, **: p≤0.01

In the eye of high dose females, bilateral retinal atrophy was observed, together with a higher incidence of lens degeneration and peripheral bilateral retinal atrophy, when compared to the controls. Retinal atrophy was characterized by degeneration of the outer plexiform layer, outer nuclear layer and rod/cones lamina.

No treatment-related changes were noted in the eye at all dose levels tested in males and at the mid and low doses in females.

Table 5.5-29 Incidence and severity of microscopic changes in the eye, all animals of the carcinogenicity phase

Sex	Male				Female			
Dose level (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of examined animals	**60**	**60**	**60**	**59**	**60**	**60**	**60**	**60**
Retinal atrophy: bilateral: diffuse								
Minimal	0	0	0	0	0	0	0	2
Slight	0	0	0	0	0	0	1	4
Moderate	0	0	1	0	0	0	0	19
Marked	0	0	0	0	0	0	0	2
Total	**0**	**0**	**1**	**0**	**0**	**0**	**1**	**27****
Lenticular degeneration : focal								
Minimal	2	0	0	0	2	0	1	2
Slight	0	0	0	2	0	2	1	4
Moderate	0	0	1	0	0	1	2	3
Marked	0	1	0	0	1	0	0	0
Total	**2**	**1**	**1**	**2**	**3**	**3**	**4**	**9***
Peripheral retinal atrophy: bilateral								
Minimal	1	0	0	1	3	0	1	3
Slight	0	0	0	0	0		0	2
Moderate	0	0	0	0	0	0	0	2
Total	**1**	**0**	**0**	**1**	**3**	**1**	**1**	**7**

*: p≤0.05, **: p≤0.01

Some indirect effects were noted in the testis and stomach:

In the testis, a higher incidence of arteritis/periarteritis was noted in the male high and mid dose groups, when compared to the controls (15/59 and 18/60, respectively, versus 8/60 in controls, p≤0.05). This vascular change was isolated (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 dose males, a higher incidence of regenerative non glandular hyperplasia: focal/multifocal (10/58 versus 6/58 in controls), non glandular erosion: focal/multifocal (7/58 versus 3/58 in controls), submucosal edema (10/58 versus 4/58 in controls, (p≤0.05) was noted. These minor changes were mainly observed in animals found prematurely 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.

Table 5.5-30 Incidence of microscopic neoplastic changes in the liver, all animals of the carcinogenicity phase

Sex	Male				Female			
Dose level (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of examined animals	**60**	**60**	**60**	**58**	**60**	**60**	**60**	**59**
M-Hepatocllular carcinoma								
Incidental	0	0	0	0	0	0	2	3
Total	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**3**
B-Hepatocellular adenoma								
Incidental	2	1	2	1	2	2	0	9
Total	**2**	**1**	**2**	**1**	**2**	**2**	**0**	**9***
Hepatocellular adenoma + carcinoma								
Total	**2**	**1**	**2**	**1**	**2**	**2**	**2**	**11+****

+ One animal had both adenoma and carcinoma
*: p≤0.05, **: p≤0.01

I. Deficiencies

None

III. Conclusions

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 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).

Assessment and conclusion by applicant:

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

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 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).

Data Point:	KCA 5.5/02
Report Author:	[redacted]
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:	M-295688-01-1
Guideline(s) followed in study:	OECD 451 (1981); EEC Directive 88/302/EEC – Annex V - Method B.32 (1987); EPA Health Effects Test Guideline (OPPTS 870.4200; 1998); M.A.F.F. in Japan notification 12 Nousan-8147 (2000) guidelines.
Deviations from current test guideline:	current guideline: 0ECD 451, 2018 Deviation: No significant deviations.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

Groups of 60 male and 60 female C57BL/6J mice were fed diet containing 0, 30, 150 or 750 ppm of AE C656948 (Mix-Batch 08528/0002) for 52 weeks. After 52 weeks, 10 males and 10 females from each group allocated to the chronic phase of the study were necropsied at the scheduled 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, 4.2, 20.9 and 105 mg/kg/day in males and 0, 5.3, 26.8 and 129 mg/kg/day in females, at 0, 30, 150 and 750 ppm, respectively. Mortality and clinical signs were checked daily. Additionally, detailed physical examinations including palpation for masses were performed weekly throughout treatment. 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 12 and 18 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.

There was no treatment-related effect on mortality, 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 points during this period ($p \leq 0.05$ or $p \leq 0.01$). 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 Month 13 (+25%, $p \leq 0.01$) and Month 19 (+22%, $p \leq 0.01$), 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 carcinogenicity 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% ($p \leq 0.01$) in males and 35 to 38% ($p \leq 0.01$) in females. In addition, mean kidney to body weight ratio in females was 5% ($p \leq 0.01$) lower 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 change consisted of a higher incidence of follicular cell adenoma in the thyroid gland in males (7/50), compared with the control group (1/50), this effect was statistically significant ($p \leq 0.05$). Non-neoplastic changes 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 hepatocellular cholestasis, hepatocellular single cell degeneration/necrosis, interstitial mixed cell infiltrate, eosinophilic inclusion bodies and multinucleated hepatocytes were noted. In females, a higher incidence of eosinophilic foci of altered hepatocytes was observed. In the kidney, a higher incidence and/or severity of bilateral cortical basophilic tubules, hyaline cast(s) and interstitial mononuclear cell infiltrate, together with a higher incidence of glomerular congestion/hemorrhage(s), associated with higher severity of amyloid deposition (mainly observed within glomerular interstitium) 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% ($p \leq 0.05$ or 0.01) in males and 17 to 20% ($p \leq 0.05$ or 0.01) in females. At the macroscopic examination, enlarged liver was observed in 2/10 males. At the microscopic examination of the thyroid gland, follicular cell hyperplasia was seen in 2/9 males.

At the 18-month terminal sacrifice, mean absolute and relative liver weights were increased by between 14 to 15% ($p \leq 0.01$) in males and 13 to 17% ($p \leq 0.01$) 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 sexes 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 liver weight of between 6 to 8% ($p \leq 0.05$ 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 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 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)

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Beige powder
Lot / Batch #:	Mix-batch 08528/0002
Purity:	94.5% (January, 2005); 94.7% (May, 2005)
CAS #	658066-35-4
Stability of test compound:	Stable in rodent diet at 20 and 10000 ppm over a 105-day period at ambient temperature or over a 95-day freezing period followed by 10 days at ambient temperature
2. Vehicle and / or positive control:	none
3. Test animals:	
Species:	Mouse
Strain:	C57BL/6J
Age:	Approximately 6 weeks of age
Weight at dosing:	20.7 – 21.1g for males; 17.6 - 17.9 g for females
Source:	[redacted]
Acclimation period:	13 days
Diet:	A04C P1-10 from SAFE, Augy, France, *ad libitum*
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages. During the first week of acclimatization, the animals were housed by sex in groups of 3.
Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	55 ± 15%
Air changes:	Approximately 10-15/hour
Photoperiod:	12 hours light, 12 hours dark

B. Study design

1. In life dates: 20 April 2005 to 15 November 2006

2. Dose level selection

The dose levels 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 sexes. At 150 ppm, only minimal to slight hypertrophy of centrilobular hepatocytes was observed in all males and some females, therefore a dose level of 150 ppm 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 males and in 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 ± 20% of the mean body weight on the day of randomization. Any animal deemed 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.

Table 5.5-31 Study design and group sizes

Test Group	Diet Concentration (ppm)	Achieved dose (mg/kg/day)	Main Study 78 weeks		Interim Sac. 52 weeks	
			Male	Female	Male	Female
Control (1)	0	0	50	50	10	10
Animal identity			PT1M2399 to 2448	PT1F2459 to 2508	PT1M2389 to 2398	PT1F2449 to 2458
Low (2)	30	M: 4.2 F: 5.3	50	50	10	10
Animal identity			PT2M2519to2568	PT2F2579 to 2628	PT2M2509to2518	PT2F2569 to 2578
Mid (3)	150	M: 20.9 F: 26.8	50	50	10	10
Animal identity			PT3M2639 to 2688	PT3F2699 to 2748	PT3M2629 to 2638	PT3F2689 to 2698
High (4)	750	M: 105 F: 129	50	50	10	10
Animal identity			PT4M2759 to 2808	PT4F2819to2868	PT4M2749 to 2758	PT4F2809to2818

4. Diet preparation and analysis

AE C656948 was incorporated into the diet to provide the required dietary concentrations. The test substance formulations were prepared approximately every 8 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 C656948 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 temperature.

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 at each dietary level was verified prior to administration to the animals for the following formulations: F1, F3, F6, F9 and F10 (at 30 and 750 ppm only).

5. Statistics

Means and standard deviations 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 ($p \leq 0.05$) (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 (2-sided) if the Kruskal-Wallis test indicated significance.

- If the Bartlett test was significant ($p \leq 0.05$) (for body weight and average 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 ($p \leq 0.05$) 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 ($p \leq 0.05$) (for hematology 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 ($p \leq 0.05$) even after square root transformation, group means were compared using the non-parametric Kruskal-Wallis which was followed by the Dunn test (2-sided), if Kruskal-Wallis test indicated significance.

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 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 accidents (accidental trauma, 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 dose-related trend in survival were assessed using Cox's and Tarone's tests on life table data. Probability values presented were two-sided for pairwise comparisons and trend test. Group mortality rates were compared at the 5% and 1% levels of significance. Survival analyses were performed on the carcinogenicity 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 incidences of the 30 and 150 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 analyses:

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, each tumour was categorized as fatal if the tumour was a factor contributing towards the death of 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 diagnosed 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 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 thereafter and prior to necropsy. Body weight recorded prior to necropsy are referred to as terminal body weight.

3. Food consumption and compound intake

Food consumption was recorded weekly during the first 13 weeks of treatment, and once approximately every 4 weeks thereafter. The weekly mean achieved dosage intake in mg/kg body weight/day for Weeks 1 to 13, then 1 week per month thereafter was calculated as follows:

$$\text{Test substance intake (mg/kg/day)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day)}}{\text{Group mean body weight (g) at the end of the food consumption period}}$$

The monthly and overall mean achieved dosage intake for the 18 months of treatment were derived from the weekly data.

4. Hematology

Hematology was performed on all the surviving 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 53 or 54 and prior to necropsy on Week 79 or 80. The following parameters were measured: hematocrit, hemoglobin, leukocyte count, erythrocyte count, platelet count, leukocyte differential count, mean corpuscular 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.

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 & OECD 451.

7. Sacrifice and pathology

On study Days 366 to 368 for the 12-month interim 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 exsanguination under deep anesthesia (Isoflurane). Animals were fasted overnight prior to sacrifice.

All animals, including animals at scheduled sacrifice, found dead or sacrificed 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 sampled and/or weighed at necropsy:

	Digestive system		**Cardiovasc. / Hemat.**		**Neurologic**
X	Tongue	X	Aorta, thoracic*	XX	Brain (3 sections)*+
X	Submandibular (salivary) gland	XX	Heart*+	X	Sciatic nerve*
X	Esophagus*	X	Bone marrow*, sternum	X	Spinal cord (cervical, thoracic, lumbar)*
X	Stomach*	X	Lymph node*, mesenteric	X	Eyes (retina)*
X	Duodenum*	X	Lymph node, submaxillary	X	Optic nerves*
X	Jejunum*	XX	Spleen*+		
X	Ileum*	X	Thymus		**Glandular**
X	Cecum*			X	Pituitary gland*
X	Colon*		**Urogenital**	XX	Adrenal gland*+
X	Rectum*	XX	Kidney*+	X	Parathyroid gland*
XX	Liver*+	X	Urinary bladder*	X	Thyroid gland*
X	Gall bladder*	XX	Testis*+	X	Harderian gland
X	Pancreas*	XX	Epididymis*+	^	Lacrymal exorbital gland
		X	Prostate gland*		
	Respiratory	X	Seminal vesicle*		
X	Trachea*	XX	Ovary*+		**Other**
X	Lung*+	XX	Uterus (with cervix)*+	X	Bone (sternum)
^	Pharynx*	X	Mammary gland*	X	Skeletal muscle
^	Larynx*	X	Vagina	X	Skin*
^	Nasal cavities*			X	All gross lesions and masses*
				X	Articular surface (femorotibial joint)

* required for carcinogenicity studies based on US EPA guideline 870.4200
\+ organ weights required for rodent studies
X tissues were collected for histological examinations
XX 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 eye and optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative.

II. Results and discussion

A. Mortality

There were no treatment-related mortalities at any dose level in either sex during the course of the study.

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 censored in the statistical analysis of the survival rate.

During the whole study period (at least 78 weeks), the mortality rate in animals 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 - Unscheduled deaths

Dose group (ppm)	First 53 weeks (up to study Day 370) All animals (n=60)		Whole study period Carcinogenicity phase animals (n=50)	
	Male	Female	Male	Female
0	2 (3.3%)	3 (5.0%)	6 (12.0%)	11 (22.0%)
30	3 (5.0%)	7 (11.7%)	9 (18.0%)	13 (26.0%)
150	2 (3.3%)	7 (11.7%)	9 (18.0%)	10 (20.0%)
750	2 (3.3%)	5 (8.3%)	8 (16.0%)	12 (24.0%)

Percentage mortality in parentheses
No statistical analysis was done on these data

B. Clinical observations

There were no treatment-related clinical signs observed at any dose level in either sex throughout the course of the study.

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 ($p \leq 0.05$ 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)

Dose group (ppm)	0	30	150	750
Males				
Initial BW (Day 1) (%C)	**20.8 ± 1.1**	**21.1 ± 1.0 (101)**	**20.8 ± 0.9 (100)**	**20.7 ± 1.1 (100)**
BW Week 2 (Day 8) (%C)	21.9 ±1.1	21.9 ± 0.9 (100)	22.0 ± 1.1 (100)	21.7 ± 1.1 (99)
BW Week 14 (Day 92) (%C)	27.4 ± 1.5	27.9 ± 1.3 (102)	27.9 *± 1.1 (102)	27.5 ± 1.1 (100)
BW Week 26 (Day 176) (%C)	28.8 ±1.8	29.4 ±1.6 (102)	29.1 ± 1.5 (101)	28.4 ± 1.3 (99)
BW Week 54 (Day 372) (%C)	31.3 ±1.9	31.5± 1.4 (101)	30.8 ± 1.7 (98)	29.8 ** ± 1.3 (95)
Final BW Week 78 (Day 540) (%C)	31.8 ± 1.7	32.2 ±1.6 (101)	31.6 ± 1.8 (99)	31.1 ± 1.8 (98)
BWG Weeks 1-2 (Days 1 to 8) (%C)	**1.0 ± 0.6**	**0.8 ± 0.5 (80)**	**1.2 ± 0.7 (120)**	**1.0 ± 0.5 (100)**
BWG Weeks 1-14 (Days 1 to 92) (%C)	6.5 ± 0.9	6.8 ± 0.8 (105)	7.1 **± 0.9 (109)	6.8 ± 0.9 (105)
BWG Weeks 14-26 (Days 92 to 176) (%C)	1.5 ± 1.1	1.5 ± 0.7 (100)	1.1 ± 1.0 (73)	0.8 **± 0.9 (53)
BWG Weeks 26-54 (Days 176 to 372) (%C)	2.5 ± 0.8	2.1 ± 0.8 (84)	1.8**± 0.8 (72)	1.3** ± 0.9 (52)
BWG Weeks 54-78 (Days 372 to 540) (%C)	0.3 ± 0.8	0.7 ± 1.1 (233)	0.8**± 0.9 (267)	1.3 ** 0.9 (433)
Overall BWG (Days 1 to 540) (%C)	**10.9 ± 1.4**	**11.1 ± 1.4 (102)**	**10.8 ± 1.6 (99)**	**10.4 ± 1.5 (95)**
Females				
Initial BW (Day 1) (%C)	**17.9 ± 0.9**	**17.7 ± 0.8 (99)**	**17.7 ± 0.9 (99)**	**17.6 ± 0.9 (98)**
BW Week 2 (Day 8) (%C)	18.3 ± 0.8	18.5 ± 0.8 (101)	18.1 ± 0.9 (99)	18.1 ± 0.9 (99)
BW Week 14 (Day 92) (%C)	22.5 ± 1.0	22.9 ± 1.1 (102)	22.7 ± 0.9 (101)	22.7 ± 0.9 (101)
BW Week 26 (Day 176) (%C)	24.2 ±1.3	24.5 ± 1.7 (101)	24.3 ± 1.1 (100)	23.7 ± 0.9 (98)
BW Week 54 (Day 372) (%C)	26.7 ± 2.0	26.8 ± 2.3 (100)	26.7 ± 1.9 (100)	25.8 ± 1.3 (97)
Final BW Week 78 (Day 540) (%C)	27.1 ± 1.8	27.8 ± 2.1 (103)	27.6 ± 1.7 (102)	27.0 ± 1.4 (100)
BWG Weeks 1-2 (Days 1 to 8) (%C)	**0.4 ± 0.4**	**0.8 **± 0.6 (200)**	**0.4 ± 0.7 (100)**	**0.5 ± 0.7 (125)**
BWG Weeks 1-14 (Days 1 to 92) (%C)	4.6 ± 0.7	5.2 **± 1.0 (113)	5.0 * ± 1.0 (109)	5.1 ** ± 0.9 (111)
BWG Weeks 14-26 (Days 92 to 176) (%C)	1.7 ± 0.9	1.7 ± 1.1 (100)	1.6 ± 0.6 (94)	1.0 **± 0.6 (59)
BWG Weeks 26-54 (Days 176 to 372) (%C)	2.7 ± 1.3	2.3 ± 1.1 (85)	2.5 ± 1.2 (93)	2.1 ± 0.9 (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 (150)	1.1 ± 1.0 (183)	1.0 ± 1.1 (167)
Overall BWG (Days 1 to 540) (%C)	**9.4 ± 1.6**	**10.0 ± 1.9 (106)**	**9.8 ± 1.6 (104)**	**9.4 ± 1.4 (100)**

%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 either sex, throughout the course of the study.

E. Achieved intake

The mean achieved dietary intakes of AE C656948 expressed in mg/kg body weight/day received by the animals during the study were as follows:

Table 5.5-34 Mean achieved intake (mg/kg/day)

Sex	Males			Females		
Dose group (ppm)	**30**	**150**	**750**	**30**	**150**	**750**
Week periods 1-13	5.1	25.5	128	6.4	32.0	156
Week periods 1-52	4.4	22.2	112	5.7	28.6	138
Week periods 1-80	4.2	20.9	105	5.3	26.8	129

F. Hematology

1. Hematology

Slightly higher mean platelet counts were noted at 750 ppm in males, at Month 13 (+25%, p≤0.01) and Month 19 (+22%, p≤0.01).

G. Sacrifice and pathology

1. Terminal body weight and organ weight

12-month sacrifice

There was no relevant change in terminal body weight of treated animals when compared to control animals.

At 750 ppm and 150 ppm, mean absolute and relative liver weights were statistically significantly higher when compared to controls, in both sexes. Liver weight changes were found to be dose-related. No effect was seen on liver weight at 30 ppm.

Table 5.5-35 Liver weight changes (± SD) at scheduled sacrifice, chronic phase (% change when compared to controls)

Sex	Males				Females			
Dose group (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Mean absolute liver weight (g)	1.26 ± 0.10	1.24 ± 0.09 (-2%)	1.40* ± 0.15 (+11%)	1.47** ± 0.13 (+17%)	1.09 ± 0.13	1.07 ± 0.18 (-2%)	1.31** ± 0.12 (+20%)	1.40** ± 0.14 (+28%)
Mean liver to body weight ratio (%)	4.53 ± 0.366	4.52 ± 0.232 (0%)	5.20** ± 0.503 (+15%)	5.68** ±0.235 (+25%)	4.7 ± 0.404	4.61 ± 0.688 (-2%)	5.51** ± 0.365 (+17%)	6.12** ± 0.325 (+30%)
Mean liver to brain weight ratio (%)	271.96 ± 17.276	269.65 ± 18.272 (-1%)	8.59** ± 30.464 (+13%)	324.60** ± 23.541 (+19%)	235.58 ± 33.240	226.49 ± 39.824 (-4%)	276.48* ± 26.136 (+17%)	307.67** ± 22.751 (+31%)

*: 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	Males				Females			
Dose group (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Mean absolute kidney weight (g)	0.553 ± 0.064	0.538 ± 0.061 (-3%)	0.554 ± 0.063 (0%)	0.460* ± 0.077 (-17%)	0.398 ± 0.056	0.385 ± 0.028 (-3%)	0.388 ± 0.037 (-3%)	0.342** ± 0.032 (-14%)
Mean kidney to body weight ratio (%)	1.99 ± 0.173	1.96 ± 0.195 (-2%)	2.06 ± 0.168 (+3%)	1.78* ± 0.191 (-11%)	1.72 ± 0.243	1.67 ± 0.134 (-3%)	1.63 ± 0.131 (-5%)	1.49** ± 0.083 (-13%)
Mean kidney to brain weight ratio (%)	119.69 ± 11.654	116.80 ± 12.203 (-2%)	121.96 ± 10.907 (+2%)	101.38** ± 14.739 (-15%)	85.91 ± 12.823	81.45 ± 5.380 (-5%)	81.68 ± 7.124 (-5%)	75.03** ± 5.046 (-13%)

*: p≤0.05; **: p≤0.01

18-month sacrifice

There was no relevant change in terminal body weights of treated males 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 750 ppm and 150 ppm.

Mean absolute and relative liver weights were slightly higher when compared to controls in males at 30 ppm but no concomitant microscopic hepatocellular hypertrophy was noted.

Therefore liver weight changes were considered to be adverse at 750 and 150 ppm only.

Table 5.5-37 Liver weight changes (± SD) at terminal sacrifice, carcinogenicity phase (% change when compared to controls)

Sex	Males				Females			
Dose group (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Mean absolute liver weight (g)	1.17 ± 0.12	1.26* ± 0.11 (+8%)	1.34** ± 0.13 (+15%)	1.49** ± 0.16 (+27%)	1.26 ± 0.17	1.31 ± 0.16 (+4%)	1.45** ± 0.21 (+15%)	1.70** ± 0.23 (+35%)
Mean liver to body weight ratio (%)	4.23 ± 0.358	4.50** ± 0.352 (+6%)	4.83** ± 0.358 (+14%)	5.53** ± 0.363 (+31%)	5.24 ± 0.473	5.36 ± 0.387 (+2%)	5.92** ± 0.602 (+13%)	7.14** ± 0.957 (+36%)
Mean liver to brain weight ratio (%)	257.14 ± 26.290	272.71* ± 25.272 (+6%)	294.43** ± 28.213 (+15%)	328.69** ± 33.960 (+28%)	265.02 ± 35.514	272.46 ± 31.668 (+3%)	310.63** ± 47.983 (+17%)	366.05** ± 48.110 (+38%)

*: p≤0.05;**: p≤0.01

Mean absolute and relative kidney weights were statistically significantly lower when compared to controls in males 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.

Table 5.5-38 Kidney weight changes at terminal sacrifice, carcinogenicity phase (% change when compared to controls)

Sex	Males				Females			
Dose group (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Mean absolute kidney weight (g)	0.542 ± 0.0501	0.557 ± 0.063 (+3%)	0.556 ± 0.058 (+3%)	0.491** ± 0.055 (-9%)	0.432 ± 0.053	0.447 ± 0.050 (+3%)	0.435 ± 0.038 (+1%)	0.408 ± 0.043 (-6%)
Mean kidney to body weight ratio (%)	1.95 ± 0.111	1.99 ± 0.181 (+2%)	2.01 ± 0.155 (+3%)	1.82** ± 0.156 (-7%)	1.80 ± 0.131	1.83 ± 0.144 (+2%)	1.78 ± 0.107 (-1%)	1.71** ± 0.146 (-5%)
Mean kidney to brain weight ratio (%)	118.59 ± 10.248	120.57 ± 11.792 (+2%)	122.10 ± 11.328 (+3%)	108.17** ± 11.754 (-9%)	90.05 ± 10.841	92.93 ± 9.699 (+2%)	92.96 ± 8.219 (+2%)	87.80 ± 9.171 (-3%)

**: p≤0.01

Mean absolute and relative heart weights were statistically significantly higher when compared to controls in females at 750 ppm (+ 17 to 19%, p≤0.01). Mean heart to body weight ratio was statistically significantly higher when compared to controls in males at 750 ppm (+8%, p≤0.05). These changes were considered not to be adverse since there were no relevant associated microscopic findings.

Mean absolute and relative adrenal gland weights were statistically significantly higher when compared to controls in females at 750 ppm (+ 23 to 26%, p≤0.01). 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 since 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 trauma on Day 301. No relevant macroscopic changes were observed except for a blood clot on the submaxillary glands.

At 750 ppm, enlarged liver was found in 1/10 males and 2/10 females.

At 150 ppm, enlarged liver was found in 2/10 males.

18-month sacrifice

Seventy-eight animals died prematurely before the end of the study. No treatment-related findings were noted.

At 750 ppm, enlarged liver and dark liver was found in some males and females.

At 150 ppm, enlarged liver was found in some females.

These findings were correlated with relevant histopathological findings.

At 750 ppm, prominent lobulation in liver was found in some males and females but since this gross observation was not correlated with relevant 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	Males				Females			
Dose group (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Obviously large	0/44	0/41	0/41	3/42	1/39	2/37	7/40	30/38
Dark	1/44	0/41	0/41	14/42	1/39	0/37	0/40	4/38
Prominent lobulation	1/44	0/41	1/41	5/42	0/39	0/37	0/40	8/38

3. Microscopic findings

12-month sacrifice

Due to an increased incidence of follicular cell adenomas observed in thyroid gland of the high dose 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 males at 750 and 150 ppm, respectively.

Table 5.5-40 Incidence and severity of microscopic changes in the thyroid gland, all animals, chronic phase

Sex	Males			
Dose group (ppm)	**0**	**30**	**150**	**750**
Follicular cell hyperplasia: focal/multifocal				
Number of animals examined	9	10	9	10
Minimal	0	0	2	1
Slight	0	0	0	1
Total	**0**	**0**	**2**	**2**

18-month sacrifice

No treatment-related cause of death was established for the few animals 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 eosinophilic foci of altered hepatocytes was observed in females at 750 ppm. Centrilobular to panlobular hypertrophy was observed in both sexes with a dose-related effect at 750 and 150 ppm. Hepatocellular 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 mixed cell infiltrate, eosinophilic inclusion bodies and multinucleated hepatocytes in males at 750 ppm only.

At 750 ppm, there was a markedly lower incidence 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 dose 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

Sex	Males				Females			
Dose group (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals examined	**49**	**49**	**49**	**50**	**48**	**50**	**50**	**50**
Eosinophilic focus(i) of altered hepatocytes: focal/multifocal								
Minimal	1	0	0	0	0	1	2	0
Slight	0	0	0	2	0	0	0	2
Moderate	0	0	0	0	0	0	0	2
Marked	0	0	0	0	0	0	0	1
Total	**1**	**0**	**0**	**2**	**0**	**1**	**2**	**5***
Centrilobular to panlobular hepatocellular hypertrophy: diffuse								
Minimal	0	0	16	3	0	0	18	24
Slight	0	0	22	13	0	0	0	2
Moderate	0	0	0	36	0	0	0	0
Total	**0**	**0**	**38****	**50****	**0**	**0**	**18****	**26****
Number of animals	49	49	49	50	48	50	50	50
Hepatocellular cholestasis: focal/multifocal								
Minimal	0	0	2	29	0	0	0	0
Slight	0	0	0	2	0	0	0	0
Total	**0**	**0**	**2**	**31****	**0**	**0**	**0**	**0**
Hepatocellular single cell degeneration/necrosis: focal/multifocal								
Minimal	1	2	7	28	1	1	0	1
Slight	0	0	0	12	0	0	0	0
Moderate	0	0	0	0	0	0	0	1
Total	**1**	**2**	**7***	**40****	**1**	**1**	**0**	**2**
Interstitial mixed cell infiltrate: focal/multifocal								
Minimal	18	15	19	39	8	8	10	8
Slight	0	1	0	1	2	3	2	0
Total	**18**	**16**	**19**	**40****	**10**	**11**	**12**	**8**
Eosinophilic inclusion bodies: focal/multifocal								
Minimal	2	3	5	18	0	0	0	0
Slight	0	0	0	1	0	0	0	0
Total	**2**	**3**	**5**	**19****	**0**	**0**	**0**	**0**
Multinucleated hepatocytes: focal/multifocal								
Minimal	3	1	3	25	1	0	0	0
Slight	0	0	1	2	0	0	0	0
Total	**3**	**1**	**4**	**27****	**1**	**0**	**0**	**0**
Hepatocellular vacuolation: mainly centrilobular: diffuse								
Minimal	21	15	12	0	3	4	1	0
Slight	6	19	11	2	2	5	0	1
Moderate	0	4	3	1	0	1	0	0
Total	**27**	**38***	**27**	**3****	**5**	**10**	**1**	**1**

*: p≤0.05; **: p≤0.01

In the kidney, higher incidences and/or severities of bilateral cortical basophilic tubules, hyaline casts(s) and interstitial 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 glomerular 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

Sex	Males				Females			
Dose group (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals examined	**50**	**50**	**50**	**50**	**48**	**50**	**50**	**50**
Cortical basophilic tubules: bilateral								
Minimal	27	33	30	20	17	11	18	17
Slight	9	2	2	1	2	2	3	14
Moderate	0	0	1	1	0	1	0	3
Total	36	**35**	**33**	**22****	**19**	**14**	**21**	**34****
Glomerular congestion/hemorrhage(s): focal/multifocal								
Minimal	0	0	0	0	2	4	3	24
Slight	0	0	0	1	0	0	0	3
Moderate	0	0	0	0	0	1	0	0
Total	**0**	**0**	**0**	**1**	**2**	**2**	**3**	**27****
Amyloid deposition: focal/multifocal								
Minimal	13	15	16	19	27	23	15	2
Slight	18	4	7	8	14	15	17	4
Moderate	1	1	0	2	2	2	8	27
Marked	0	0	0	0	0	0	0	8
Total	**32**	**20**	**23**	**29**	**43**	**40**	**40**	**41**
Hyaline cast(s): focal/multifocal								
Minimal	3	1	2	0	0	0	1	8
Slight	0	0	0	1	0	1	0	3
Moderate	0	0	1	0	0	1	0	0
Total	**3**	**1**	**3**	**1**	**0**	**2**	**1**	**11****
Interstitial mononuclear cell infiltrate: focal/multifocal								
Minimal	27	27	25	25	33	27	26	16
Slight	5	2	3	2	9	11	12	25
Moderate	0	0	1	0	0	0	1	1
Total	**32**	**29**	**29**	**27**	**42**	**38**	**39**	**42**

**: p≤0.01

In the thyroid gland, a higher incidence of follicular cell hyperplasia was noted in both sexes at 750 ppm and as well in males at 150 ppm.

Table 5.5-43 Incidence and severity of microscopic changes in the thyroid gland, all animals, carcinogenicity phase

Sex	Males				Females			
Dose group (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals examined	**50**	**50**	**50**	**50**	**48**	**50**	**50**	**50**
Follicular cell hyperplasia: focal/multifocal								
Minimal	0	2	10	18	11	4	10	10
Slight	3	2	5	6	5	2	5	12
Moderate	1	2	2	5	1	2	3	6
Marked	0	0	3	3	0	0	1	4
Severe	0	0	1	0	0	0	0	1
Total	**4**	**6**	**21****	**32****	**17**	**8***	**19**	**33****

**: p≤0.01

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 5.5-44 Incidence of neoplastic microscopic changes in the thyroid gland, all animals, carcinogenicity phase

Sex	Males				Females			
Dose group (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals examined	**50**	**50**	**50**	**50**	**48**	**50**	**50**	**50**
Follicular cell adenoma								
Incidental	1	1	3	7	3	1	3	1
Total	**1**	**1**	**3**	**7***	**3**	**1**	**3**	**1**

*: p≤0.05

H. Deficiencies

No deficiencies are identified in this study which complied with the requirement of tg oecd 451 in force when the study was carried out.

III. Conclusions

In conclusion, dietary administration of AE C656948 over an 18-month period to the C57BL/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 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).

Assessment and conclusion by applicant:

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

Dietary administration of AE C656948 over an 18-month period to the C57BL/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 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).

Mechanism of action and supporting data

Supporting data regarding female rat liver tumors

In the rat carcinogenicity study (M-298339-01-1), 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 neoplastic 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 mitoses) 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 (M-085510-01-1), 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., 1996[1]) 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 rat in 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. 2003[2]), liver cell proliferation would only occur in rodents and not in humans, this is 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 effects (IARC, 2001[3]). For these reasons, the mode of action for phenobarbital-like P-450 induction is considered not to be relevant to humans (Holsapple et al., 2006[4]).

In order to test this hypothesis, an intial explanatory study was conducted with AE C656948 in female 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/Pxr 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 total cytochrome P-450 content, specific cytochrome P-450 enzyme activities and UDPGT-species isoenzyme profiles and corresponding gene transcript expression, which acted as markers for activation of the Car/Pxr receptor, liver cell proliferation together with histopathological changes in the liver. Reversibility of changes seen following 28 days of treatment with AE C656948 was also assessed. In both studies, phenobarbital was included as a positive control. These explanatory studies are summarized 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 mouse 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 demonstrate 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 C656948 ([redacted] (2020), M-759019-01-1, KCA 5.5/22), was conducted to address the T-modality 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 ≥10 µM 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, CYP3A4 and UGT1A1 inducer in human hepatocytes, 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 rodents and humans (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. Toxicol. Pathol. 38: 487-501, 2010), KIIA 5.5.4 /36; Cohen, S. M.; 2010; M-367547-01-1) those highlighted in bold in the list below are likely to be relevant to humans:

I. DNA reactivity
II. Increased cell proliferation
  a. Receptor mediated
    i. Ppar (peroxisome proliferation)
    ii. Enzyme induction (Car, Pxr, AhR)
    iii. Estrogen
    iv. Statins
    v. Cytotoxicity
    vi. Other
  b. Non-receptor mediated
    i. Cytotoxicity
    ii. Infectious
    iii. Iron (copper) overload
    iv. Increased apoptosis (e.g., fumonisin B1)
    v. Other

DNA reactivity is the first broad category of a MoA for hepatocellular carcinogens. A battery of *in-vitro* genotoxicity studies, the bacterial reverse mutation test (Ames test), mammalian chromosome aberration test, and a mammalian 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 tumors in rats or mice.

For non-DNA-reactive rodent liver carcinogens, several MoAs have been identified that act by stimulating hepatocellular proliferation through either a receptor- or non-receptor-mediated mechanism. The MoA studies in rats and mice with AE C656948 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 (Pparα, AhR, ER, statins, and cytotoxicity) and non-receptor-mediated (cytotoxicity, infections, iron overload, and increased apoptosis) involvement in the key events leading to AE C656948-induced liver tumors.

Overall, the mechanistic studies together with the standard repeat dose studies, clearly demonstrate the MoA for the rat 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 C656948 as compared to rat hepatocytes, provide convincing evidence that the liver tumors in the rat are non-relevant to humans. Consequently, the mode of 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 tumors following exposure to fluopyram (AE C656948) are presented in the following Expert Summary documents:

Position Paper

Fluopyram: Mode of Action and Human Relevance Analysis of Rodent Liver and Thyroid Tumors, MIIA Sec 3 /03; [redacted] 2013; M-465168-01-2

Expert Summary Report

Fluopyram: Mode of Action and Human Relevance Framework Analysis for Fluopyram-Induced Rodent Liver and Thyroid Tumors, MIIA Sec 3 /02; [redacted];2013; M-454439-02-1

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 Ther, 71 (1/2), 153-191. KIIA 5.5.4 /06; Whysner, J.; Ross, P. M.; Williams, G. M.; 1996; M-300847-01-1

2 Moore J.T., Moore L.B., Maglish J.M. and Kliewer S.A. (2003) Functional and structural comparison of PXR and CAR, Biochem. Biophys. Acta, Feb 17, 1919 (3) 235-238. KIIA 5.5.4 /07; Moore, J. T.; Moore, L. B.; Maglich, J. M.; Kliewer, S. A.; 2003; M-300852-01-1

3 IARC (2001) (Anon.), Phenobarbital and its sodium salts in "Some thyrotropic agents - Summary of data reported and evaluation", IARC Monograph Vol 79, IARC Press Lyon-France, pp 161-288. KIIA 5.5.4 /08; Anon.; 2001; M-300830-01-1

4 Holsapple M.P., Pitot H.C., Cohen S.H., Boobis A.R., Klaunig J.E., Pastoor T., Dellarco V.L. and Dragan Y.P. (2006) Mode of action in relevance of rodent liver tumors to human cancer risk. Toxicol. Sci. 89(1), 51-56. KIIA 5.5.4 /09; Holsapple, M.; Pitot, H. C.; Cohen, S. H.; Boobis, A. R.; Klanig, J. E.; Pastoor, T.; Dellarco, V. L.; Dragan, Y. P.; 2005; M-300828-01-1

Data Point:	KCA 5.5.03
Report Author:	[redacted]
Report Year:	2008
Report Title:	Fluopyram (AE C 656948): 7-day mechanistic study in the female Wistar rat by dietary administration
Report No:	SA 07323
Document No:	M-299274-01-1
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
Acceptability/Reliability:	Yes

Executive Summary

AE C656948 (Batch number Mix-batch:08528/0002, 94.7% w/w purity), was administered continuously in the diet to groups 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 findings (enlarged and dark livers) in 13/15 females compared to no incidences in the controls.

At histological examination, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was found in all treated animals. A markedly decreased incidence of periportal vacuolation was also noted.

Assessment of cell proliferation in the liver revealed a four fold increase in mean BrdU labeling index in both the perilobular and centrilobular areas of the hepatic lobule in treated animals, when compared to the controls.

Assessment of total cytochrome P-450 content and microsomal 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 UDPGT activity (all statistically different from the control group ($p \leq 0.01$)). 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 proliferation in the liver.

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Light beige powder
Lot / Batch #:	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. Vehicle and / or positive control:	none
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN) – Female only
Age:	11 weeks approximately at start of treatment
Weight at dosing:	228 -254 g
Source:	[redacted]
Acclimation period:	12 days
Diet:	Certified rodent powdered and irradiated diet A04C-10P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), ad libitum
Water:	Tap water, ad libitum
Housing:	Animals were caged individually in suspended stainless steel 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 females 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, 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".

Control animals were fed control diet for 7 days. Test animals were fed diet containing fluopyram (AE C656948) at 3000 ppm for 7 days. A solution of BrdU at 80 mg of BrdU/100 mL of drinking water was administered to all animals during 7 days to allow liver 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 ppm 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.

Table 5.5-45 Study design

Test group	Concentration in diet (ppm)	Dose per animal (study averages)	Animals assigned
		Female (mg/kg bw/day)	Female
1	0	0	15
2	3000	193	15

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-sided).

If the F test was significant ($p\leq0.05$), 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 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 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 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 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 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 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 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. 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 BrdU 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 samples were taken from all animals in all groups by puncture of the abdominal aorta. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane. Blood 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 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 isoenzyme activities.

Histotechnology - Histopathology – Cell proliferation assessment

Duodenum and the two central sections of the liver were embedded in paraffin wax.

Histological sections, stained with hematoxylin and eosin, were prepared and examined from all the animals.

For cell proliferation assessment, an immunohistochemical staining demonstrating theincorporation 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 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 hematoxylin.

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 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 index were performed on all animals showing sufficient BrdU incorporation (estimated by water consumption or duodenum BrdU labeling).

7. 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

There were no treatment-related clinical signs observed in any group.

2. Mortality

There was no mortality in any group during the course of the study.

B. Body weight and body weight gain

There was no relevant change in 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)	0	3000
Liver weight Absolute (g)	5.61 ± 0.49	7.86 ± 0.93** (+40%)
Bodyweight-relative	2.53 ± 0.13	3.63 ± 0.320** (+43%)
Brain-relative	299.50 ± 30.26	421.3 ± 40.62** (+41%)
Microscopic pathology		
Diffuse centrilobular to panlobular hepatocellular hypertrophy	0/15	15/15
Diffuse mainly periportal hepatocellular vacuolation	11/15	1/15

**: p≤0.01; Figures in parentheses are % differences from control

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 BrdU labeling indexes were found to be approximately 4 times higher in treated animals (p≤0.01), when compared to controls.

Table 5.5-47: Mean BrdU labeling index in the liver after 7 days of treatment with AE C656948 at 3000 ppm (mean±SD)

Dosage level (ppm)	0	3000
Number of animals	14	14
BrdU positive cells in the centrilobular zone	44.54 ± 22.31	179.68 ± 95.18**
BrdU positive cells in the periportal zone	28.55 ± 16.80	112.94 ± 58.19**
Overall BrdU positive cells	**36.54 ± 18.70**	**146.31 ± 70.26****

**: p≤0.01

2. Hepatotoxicity testing

Changes that were observed are 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 activity, a moderate increase in mean PROD and BROD activities and a marked increase in mean UDPGT activity.

Table 5.5-48 Results of the hepatotoxicity testing after 7 day of treatment with AE C656948 at 3000 ppm

Dosage level (ppm)	0	3000
Number of animals	10	10
Total P-450 (nmol/mg prot.)	0.91 ± 0.17	1.23 ± 0.20 ** (+35 %)
EROD (pmol/min/mg prot.)	47.99 ± 3.73	103.18 ± 13.74 ** (+115 %)
PROD (pmol/min prot.)	6.65 ± 0.70	28.55 ± 14.12** (+329 %)
BROD (pmol/min/mg prot.)	6.39± 1.12	74.51 ± 50.89 ** (+1066 %)
UDPGT (nmol/min /mg prot.)	6.42 ± 0.61	30.69 ± 1.94 ** (+378 %)

**: p≤0.01
(%) as compared to control

E. Deficiencies

No specific deficiencies were noted in the study.

III. Conclusions

These data showed that ae c656948 has the ability to induce moderately phenobarbital-inducible hepatic enzymes (total cytochrome P-450, BROD, PROD and UDPGT) 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 fluopyram in the rat.

These data showed that AE C656948 has the ability to induce moderately phenobarbital-inducible hepatic enzymes (total cytochrome P-450, BROD, PROD and UDPGT) as well as liver hypertrophy and cell proliferation in the liver.

Data Point:	KCA 5.5/04
Report Author:	[redacted]
Report Year:	2008
Report Title:	Phenobarbital 7-day mechanistic study in the female Wistar rat by gavage
Report No:	SA 07325
Document No:	M-299491-01-1
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
Acceptability/Reliability:	Yes

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 days at a concentration of 80 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 BrdU administration period. All animals were subjected to necropsy. Brain and liver 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.

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: $p \leq 0.01$). At macroscopic examination, dark liver was found in 5/14 females and enlarged liver in 3/14 females compared to no incidences in the controls. At histological examination, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was found in all treated animals. A decreased incidence of periportal vacuolation was also noted. Assessment of cell proliferation in the liver revealed a 2 fold increase in mean BrdU labeling index 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 $p \leq 0.01$). 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.

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:	Stable in rodent diet for a period covering the study duration
2. Vehicle and / or positive control:	Methylcellulose 400
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj WI (IOPS HAN) – Female only
Age:	11 weeks approximately at start of treatment
Weight at dosing:	226 – 263 g
Source:	[redacted]
Acclimation period:	12 days
Diet:	Certified rodent powdered and irradiated diet A04C-10P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), ad libitum
Water:	Tap water, ad libitum
Housing:	Animals were caged individually in suspended stainless steel 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

Phenobarbital was administered once daily by oral gavage 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 phenobarbital. A negative 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 mL of drinking water was administered to all animals for 7 days to allow liver cell proliferation evaluation.

Table 5.5.49 Study design

Test group	Treatment	Dose level (mg/kg bw/day)	Animals assigned
1	Control	0	15
2	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 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-sided).

If the F test was significant ($p\leq0.05$), 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 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 t-test (2-sided).

If the F test was significant ($p\leq0.05$), 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 ($p\leq0.05$) 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 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 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 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 scheduled 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, 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 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 isoenzyme activities.

5.2 Histotechnology - Histopathology – Cell proliferation assessment

Duodenum and the two central sections of the liver were embedded in paraffin wax.

Histological sections, stained with hematoxylin and eosin, were prepared and examined from all the animals.

For cell proliferation assessment, an immunohistochemical 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 immunohistochemical 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 hematoxylin.

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 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 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 motor activity.

2. Mortality

One animal was found dead on day 5 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 to 7 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 Liver changes after 7 days of treatment with phenobarbital at 80 mg/kg bw/d (mean±SD)

Dosage level (ppm)	0	80
Liver weight Absolute (g)	5.55±0.40	6.63±0.98** (+19%)
Bodyweight-relative	2.47±0.16	3.02±0.36** (+22%)
Brain-relative	296.35±20.81	358.66±49.18** (+21%)
Microscopic pathology		
Diffuse centrilobular to panlobular hepatocellular hypertrophy	0/15	14/14
Diffuse mainly periportal hepatocellular vacuolation	7/15	3/14

**: p≤0.01

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 BrdU labeling indexes were found to be 2

fold higher in treated animals, when compared to controls (p≤0.01). Centrilobular index was higher than periportal index in treated animals.

Table 5.5-51 Mean BrdU labeling index in the liver after 7 days of treatment with phenobarbital at 80 mg/kg bw/d (mean±SD)

Dosage level (ppm)	0	80
Number of animals	15	14
BrdU positive cells in the centrilobular zone	21.73 ± 16.34	55.21 ± 43.31**
BrdU positive cells in the periportal zone	16.70 ± 10.02	33.19 ± 18.22**
Overall BrdU positive cells	19.22 ± 12.52	44.20 ± 27.39**

**: p≤0.01

2. Hepatotoxicity testing

Changes that were observed are described in Table 5.5.4-8. 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≤0.01).

Table 5.5-52 Results of the hepatotoxicity testing after 7 day of treatment with phenobarbital at 80 mg/kg bw/d (mean±SD)

Dosage level (ppm)	0	80
Number of animals	10	10
Total P-450 (nmol/mg prot.)	0.95 ± 0.20	1.49 ± 0.38 ** (+ 57 %)
EROD (pmol/min/mg prot.)	38.25 ± 6.42	47.56 ± 9.75 * (+ 24 %)
PROD (pmol/min prot.)	4.89 ± 0.61	26.36 ± 17.55 ** (+ 439 %)
BROD (pmol/min/mg prot.)	4.91 ± 0.70	94.43 ± 62.94 ** (+ 1823 %)
UDPGT (nmol/min /mg prot.)	6.99 ± 0.52	13.47 ± 1.66 ** (+ 93 %)

* : p≤0.05; **: p≤0.01
(%) as compared to control

E. Deficiencies

No specific deficiencies were noted in the study.

III. 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 P-450, PROD, BROD and UDPGT activities as well as liver hypertrophy and cell proliferation in the liver

Data Point:	KCA 5.5/05
Report Author:	[redacted]
Report Year:	2011
Report Title:	Fluopyram (AE C 656948): Mechanistic investigation in the female rat by dietary administration for up to 7 days
Report No:	SA 10240
Document No:	M-408029-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evaluated
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Fluopyram, (batch number: Mix-batch-08528/0002; a light beige solid, 94.7% w/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 levels of 30, 75, 150, 600 and 1500 ppm. These doses equated to 2.4, 6.2, 12.0, 46.1 and 117.6 mg/kg/day, respectively for those animals sacrificed after 3 days of treatment. For those animals sacrificed following 7 days of treatment the doses corresponded to 2.3, 5.6, 11.6, 44.1 and 118.5 mg/kg/day, respectively. A group of animals dosed by oral gavage with 80 mg/kg/day phenobarbital (a CAR/PXR nuclear receptor activator) acted as a positive control for the parameters investigated in this present study. A control group received untreated diet. Animals were observed daily for mortality and clinical 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 liver 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 a positive 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 microsomal 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 sacrifice 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 treatment-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 75 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 150 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 191.7% compared to 106% at 150 ppm. A slight but statistically significant increase in liver weight (absolute and relative to body weight) was recorded following 3 days treatment and in one female minimal centrilobular to panlobular hepatocellular hypertrophy was observed following 7 days treatment. Statistically significant increases in enzyme activity (BROD, PROD and both isoforms of UDPGT) were also recorded 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 (Udpgtr2; Gstm4 and Ephx1). 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 proliferation 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 191.7% at 600 ppm. In addition, an increased number of mitoses was observed in 4/15 females dosed for 3 days treatment with fluopyram. Furthermore a slight, though statistically significant increase 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 centrilobular to panlobular hypertrophy observed in 6/15 females following 3 day treatment (minimal) and in 14/15 females following 7 days treatment (minimal to slight). 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 changes 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 (+217.7% 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 3/15 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). BROD, 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 Cyp2b1, Cyp3a3 and Udpgtr2 were recorded (at both time points).

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 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 Cyp3a3 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:	Stable in rodent diet for a period covering the study duration
2. Vehicle and / or positive control:	Phenobarbital (positive control)
Description	White crystalline powder
Lot / Batch #:	Lot No. 06100228
Purity:	99.6%
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:	Rat
Strain:	Wistar Rj: WI (IOPS HAN) – Female only
Age:	10 weeks approximately at start of treatment
Weight at dosing:	218 - 263 g
Source:	[redacted]
Acclimation period:	12 days
Diet:	Certified rodent powdered and irradiated diet A04C-10P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), ad libitum
Water:	Tap water, ad libitum
Housing:	Animals were caged individually in suspended stainless steel 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: 12 July 2010 - 22 July 2010

2. Animal assignment and treatment

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.

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.

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".

Table 5.5-53 Study design

Test group	Concentration in diet (ppm) of AE C656948	Dose per animal (study averages)		Animals assigned	
		Females Subgp 1 Days 1-3 (mg/kg bw/day)	Females Subg 2 Days 1-7 (mg/kg bw/day)	Females Subgp 1	Females Subgp 2
1	0	0	0	15	15
2	30	2.4	2.3	15	15
3	75	6.2	5.6	15	15
4	150	12.0	11.6	15	15
5	600	46.1	44.1	15	15
6	750	112.6	118.5	15	15
7	Phenobarbital 80 mg/kg bw/day by gavage	-	-	15	15

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 covered 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 value obtained in homogeneity check was used as measured concentration.

4. Statistics

4.1 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
- 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 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 will be performed. If the ANOVA was significant (p≤0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (2-sided).

If the Bartlett test was significant (p≤0.05) 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≤0.05), means of the exposed groups were compared to the mean of the control group using the Dunn test (2-sided).

- Body weight and average food consumption/day parameters
- Total cytochrome P450 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 group using the Dunnett test (2-sided).

If the Bartlett test was significant (p≤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≤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.
- If the Bartlett test was significant (p≤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≤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 to 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 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≤0.05) even after log transformation, means of the exposed groups were compared to the mean of the control group using the Dunn test (1-sided).

If one or more group variance(s) equal 0, means were compared using non-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≤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 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 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≤0.05), the data were transformed using log transformation. If the F test on log transformed data 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≤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 proliferation 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 ($p \leq 0.05$), 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 ($p \leq 0.05$), 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 non-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 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 during the acclimatization period. Body weights were also measured on study Day 1 and 3 for Subgroup 1 and on study Day 1 and 7 for Subgroup 2. Additionally, diet fasted 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 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 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 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. From the Subgroup 2 animals, 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 paraffin wax.

Histological sections, stained with hematoxylin and eosin, were prepared for each animal in all groups.

4.2.2 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

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-benzidine (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 labeling index was 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 Ki67-positive hepatocytes per thousand cells, were measured separately on random fields comprising of at least 1000 centrilobular 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 females per treatment group (Subgroup 2) were weighed and 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) and UDPGT specific isoenzyme 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

Table 5.5-54 Cytochrome P-450s and typical inducing agents

Family	Enzymatic activity	Activity	Typical inducing agents
CYP 1A1 1A2	} EROD	activation of mutagens and carcinogens	β-naphtoflavone
CYP 2 2B1 2B2 2E	} PROD	detoxication of drugs and chemicals activation of nitrosamines	Phenobarbital isoniazid
CYP 3A1 & 3A2	BROD	detoxication of drugs and chemicals	Pregnenolone 16 α carbonitrile phenobarbital

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry (SAFAS SP2000 version 6.10.7.4) using the following substrates:

- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)
- benzoxyresorufin (BROD)

Ethoxyresorufin 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 individual control and treated animals using RNeasy Midi kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

4.6.2 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, 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.

Table 5.5-55 The list of Taqman assays used is as follows :

Gene (Major function)	Abbreviation	Refset ID	Taqman assay ID (Applied Biosystems)
METABOLISM: Phase I			
P450 (cytochrome)oxidoreductase	POR	NM_031576.1	Rn00580820_m1
Cytochrome P450 1a1	Ja1	NM_012540.2	Rn00487218_m1
Cytochrome P450 2b1	Cyp2b2	275205*	Cyp2b1_tc5
Cytochrome P450 3a3	Cyp3a3 (Cyp3a23)	NM_013105.2	Rn01640761_g1
Cytochrome P450 4a1	Cyp4a1	NM_175837.1	Rn00598510_m1
METABOLISM: Phase II			
Glutathione S-transferase A2	Gsta2	NM_017013.4	Rn00566636_m1
Glutathione S-transferase mu3	Gstm4	NM_020540.1	Rn01789233_m1
UDP glucoronosyltransferase 2 family, polypeptide B1	Udpgtr2	NM_173295.1 NM_057105.3	Rn00756519_m1
Epoxide hydrolase 1, microsomal	Ephx1	NM_012844.2	Rn00563349_m1
Epoxide hydrolase 2, cytoplasmic	Ephx2	NM_022936.1	Rn00576023_m1
Sulfotranferase family 1E, member 1	Sult1e1	NM_012883.1	Rn00820646_g1
CELL PROLIFERATION/APOPTOSIS			
Epithelial cell adhesion molecule	Tacstd1	NM_138541.1	Rn01473202_m1
Growth arrest and DNA-damage-inducible 45 beta	Gadd45b	NM_001008321.1	Rn01452530_g1
Retinoblastoma 1	Rb1	XM_344434.3	Rn01753308_m1

*: Transcript made to order

Beta-microglobulin (B2m; Refset ID: NM_012512.1; Taqman 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:

$\Delta\Delta Ct = (Cttest - CtB2m)_{treated} - (Cttest - CtB2m)_{control}$

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. 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 as 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 treatment 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 Day 3 of treatment.

Seven day treatment (Subgroup 2):

Fluopyram: There were no treatment-related clinical signs during the seven day treatment period.

Phenobarbital: All females displayed reduced motor activity starting from Day 1 of treatment. Two females had ocular discharge starting from Day 2 of treatment.

2. Mortality

There was no mortality in any group during the course of the study.

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.

Phenobarbital: There were no relevant changes in any of the body weight parameters between Day 1 and Day 3 compared to the controls.

Seven day treatment (Subgroup 2):

Fluopyram: There were no relevant changes in any of the body weight parameters between Day 1 and Day 7 compared to the controls.

Phenobarbital: Between Days 1 and 7 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 treatment-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 study were as follows:

Table 5.5-56 Mean achieved intake of fluopyram

Mean achieved dietary intake of fluopyram		
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
150	12.0	11.6
600	46.1	44.1
1500	117.6	118.5

D. Sacrifice and pathology

1. Terminal body weight and organ weight

Three day treatment (Subgroup 1):

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls. The mean absolute and mean liver to body weight ratio were statistically significantly 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 controls. These liver weights changes were considered to be toxicologically relevant.

Phenobarbital: 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 when compared to controls. These liver weights changes were considered to be toxicologically relevant.

Table 5.5-57 Mean absolute and relative liver weight changes following 3 day treatment with fluopyram or phenobarbital

Mean liver weight ± SD at scheduled 3 Day sacrifice (% change when compared to controls)							
Sex	**Females**						
Dose group (ppm)	**0**	**30**	**75**	**150**	**600**	**1500**	**PB (80 mg/kg)**
Mean absolute liver weight (g)	5.84 ±0.30	5.93 ±0.35 (+2%)	5.97 ±0.40 (+2%)	6.16 ±0.26 (+5%)	6.23* ±0.49 (+7%)	6.84** ±0.38 (+17%)	6.36** ±0.57 (+9%)
Mean liver to body weight ratio (%)	2.69 ±0.08	2.70 ±0.15 (0%)	2.75 ±0.13 (+2%)	2.79 ±0.10 (+4%)	2.83* ±0.17 (+5%)	3.15** ±0.17 (+17%)	2.93** ±0.22 (+9%)
Mean liver to brain weight ratio (%)	313.50 ±17.26	318.52 ±18.34 (+2%)	321.00 ±25.37 (+2%)	331.36 ±18.03 (+6%)	333.10 ±25.17 (+6%)	365.96** ±32.54 (+17%)	341.02** ±28.83 (+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.

Phenobarbital: 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. This change was considered to be toxicologically relevant.

Table 5.5-58 Mean absolute and relative liver weight changes following 7 day treatment with fluopyram or phenobarbital

Mean liver weight ± SD at scheduled 7 Day sacrifice (% change when compared to controls)							
Sex	**Females**						
Dose group (ppm)	**0**	**30**	**75**	**150**	**600**	**1500**	**PB (80 mg/kg)**
Mean absolute liver weight (g)	6.18 ±0.91	5.88 ±0.53 (-5%)	5.96 ±0.43 (-4%)	5.96 ±0.35 (-4%)	6.29 ±0.49 (+2%)	7.17** ±0.51 (+16%)	6.66 ±0.37 (+8%)
Mean liver to body weight ratio (%)	2.67 ±0.34	2.52 ±0.13 (-5%)	2.59 ±0.14 (-3%)	2.61 ±0.20 (-2%)	2.76 ±0.16 (+3%)	3.16** ±0.16 (+18%)	2.95** ±0.17 (+11%)
Mean liver to brain weight ratio (%)	332.98 ±53.94	317.53 ±27.19 (-5%)	321.09 ±23.25 (-4%)	320.04 ±23.75 (-4%)	336.49 ±22.32 (+1%)	392.26** ±29.24 (+18%)	361.88 ±20.36 (+9%)

** p≤0.01

2. Gross pathology

Three day treatment (Subgroup 1):

Enlarged liver was found in 2/15 females at 1500 ppm fluopyram. There were no macroscopic changes recorded for the phenobarbital treated females.

Table 5.5-59 Macroscopic changes in the liver following 3 days treatment with fluopyram or phenobarbital

Incidence and severity of macroscopic changes in the liver- scheduled 3 day sacrifice							
Sex	**Females**						
Dose group (ppm)	**0**	**30**	**75**	**150**	**600**	**1500**	**PB (80 mg/kg)**
Number examined	15	15	15	15	15	15	15
Enlarged	0	0	0	0	0	2	0

Seven day treatment (Subgroup 2):

Enlarged liver was found in 3/15 females at 1500 ppm fluopyram. There were no macroscopic changes recorded for the phenobarbital treated females.

Table 5.5-60 Macroscopic changes in the liver following 7 days treatment with fluopyram or phenobarbital

Incidence and severity of macroscopic changes in the liver- scheduled 7 day sacrifice							
Sex	**Females**						
Dose group (ppm)	**0**	**30**	**75**	**150**	**600**	**1500**	**PB (80 mg/kg)**
Number examined	15	15	15	15	15	15	15
Enlarged	0	0	0	0	0	3	0

3. Microscopic pathology

Three day treatment (Subgroup 1):

Fluopyram: Minimal centrilobular to panlobular hepatocellular hypertrophy was found for 6/15 females at 1500 ppm. 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

Incidence and severity of microscopic changes in the liver- scheduled 3 day sacrifice							
Sex	**Females**						
Dose group (ppm)	**0**	**30**	**75**	**150**	**600**	**1500**	**PB (80 mg/kg)**
Number examined	15	15	15	15	15	15	15
Hepatocellular hypertrophy: centrilobular to panlobular							
Minimal	**0**	**0**	**0**	**0**	**0**	**6**	**3**
Total	0	0	0	0	0	6	3
Increased number of mitoses							
Present	**0**	**0**	**0**	**0**	**0**	**4**	**3**
Total	0	0	0	0	0	4	3

Seven day treatment (Subgroup 2):

Fluopyram: Minimal to slight centrilobular to panlobular hepatocellular hypertrophy was found from 600 ppm (1/15 females at 600 ppm; 14/15 females at 1500 ppm).

Phenobarbital: Minimal to slight centrilobular to panlobular hepatocellular hypertrophy in 9/15 females was found as well as an increased number of mitoses in hepatocytes in 3/15 females.

Table 5.5-62 Microscopic changes in the liver following 7 days treatment with fluopyram or phenobarbital

Incidence and severity of microscopic changes in the liver- scheduled 7 day sacrifice							
Sex	**Females**						
Dose group (ppm)	**0**	**30**	**75**	**150**	**600**	**1500**	**PB (80 mg/kg)**
Number examined	15	15	15	15	15	15	15
Hepatocellular hypertrophy: centrilobular to panlobular							
Minimal	0	0	0	0	1	6	6
Slight	0	0	0	0	0	8	3
Total	**0**	**0**	**0**	**0**	**1**	**14**	**9**
Increased number of mitoses							
Present	0	0	0	0	0	0	3
Total	**0**	**0**	**0**	**0**	**0**	**0**	**3**

4. Cell proliferation

Three day treatment (Subgroup 1):

Fluopyram: Dose-related increases in the centrilobular, perilobular and, therefore, the global proliferation indexes were observed, which were statistically significantly higher from 150 ppm when compared to controls.

Table 5.5-63 Mean cell proliferation index following 3 days treatment with fluopyram

Mean cell proliferation index ± SD following three days treatment (% change when compared to controls)					
Test Substance	**Dose**	**Metric**	**Centrilobular**	**Perilobular**	**Total**
Control	0 ppm	N	15	15	15
		Mean ± SD	14.6 ± 7.4	11.1 ± 5.1	12.8 ± 5.8
Fluopyram	30 ppm	N	14	14	14
		Mean ± SD	11.8 ± 8.0	10.7 ± 5.8	11.2 ± 6.4
		% Change	-19%	-3.5%	-12.5%
	75 ppm	N	15	15	15
		Mean ± SD	13.4 ± 5.9	15.4 ± 8.2	14.4 ± 5.8
		% Change	-8.2%	39.3%	12.3%
	150 ppm	N	15	15	15
		Mean ± SD	25.1 ± 14.1*	22.6 ± 13.4**	23.8 ± 10.6**
		% Change	71.9%	104.2%	86.0%
	600 ppm	N	15	15	15
		Mean ± SD	57.3 ± 20.1**	36.6 ± 15.7**	47.0 ± 15.8**
		% Change	293.8%	230.7%	266.6%
	1500 ppm	N	15	15	15
		Mean ± SD	99.5 ± 62.3**	67.4 ± 38.0**	83.4 ± 38.3**
		% Change	583.2%	509.0%	551.2%

* p≤0.05; ** p≤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, perilobular and, therefore the global proliferation indexes were statistically significantly higher, when compared to controls. 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 (58% increase compared to the corresponding controls).

Table 5.5-64 Mean cell proliferation index following 3 days treatment with phenobarbital

Mean cell proliferation index ± SD following three days treatment (% change when compared to controls)					
Test Substance	**Dose**	**Metric**	**Centrilobular**	**Perilobular**	**Total**
Control	0 ppm	N	15	15	15
		Mean ± SD	14.6 ± 7.4	11.1 ± 5.1	12.8 ± 5.8
Phenobarbital	80 mg/kg/day	N	14	14	14
		Mean ± SD	46.25 ± 36.24**	17.48 ± 8.95*	31.87 ± 19.80**
		% Change	217.7%	58.0%	148.8%

* p≤0.05; ** p≤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):

Fluopyram: Centrilobular, perilobular and, therefore, the global proliferation indexes were statistically significantly higher from 150 ppm when compared to controls.

Table 5.5-65 Mean cell proliferation index following 7 days treatment with fluopyram

Mean cell proliferation index ± SD following seven days treatment (% change when compared to controls)					
Test substance	Dose	Metric	Centrilobular	Perilobular	Total
Control	0 ppm	N	15	15	15
		Mean ± SD	8.3 ± 5.3	10.5 ± 6.1	9.4 ± 5.1
Fluopyram	30 ppm	N	15	15	15
		Mean ± SD	11.2 ± 7.6	15.3 ± 11.2	13.2 ± 8.6
		% Change	34.5%	45.6%	40.7%
	75 ppm	N	15	15	15
		Mean ± SD	12.0 ± 6.1	10.3 ± 5.1	11.1 ± 5.1
		% Change	43.5%	NC	18.5%
	150 ppm	N	15	15	15
		Mean ± SD	20.4 ± 13.0**	18.4 ± 8.1**	19.4 ± 9.8**
		% Change	144.5%	75.2%	106.0%
	600 ppm	N	15	15	15
		Mean ± SD	27.8 ± 12.1**	27.0 ± 13.0**	27.4 ± 9.8**
		% Change	234.1%	157.6%	191.7%
	1500 ppm	N	15	15	15
		Mean ± SD	32.2 ± 19.6**	34.9 ± 17.2**	33.5 ± 15.0**
		% Change	286.3%	232.5%	256.6%

NC: no change; ** p≤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 proliferation indexes were statistically significantly higher when compared to controls. The magnitude of response was greater in the centrilobular region (603.4% increase compared to controls) compared to that recorded for the perilobular region (54.7% increase compared to controls).

Table 5.5-66 Mean cell proliferation index following 7 days treatment with phenobarbital

Test Substance	Dose	Metric	Centrilobular	Perilobular	Total
Control	0 ppm	N	15	15	15
		Mean ± SD	8.3 ± 5.3	10.5 ± 6.1	9.4 ± 5.1
Phenobarbital	80 mg/kg/day	N	15	15	15
		Mean ± SD	58.6 ± 33.9**	16.2 ± 10.2	37.4 ± 19.6**
		% Change	603.4%	54.7%	297.9%

* p≤0.05; ** p≤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.

5. Hepatotoxicity testing

Investigation of hepatotoxicity was only conducted on 5 randomly chosen females/group from those animals dosed for 7 days (Subgroup 2).

Fluopyram: No statistically significant changes in total P450 content or enzyme activity was observed up to 150 ppm. Statistically significant increases in PROD (p≤0.05 at 600 ppm and p≤0.01 at 1500 ppm), BROD and both UDPGT isoforms (p≤0.01) were observed from 600 ppm. At 1500 ppm, total P450 content was also statistically significantly increased (p≤0.05). In addition, EROD activity was statistically significantly increased (p≤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 ($p \leq 0.05$) in total P450 was recorded as well as statistically significant increases BROD ($p \leq 0.05$), PROD ($p \leq 0.01$) and UDPGT-nitrophenol ($p \leq 0.01$) activity.

Table 5.5-67 Mean cytochrome P-450 content and enzymatic activities in the liver following 7 days treatment with fluopyram or phenobarbital

Fluopyram level (ppm)	Mean Increase Compared to Controls (Statistical Significance)					
	Total P450 Content	EROD	BROD	PROD	UDPGT-4-nitrophenol	UDPGT-bilirubin
30	x 0.93	x 1.05	x 1.09	x 1.34	x 1.05	x 1.05
75	NC	x 0.94	x 1.17	x 1.46	x 1.10	x 1.28
150	x 1.11	x 1.02	x 1.44	x 1.41	x 1.27	x 1.44
600	x 1.02	x 1.12	x 2.42 (**)	x 2.16 (*)	x 1.52 (**)	x 1.91 (**)
1500	x 1.34 (*)	x 1.62 (**)	x 5.89 (**)	x 4.56 (**)	x 3.01 (**)	x 2.65 (**)
Phenobarbital 80 mg/kg/day	x 1.65 (*)	x 1.12	x 27.7 (*)	x 10.59 (**)	x 1.86 (**)	x 1.37

NC: no change; * $p \leq 0.05$; ** $p \leq 0.01$

6. Gene Transcript Analyses

Three day treatment (Subgroup 1)

Fluopyram: At 30 ppm, no statistically significant changes in the expression of any of the genes investigated were observed. At 75 ppm a marginal but statistically significant increase in the expression of Cyp3a3 (+48.4%; $p \leq 0.05$) was recorded. From 150 ppm, clear dose-related increases were recorded for the expression of the Phase I genes Cyp2b1 (+230.7% 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. Gsta2, Gstm4, Udpgtr2 and Ephx1) were statistically significantly increased. In addition, Cyp1a1 was statistically significantly increased (+631.1%; $p \leq 0.01$) from this dose level. A marginal, though statistically significant increase (+45%; $p \leq 0.05$) 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 (Tacstd 1, Gadd 45b) were recorded.

There were no clear effects on the expression of POR, Cyp4a1, Ephx2 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 (61/90 control and fluopyram treated liver samples) was greater than or equal to the cut-off limit of 35.

Table 5.5-68 Mean gene transcript analyses following 3 day treatment with fluopyram

Mean Relative Quantity ± standard deviation of gene transcripts (3 day treatment) (% change compared to control mean values)						
Gene transcripts	**Control**	**30 ppm**	**75 ppm**	**150 ppm**	**600 ppm**	**1500 ppm**
METABOLISM: Phase I						
POR	0.87 ± 0.24	0.82 ± 0.29 NC	0.96 ± 0.37 (+10.3)	0.71 ± 0.21 (-18.4)	0.95 ± 0.30 (+9.2)	0.98 ± 0.39 (+12.6)
Cyp1a1	1.35 ± 1.14	1.12 ± 0.68 (-17)	1.51 ± 1.61 (+11.9)	2.3 ± 2.3 (+70.4)	9.87 ± 8.29** (+631.1)	84.6 ± 51.6** (+6167)
Cyp2b1	1.27 ± 1.87	0.81 ± 0.56 (-36.2)	1.44 ± 0.86 (+13.4)	4.2 ± 3.38** (+230.7)	63.0 ± 71.2** (+4861)	309.5 ± 176.7** (+24270)
Cyp3a3	0.93 ± 0.49	1.01 ± 0.50 (+8.6)	1.38 ± 0.68* (+48.4)	2.41 ± 0.89** (+159.1)	7.64 ± 2.23** (+721.5)	20.0 ± 6.45** (+2051)
Cyp4a1	1.46 ± 0.33	1.37 ± 0.38 (-6.2)	1.51 ± 0.4 (+3.4)	1.3 ± 0.4 (-11.0)	1.43 ± 0.46 (NC)	1.14 ± 0.27 (-21.9)
METABOLISM: Phase II						
Gsta2	0.51 ± 0.45	0.58 ± 0.60 (+13.7)	0.52 ± 0.34 (NC)	0.83 ± 0.54 (+62.7)	1.02 ± 0.67** (+100)	2.35 ± 1.31** (+360.9)
Gstm4	0.83 ± 0.35	1.05 ± 0.69 (+26.5)	1.24 ± 0.78 (+49.4)	1.01 ± 0.99 (+21.7)	2.15 ± 1.23** (+159.0)	3.86 ± 2.09** (+365)
Udpgtr2	1.77 ± 0.93	1.68 ± 0.79 (-5.1)	2.0 ± 0.89 (+13)	3.0 ± 2.13 (+69.5)	4.27 ± 2.03** (+141.2)	6.66 ± 3.68** (+276.3)
Ephx1	0.85 ± 0.24	0.91 ± 0.30 (+7.1)	0.84 ± 0.31 (NC)	1.0 ± 0.36 (+17.6)	1.73 ± 0.44** (+103.5)	3.12 ± 0.93** (+267.1)
Ephx2	1.14 ± 0.31	0.92 ± 0.29 (-19.3)	0.94 ± 0.21 (-17.5)	1.02 ± 0.26 (-10.5)	1.18 ± 0.33 (NC)	0.95 ± 0.32 (-16.7)
Sult1e1	ND	ND	ND	ND	ND	ND
CELL PROLIFERATION/APOPTOSIS						
Tacstd1	1.19 ± 0.17	1.1 ± 0.16 (-7.6)	1.1 ± 0.21 (-7.6)	1.12 ± 0.23 (-5.9)	1.26 ± 0.22 (+5.9)	1.66 ± 0.34** (+39.5)
Gadd45b	0.8 ± 0.26	0.8 ± 0.29 (NC)	0.84 ± 0.36 (+5)	0.9 ± 0.23 (+12.5)	1.16 ± 0.45* (+45)	1.34 ± 0.57** (+67.5)
Rb1	1.01 ± 0.15	0.93 ± 0.15 (-7.9)	0.96 ± 0.16 (-5)	1.05 ± 0.3 (+4)	1.04 ± 0.26 (+3)	0.82 ± 0.14 * (-18.8)

NC: no change; *p≤0.05; ** p≤0.01; 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: 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 glutathione (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 expression. 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**.**

Table 5.5-69: Mean gene transcript analyses following 3 day treatment with phenobarbital

Mean Relative Quantity ± standard deviation of gene transcripts (3 day treatment) (% change compared to control mean values)		
Gene transcripts	**Control**	**Phenobarbital (80 mg/kg/day)**
METABOLISM: Phase I		
POR	0.87 ± 0.24	0.7 ± 0.24 (-19.5)
Cyp1a1	1.35 ± 1.14	1.65 ± 2.05 (+22.3)
Cyp2b1	1.27 ± 1.87	962.8 ± 892.5** (+75711)
Cyp3a3	0.93 ± 0.49	10.12 ± 7.18** (+988.2)
Cyp4a1	1.46 ± 0.33	0.52 ± 0.16** (-64.4)
METABOLISM: Phase II		
Gsta2	0.51 ± 0.45	2.22 ± 1.63** (+335.3)
Gstm4	0.83 ± 0.35	3.62 ± 3.82* (+336.1)
Udpgtr2	1.77 ± 0.96	7.83 ± 5.43** (+342. 4)
Ephx1	0.85 ± 0.24	3.3 ± 2.3** (+288.2)
Ephx2	1.14 ± 0.31	0.53 ± 0.23** (-53.5)
Sult1e1	ND	ND
CELL PROLIFERATION/APOPTOSIS		
Tacstd1	1.19 ± 0.17	1.08 ± 0.18 (-9.2)
Gadd45b	0.8 ± 0.26	1.31 ± 0.67** (+63.75)
Rb1	1.01 ± 0.15	0.82 ± 0.15** (-18.8)

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.

Seven day treatment (Subgroup 2):

Fluopyram:

A similar gene expression profile was observed following 7 days treatment with fluopyram as that observed following 3 days treatment.

At 30 ppm, no significant changes in the expression of any of the genes investigated were observed.

At 75 ppm, a marginal but statistically significant increase in the expression of Cyp3a3 (+95%; p≤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≤0.05) in Gadd45b was recorded. Cyp4a1 (-37%; p≤0.01) and Ephx2 (-39.5%; p≤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.

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) (% change compared to control mean values)						
Gene transcripts	**Control**	**30 ppm**	**75 ppm**	**150 ppm**	**600 ppm**	**1500 ppm**
METABOLISM: Phase I						
POR	0.85 ± 0.35	0.81 ± 0.29 (-4.7)	0.81 ± 0.26 (-4.7)	0.91 ± 0.3 (+7.1)	1.03 ± 0.42 (+21.2)	0.99 ± 0.36 (+16.5)
Cyp1a1	2.26 ± 1.3	3.07 ± 3.87 (+35.8)	4.0 ± 3.87 (+77)	10.31 ± 11.01** (+356.2)	143.7 ± 81.0** (+6258)	503.8 ± 470.5** (+22192)
Cyp2b1	0.95 ± 0.74	2.43 ± 1.83 (+155.8)	2.93 ± 3.87 (+208.4)	13.64 ± 12.06** (+1336)	310.2 ± 322.0** (+32553)	1362.3 ± 1422.2** (+143300)
Cyp3a3	0.99 ± 0.59	1.46 ± 0.84 (+47.5)	4.93 ± 0.79** (+395)	3.59 ± 1.65** (262.6)	12.32 ± 3.35** (+1144)	28.27 ± 10.15** (+2756)
Cyp4a1	0.73 ± 0.26	0.70 ± 0.22 (-4.1)	0.62 ± 0.14 (-15.1)	0.64 ± 0.24 (-12.3)	0.64 ± 0.19 (-12.3)	0.46 ± 0.11** (-37.0)
METABOLISM: Phase II						
Gsta2	2.08 ± 1.7	1.37 ± 0.56 (-34.1)	1.97 ± 1.45 (-5.3)	2.74 ± 1.78 (+31.7)	3.6 ± 1.83** (+73.1)	7.03 ± 4.57** (+238)
Gstm4	2.02 ± 1.02	2.42 ± 1.76 (+19.8)	1.81 ± 0.97 (-10.4)	2.78 ± 1.54 (+37.6)	4.2 ± 1.82* (+108)	11.3 ± 19.5** (+459.4)
Udpgtr2	2.53 ± 1.28	3.58 ± 4.32 (+41.5)	3.91 ± 1.16** (+54.5)	3.46 ± 1.5 (+36.8)	7.33 ± 3.22** (+189.7)	12.01 ± 4.25** (+374.7)
Ephx1	1.2 ± 0.41	1.46 ± 0.25 (+21.7)	1.34 ± 0.32 (+11.7)	1.2 ± 0.39 (NC)	2.12 ± 0.69** (+77)	4.21 ± 2.34** (+250.8)
Ephx2	1.19 ± 0.44	1.18 ± 0.38 (NC)	1.09 ± 0.48 (-8.4)	1.03 ± 0.43 (-13.4)	0.95 ± 0.38 (-20.2)	0.72 ± 0.35 * (-39.5)
Sult1e1	ND	ND	ND	ND	ND	ND
CELL PROLIFERATION/APOPTOSIS						
Tacstd1	1.41 ± 0.3	1.55 ± 0.37 (+10)	1.36 ± 0.23 (-3.5)	1.1 ± 0.26 (-22.0)	1.38 ± 0.45 (-2.1)	1.51 ± 0.34 (+7.1)
Gadd45b	1.2 ± 0.45	1.41 ± 0.43 (+17.5)	1.13 ± 0.34 (-5.8)	1.25 ± 0.65 (+4.2)	1.55 ± 0.53 (+29.2)	2.19 ± 1.27* (+82.5)
Rb1	0.90 ± 0.18	0.98 ± 0.15 (+8.9)	0.89 ± 0.13 (NC)	0.83 ± 0.17 (-7.8)	0.87 ± 0.14 (-3.3)	0.79 ± 0.15 (-12.2)

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 enzymes (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 ($p \leq 0.01$) down-regulation of Cyp4a1 and Ephx2 expression and the marker for cell proliferation (Gadd45b) was also marginally but statistically significantly ($p \leq 0.05$) 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 (8/15 phenobarbital treated liver samples) was greater than or equal to the cut-off limit of 35.

Table 5.5-71 Mean gene transcript analyses following 7 day treatment with phenobarbital

Mean Relative Quantity ± standard deviation of gene transcripts (7 day treatment) (% change compared to control mean values)		
Gene transcripts	**Control**	**Phenobarbital (80 mg/kg/day)**
METABOLISM: Phase I		
POR	0.85 ± 0.35	0.66 ± 0.20 (-22.4)
Cyp1a1	2.26 ± 1.30	1.83 ± 1.24 (-19.0)
Cyp2b1	0.95 ± 0.74	2776.8 ± 1842.7** (+292195)
Cyp3a3	0.99 ± 0.59	16.26 ± 10.45** (+1542.4)
Cyp4a1	0.73 ± 0.26	0.40 ± 0.08** (-45.2)
METABOLISM: Phase II		
Gsta2	2.08 ± 1.70	4.8 ± 2.3** (+130.8)
Gstm4	2.02 ± 1.02	18.3 ± 11.8** (+805.9)
Udpgtr2	2.53 ± 1.28	13.1 ± 7.8** (+418)
Ephx1	1.2 ± 0.41	4.76 ± 2.43** (+297)
Ephx2	1.19 ± 0.44	0.81 ± 0.15** (-31.9)
Sult1e1	ND	ND
CELL PROLIFERATION/APOPTOSIS		
Tacstd1	1.41 ± 0.32	1.13 ± 0.18** (-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 change; * $p \leq 0.05$; ** $p \leq 0.01$; 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 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 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 283/2013 as it provides mechanistic information on the effects and target organs of fluopyram in the rat.

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 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 cyp3a3 at this dose level) and 30 ppm as a NOEL.

Data Point:	KCA 5.5/06
Report Author:	[redacted]
Report Year:	2012
Report Title:	Fluopyram (AE C 656948): Mechanistic investigations in the liver of female rats following dietary administration
Report No:	SA 11104
Document No:	M-427431-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evaluated
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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 enzymatic 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, phenobarbital, was administered by oral gavage and was used as a positive control for the liver effects. The reversibility 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 conjunctiva 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 ($p \leq 0.05$) and week 4 ($p \leq 0.01$) 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 ($p \leq 0.05$ for weeks 1 and 4; $p \leq 0.01$ for week 2).

Fluopyram had no effect on terminal body weight but induced treatment-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, a statistically significant ($p \leq 0.01$) increase in the expression of Cyp3a13 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 liver was observed in 3/15 females and centrilobular cell proliferation was marginally, but statistically significantly ($p \leq 0.05$), increased (+47%) compared to the controls.

At 150 ppm, slight but statistically significant increases in mean liver weight relative to body weight (+7%; $p \leq 0.01$) and brain weight (+11%; $p \leq 0.05$) were recorded. This increased liver weight could be associated with enlarged liver in 5/15 females. Centrilobular and global hepatic cell proliferation (+106% and +70% respectively) were statistically significantly ($p \leq 0.01$) increased compared to the controls. In addition, statistically significant ($p \leq 0.01$) increases were recorded for the expression of the Phase I genes Cyp1a1 (+711%), Cyp2b1 (+990%) and for the Phase II genes Gstm4 (+136%) and Udpgtr2 (+64%). Statistically significantly ($p \leq 0.01$) increased hepatic enzyme activity was, however, only observed for UDPGT-bilirubin (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 liver weights (+10 to +15%), coupled with enlarged liver in 4/15 animals and minimal hepatocellular hypertrophy in 6/15 females. However, despite these macroscopic and microscopic changes, hepatic cell proliferation was similar to that observed at 150 ppm with centrilobular and global proliferation being statistically significantly increased (+106% and +67% respectively; $p \leq 0.01$). 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%; $p \leq 0.01$), increased expression of the marker for cell proliferation (Gadd 45b) was also observed. Increased activity of BROD ($p \leq 0.01$), PROD ($p \leq 0.05$) and UDPGT-4-nitrophenol ($p \leq 0.01$) were recorded from this dose level.

At 1500 ppm, 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/PXR nuclear receptors. Liver weight (absolute and relative to brain and body weight) was statistically significantly increased following phenobarbital treatment and enlarged liver was noted in 10/15 animals, which could be associated with the centrilobular to panlobular hypertrophy observed in 12/15 females and an 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) than in the perilobular region (+23% compared to controls). Furthermore, the gene expression of Gadd45b was statistically significantly increased (+68%; $p \leq 0.01$). Statistically significant ($p \leq 0.01$) 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; $p \leq 0.01$), BROD (+88-fold; $p \leq 0.01$) and UDPGT-bilirubin (+1.38-fold; $p \leq 0.05$) respectively. Enzyme activity of the second isoform of UDPGT (UDPGT-4-nitrophenol) was also statistically significantly increased (+1.81-fold; $p \leq 0.01$) and increased expression of genes coding for additional Phase II enzymes (Gstm4, Gsta2 and Ephx1) was also recorded following phenobarbital treatment.

Recovery Phase:

For the females previously treated with 1500 ppm fluopyram, there were no relevant changes in any of the body weight parameters during the recovery phase. A statistically significant reduction ($p \leq 0.05$) 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 microscopic changes recorded in the liver. Centrilobular and global hepatic cell proliferation were still statistically significantly increased compared to the controls (+81%, $p \leq 0.01$ and +51%, $p \leq 0.05$, respectively); however these observed increases were lower than those observed immediately following treatment with 1500 ppm fluopyram (215% and 188% for centrilobular and global cell proliferation, respectively). Hepatic molecular and enzymatic changes were still apparent at the end of the recovery phase as evidenced by increased enzyme activity/gene expression for BROD/Cyp3a3, PROD/Cyp2b1, EROD/Cyp1a1 and UDPGT-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.

A similar profile was observed for the females previously treated with 80 mg/kg/day phenobarbital. In these animals a loss or a reduction in body weight gain was recorded 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 significantly increased compared to the controls; however the magnitude of the increases were generally lower than those observed immediately following treatment with phenobarbital. Hepatic 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 cell proliferation parameters, the magnitude of these molecular and enzymatic changes was generally lower than those recorded immediately following treatment.

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 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 fluopyram following the recovery period.

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Light beige powder
Lot / Batch #:	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. Vehicle and / or positive control:	Phenobarbital (positive control)
Description	White crystalline powder
Lot / Batch #:	Lot No. 09050075
Purity:	99.6%
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:	Rat
Strain:	Wistar Rj: WI (IOPS HAN) – Female only
Age:	10 weeks approximately at start of treatment
Weight at dosing:	215 - 261 g
Source:	[redacted]
Acclimation period:	6-8 days
Diet:	Certified rodent powdered and irradiated diet A04C-10P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), ad libitum
Water:	Tap water, ad libitum
Housing:	Animals were caged individually in suspended stainless steel 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: Dosing period: 10 May 2011 – 09 June 2011

Recovery period: 09 June 2011 – 08 July 2011

2. Animal assignment and treatment

Animals were assigned to dose groups using a randomization procedure by weight.

Seven groups of female rats were dosed for at least 28 days with 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 at the appropriate dietary concentration (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 the compound 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.

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 diet (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 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.

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".

Table 5.5-72 Study design

Group	Test substance	Dose level	Number of animals per group
1	Control	0	15+15*
2	Fluopyram	30 ppm	15
3		75 ppm	15
4		150 ppm	15
5		600 ppm	15
6		1500 ppm	15+15*
7	Phenobarbital	80 g/kg/day	15+15*

*Animals allocated to recovery phase

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.

Two formulations of phenobarbital 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 usage 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

4.1 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

- Total cytochrome P450 content and liver enzyme activities
- Cell proliferation parameters
- Gene transcript analysis

4.2 Statistical methods

Mean and standard deviation were calculated for each group.

Group means were compared at the 5% and 1% levels of significance.

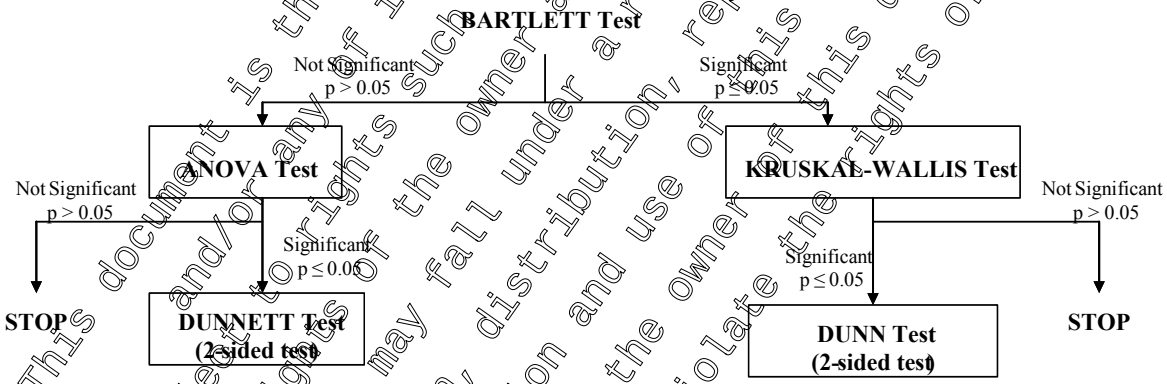
The statistical analyses of the cell proliferation data were carried out by the statistician using the methods described in the appropriate paragraphs.

All other statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

Dosing Phase: comparison between fluopyram groups and control group

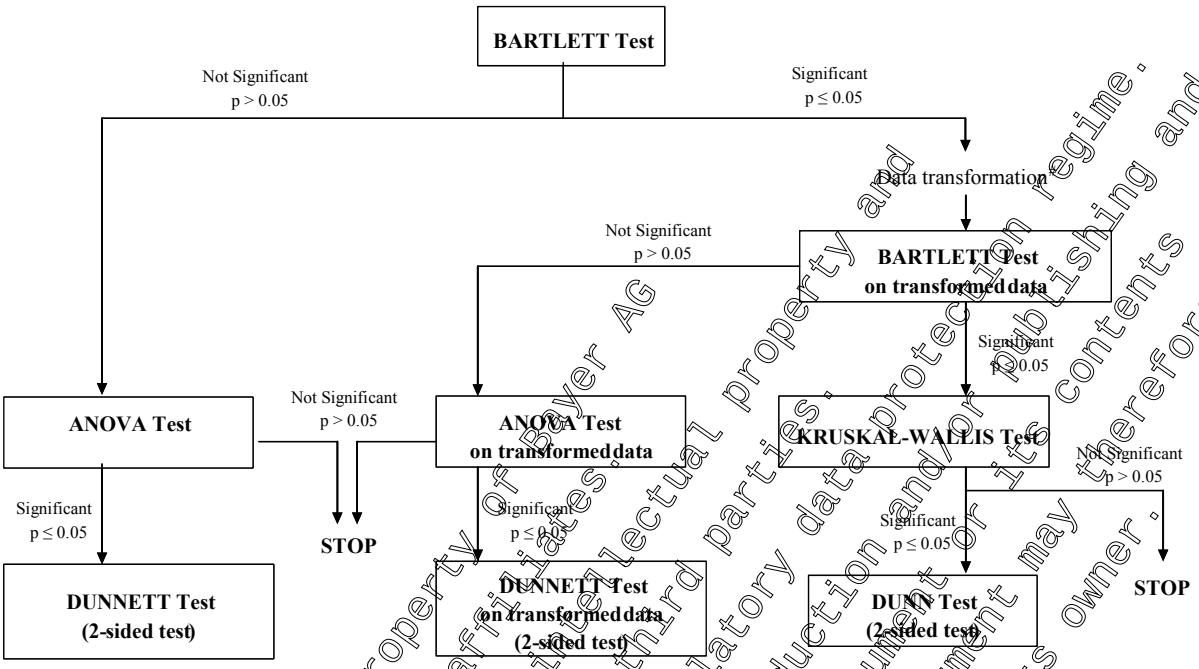
- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters.

The mean and standard deviation will be calculated for each group and per time period for body weight change parameters.



- Body weight and average food consumption/day parameters
- Total cytochrome P450 content and liver enzyme activities
- Gene transcript analyses.

The mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.



[#] Data will be transformed using the log transformation for body weight parameters

- Cell proliferation parameters

The Levene test was performed to compare the homogeneity of group variances. As the Bartlett test of homogeneity of variances is very sensitive to non-normality of data, the Levene test was preferred.

If the Levene test was not significant (p>0.05), means of the exposed groups were compared to 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 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≤0.05) even after log transformation, means of the exposed groups were compared to the mean of the control group using the Dunn test (1-sided).

If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

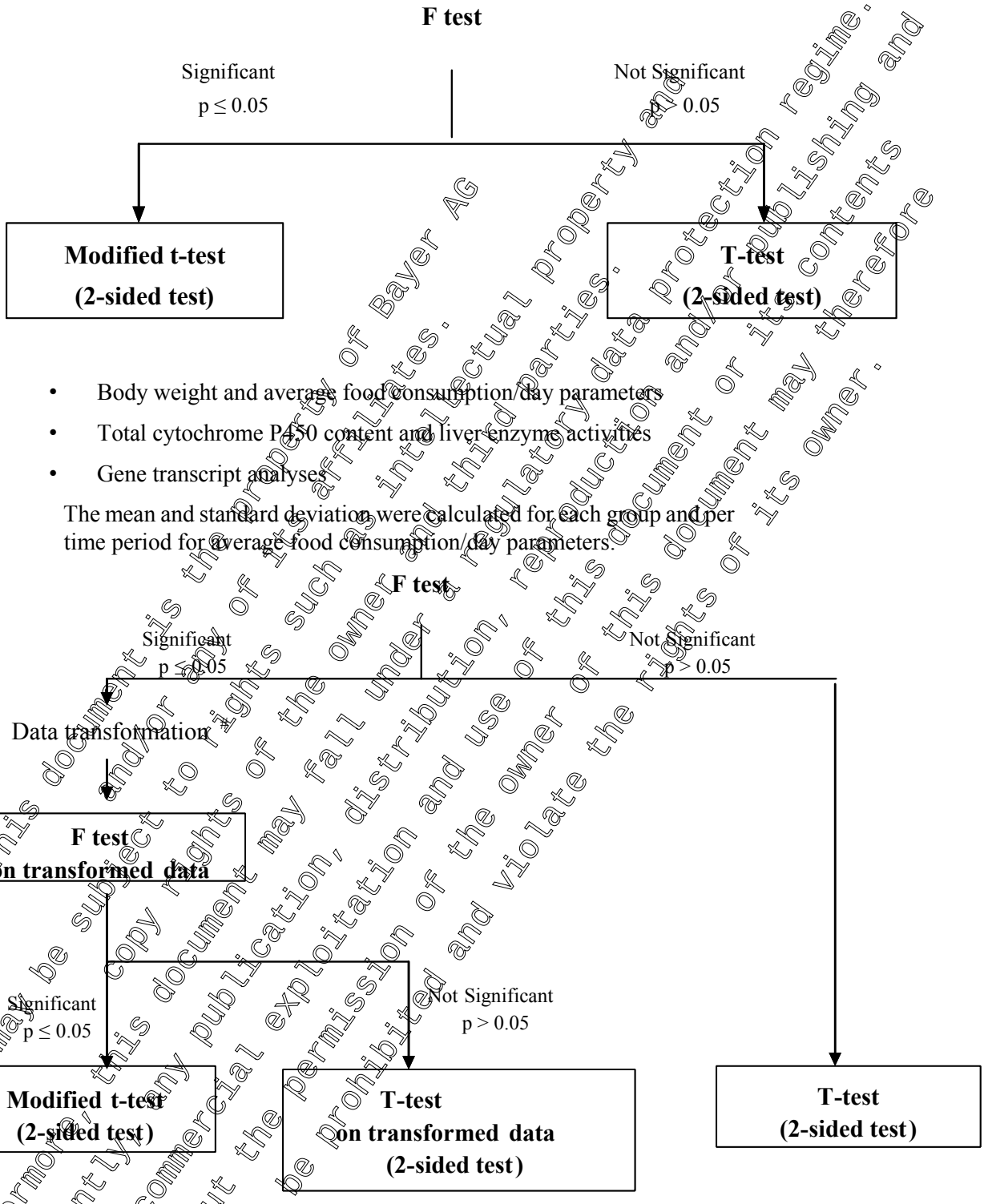
Dosing Phase: comparison between phenobarbital group and control group

Recovery Phase: comparison between high dose fluopyram group and control group

Recovery Phase: comparison between phenobarbital group and control group

- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters.

The mean and standard deviation were calculated for each group and per time period for body weight change parameters.



- Body weight and average food consumption/day parameters
- Total cytochrome P450 content and liver enzyme activities
- Gene transcript analyses

The mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.

Data will be transformed using the log transformation for body weight parameters.

- Cell proliferation 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 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≤0.05), even after log transformation, mean of the test group were compared to the mean of the control group using the exact Mann-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 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.

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 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).

3. Food consumption and compound intake

The weight of food supplied was measured on Day 1 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 fluopyram 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 exsanguination 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 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.

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 paraffin wax.

Histological sections, stained with hematoxylin and eosin, were prepared for these two organs for each animal in all groups.

4.2.2 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 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-benzidine (DAB) and nuclear counter-staining with hematoxylin.

4.3 Histopathology

Histopathological examinations were performed on the liver samples from all animals in all groups at both scheduled sacrifices

4.4 Cell proliferation assessment

The immunohistochemical staining for Ki67 and determination of the labeling index was 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 Ki67-positive hepatocytes per thousand cells, were measured separately on random fields comprising of at least 1000 centrilobular and perilobular cells using an automatic image analysis system. The mean and standard deviation were calculated for each group.

4.5. Hepatotoxicity testing

At both scheduled sacrifice times, the remaining portions of the liver from five randomly selected females per group were weighed and 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 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 (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

Table 5.5-73 Cytochrome P-450s and typical inducing agents:

Family	Enzymatic activity	Activity	Typical inducing agents
CYP 1A1 1A2	} EROD	activation of mutagens and carcinogens	β-naphtoflavone
CYP 2 2B1 2B2 2E	} PROD	detoxication of drugs and chemicals activation of nitrosamines	Phenobarbital isoniazid
CYP 3A1 & 3A2	BROD	detoxication of drugs and chemicals	Pregnenolone 16 α carbonitrile phenobarbital

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry (SAFAS SP2000 version 6.10.7.4) using the following substrates:

- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)
- benzoxyresorufin (BROD)

Ethoxyresorufin 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 individual control and treated animals using RNeasy Midi kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

4.6.2 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, 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.

Table 5.5-74 The list of Taqman assays used is as follows:

Gene (Major function)	Abbreviation	Refset ID	Taqman assay ID (Applied Biosystems)
METABOLISM: Phase I			
P450 (cytochrome)oxidoreductase	POR	NM_031576.1	Rn00580820_m1
Cytochrome P450 1a1	Cyp1a1	NM_012540.2	Rn00487218_m1
Cytochrome P450 2b1	Cyp2b1	4331248*	Cyp2b1_tc5
Cytochrome P450 3a3	Cyp3a3 (Cyp3a23)	NM_013105.2	Rn01640761_g1
Cytochrome P450 4a1	Cyp4a1	NM_175837.1	Rn00598510_m1
METABOLISM: Phase II			
Glutathione S-transferase A2	Gsta2	NM_017013.4	Rn00566636_m1
Glutathione S-transferase mu3	Gstm4	NM_020540.1	Rn01789233_m1
UDP glucoronosyltransferase 2 family, polypeptide B1	Udpgtr2	NM_173295.1 NM_057105.3	Rn00756519_m1
Epoxide hydrolase 1, microsomal	Ephx1	NM_012844.2	Rn00563349_m1
Epoxide hydrolase 2, cytoplasmic	Ephx2	NM_022936.1	Rn00576023_m1
Sulfotranferase family 1E, member 1	Sult1e1	NM_012883.1	Rn00820646_g1
CELL PROLIFERATION/APOPTOSIS			
Epithelial cell adhesion molecule	Tacstd1	NM_138541.1	Rn01473202_m1
Growth arrest and DNA-damage-inducible 45 beta	Gadd45b	NM_001008321.1	Rn01452530_g1
Retinoblastoma 1	Rb1	XM_344434.3	Rn01753308_m1

*: Transcript made to order

Beta-microglobulin (B2m; Refset ID: NM_012512.1; Taqman 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:

$\Delta\Delta Ct = (Cttest - CtB2m)_{treated} - (Cttest - CtB2m)_{control}$

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. 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 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 phase:

Fluopyram: 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.

Phenobarbital: 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 not to be treatment-related.

Fluopyram: Two females at 1500 ppm exhibited soiled fur around the head region during Week 2 of the recovery phase.

Phenobarbital: 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 study.

B. Body weight and body weight gain

Treatment phase:

Fluopyram: There were no relevant treatment-related changes in any of the body weight parameters compared to the controls during the treatment phase.

Phenobarbital: There were no relevant changes in any of the body weight parameters compared to the controls during the treatment phase.

Recovery phase:

Fluopyram: There were no relevant changes in any of the body weight parameters during the recovery phase compared to the controls.

Phenobarbital: 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 +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 (5g compared to 10g in 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:

Fluopyram: Food consumption was statistically significantly reduced at 1500 ppm during week 3 (p≤0.05) and week 4 (p≤0.01) of treatment. All other changes were considered non-relevant.

Phenobarbital: Food consumption was increased by up to 12% compared to the controls for all periods during the treatment phase. This increase was statistically significant for the first (p≤0.05), second (p≤0.01) and fourth (p≤0.05) week of treatment.

Recovery phase:

Fluopyram: A marginal, though statistically significant reduction (p≤0.05) in food consumption was recorded during the second and third week of the recovery phase.

Phenobarbital: A significant reduction (p≤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 5.5-75 Mean achieved intake of fluopyram

Dose group (ppm)	Mean achieved dietary intake of fluopyram (mg/kg/day) for weeks 1-4
30	2.2
75	5.6
150	11.3
600	44.5
1500	111.4

D. Sacrifice and pathology

1. Terminal body weight and organ weight

Treatment phase:

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls.

At 600 and 1500 ppm, mean absolute and relative liver weight were statistically significantly higher when compared to controls.

At 150 ppm, mean liver to body weight ratio and mean liver to brain weight ratio were statistically significantly higher when compared to controls.

These changes were considered to be treatment-related.

Table 5.5-76 Mean absolute and relative liver weight changes following 28 days treatment with fluopyram

Mean liver weight ± SD at scheduled 3 day sacrifice (% change when compared to controls)						
Dose level (ppm)	Fluopyram					
	0	30	75	150	600	1500
Absolute liver weight (g)	6.14±0.33	6.10±0.26 (-1%)	6.48±0.46 (+6%)	6.64±0.77 (+8%)	6.78±0.49* (+10%)	7.90±0.53** (+29%)
Liver to body weight ratio (%)	2.38±0.16	2.42±0.11 (+2%)	2.49±0.13 (+5%)	2.55±0.11** (+7%)	2.69±0.15** (+13%)	3.16±0.14** (+33%)
Liver to brain weight ratio (%)	319.44 ±31.96	319.41 ±18.44 (0%)	342.46 ±24.12 (+7%)	353.35 ±39.95* (+11%)	368.06 ±28.60** (+15%)	424.55 ±30.85** (+33%)

*** p≤0.05; ** p≤0.01**

The few other organ weight changes were considered to be incidental.

Phenobarbital: There was no change in mean terminal body weight in treated animals when compared to the controls.

Mean absolute and relative liver weight were statistically significantly higher when compared to controls following phenobarbital treatment.

Table 5.5-77 Mean 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	**Phenobarbital**	
	0 ppm	80 mg/kg
Absolute liver weight (g)	6.14 ± 0.53	7.48 ± 1.01** (+22%)
Liver to body weight ratio (%)	2.382 ± 0.160	2.936 ± 0.352** (+23%)
Liver to brain weight ratio (%)	319.44 ± 31.96	412.05 ± 51.53** (+29%)

**: p≤0.01

Recovery phase:

Fluopyram: There was no change in mean terminal 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 in treated animals when compared to the controls.

The few organ weight changes were considered to be incidental.

2. Gross pathology

Treatment phase:

Fluopyram: Enlarged liver 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).

Table 5.5-78 Macroscopic changes in the liver following 28 days treatment with fluopyram

Incidence of macroscopic changes in the liver, scheduled sacrifice						
Dose-level (ppm)	**Fluopyram**					
	0	**30**	**75**	**150**	**600**	**1500**
Enlarged	0/15	0/15	3/15	5/15	4/15	14/15

Phenobarbital: Enlarged livers were noted in 10/15 animals treated with phenobarbital.

Other changes were considered as incidental and not treatment-related.

Recovery phase:

Fluopyram: All changes were considered as incidental and not treatment-related.

Phenobarbital: All changes were considered as incidental and not treatment-related.

3. Microscopic pathology

Treatment phase:

Fluopyram: 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.

Table 5.5-79 Microscopic changes in the liver following 28 days treatment with fluopyram or phenobarbital

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**	**PB (80 mg/kg)**
Number of examined animals	15	15	15	15	15	15	15
Hepatocellular hypertrophy: centrilobular to panlobular							
Minimal	0	0	0	0	6	7	6
Slight	0	0	0	0	0	6	5
Moderate	0	0	0	0	0	1	1
Total	0	0	0	0	6	14	12
Increased number of mitoses							
Present	0	0	0	0	0	0	1
Total	0	0	0	0	0	0	1

Recovery phase:

Fluopyram: All changes were considered as incidental and not treatment-related.

Phenobarbital: All changes were considered as incidental and not treatment-related.

4. Cell proliferation

Treatment phase:

Fluopyram: Centrilobular and global (referred to as "Total" in tables below) proliferation indexes were statistically significantly higher from 150 ppm when compared to controls. Periportal proliferation index was also significantly higher at 1500 ppm when compared to controls. Centrilobular proliferation was statistically significantly increased at 75 ppm.

Phenobarbital: Centrilobular and global proliferation indexes were statistically significantly higher when compared to controls.

Table 5.5-80 Mean cell proliferation index following 28 days treatment with fluopyram or phenobarbital

Compound	Dose	Mean ± SD[A] (% change compared to control mean values)		
		Centrilobular	**Periportal**	**Total**
Control	0	4.93 ± 3.11	8.37 ± 4.75	6.65 ± 3.19
Fluopyram	30 ppm	4.23 ± 2.42 (-14%)	7.62 ± 3.66 (-9%)	5.93 ± 2.82 (-11%)
	75 ppm	7.23 ± 3.55* (+47%)	8.51 ± 3.86 (+2%)	7.87 ± 2.65 (+18%)
	150 ppm	10.16 ± 3.86** (+106%)	12.51 ± 3.97* (+49%)	11.33 ± 3.30** (+70%)
	600 ppm	10.14 ± 5.27** (+106%)	12.10 ± 8.36 (+45%)	11.12 ± 6.50** (+67%)
	1500 ppm	15.54 ± 7.33** (+215%)	22.80 ± 10.49** (+172%)	19.17 ± 7.20** (+188%)
Phenobarbital	80 mg/kg	21.46 ± 17.90** (+335%)	10.28 ± 7.24 (+23%)	15.87 ± 11.74** (+139%)

A: n = 15 females/group for cell proliferation determinations; *: p≤0.05; **: p≤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.

Phenobarbital: Centrilobular, periportal and global proliferation indexes were significantly higher when compared to the control group.

Table 5.5-81 Mean cell proliferation index following Recovery Phase

Group	Mean ± SD[A]		
	Centrilobular	Periportal	Total
Control	4.59 ± 2.44	8.25 ± 4.60	6.42 ± 3.29
1500 ppm	8.30 ± 3.75** (+81%)	11.12 ± 6.87 (+35%)	9.71 ± 4.77** (+51%)
PB (80 mg/kg)	6.91 ± 3.44* (+51%)	16.92 ± 9.83** (+105%)	11.92 ± 6.47** (+86%)

A: n = 15 females/group for cell proliferation determinations with the exception of the control group where n=14 instead of 15; *: p≤0.05; **: p≤0.01. Differences in data between table above and in Appendix K are due to rounding up.

5. Hepatotoxicity testing

Treatment phase:

Fluopyram: A marginal, though not statistically significant increase in total P450 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≤0.01) 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 considered 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 cytochrome P-450 content and enzymatic activities in the liver following 28 days treatment with fluopyram or phenobarbital

Fluopyram dose	Mean Fold Change Relative to Controls					
	Total P450 Content	EROD	BROD	PROD	UDPGT-4-nitrophenol	UDPGT-bilirubin[A]
30 ppm	0.94	1.13	1.24	0.92	0.82	1.09
75 ppm	0.97	1.08	1.44	1.27	0.95	1.21
150 ppm	1.24	1.34**	2.89	1.53	1.08	1.57**
600 ppm	1.05	1.31*	9.12**	1.87*	1.83**	2.14**
1500 ppm	1.42	1.96**	39.09**	4.76**	3.21**	2.76**
Phenobarbital 80 mg/kg	1.51**	1.07	87.4**	8.44*	1.81**	1.38*

a: n=4 instead of 5 for the control group due to one outlier animal. *: p≤0.05; **: p≤0.01

Recovery phase:

Fluopyram: EROD (p≤0.05), BROD (p≤0.05), PROD (p≤0.01) and UDPGT-bilirubin (p≤0.05) activity were statistically significantly increased following 1 month on control diet; however the increases were much lower than those recorded immediately following treatment.

Phenobarbital: PROD and UDPGT-bilirubin activity were statistically significantly increased (p≤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≤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 activities in the liver following Recovery Phase

Dose	Mean fold change relative to controls					
	Total P450 Content	EROD	BROD	PROD	UDPGT-4-nitrophenol	UDPGT-bilirubin
Fluopyram 1500 ppm	1.03	1.21*	1.47*	1.54**	1.01	1.39*
Phenobarbital 80 mg/kg/day	1.01	1.28**	0.85	1.93**	0.98	1.56**

*: p≤0.05; **: p≤0.01

6. Gene Transcript Analyses

Treatment phase:

Fluopyram: At 30 ppm, a clear and statistically significant (p≤0.01) dose-related increase in the expression of Cyp3a3 was recorded (+81% at 30 ppm up to +4943% at 1500 ppm).

From 150 ppm, clear dose-related and statistically significant (p≤0.01) increases were recorded 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≤0.01) increase was recorded for the expression of genes coding for the Phase II enzymes Gstm4 (+136% at 150 ppm up to 1289% at 1500 ppm) and Udpgtr2 (+64% at 150 ppm up to +304% at 1500 ppm).

From 600 ppm, genes coding for additional Phase II enzymes were statistically significantly increased. Specifically, Gsta2 expression was increased by 53% (p≤0.05) at this dose and Ephx1 expression was increased by +116% (p≤0.01). A marginal, though statistically significant (+75%; p≤0.01), increased expression of the marker for cell proliferation (Gadd 45b) was also observed.

Finally, the expression of Ephx2 demonstrated a weak but dose-related decrease (-3%, -10%, -13%, 27% at 30, 75, 150 and 600 ppm respectively), which was statistically significant at 1500 ppm (-43%; p≤0.01).

There were no clear effects on the expression of POR, Cyp4a1, Tacstd1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which was greater than or equal to the cut-off limit of 35 for the majority of samples (59/90 control and fluopyram treated liver samples).

Table 5.5-84 Mean gene transcript analyses following 28 days treatment with fluopyram

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 ppm	1500 ppm
METABOLISM: Phase I						
POR	0.78 ± 0.34	0.70 ± 0.29 (-10%)	0.67 ± 0.29 (-14%)	0.77 ± 0.27 (NC)	0.81 ± 0.24 (+4%)	0.89 ± 0.30 (+14%)
Cyp1a1	1.06 ± 0.70	1.87 ± 1.25 (+76%)	2.43 ± 1.48 (+129%)	8.60 ± 7.02** (+711%)	107.0 ± 28.03** (+999%)	376.0 ± 130.43** (+35372%)
Cyp2b1	1.26 ± 1.15	3.38 ± 7.17 (+168%)	2.09 ± 1.91 (+66%)	13.74 ± 10.57** (+990%)	267.69 ± 193.39** (+21145%)	1945.13 ± 1518.28** (+154275%)
Cyp3a3	1.66 ± 0.65	3.01 ± 1.34** (+81%)	6.19 ± 2.66** (+273%)	8.72 ± 2.69** (+425%)	28.37 ± 7.22** (1609%)	83.72 ± 27.02** (+4943%)
Cyp4a1	0.78 ± 0.30	0.65 ± 0.25 (-17%)	0.69 ± 0.20 (-12%)	0.77 ± 0.16 (NC)	0.63 ± 0.18 (-19%)	0.55 ± 0.08 (-30%)
METABOLISM: Phase II						
Gsta2	3.62 ± 2.63	3.76 ± 2.19 (+4%)	3.43 ± 2.24 (-5%)	3.28 ± 2.37 (-9%)	5.54 ± 2.54* (+53%)	10.85 ± 6.21** (+200%)
Gstm4	0.56 ± 0.39	0.60 ± 0.36 (+7%)	0.92 ± 0.62 (+64%)	1.32 ± 0.48** (+136%)	3.42 ± 2.82** (+511%)	7.78 ± 6.64** (+1289%)
Udpgtr2	0.90 ± 0.40	1.08 ± 0.40 (+20%)	1.04 ± 0.58 (+16%)	1.48 ± 0.51** (+64%)	2.29 ± 0.92** (+154%)	3.64 ± 1.51** (+304%)
Ephx1	1.06 ± 0.22	1.34 ± 0.60 (+26%)	1.18 ± 0.31 (+11%)	1.49 ± 0.35 (+41%)	2.29 ± 0.58** (+116%)	4.49 ± 2.04** (+324%)
Ephx2	1.73 ± 0.64	1.67 ± 0.64 (-3%)	1.55 ± 0.74 (-10%)	1.51 ± 0.66 (-13%)	1.27 ± 0.54 (-27%)	0.98 ± 0.29** (-43%)
Sult1e1	ND	ND	ND	ND	ND	ND
CELL PROLIFERATION/APOPTOSIS						
Tacstd1	1.09 ± 0.31	1.30 ± 0.38 (+19%)	1.16 ± 0.23 (+6%)	1.35 ± 0.25 (+24%)	1.23 ± 0.28 (+13%)	1.35 ± 0.32 (+24%)
Gadd45b	0.79 ± 0.23	0.85 ± 0.44 (+8%)	0.76 ± 0.20 (-4%)	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)	1.10 ± 0.19 (-4%)	1.21 ± 0.18 (+5%)	1.16 ± 0.19 (NC)	1.16 ± 0.23 (NC)

NC: no change; *: $p \leq 0.05$; **: $p \leq 0.01$; ND: considered as not detected due to the variable and high Ct values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (59/90 control and fluopyram treated liver samples). 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: 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 (Gstm4 and Gsta2), Ephx1 and Udpgt were all statistically significantly increased. In contrast there was a statistically significant ($p \leq 0.01$) 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).

Table 5.5-85 Mean gene transcript analyses following 28 days treatment with phenobarbital

Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (28 day treatment) (% change compared to control mean values)	
	Control	Phenobarbital (80 mg/kg/day)
METABOLISM: Phase I		
POR	0.78 ± 0.34	0.60 ± 0.25 (-23%)
Cyp1a1	1.06 ± 0.70	0.80 ± 0.41 (-25%)
Cyp2b1	1.26 ± 1.15	2930.45 ± 2678.47** (+232475%)
Cyp3a3	1.66 ± 0.65	54.21 ± 43.44** (+32%)
Cyp4a1	0.78 ± 0.30	0.41 ± 0.08** (-47%)
METABOLISM: Phase II		
Gsta2	3.62 ± 2.69	6.33 ± 4.30* (+75%)
Gstm4	0.56 ± 0.39	13.85 ± 11.39** (+2373%)
Udpgtr2	0.90 ± 0.40	3.92 ± 2.66** (+336%)
Ephx1	1.06 ± 0.22	3.67 ± 1.77** (+246%)
Ephx2	1.73 ± 0.64	0.95 ± 0.27** (-45%)
Sult1e1	ND	ND
CELL PROLIFERATION/APOPTOSIS		
Tacstd1	1.09 ± 0.31	1.16 ± 042 (+6%)
Gadd45b	0.79 ± 0.23	1.33 ± 0.59** (+68%)
Rb1	1.15 ± 0.26	1.28 ± 0.23 (+11%)

NC: no change; *: p≤0.05; **: p≤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 of 35 for the majority of samples (19/30 for controls and phenobarbital). In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Recovery phase:

Fluopyram: The expression of genes coding for the Phase I enzymes Cyp1a1 (+83%), Cyp2b1 (+70%) and Cyp3a3 (+157%; p≤0.01) and the Phase II enzyme Gstm4 (+73%; p≤0.05) were still increased compared to the controls. Gadd45b (+42%; p≤0.01) and Rb1 (+13%; p≤0.05) were also statistically significantly increased compared to the controls. In all cases however; the % changes were much lower than those recorded immediately following treatment.

Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which were greater than the cut-off limit of 35 for the majority of samples (25/30 control and fluopyram treated liver samples).

Table 5.5-86 Mean gene transcript analyses following Recovery Phase in fluopyram treated group

Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (Recovery Phase) (% change compared to control mean values)	
	Control	1500 ppm
METABOLISM: Phase I		
POR	0.54 ± 0.24	0.62 ± 0.18 (+15%)
Cyp1a1	0.72 ± 0.23	1.32 ± 1.11 (+83%)
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	0.76 ± 0.14 (+12%)
METABOLISM: Phase II		
Gsta2	1.55 ± 1.37	1.67 ± 1.12 (+8%)
Gstm4	0.74 ± 0.48	1.28 ± 0.95* (+73%)
Udpgtr2	0.83 ± 0.33	0.65 ± 0.25 (-22%)
Ephx1	0.80 ± 0.19	0.86 ± 0.21 (+8%)
Ephx2	0.85 ± 0.26	1.11 ± 0.29* (+31%)
Sult1e1	ND	ND
CELL PROLIFERATION/APOPTOSIS		
Tacstd1	1.21 ± 0.29	1.31 ± 0.27 (+8%)
Gadd45b	1.38 ± 0.42	1.96 ± 0.57** (+42%)
Rb1	1.02 ± 0.14	1.15 ± 0.17* (+13%)

NC: no change; *: p≤0.05; **: p≤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 of 35 for the majority of samples (25/30 for controls and fluopyram). 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: The expression of genes coding for the Phase I enzymes Cyp2b1 (+107%; p≤0.01), Cyp3a3 (+179%; p≤0.01) and Cyp4a1 (+46%; p≤0.01) and the Phase II enzyme Gstm4 (+181%; p≤0.01) were still increased compared to the controls. Ephx1 (+21%; p≤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.

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 (24/30 control and phenobarbital treated liver samples).

Table 5.5-87: Mean gene transcript analyses following Recovery Phase in phenobarbital treated group

Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (Recovery Phase) (% change compared to control mean values)	
	Control	Phenobarbital (80 mg/kg/day)
METABOLISM: Phase I		
POR	0.54 ± 0.24	0.76 ± 0.23* (+41%)
Cyp1a1	0.72 ± 0.23	0.87 ± 0.45 (21%)
Cyp2b1	0.30 ± 0.24	0.62 ± 0.46** (+107%)
Cyp3a3	2.67 ± 1.61	7.46 ± 5.02** (+179%)
Cyp4a1	0.68 ± 0.29	0.99 ± 0.29** (+46%)
METABOLISM: Phase II		
Gsta2	1.55 ± 1.37	1.98 ± 2.11 (+28%)
Gstm4	0.74 ± 0.48	2.08 ± 1.22** (+181%)
Udpgtr2	0.83 ± 0.33	0.69 ± 0.38 (-17%)
Ephx1	0.80 ± 0.19	0.97 ± 0.22* (+21%)
Ephx2	0.85 ± 0.26	1.03 ± 0.40 (+21%)
Sult1e1	ND	ND
CELL PROLIFERATION/APOPTOSIS		
Tacstd1	1.21 ± 0.29	1.20 ± 0.26 (NC)
Gadd45b	1.38 ± 0.42	1.57 ± 0.41 (+14%)
Rb1	1.02 ± 0.14	1.16 ± 0.22 (+14%)

NC: no change; *: $p \leq 0.05$; **: $p \leq 0.01$; ND: considered as not detected due to the variable and high Ct values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (24/30 for controls and phenobarbital). 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, 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 hepatic 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.

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 hepatic 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 5.5/07
Report Author:	[redacted]
Report Year:	2013
Report Title:	Fluopyram: Enzyme and DNA-synthesis induction in cultured human hepatocytes
Report No:	CXR1241
Document No:	M-450156-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evaluated
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

• Female human 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 µM with a slight decrease followed by a marked decrease at 300 µM fluopyram was observed. This was indicative of cytotoxicity. Fluopyram was not cytotoxic at 30 µM.

• 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 human hepatocytes with fluopyram resulted in an increase in pentoxyresorufin-*O*-depentylation (PROD) activity of up to a maximum of 1.9–fold at 100 µM. This indicates that fluopyram is an inducer of CYP2B.

• Culturing primary human hepatocytes with fluopyram resulted in an increase in benzyloxyresorufin-*O*-debenzylation (BROD) activity, up to a maximum 2–fold at 300 µM. This indicates that fluopyram is an inducer of CYP2B/CYP3A.

• Culturing primary human hepatocytes with fluopyram resulted in an increase in benzyloxyquinoline-*O*-debenzylation (BQ) activity up to a maximum of 1.8–fold at 10 µM. This indicates that fluopyram is an inducer 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

1. Test material:	Fluopyram (AE C656948)
Description	Beige powder
Lot / Batch #:	EDFL013235
Purity:	98.7%
CAS #	658066-35-4
Stability of test compound:	Stable in 0.1% (v/v), 1 µL Dimethyl sulfoxide (DMSO)/mL medium, prepared daily
2. Vehicle and / or positive control:	Phenobarbital sodium salt (PB), positive control
Lot / Batch #:	P-5178
Supplier:	Sigma Chemicals
CAS #	50-06-6
Stability of test compound:	Stable in 0.1% (v/v), 1 µL Dimethyl sulfoxide (DMSO)/mL medium, prepared daily
3. Vehicle and / or positive control:	Epidermal growth factor (EGF), positive control
Lot / Batch #:	E-9644
Supplier:	Sigma Chemicals
Stability of test compound:	Stable in 0.1% (v/v), 1 µL Dimethyl sulfoxide (DMSO)/mL medium, prepared daily
4. Test system:	
Species:	Primary female human hepatocytes, plateable cryopreserved
Viability:	In excess of 70% (one donor was used)
Source:	Invitrogen, 7 Kingsland Grange, Warrington, Cheshire, UK

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 human hepatocytes. Stimulation of CYP2B activity, CYP3A activity and cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) were determined in primary cultures of isolated female human hepatocytes. Phenobarbital (PB) and epidermal growth factor (EGF) were included as positive control reference items for induction of CYP2B/3A activities (phenobarbital only) and cell proliferation.

Table 5.5-88 Study design

Group #	Treatment	BrdU for Sphase	CYP Enzyme Activity	ATP
1	Control	✓	✓	✓
2	PB 10µM	✓	✓	✓
3	PB 100µM	✓	✓	✓
4	PB 1000µM	✓	✓	✓
5	Fluopyram 1µM	✓	✓	✓
6	Fluopyram 3µM	✓	✓	✓
7	Fluopyram 10µM	✓	✓	✓
8	Fluopyram 30µM	✓	✓	✓

Group #	Treatment	BrdU for Sphase	CYP Enzyme Activity	ATP
9	Fluopyram 100µM	✓	✓	✓
10	Fluopyram 300µM	✓	✓	✓
11	EGF (+ve control BrdU)	✓	-	

3. Statistics

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 collagen coated 25 cm2 flasks, 96- and 6-well 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 Cryopreserved Hepatocyte Recovery Medium (CHRM®) then cultured in Cryopreserved Hepatocyte Plating Medium (CHPM) for approximately 6 h to allow adherence. Following a visual check for adhesion, the medium was then changed to Leibowitz HCL15 (Laboratory Method Sheet (LMS) Tic-002) medium and the hepatocytes were exposed to PB at 3 concentrations (10, 100 and 1000 µM), to fluopyram 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 or reference items, was replenished daily for a further 3 days. 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% (v/v), 1 µL DMSO/mL medium.

2. Assays

After 96 hours in culture, hepatocytes were fixed or harvested by scraping them into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH7.4), sonicating the mixture and storing it at approximately –70oC until analysis. Protein was determined by the method of Lowry *et al* (1951) J. Biol. Chem. 193, 265-275, (LMS Spec-001).

2.1 Adenosine 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 luminescent cell viability assay).

Results are expressed as a percentage 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. Immunostaining 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.

2.3 Pentoxyresorufin-*O*-dependentylation (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, 18:3337-3345, 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 spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline as described by GENTEST HTS technical bulletin, according to LMS Fluor-005.

II. Results and discussion

1. Adenosine 5'-Triphosphate (ATP)

Fluopyram (1 - 30 µM) had little, or no effect on cellular ATP concentrations after 96 hours. However, at a concentration of 100 µM a slight decrease in in ATP concentration was observed whilst at 300 µM a marked decrease in ATP concentration was observed, indicating the start of cytotoxicity at 100 µM, which was clearly evident at 300 µM.

Table 5.5-89 Adenosine 5'-Triphosphate

Treatment	ATP (% Control)
Vehicle Control	100.0 ± 8.7[a]
PB 10 µM	110.0 ± 5.2*
PB 100 µM	103.4 ± 7.1
PB 1000 µM	110.8 ± 9.5
Fluopyram 1 µM	108.9 ± 1.6*
Fluopyram 3 µM	113.0 ± 6.3*
Fluopyram 10 µM	111.5 ± 11.5
Fluopyram 30 µM	126.3 ± 8.6***
Fluopyram 100 µM	91.9 ± 6.9
Fluopyram 300 µM	29.1 ± 1.4***

[a] Values are mean % control ± SD, n = 6 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

Figure 5.5-1 Adenosine 5'-Triphosphate

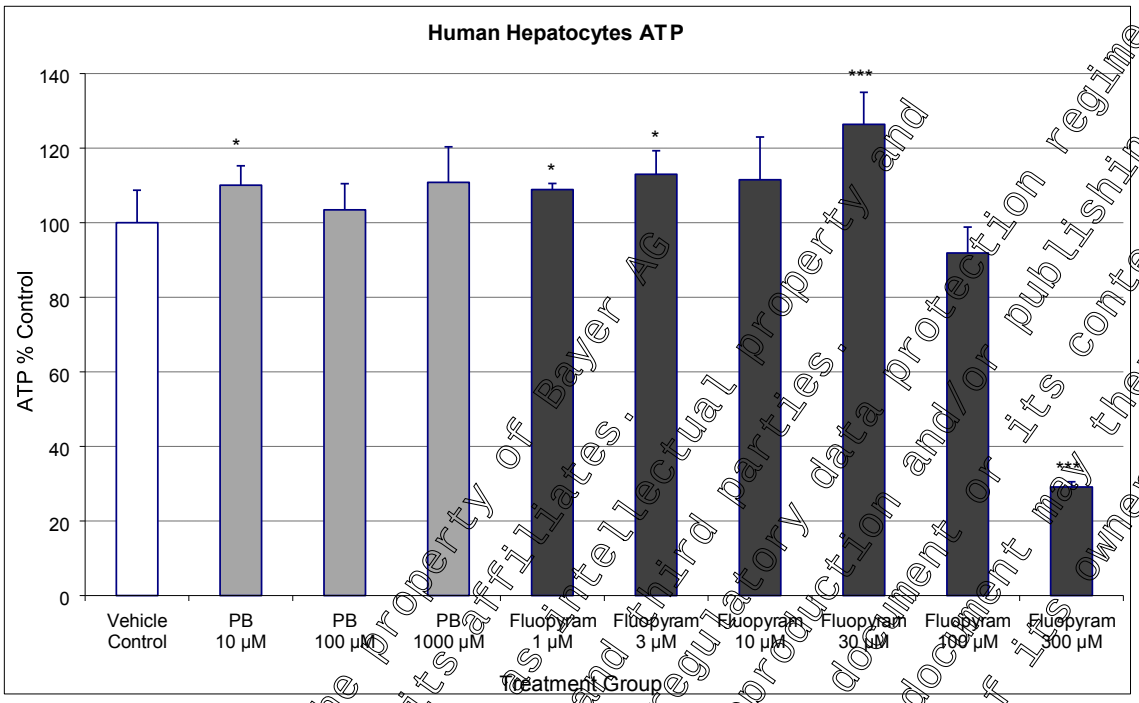
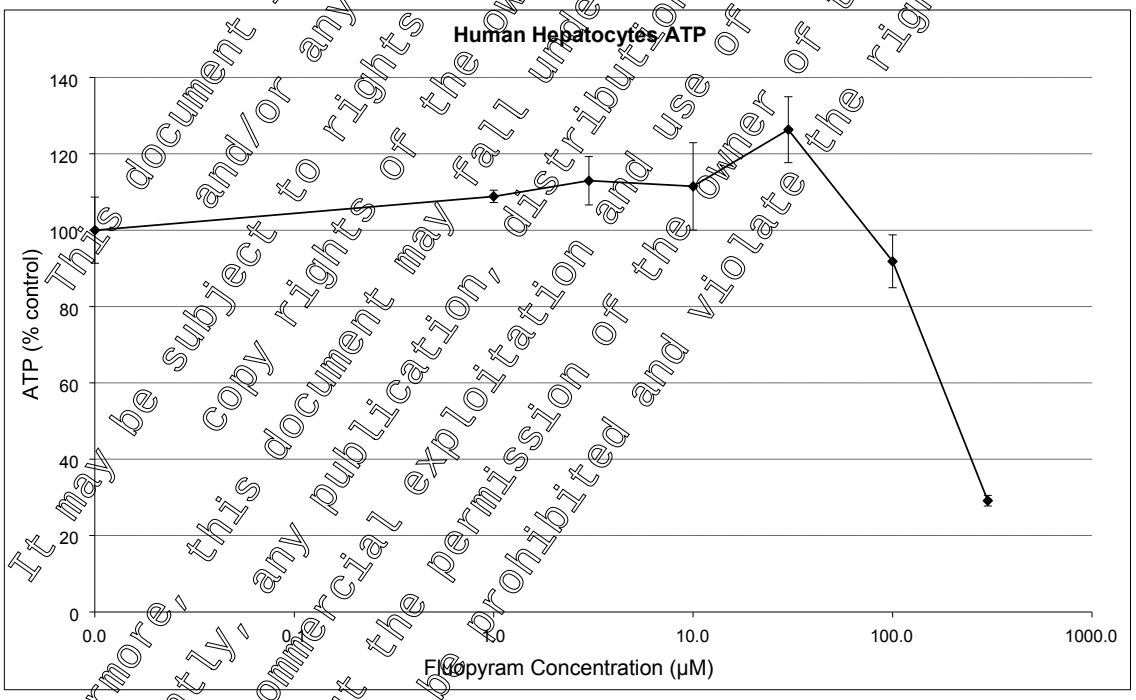


Figure 5.5-2 Adenosine 5'-Triphosphate, Fluopyram Dose Response



2. Replicative DNA Synthesis (S-Phase)

Treatment with 25ng/mL EGF 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.

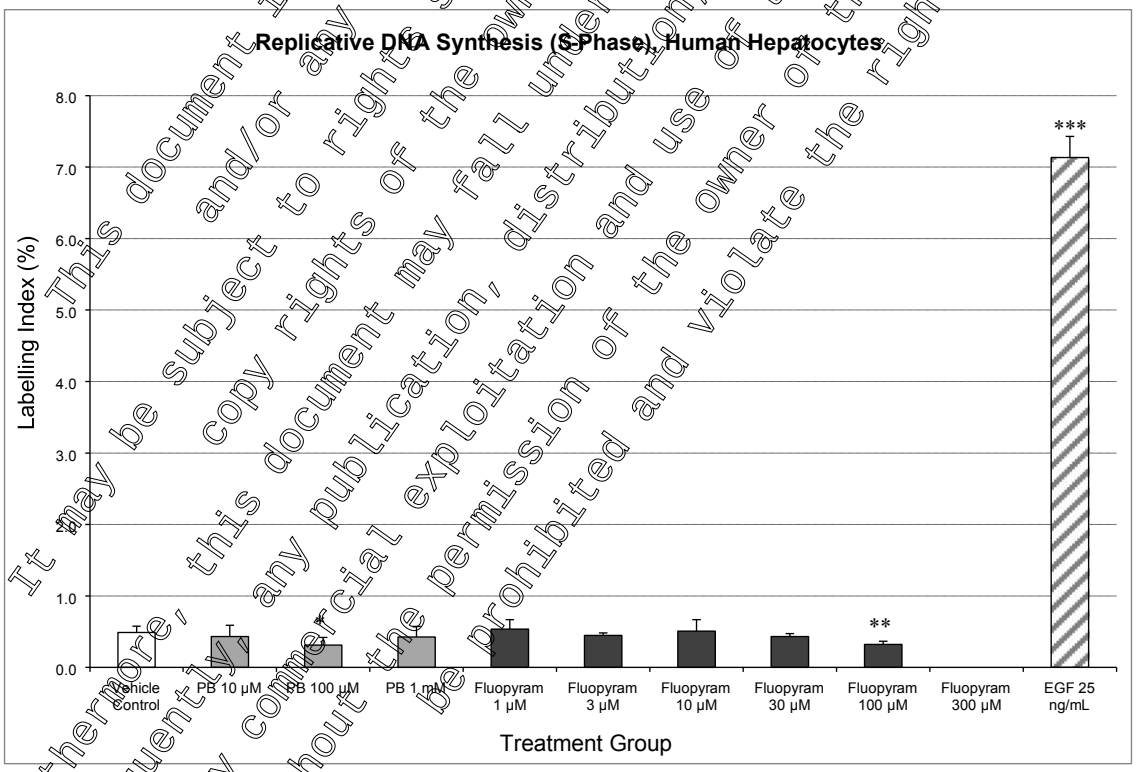
No biologically significant changes in replicative DNA synthesis were observed following treatment with either PB (10 – 1000 µM) or fluopyram (1 - 30 µM). However, at a concentration of 100 µM fluopyram, there was a decrease in hepatocyte confluency, with fewer adherent hepatocytes present, possibly indicating the beginning of dose-dependent toxicity. Analysis of the cells exposed to 300 µM fluopyram was precluded due to extensive cytotoxicity.

Table 5.5-90 Replicative DNA Synthesis

Treatment	Labelling Index (%)
Vehicle Control	0.49 ± 0.09 (100.0 ± 18.5)[a]
PB 10 µM	0.43 ± 0.16 (88.4 ± 32.4)
PB 100 µM	0.31 ± 0.11* (63.8 ± 22.2)
PB 1000 µM	0.43 ± 0.15 (87.4 ± 30.3)
Fluopyram 1 µM	0.54 ± 0.13 (109.9 ± 27.2)
Fluopyram 3 µM	0.45 ± 0.03 (91.7 ± 7.1)
Fluopyram 10 µM	0.51 ± 0.16 (103.7 ± 33.2)
Fluopyram 30 µM	0.43 ± 0.04 (88.7 ± 7.9)
Fluopyram 100 µM	0.32 ± 0.04** (66.1 ± 9.1) [b]
Fluopyram 300 µM	[c]
EGF 25ng/mL	7.13 ± 0.30*** (1461.4 ± 60.7)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD n = 5 per group. [b] Decrease in hepatocyte confluency, fewer adherent hepatocytes present. [c] Analysis unable to be performed due to excessive cytotoxicity. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

Figure 5.5-3 Replicative DNA Synthesis



3. Pentoxyresorufin-*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 µM. This is consistent with historical data.

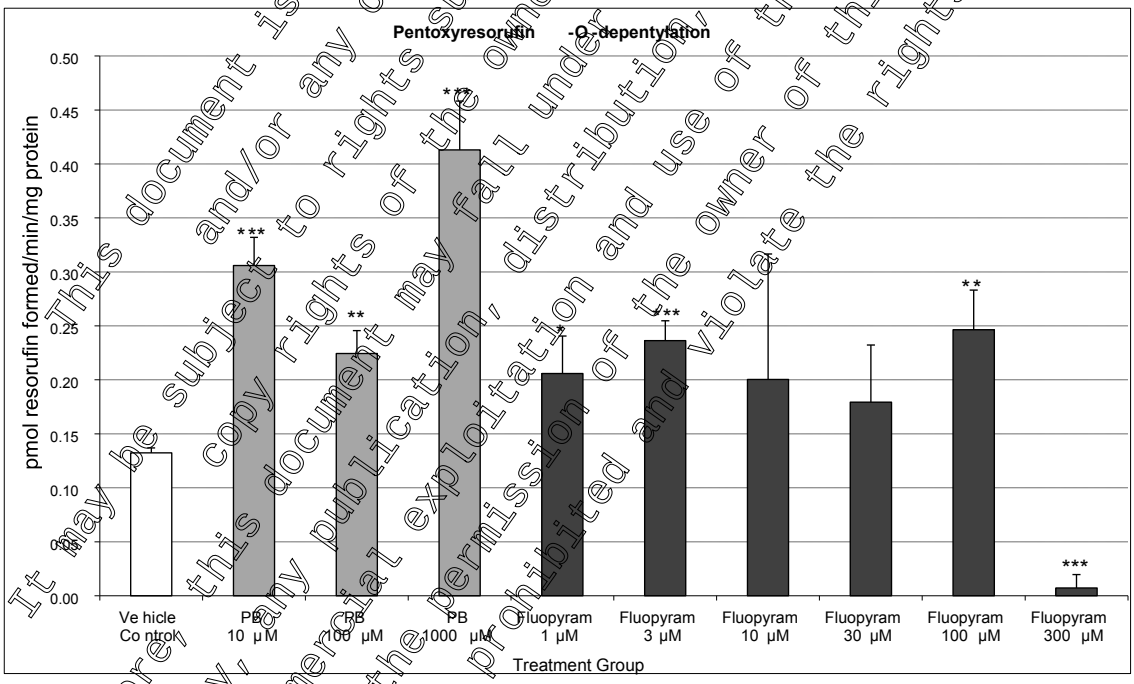
Addition of fluopyram to the hepatocytes resulted in increases in PROD activity to a maximum 1.9 fold of control value at 100 µM. Treatment with 300 µM fluopyram reduced PROD activity to 5% of the control value, reflecting the cytotoxicity shown by the ATP and S-phase results (Table 4 and Figure 4). Clear dose-dependent induction by fluopyram was not seen, suggesting that maximal effects were seen at the low concentrations of fluopyram.

Table 5.91 Pentoxyresorufin-*O*-Depentylation

Treatment	PROD (pmol resorufin formed/min/mg protein)
Vehicle Control	0.132 ± 0.005 (100.0 ± 3.5)[a]
PB 10 µM	0.306 ± 0.026*** (231.1 ± 19.8)
PB 100 µM	0.224 ± 0.021** (169.6 ± 16.0)
PB 1000 µM	0.413 ± 0.045*** (312.0 ± 34.2)
Fluopyram 1 µM	0.206 ± 0.035* (155.6 ± 26.2)
Fluopyram 3 µM	0.236 ± 0.018*** (178.6 ± 13.9)
Fluopyram 10 µM	0.200 ± 0.116 (151.4 ± 87.7)
Fluopyram 30 µM	0.179 ± 0.053 (135.5 ± 40.0)
Fluopyram 100 µM	0.246 ± 0.037** (186.1 ± 27.8)
Fluopyram 300 µM	0.007 ± 0.012*** (5.4 ± 9.4)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 3 per group. A Student's t-test (2sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

Figure 5.5-4 Pentoxyresorufin-*O*-Depentylation



4. Benzyloxyresorufin-*O*-Debenzylation (BROD)

Culturing primary human hepatocytes, for 96 hours, with phenobarbital resulted in increases in BROD activity up to a maximum 4-fold increase at 1000 µM. This is consistent with historical data.

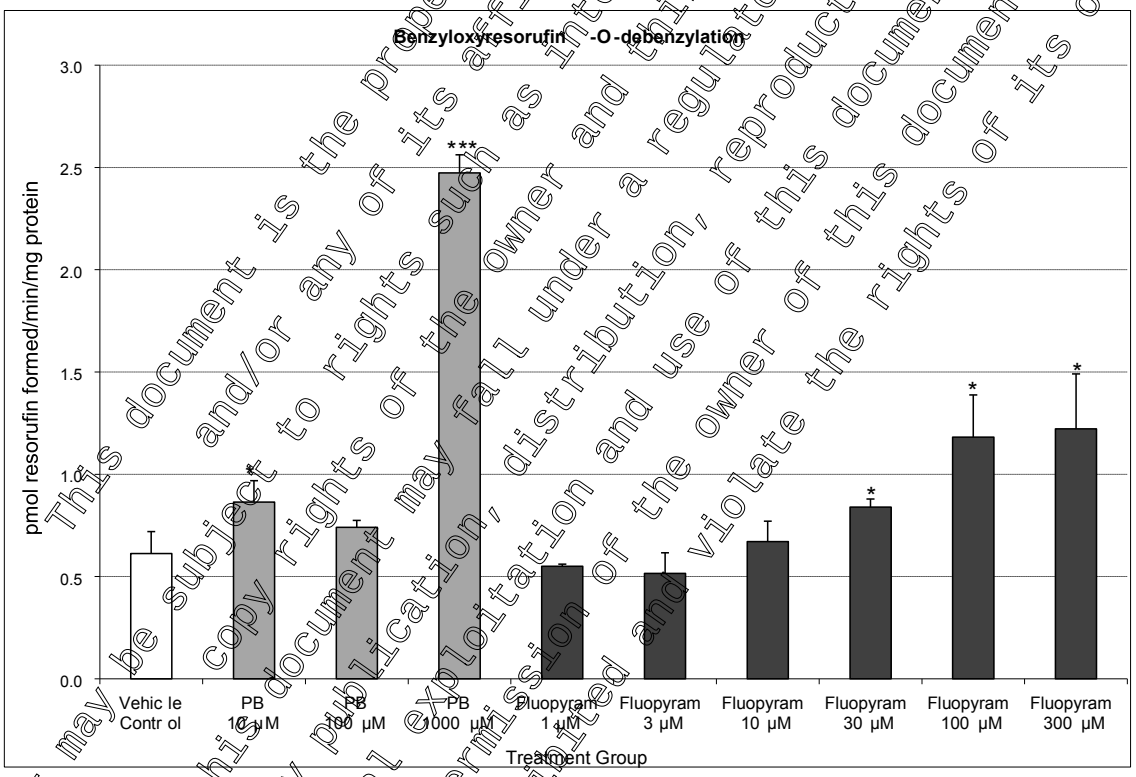
Culturing hepatocytes with fluopyram resulted in a dose-dependent increase in BROD activity, up to a maximum increase of 2-fold at 300 µM. This level of induction could be affected by the cytotoxicity shown by the ATP and S-phase and PROD results.

Table 5.5-92 Benzyloxyresorufin-*O*-Debenzylation

Treatment	BROD (pmol resorufin formed/min/mg protein)
Vehicle Control	0.612 ± 0.107 (100.0 ± 17.5)[a]
PB 10 µM	0.865 ± 0.105* (141.2 ± 17.1)
PB 100 µM	0.741 ± 0.033 (121.0 ± 5.5)
PB 1000 µM	2.473 ± 0.089*** (403.8 ± 14.5)
Fluopyram 1 µM	0.550 ± 0.011 (89.9 ± 1.8)
Fluopyram 3 µM	0.515 ± 0.102 (84.1 ± 16.6)
Fluopyram 10 µM	0.671 ± 0.100 (109.5±)16.4
Fluopyram 30 µM	0.840 ± 0.040* (137.1 ± 6.5)
Fluopyram 100 µM	1.182 ± 0.206* (193.0 ± 33.6)
Fluopyram 300 µM	1.222 ± 0.269* (199.5 ± 43.9)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 3 per group. A Student's t-test (2sided) was performed on the results; *statistically different from control $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Figure 5.5-5 Benzyloxyresorufin-*O*-Debenzylation



5. Benzyloxyquinoline-*O*-Debenzylation (BQ)

Culturing primary human hepatocytes, for 96 hours, with phenobarbital resulted in a dosedependent increase in BQ activity of up to 5.2-fold at 1000 µM. This is consistent with historical data.

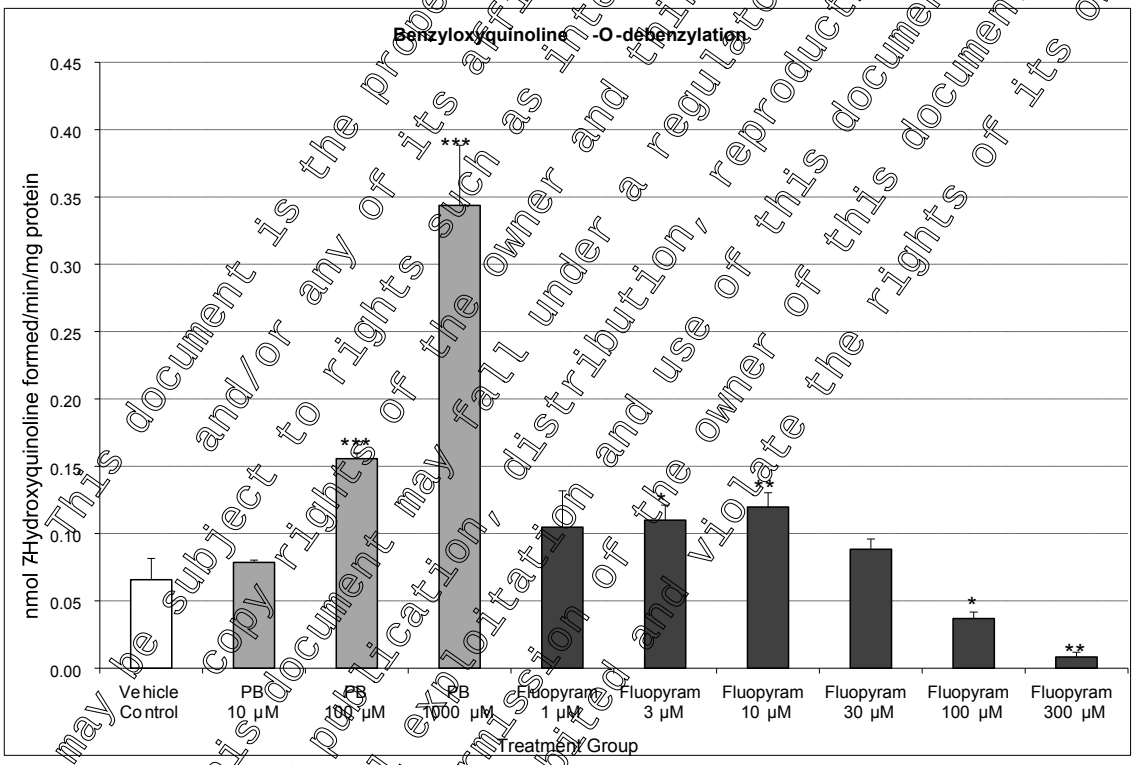
Exposure of hepatocytes to fluopyram resulted in an increase in benzyloxyquinoline-*O*debenzylation (BQ) activity up to a maximum of 1.8–fold at 10 µM. This indicates that fluopyram is an inducer of CYP3A at low concentrations. Higher concentrations led to decreases in activity, with cytotoxicity commencing at 100 µM and clearly evident at 300 µM.

Table 5.5-93 Benzyloxyquinoline-*O*-Debenzylation

Treatment	BQ (nmol 7-Hydroxyquinoline formed/min/mg protein)
Vehicle Control	0.066 ± 0.016 (100.0 ± 24.2)[a]
PB 10 µM	0.079 ± 0.002 (119.6 ± 2.5)
PB 100 µM	0.156 ± 0.004*** (236.8 ± 6.5)
PB 1000 µM	0.344 ± 0.045*** (523.3 ± 67.9)
Fluopyram 1 µM	0.105 ± 0.027 (159.4 ± 41.1)
Fluopyram 3 µM	0.110 ± 0.011* (167.5 ± 16.9)
Fluopyram 10 µM	0.120 ± 0.011** (182.2 ± 16.1)
Fluopyram 30 µM	0.088 ± 0.008 (134.4 ± 11.6)
Fluopyram 100 µM	0.037 ± 0.005* (56.2 ± 7.2)
Fluopyram 300 µM	0.008 ± 0.003** (12.7 ± 5.3)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n= 3 per group. A Student's t-test (2sided) was performed on the results; *statistically different from control p<0.05; **p<0.01; ***p<0.001.

Figure 5.5-6 Benzyloxyquinoline-*O*-Debenzylation



A. Deficiencies

No specific deficiencies were noted in the study.

III. Conclusions

Fluopyram and phenobarbital appeared to exhibit similar properties in human hepatocytes, inducing cytochromes P450 *via* the pregnane x receptor (PXR) and the constitutive androstane receptor (CAR). The increases in prod activity 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.

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 no 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 mechanistic information on the effects and target organs of fluopyram in the rat.

Fluopyram and phenobarbital appeared to exhibit similar properties in human hepatocytes, inducing cytochromes P450 *via* the pregnane x receptor (PXR) and the constitutive androstane receptor (CAR). The increases in PROD activity 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.
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 no compound-stimulated DNA synthesis in human hepatocytes.

Data Point:	KCA 5.5.08
Report Author:	[redacted]
Report Year:	2013
Report Title:	Fluopyram: Enzyme and DNA-synthesis induction in cultured rat hepatocytes
Report No:	CXR1242
Document No:	M-450157-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evaluated
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

• Female rat hepatocytes were cultured in the presence of the test item fluopyram, and phenobarbital or EGF as two positive control compounds.

• A decrease in ATP concentration at 300 µM fluopyram was observed. This was indicative of cytotoxicity. Fluopyram was not cytotoxic at 100 µM.

• Culturing primary female rat hepatocytes with fluopyram stimulated replicative DNA synthesis in a dose-dependent manner. At 100 µM, 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 µM).

• Culturing primary rat hepatocytes with fluopyram resulted in an increase in pentoxyresorufin-*O*-dependentylation (PROD) activity of up to a maximum of 2.8–fold at 30 µM. This indicates that fluopyram is an inducer of CYP2B.

• Culturing primary rat hepatocytes with fluopyram resulted in an increase in benzyloxyresorufin-*O*-debenzylation (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 µM. This 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 (AE C656948)
Description	Beige powder
Lot / Batch #:	EDFL013235
Purity:	98.7%
CAS #	658066-35-4
Stability of test compound:	Stable in 0.1% (v/v), 1 µL Dimethyl sulfoxide (DMSO)/mL medium, prepared daily
2. Vehicle and / or positive control:	Phenobarbital sodium salt (PB), positive control
Lot / Batch #:	P-5178
Supplier:	Sigma Chemicals
CAS #	50-06-6
Stability of test compound:	Stable in 0.1% (v/v), 1 µL Dimethyl sulfoxide (DMSO)/mL medium, prepared daily
3. Vehicle and / or positive control:	Epidermal growth factor (EGF), positive control
Lot / Batch #:	E-9644
Supplier:	Sigma Chemicals
Stability of test compound:	Stable in 0.1% (v/v), 1 µL Dimethyl sulfoxide (DMSO)/mL medium, prepared daily
4. Test animals:	
Species:	Rat – (Female only)
Strain:	Han Wistar (out-bred HsdHanTM: WIST)
Age:	8 weeks approximately at start of treatment
Weight at dosing:	150 to 200 g at arrival
Source:	Harlan UK Ltd, Shaw's Farm, Blackthorn, Oxon, UK
Acclimation period:	At least 5 days
Diet:	RM1pelleted diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were housed in groups on saw-dust in solid-bottom, polypropylene cages.
Environmental conditions:	
Temperature:	19-23°C
Humidity:	40-70%
Air changes:	14-15 air changes per hour
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, CYP3A activity and cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) 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 CYP2B/3A activities (phenobarbital only) and cell proliferation.

Table 5.5-94 Study design

Group #	Treatment	BrdU for S-phase	CYP Enzyme Activity	ATP
1	Control	✓	✓	✓
2	PB 10µM	✓	✓	✓
3	PB 100µM	✓	✓	✓
4	PB 1000µM	✓	✓	✓
5	Fluopyram 1µM	✓	✓	✓
6	Fluopyram 3µM	✓	✓	✓
7	Fluopyram 10µM	✓	✓	✓
8	Fluopyram 30µM	✓	✓	✓
9	Fluopyram 100µM	✓	✓	✓
10	Fluopyram 300µM	✓	✓	✓
11	EGF (+ve control BrdU)	✓	-	-

3. Statistics

Statistical comparisons between treated hepatocytes and their control group were undertaken for all numerical data sets using a 2-tailed Student's t-test.

A. Methods

1. Hepatocyte Isolation

Rats were terminally anaesthetised, using euthatal, 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 trypan 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 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 cultured in Leibowitz CL15 (Laboratory Method Sheet (LMS) TIC001) medium for approximately 4h 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), fluopyram 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 (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4), sonicating the mixture and storing it at –70°C until analysis. Protein was determined by the method of Lowry *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 ATP depletion. The ATP released from viable somatic cells was determined by luminometry according to LMS FLUOR003, using an assay kit supplied by Promega (CellTitre-Glo luminescent 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 hepatocytes that have incorporated BrdU).

EGF (25 ng/mL, n=5) was included as a positive control for induction of replicative DNA synthesis.

6. 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. Pharmacol. **34**, 18. 3337-3345, according to LMS Fluor-002.

7. 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.

8. Benzyloxyquinoline-*O*-debenzylation (BQ)

The activity of CYP3A in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline, as described by GENTEST HTS technical bulletin, according to LMS Fluor-005.

II. Results and discussion

A. Adenosine 5'-Triphosphate (ATP)

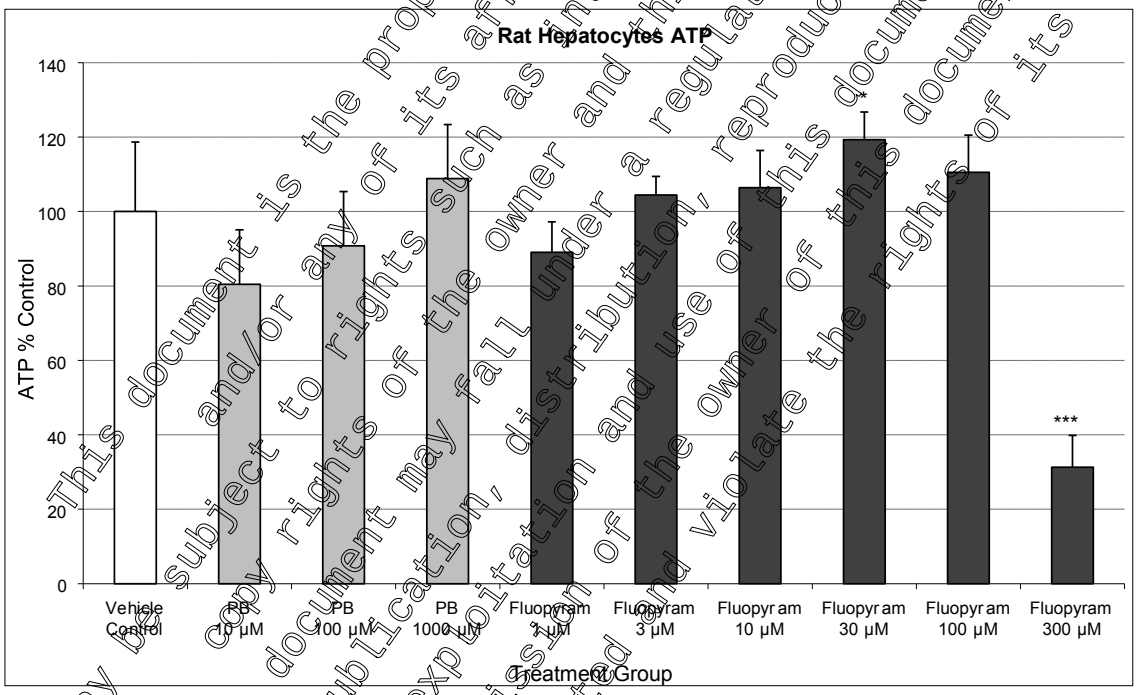
Fluopyram (1 – 100 µM) had little, or no, effect on cellular ATP concentrations after 96 hours. However, at a concentration of 300 µM a marked decrease in ATP concentration was observed, demonstrating cytotoxicity.

Table 5.5-95: Adenosine 5'-Triphosphate

Treatment	ATP (% Control)
Vehicle Control	100.0 ± 18.7[a]
PB 10 µM	80.5 ± 14.6
PB 100 µM	90.8 ± 14.6
PB 1000 µM	108.9 ± 14.5
Fluopyram 1 µM	89.0 ± 8.2
Fluopyram 3 µM	104.4 ± 5.0
Fluopyram 10 µM	106.4 ± 10.0
Fluopyram 30 µM	119.3 ± 7.4*
Fluopyram 100 µM	110.5 ± 10.0
Fluopyram 300 µM	31.3 ± 8.6***

[a] Values are mean % control ± SD. n = 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<0.001.

Figure 5.5-7 Adenosine 5'-Triphosphate



B. Replicative DNA Synthesis (S-Phase)

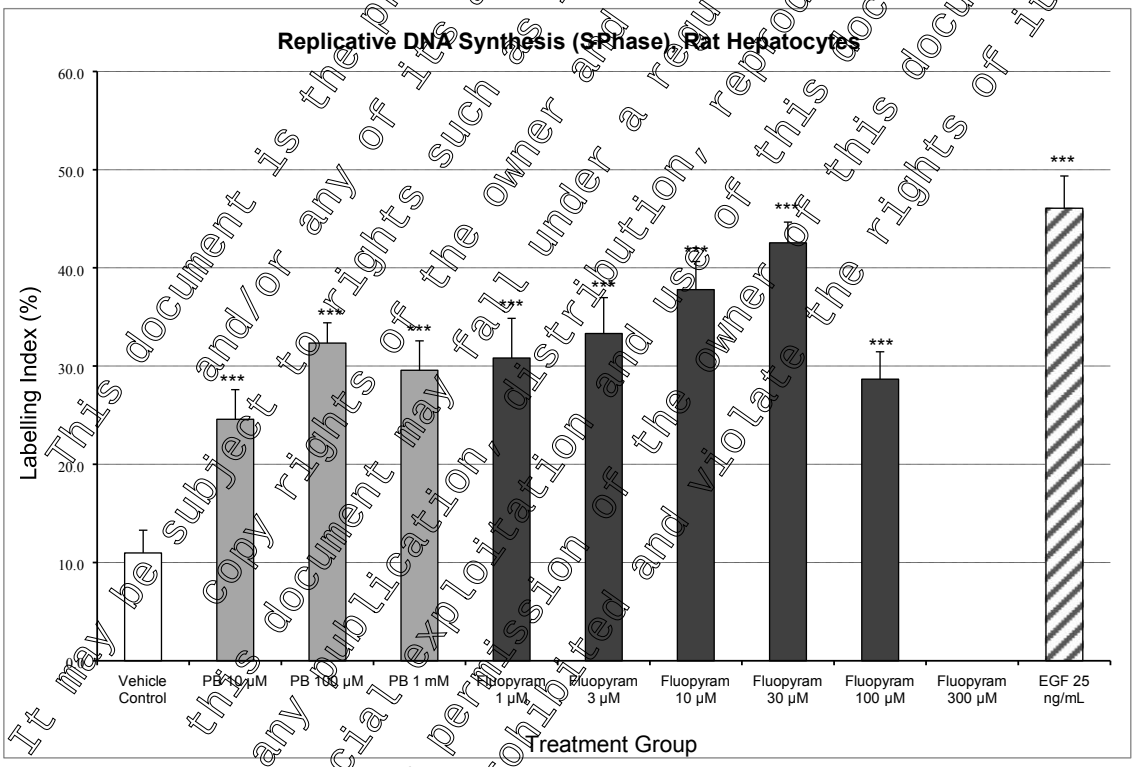
Culturing primary rat hepatocytes for 96 hours with phenobarbital resulted in a dose dependent increase in replicative DNA synthesis up to a maximum of 2.9-fold. Similarly, a dose-dependent increase of S-phase was observed following treatment with fluopyram, with maximal stimulation at 30-fold). Exposure of hepatocytes to higher concentrations resulted in less M fluopyram (3.9 stimulation of S-phase. 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 Replicative DNA Synthesis

Treatment	Labelling Index (%)
Vehicle Control	10.97 ± 2.32 (100.0 ± 21.1)[a]
PB 10 µM	24.59 ± 3.00*** (224.3 ± 27.4)
PB 100 µM	32.34 ± 2.07*** (294.9 ± 18.8)
PB 1000 µM	29.57 ± 2.99*** (269.7 ± 27.3)
Fluopyram 1 µM	30.82 ± 4.04*** (281.0 ± 36.8)
Fluopyram 3 µM	33.31 ± 3.66*** (303.8 ± 33.4)
Fluopyram 10 µM	37.79 ± 2.386*** (344.6 ± 26.0)
Fluopyram 30 µM	42.56 ± 2.09*** (388.1 ± 19.1)
Fluopyram 100 µM	28.67 ± 2.80*** (261.4 ± 25.5)
Fluopyram 300 µM	b
EGF 25ng/mL	46.07 ± 3.29*** (420.1 ± 30.0)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD, n = 5 per group. [b] Analysis unable to be performed due to excessive cytotoxicity. A Student's t-test (2-sided) was performed on the results, *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

Figure 5.5-8 Replicative DNA Synthesis



C. Pentoxyresorufin-*O*-Depentylation (PROD)

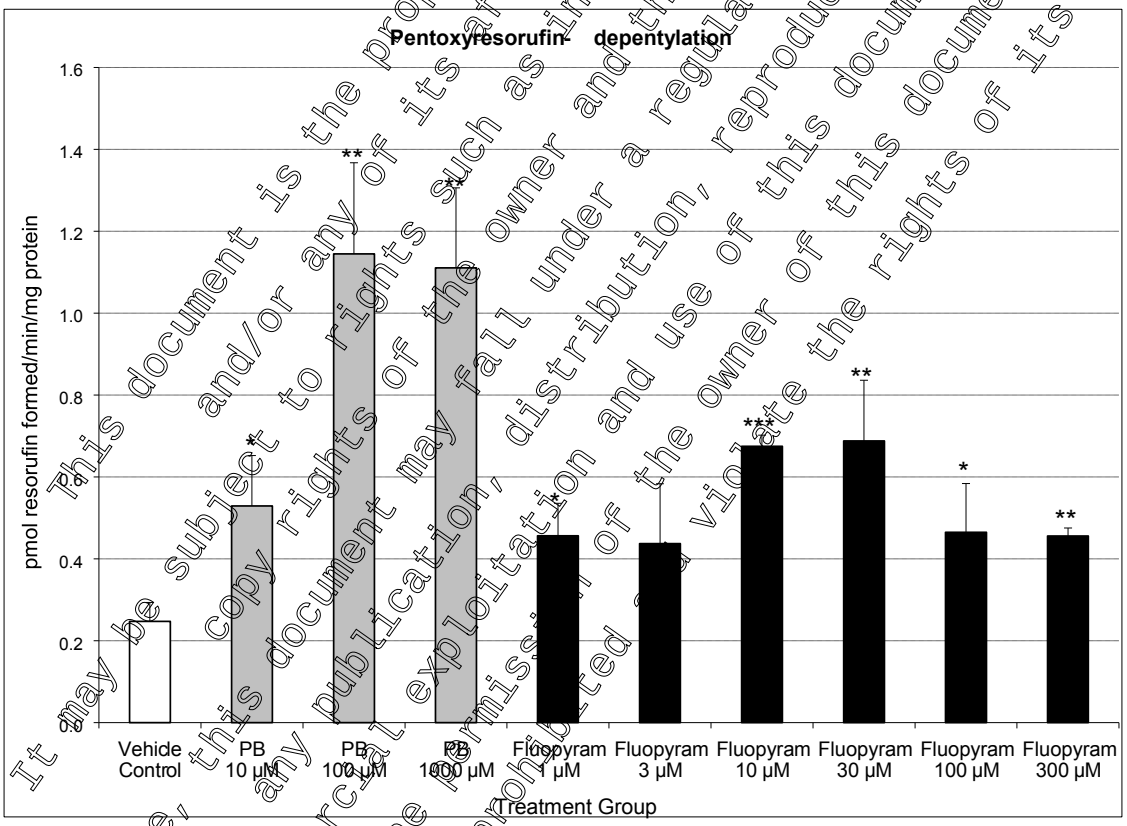
Culturing primary rat hepatocytes for 96 hours in the presence of phenobarbital resulted in a dosedependent increase in PROD activity to a maximum 4.6-fold at 100 µM. This is consistent with historical data. Addition of fluopyram to the hepatocytes resulted in a dose-dependent increase in PROD activity to a maximum 2.8-fold of control value at 30 µM. The degree of induction decreased as the concentration of fluopyram was increased to 100 and 300 µM .

Table 5.5-97 Pentoxyresorufin-*O*-Depentylation

Treatment	PROD (pmol resorufin formed/min/mg protein)
Vehicle Control	0.247 ± 0.046 (100.0 ± 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 ± 0.196** (448.9 ± 79.1)
Fluopyram 1 µM	0.456 ± 0.079* (184.5 ± 32.0)
Fluopyram 3 µM	0.437 ± 0.146 (176.7 ± 59.1)
Fluopyram 10 µM	0.675 ± 0.027*** (272.9 ± 11.0)
Fluopyram 30 µM	0.688 ± 0.148** (278.2 ± 59.9)
Fluopyram 100 µM	0.465 ± 0.119* (187.9 ± 48.2)
Fluopyram 300 µM	0.456 ± 0.019** (184.4 ± 7.8)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 3 per group. A Student's t-test (2sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

Figure 5.5-9 Pentoxyresorufin-*O*-Depentylation



D. Benzyloxyresorufin-*O*-Debenzylation (BROD)

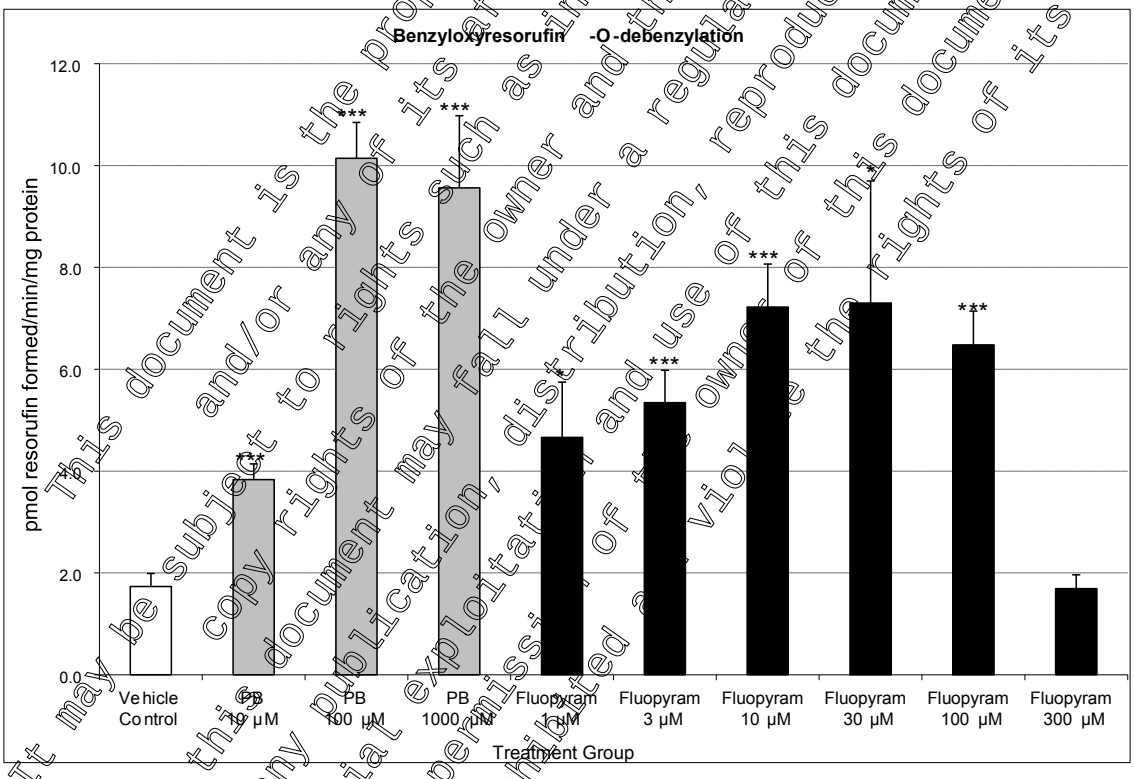
Culturing primary rat hepatocytes, for 96 hours, with phenobarbital resulted in a dosedependent increase in BROD activity up to a maximum 5.8-fold increase at 100 µM. This is consistent with historical data. Culturing hepatocytes with fluopyram resulted in a dosedependent increase in BROD activity, up to a maximum increase of 4.2-fold at 30 µM.

Table 5.5-98 Benzyloxyresorufin-*O*-Debenzylation

Treatment	BROD (pmol resorufin formed/min/mg protein)
Vehicle Control	1.734 ± 0.255 (100.0 ± 14.7)[a]
PB 10 µM	3.835 ± 0.309*** (221.1 ± 17.8)
PB 100 µM	10.142 ± 0.704*** (584.8 ± 40.6)
PB 1000 µM	9.560 ± 1.419*** (551.3 ± 81.8)
Fluopyram 1 µM	4.661 ± 1.087* (268.7 ± 62.7)
Fluopyram 3 µM	5.343 ± 0.639*** (308.1 ± 36.8)
Fluopyram 10 µM	7.223 ± 0.844*** (416.5 ± 48.7)
Fluopyram 30 µM	7.299 ± 2.398** (420.9 ± 138.3)
Fluopyram 100 µM	6.480 ± 0.662*** (373.7 ± 38.1)
Fluopyram 300 µM	1.693 ± 0.217 (97.7 ± 15.7)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 3 per group. A Student's t-test (2sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

Figure 5.5-10 Benzyloxyresorufin-*O*-Debenzylation



E. Benzyloxyquinoline-*O*-Debenzylation (BQ)

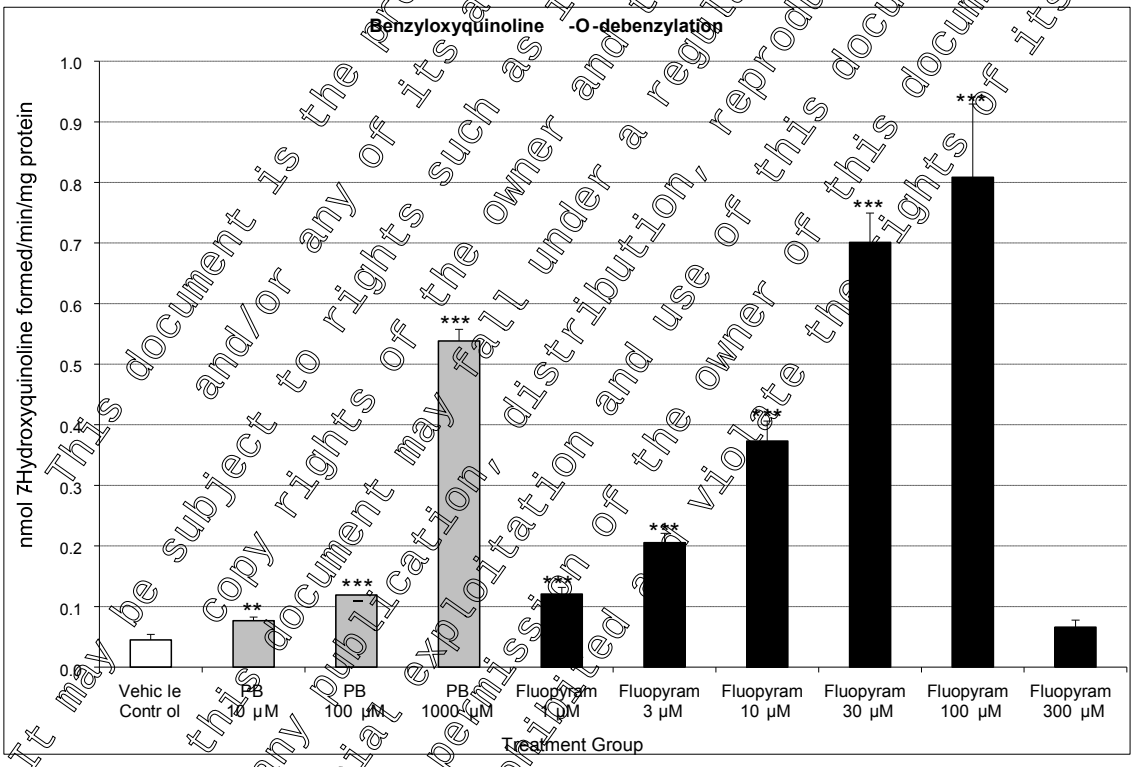
Culturing primary rat hepatocytes for 96 hours, with phenobarbital resulted in a dose-dependent increase in BQ activity of up to 12-fold at 1000 µM. This is consistent with historical data. Exposure of hepatocytes to fluopyram resulted in a large, dose dependent increase in BQ activity of up to 18-fold at 100 µM.

Table 5.5-99 Benzyloxyquinoline-*O*-Debenzylation

Treatment	BQ (nmol 7-Hydroxyquinoline formed/min/mg protein)
Vehicle Control	0.045 ± 0.009 (100.0 ± 20.5)[a]
PB 10 µM	0.077 ± 0.006** (170.8 ± 13.4)
PB 100 µM	0.119 ± 0.004*** (265.5 ± 8.4)
PB 1000 µM	0.538 ± 0.019*** (1198.8 ± 43.2)
Fluopyram 1 µM	0.121 ± 0.011*** (268.7 ± 24.3)
Fluopyram 3 µM	0.206 ± 0.015*** (457.9 ± 34.1)
Fluopyram 10 µM	0.373 ± 0.033*** (831.1 ± 73.8)
Fluopyram 30 µM	0.701 ± 0.048*** (1561.5 ± 107.8)
Fluopyram 100 µM	0.808 ± 0.121*** (1800.9 ± 269.2)
Fluopyram 300 µM	0.066 ± 0.011 (147.2 ± 25.5)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 3 per group. A Student's t-test (2sided) was performed on the results; *statistically different from control $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Figure 5.5-11 Benzyloxyquinoline-*O*-Debenzylation



B. Deficiencies

No specific deficiencies were noted in the study.

III. Conclusions

The clear increases in prod activity seen after exposure to fluopyram indicate that fluopyram is an 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) 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 an 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) in the hepatocytes.

In conclusion, these data suggest that fluopyram is an activator of both CAR and PXR.

Supporting data regarding male mouse thyroid tumors

In the mouse carcinogenicity study (M-295688-01-1), a higher incidence of thyroid follicular cell tumors was observed in male mice exposed to AE C656948 at 750 ppm (105 mg/kg bw/d) for 18 months compared to the corresponding controls. There were 7/50 animals 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-neoplastic lesion follicular cell hyperplasia. Retrospective examination of the thyroid gland of males sacrificed after 1 year of treatment revealed 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 panlobular hepatocellular hypertrophy, hepatocellular cholestasis, single cell degeneration/necrosis, interstitial mixed cell infiltrates, eosinophilic inclusion bodies and multinucleated hepatocytes. AE C656948 did not show any genotoxic potential (see section 5.4).

These data alone support the hypothesis of a non-genotoxic threshold mechanism for the thyroid follicular cell adenoma formation.

In addition, AE C656948 being a moderate phenobarbital-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 hormone metabolism through different enzymatic activities (Kelly, 2000[5]). In order to test this hypothesis, a series of studies were conducted including:

(1) an *in vivo* study on potential interaction of AE C656948 on thyroid peroxidase-catalysed reactions i.e. direct effect on thyroid hormone synthesis,

(2) an *in vivo* study to investigate the effects of AE C656948 on liver enzymes and on pituitary/thyroid hormone balance in the male mouse.

(3) two *in vivo* studies to investigate the effects of AE C656948 on the pharmacokinetics of intravenously administered 125I-thyroxine in the male mouse.

(4) an *in vivo* 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 *in vivo* 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 excessive 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 C656948 did not affect the thyroid hormone synthesis at the thyroid 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 BROD and PROD activities.

• AE C656948 is able to modify the normal thyroid hormone levels in male mice, in particular by causing a decrease in T4 level associated with an increased level of TSH after only 3 days of treatment but also consistently after 2 weeks of treatment.

• AE C656948 induced a more rapid clearance of T4 from the blood than in the corresponding controls over the 24 hour period following 125I-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 levels and this constant stimulation at mid- or long-term is known to induce follicular cell hypertrophy/hyperplasia (observed in the mouse oncogenicity study already after 12 months in a few animals and clearly after 18 months in a large number 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 β-D-glucuronyl transferases which catalyse the metabolism of thyroxine to its glucuronide conjugate in the rodent (Bastomsky, 1976). In the qPCR analysis assay, the gene transcrips in the liver that were up-regulated following treatment 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 mice following life-time exposure to AE C656948 at the high dose level of 750 ppm, a series of 28-day feeding studies 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 assessed: specific cytochrome P-450 enzyme activities which acted as markers for activation of the Car/Pxr nuclear 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 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 (M-449890-01-1) 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 end points of the thyroid gland. Toxicol. Pathol. 25(1):39-48: KIIA 5.5.4 /37;Capen, C. C.;1997; M-435031-01-1 and Hurley *et al.*, 1998) Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents. Environ. Health Perspect. 106(8):437-45, KIIA 5.5.4 /38;Hurley, P. M.; Hill, R. N.; Whiting, R. J.;1998; M-086436-01-1.

I. DNA reactivity

II. Inhibition of the active transport of inorganic iodide into the follicular cell (iodide pump)

III. Inhibition of thyroid peroxidase that converts inorganic iodide into organic iodide 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 T4 to T3 by 5'-monodeiodinase at various sites in the body

VII. Enhancement of the metabolism and excretion of thyroid hormone by the liver, largely through the action of UGT

Concerning MoA I; *DNA reactivity*, this mechanism can be ruled out based on *in-vitro* and *in-vivo* genotoxicity studies which show that fluopyram is not a genotoxic chemical. 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 thyroid peroxidase*, mechanistic studies using hog thyroid microsomes showed fluopyram did not affect thyroid peroxidase. With regard to MoA IV; *Damage to follicular cells*, microscopic examination of the thyroid gland in the mouse and rat have never shown overt cytotoxicity, only hyperplasia/adenoma in the mouse following chronic 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 T3 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 tumor induction and is strongly supported by the mechanistic data.

Sex- and species differences

Although the basic functions of the hypothalamic-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. Rats 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, 1992[7]). By contrast, humans are much less sensitive to elevated TSH levels or alteration of the thyroid function which are usually not associated with increase in thyroid cancer incidences (Hill *et al*. 1998[8]). 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.,* 1989[9]; Curran and De Groot, 1991[10]). 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 bound preferably to albumin and postalbumin, whereas in humans T4 is bound to thyroxine binding globulin (van Raaij, 2002[11]). Consequently, the proportion of unbounded T4 (therefore more susceptible to metabolism and excretion) is greater in rodents than in humans (Capen, 1992[7]). In rodents, males have higher serum TSH levels than females and are usually more sensitive to goitrogenic stimulation and thyroid carcinogenesis (Chen, 1984[12]). In humans, there is no sex difference in hormone levels although females develop thyroid tumors more frequently than males (Boring *et al.*, 1994[13]).

An *in vitro* CYP and UGT induction study in human and Wistar rat hepatocytes with AE C656948 ([redacted] (2020), M-759019-01-1, KCA 5.5/22), was conducted to address the T-modality 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 ≥10 µM 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, CYP3A4 and UGT1A1 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 clearly demonstrate the mode of action for the thyroid tumors in the mouse following a life-time exposure to fluopyram is mediated through and secondary to liver enzymes induction *via* activation of the Car/Pxr nuclear receptors. The rodent specificity of liver mediated thyroid follicular cell tumors is 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 C656948) are presented in the following Expert Summary documents:

Position Paper

Fluopyram: Mode of Action and Human Relevance Analysis of Rodent Liver and Thyroid Tumors MIIA Sec 3 /03; [redacted] 2013; M-465068-01-2

Expert Summary Report

Fluopyram: Mode of Action and Human Relevance Framework Analysis for Fluopyram-Induced Rodent Liver and Thyroid Tumors MIIA Sec 3 /02; [redacted] 2013; M-454439-02-1

5 Kelly G. (2000) Peripheral metabolism of thyroid hormones - a review. Alternative Medecine Review, Vol. 5 N°4, 306-333. KIIA 5.5.4 /10; Kelly, G.; 2000; M-300835-01-1

6 Bastomsky C.H. (1973) The biliary excretion of thyroxine and its glucoronic acid conjugate in normal and Gunn rats. Endocrinology, 92, 35-40. KIIA 5.5.4 /18;Bastomsky, C. H.;1973;M-308397-01-1

7 Capen C.C. (1992) Pathophysiology of chemical injury of the thyroid gland. Toxicology Letters., 64/65, 381-388. KIIA 5.5.4 /11; Capen, C. C.; 1992; M-300815-01-1

8 Hill R.N., Crisp T.M., Hurley P.S., Rosenthal S.L. and Singh D.V. (1998) Risk assessment of thyroid follicular cell tumors. Environmental Health Perspectives, 106 (8), 447-457. KIIA 5.5.4 /12; Hill, R. N.; Crisp, T. M.; Hurley, P. M.; Rosenthal, S. L.; Singh, D. V.; 1998; M-300844-01-1

9 McClain R.M., Levin A.A., Posh R. and Downing J.C. (1989) The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. Toxicology and Applied Pharmacology, 99, 216-228. KIIA 5.5.4 /13; McClain, R. M.; Levin, A. A.; Posch, R.; Downing, J. C.; 1989; M-103760-01-1

10 Curran P.G. and DeGroot L.J. (1991) The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid gland. Endocrine Reviews, 12 (2), 135-150. KIIA 5.5.4 /14; Curran, P. G.; DeGroot, L. J.; 1991; M-066260-01-1

11 Van Raaij 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; M-300839-01-1

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.; Squires, T. S.; Tong, T.; Montgomery, S.; 2008; M-300849-01-1

Data Point:	KCA 5.5/09
Report Author:	[redacted]
Report Year:	2008
Report Title:	AE C656948 (Fluopyram) *In vitro* studies on the potential interactions with thyroid peroxidase-catalyzed reactions
Report No:	AT04481
Document No:	M-299276-01-1
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
Acceptability/Reliability:	Yes

Executive Summary

To investigate a potential effect of AE C656948 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 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 µM, the highest concentration tested. Similarly, TPO-catalyzed iodine formation was not affected by 300 µM AE C656948.

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
Description	Light beige powder
Lot / Batch #:	Mix-Batch:08528/0002
Purity:	94.7%
CAS #	658066-35-4
Stability of test compound:	Stable for a period covering the study duration
2. Vehicle and / or positive control:	Dimethylsulfoxide

3. Positive control substances:	Amitrole (3-amino-1,2,4-triazole) from Sigma (Lot number 083K0649) ETU (ethylenethiourea, 2-imidazolidinethione) from Riedel-de-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: 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 guaiacol oxidation

Guaiacol oxidation was used as a measure for peroxidative activity. Incubations were carried out at room temperature in 0.1 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 ΔE/min, corresponds to 3.5 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 (ΔE/min) of the absorption at 470 nm was used to calculate the peroxidase activity.

2. Determination of TPO-catalyzed iodine formation

Incubations were carried out as described above, however, guaiacol was replaced by potassium iodide (100 µL of 100 mM solution in water, final concentration 10 mM).

The following final concentrations were used:

AE C656948: 3.0 – 30 – 300 µM

Amitrole: 0.1 µM

ETU: 5 µM

The initial linear increase (ΔE/min) of the absorption at 350 nm was used to calculate the enzymatic activity.

II. 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 µM 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 µM did not affect this reaction.

Table 5.5-100 Effect of AE C656948 on TPO-catalyzed guaiacol reaction

Compound	Concentration (µM)	ΔE/min. ± SD	% of control
Vehicle	-	0.121 ± 0.006	100
AE C656948	3	0.122 ± 0.002	100.8
	30	0.123 ± 0.005	101.6
	300	0.124 ± 0.001	102.5
Amitrole	1	0.054 ± 0.003	44.6

The results of the TPO-catalyzed iodine formation are summarized in Table 5.5.4-53. Up to 300 µM AE C656948 did not affect TPO-catalyzed iodine formation. Neither 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, whilst Amitrole at a concentration of 0.1 µM inhibited the initial rate of this reaction by 50%.

Table 5.5-101 Effect of AE C656948 on TPO-catalyzed iodine formation

Compound	Concentration (µM)	ΔE/min. ± SD	% of control
Vehicle	-	0.259 ± 0.012	100
AE C656948	3	0.269 ± 0.012	103.9
	30	0.246 ± 0.005	95.0
	300	0.260 ± 0.012	100.4
Amitrole	0.1	0.131 ± 0.013	50.6

III. Conclusion

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 conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on the potential interactions of fluopyram with thyroid peroxidase-catalyzed reactions.

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.

Data Point:	KCA 5.5/10
Report Author:	[redacted]
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:	M-299522-01-1
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
Acceptability/Reliability:	Yes

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 similarly constituted groups of 15 males received untreated 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 microscopic 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 T3 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. Mean absolute and relative liver weights were increased by approximately 60% when compared to control animals. At macroscopic observation, enlarged liver was found in all the treated animals. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy and an increased number of mitoses were observed in all examined treated animals. In addition, hepatocellular single cell necrosis was observed in 1/5 treated animals. No significant microscopic change was observed in the thyroid gland.

Total cytochrome P-450 content was markedly increased (+116%) by the treatment. EROD activities were marginally increased (+235%), whereas PROD and BROD activities were markedly increased (respectively +2890% and +871%) when compared to controls. No significant changes were observed in UDPGT.

After 14 days of exposure at 2000 ppm, overall the results were very similar to those obtained after 3 days of exposure. Mean T3 level was not changed whereas mean T4 level was still decreased (-27%) and mean TSH level 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, dark liver was observed in 14/15 treated animals compared to only 1 in the control animals. At 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 nominal concentration of 2000 ppm (equivalent to between 308 and 314 mg/kg bw/day) in the C57BL/6J mouse for 3 and 14 days induced a phenobarbital-like P-450 hepatic enzymatic activity profile and has the ability to modify the normal thyroid hormone balance (in particular to cause a sustainable decrease in 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
Description	Light beige powder
Lot / Batch #:	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. Vehicle and / or positive control:	none
3. Test animals:	
Species:	Mouse - Male only
Strain:	C57BL/6J
Age:	8 weeks approximately at start of treatment
Weight at dosing:	21.2 to 25.1 g
Source:	[redacted]
Acclimation period:	5 to 6 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	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 for the 3-day exposure groups: 01 October – 04 October 2007.

Dosing period for the 14-day exposure groups: 18 September – 02 October 2007

2. Animal assignment and treatment

The dose level was set after evaluation 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 all males 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

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 Européennes, L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Table 5.5-102 Study design

Test group	Treatment	Dose level in ppm (mg/kg bw/day)	Duration of treament	Animals assigned
1	Control	0	3 days	15
2	AE C656948	2000 (308)	3 days	15
3	Control	0	14 days	15
4	AE C656948	2000 (314)	14 days	15

The stability of the test substance in the diet 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 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, 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-sided).

If the F test was significant ($p\leq0.05$), 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 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 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, 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 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 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 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 then weekly during the treatment period. 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 to calculate the mean daily food consumption. Any food 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, Maurepas, 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 TSH, T3 and T4 hormone levels with specific radio-immunoassay kits (supplied by Amersham for TSH and by DIASORIN 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 paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared and examined

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 measuring UDPglucuronosyltransferase (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

There was no effect on body weights or body weight gains throughout the study.

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.

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 2 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 +7%, 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 thyroid hormones levels are low, the pituitary gland will produce TSH to stimulate the thyroid gland in order to restore the normal level of thyroid hormones.

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 exposure	
	Control	2000 ppm (308 mg/kg bw/d)	Control	2000 ppm (314 mg/kg bw/d)
T3 (nmol/L)	1.62 ± 0.15	1.64 ± 0.25 (+1%)	1.45 ± 0.18	1.52 ± 0.38 (+5%)
T4 (nmol/L)	43.7 ± 8.1	30.7** ± 6.0 (-30%)	38.1 ± 9.1	27.7** ± 8.7 (-27%)
TSH (ng/mL)	3.81 ± 0.23	4.48** ± 0.31 (+18%)	3.81 ± 0.28	4.09* ± 0.44 (+7%)

*: p≤0.05; **: p≤0.01

E. Sacrifice and pathology

As seen in other studies in the mouse (see section 5.3), liver weights were markedly increased in the treated group compared to control groups after only 3 days of exposure and after 14 days of exposure (approximately +60% in all cases). This was associated with enlarged and/or dark liver seen at the macroscopic examination and with hepatocellular hypertrophy in all examined animals and single cell necrosis on many occasions especially after 14 day of exposure. An increased number of mitoses was also observed in all animals after 3 days of exposure.

Table 5.5-104 Pathological liver effects after 3 and 14 days of exposure to AE C656948 (mean±SD)

Group	3-day exposure		14-day exposure	
	Control	2000 ppm (308 mg/kg bw/d)	Control	2000 ppm (314 mg/kg bw/d)
Liver weights Absolute weight (g)	1.24±0.12	1.97±0.17** (+59%)	1.25±0.10	1.99±0.22** (+59%)
Body weight ratio (%)	5.40±0.57	8.71±0.54** (+61%)	5.23±0.33	8.42±0.94** (+61%)
Brain weight ratio (%)	279.99±29.24	448.34±34.51** (+60%)	286.49±21.42	462.30±52.04** (+61%)
Histopathological findings				
Centrilobular to panlobular hepatocellular hypertrophy	0/5	5/5	0/5	5/5
Increase mitosis	0/5	5/5	0/5	0/5
Single cell necrosis	0/5	1/5	0/5	4/5

*: p≤0.05; **: p≤0.01

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, treatment with AE C656948 induced a 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 increased whereas 4-nitrophenol 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)

Group	3-day exposure		14-day exposure	
	Control	2000 ppm (308 mg/kg bw/d)	Control	2000 ppm (314 mg/kg bw/d)
P-450 (nmol/mg Prot.)	1.08 ± 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 ± 43.49 (+235%) **	99.02 ± 8.98	262.24 ± 72.87 (+165%) **
PROD (pmol/min/ mg Prot.)	4.93 ± 0.83	143.42 ± 57.05 (+2809%) **	4.19 ± 0.49	94.80 ± 44.77 (+2163%) **
BROD (pmol/min/ mg Prot.)	12.99 ± 2.34	1145.28 ± 262.93 (+8715%) **	12.83 ± 2.11	1175.30 ± 163.99 (+9061%) **
UDPGT (nmol/min/ mg Prot.)	16.04 ± 1.42	15.36 ± 0.53 (N.C.)	17.09 ± 0.90	14.32 ± 0.63 (-16%) **

*: p≤0.05; **: p≤0.01

F. Deficiencies

No specific deficiencies were noted in this study.

III. Conclusions

These data indicate that AE 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 particular an induction of phenobarbital-like hepatic enzymes including total cytochrome P-450, PROD, 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.

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 mouse.

These data indicate that AE 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 particular an induction of phenobarbital-like hepatic enzymes including total cytochrome P-450, PROD, 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:	[redacted]
Report Year:	2008
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:	M-299521-01-1
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
Acceptability/Reliability:	Yes

Executive Summary

Phenobarbital, a reference hepatotoxic compound (batch number: 06100228; white powder; 99.6% of purity), was administered daily by gavage to 2 groups of 15 female C57BL/6J mice for 3 or 14 days at a dose level of 80 mg/kg/day. Two similarly constituted groups of 15 females received 0.5% methycellulose for 3 or 14 days 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. Before necropsy, blood samples were taken for thyroid and pituitary gland hormone analyses (T3, T4 and TSH). At final sacrifice times, liver and brain were weighed and liver and thyroid gland sampled for the assessment of morphological changes. In addition, hepatic cytochrome P-450 and UDPGT isoenzyme activities were assessed.

After 3 days of exposure, mean T3 and T4 levels were decreased (respectively -10% and -27%) and mean TSH level was not affected when compared to controls. At 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 liver in several animals and with microscopic observations including diffuse centrilobular to panlobular hepatocellular hypertrophy in most animals.

Total cytochrome P-450 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 controls. 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 14 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 animals. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy were seen in all treated animal.

Total cytochrome 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 activities when 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

A. Materials

1. Test material:	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
2. Vehicle and / or positive control:	Methylcellulose 400
3. Test animals:	
Species:	Mouse - Male only
Strain:	C57BL/6J
Age:	8 weeks approximately at start of treatment
Weight at dosing:	19.9 to 24.2 g
Source:	Charles River Laboratories, St Germain-sur-l'Arbresle, France
Acclimation period:	6 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
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 for the 3 day exposure groups: 27 November – 30 November 2007.
Dosing period for the 14-day exposure groups: 20 November – 04 December 2007

2. Animal 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 0.5% aqueous solution of methylcellulose 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 methylcellulose 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".

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
2	Phenobarbital	80	3 days	15
3	Control	0	14 days	15
4	Phenobarbital	80	14 days	15

Before the start of the study homogeneity and concentration were checked on the test formulation at 8 g/L. The mean value obtained from the homogeneity check was taken as measured concentration. The 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 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 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-sided).

If the F test was significant (p≤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 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 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, 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 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 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 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 then weekly during the treatment period. 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. 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, Maurepas, 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 TSH, T3 and T4 hormone levels with specific radio-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 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 were used for microsome preparation.

Histotechnology - Histopathology

Liver portions and thyroid glands sampled for microscopy were embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared and examined

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 measuring UDP-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 a gain of 0.3 g in the control group. In the 14-day exposure group, there was a mean body weight loss of 0.3 g ($p \leq 0.05$) in the treated group compared with a gain of 0.4 g in the control group on study Day 7 although overall mean bodyweight was not affected at the end of treatment period.

C. Food consumption

There was a slight reduction in food consumption at the beginning of treatment but not during the second week of treatment.

D. Hormone analysis

The results of TSH, T3 and T4 levels are summarized in Table 5.5.4-59.

After 3 day of exposure, statistically significantly lower levels of both T3 and T4 were observed in the treated group compared to control while TSH level remained unaffected.

After 14 days of exposure T4 level was still significantly lower in the treated group than in the control with a concomitant significant higher level of TSH. An increased level of TSH is expected when T4 level is low as TSH will stimulate thyroid to produce T4.

Table 5.5-107 Mean levels of thyroid/pituitary hormone after 3 and 14 days of exposure to phenobarbital (mean±SD)

Group	3-day exposure		14-day exposure	
	1 Control	2 Phenobarbital	3 Control	4 Phenobarbital
T3 (nmol/L)	1.72 ± 0.25	1.54* ± 0.17 (-10%)	1.61 ± 0.20	1.57 ± 0.19 (-2%)
T4 (nmol/L)	36.7 ± 6.2	26.8** ± 3.5 (-27%)	32.4 ± 6.5	26.1* ± 6.7 (-19%)
TSH (ng/mL)	4.44 ± 0.27	4.41 ± 0.54 (-1%)	4.47 ± 0.37	4.89* ± 0.58 (+9%)

*: $p \leq 0.05$; **: $p \leq 0.01$

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 5.5-108 Pathological liver effects after 3 and 14 days of exposure to phenobarbital

Group	3-day exposure		14-day exposure	
	1 Control	2 Phenobarbital	3 Control	4 Phenobarbital
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±0.33	6.32**±0.21 (+11%)	5.38±0.50	6.65**±0.47 (+23%)
Brain weight ratio (%)	307.79±34.56	320.20±22.02 (+4%)	301.83±34.16	365.46**±47.51 (+21%)
Histopathological findings				
Centrilobular to panlobular hepatocellular hypertrophy	0/5	4/5	0/5	5/5
Increased mitosis	0/5	3/5	0/5	0/5

*: p≤0.05; **: p≤0.01

Hepatotoxicity testing

The results are described in Table 5.5.4-61. 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 increased 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.

Table 5.5-109 Cytochrome P-450 content and enzymatic activities in the liver after 3 and 14 days of exposure to phenobarbital (mean±SD)

Group	3-day exposure		14-day exposure	
	1 Control	2 Phenobarbital	3 Control	4 Phenobarbital
P-450 (nmol/mg Prot.)	0.94 ± 0.06	2.31** ± 0.20 (+ 146%)	0.98 ± 0.12	1.33* ± 0.29 (+ 36%)
EROD (pmol/min/ mg Prot.)	48.08 ± 6.87	190.65** ± 97.94 (+ 297%)	35.34 ± 10.48	167.88** ± 96.47 (+ 375%)
PROD (pmol/min/ mg Prot.)	6.01 ± 1.99	88.99** ± 29.89 (+ 1381%)	4.98 ± 0.59	71.97** ± 21.55 (+ 1345%)
BROD (pmol/min/ mg Prot.)	17.33 ± 1.58	871.66** ± 148.84 (+ 4930%)	18.82 ± 3.30	554.00** ± 119.35 (+ 2844%)
UDPGT (nmol/min/ mg Prot.)	16.24 ± 0.56	17.23 ± 0.82 N.S.	15.18 ± 1.28	12.96 ± 2.14 N.S.

*: p≤0.05; **: p≤0.01

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 concomitant 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 mechanistic information on the effects and target organs of phenobarbital in the mouse.

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 concomitant increase in TSH,
- induce liver changes including in particular induction of several hepatic enzymes as total cytochrome P-450, PROD, BROD activities.

Data Point:	KCA 5.5.12
Report Author:	[redacted]
Report Year:	2008
Report Title:	AE C656948 Mechanistic 3-day toxicity study in the male mouse (pharmacokinetic investigations of the clearance of intravenously administered ^{125}I-thyroxine)
Report No:	SA 08159
Document No:	M-308369-01-1
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:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

AE C656948, a fungicide (batch number: Mix-batch: 08528/0002: light beige powder, 94.7% purity) was administered continuously via the diet 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 of 5 males received 80 mg/kg/day Phenobarbital (batch number: 06100228: white powder, 99.6% 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 Day 4 each animal received by intravenous injection via the tail 250 µl of diluted ^{125}I-Thyroxine solution. Approximately 3 hours post-administration with ^{125}I-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 hours post ^{125}I-Thyroxine administration. The level of ^{125}I radioactivity in each sample was measured using a Cobra gamma scintillation counter. The rate of ^{125}I 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 ^{125}I-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 into 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 ^{125}I-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 ^{125}I 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 ^{125}I 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 material:	AE C656948
Description	Light beige powder
Lot / Batch #:	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. Vehicle and / or positive control:	Vehicle for AE C656948: rodent diet Positive control: Phenobarbital Vehicle for Phenobarbital: methylcellulose 400
Positive control:	Phenobarbital
Description:	White crystalline powder
Lot / Batch :	0610228
Purity:	99.6%
CAS :	50-06-6
Stability of test compound:	Stable in rodent diet for a period covering the study duration
3. Test animals:	
Species:	Mouse - Male only
Strain:	C57BL/6J
Age:	8 weeks approximately at start of treatment
Weight at dosing:	19.8 to 25.4 g
Source:	[redacted]
Acclimation period:	6 days (subgroup 1 animals), 23 days (subgroup 2 animals)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
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: Start of treatment to final sacrifice for the subgroup 1 animals: 23 August – 27 August 2008

Start of treatment to final sacrifice for the subgroup 2 animals: 05 September – 09 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 28-day 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 toxicity 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 toxicity. Males only were used in this study as an increased incidence of thyroid follicular cell adenoma was observed in this sex only. In 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 3 days by oral gavage to a group of 5 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 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".

Table 5.5-110 Study design

Test group	Treatment	Dose level	Duration of treatment	Animals assigned
1*	Control diet	0	3 days	5
2*	AE C656948	2000 ppm	3 days	5
3*	Phenobarbital	80 mg/kg/day	3 days	5
4**	Control diet	0	3 days	1
5**	AE C656948	2000 ppm	3 days	4

* subgroup 1 animals, ** subgroup 2 animals

The stability of AE C656948 in the diet 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 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.E. (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 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 ($p\leq0.05$), 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 ($p\leq0.05$) 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 or more group variance(s) equal 0, 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 V4.2.2 (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 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 ^{125}I-Thyroxine

^{125}I-Thyroxine solution (^{125}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 ^{125}I-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 ^{125}I-Thyroxine solution corresponding to 1 µCi of ^{125}I-Thyroxine per animal. Approximately 3 hours after the intravenous injection of ^{125}I-Thyroxine, each

animal received 0.1 mg of NaI in 250 µl of 0.9 % sterile saline by intraperitoneal injection. Dilution of the ^{125}I-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 ^{125}I-Thyroxine, a new dilution of the ^{125}I-Thyroxine stock solution was performed. This diluted solution was performed by mixing the same volumes of stock solution of ^{125}I-Thyroxine and 0.9 % sterile saline 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 venous 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 Isoflurane (Baxter, Maurepas, France). Samples were taken at 1 hour 20 min, 2, 4, 6, and 24 hours after the dosing with the ^{125}I-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 (^{125}I) was measured at the same time in all the blood samples generated both in subgroup 1 and subgroup 2 animals.

Radioativity (^{125}I) were measured using a Cobra gamma scintillation counter (Packard).

Radioactivity measurement was expressed as follows:

Whole blood radioactive content.

Data from animals belonging to both subgroups 1 and 2 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 groups throughout the study.

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.

C. T4 clearance

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 4 AE C656948 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 ± Standard deviation (cpm)

Time point	Control	AE C656948 (2000 ppm)	Phenobarbital (80 mg/kg/day)
Number of animals	**6**	**5**	**3**
Time point 1h20min	11434 ± 1624	4767 ±1953	5775 ±2615
Time point 2h	11025 ±1415	4686 ±1999	5905 ±2095
Time point 4h	9811±1756	4984 ±1491	5651 ± 995
Time point 6h	8692 ±1397	4566 ± 1342	6021 ±1046
Time point 24h	2686 ± 454	1955 ± 799	2309 ± 446

The results show after an intravenous injection of ^{125}I-thyroxine a decrease of the radioactivity in the blood of the AE C656948 treated animals when compared to 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 ^{125}I administration, compared with the controls.

Similarly, the decrease of the radioactivity was also observed in animals treated with phenobarbital a reference compound known to induce an increase in thyroxine clearance in the mouse through induction of thyroxine glucoronidation.

Overall these results indicate that the thyroxine clearance was increased in the AE C656948 treated animals when compared to control animals.

D. Deficiencies

No specific deficiencies were noted in this study

III. Conclusions

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 ^{125}I 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.

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, 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 ^{125}I 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.

Data Point:	KCA 5.5/13
Report Author:	[redacted]
Report Year:	2008
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:	M-308073-01-1
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:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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 administered daily to 2 groups of 10 male C57BL/6J mice for 3 days at dose levels of 2000 ppm 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 on Study Days 1 and 4. At final sacrifice, liver was weighed and sampled for gene expression analyses by quantitative Polymerase Chain Reaction (qPCR).

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 C656948 treated animals an up-regulation of sulfotransferase transcripts (from +92% to +463%, $p \leq 0.01$) and UDP 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 UDP-glucoronosyltransferase transcripts (from +82% to +119%, $p \leq 0.01$) was observed in Phenobarbital 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 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.

I. Materials and methods

A. Materials

1. **Test material:** AE C656948
   Description Light beige powder
   Lot / Batch #: 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. Vehicle and / or positive control:	Vehicle for AE C656948: rodent diet Positive control: Phenobarbital Vehicle for Phenobarbital: methylcellulose 400
Positive control:	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.
3. Test animals:	
Species:	Mouse - Male only
Strain:	C57BL/6J
Age:	8 weeks approximately at start of treatment
Weight at dosing:	19.9 to 23.4 g
Source:	[redacted]
Acclimation period:	6 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
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: Start of treatment to final sacrifice: 08 July – 11 July 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 28-day 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 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 AE C656948 without 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. In 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 3 days by oral gavage to a group of 5 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 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".

Table 5.5-112 Study design

Test group	Treatment	Dose level	Duration of treament	Animals assigned
1	Control diet	0	3 days	10
2	AE C656948	2000 ppm	3 days	10
3	Phenobarbital	80 mg/kg/day	3 days	10

The stability of AE C656948 in the diet 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 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 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 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 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*. Animals were not diet fasted 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 parameters
- qPCR data

Mean and standard deviation were calculated for each group.

• Terminal body weight, absolute and relative organ weight 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 ($p\leq0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

• Body weight

The 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 ($p\leq0.05$), 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 ($p\leq0.05$) 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).

• 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 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 PathTox System V4.2.2. (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 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 4.

3. Food consumption

Food consumption was not recorded.

4. Necropsy procedure and tissue collection

On Study Day 4 all animals 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 necropsied. The necropsy 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.

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 Chain Reaction analysis. The remaining portions of livers were discarded.

5. qPCR analysis:

Total RNA purification

Total cytoplasmic RNA was isolated from the liver of individual control and treated animals using RNeasy Midi Kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles 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 5.5-113: List of Taqman assays used

Gene family	Isoform	Refset ID	Taqman assay ID (Applied Biosystems)
Cytochrome P450	Cyp1a1	NM_009992.3	Mm00487218_m1
Cytochrome P450	Cyp2b9	NM_010000.2	Mm00657910_m1
Cytochrome P450	Cyp3a11	NM_007818.3	Mm00731567_m1
Sulfotransferase	Sulta1	NM_133670.1	Mm00467072_m1
Sulfotransferase	Sulta2	NM_009286.1	Mm02394381_g1
Sulfotransferase	Sultn	NM_016771.2	Mm00502030_m1
UDP glucuronosyltransferase	Ugt1a1	NM_201645.2	Mm02603387_m1
UDP glucuronosyltransferase	Ugt2b1	NM_152811.1	Mm00514184_m1
UDP glucuronosyltransferase	Ugt2b5	NM_009467.1	Mm01623253_s1
Beta-2 microglobulin	B2m	NM_009735.3	Mm00437762_m1

Beta-2 microglobulin (B2m) 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:

$\Delta\Delta Ct = (Cttest - CtB2_m)_{treated} - (Ct_{test} - CtB2_m)_{control}$

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal.

Each RQ value obtained for a given gene was normalized by dividing by the RQ value obtained for the control animal ST1M2625.

II. Results 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 Phenobarbital treated 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.

Table 5.5-114 Liver weight changes (% change when compared to controls)

Sex	Males		
Test substance	Control Diet	AE C656948 (2000 ppm)	Phenobarbital (80 mg/kg/day)
Mean absolute liver weight (g)	1.20±0.10	1.93**±0.12 (+61%)	1.40**±0.13 (+17%)
Mean liver to body weight ratio (%)	5.34±0.36	8.55**±0.40 (+60%)	6.34**±0.29 (+19%)

** p≤0.01

QPCR analysis

Table 5.5-115 Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)

Gene transcripts	Control	AE C656948 (2000 ppm)	Phenobarbital (80 mg/kg/day)
Cyp1a1	1.29 ± 0.30	4.81** ± 1.19 (+272%)	1.20 ± 0.24 NS (-7%)
Cyp2b9	14.71 ± 30.17	48.61* ± 91.21 (+230%)	21.11 ± 30.19 NS (+43%)
Cyp3a11	1.51 ± 0.56	43.59** ± 34.38 (+2783%)	7.76** ± 4.11 (+413%)
Sult1a1	1.19 ± 0.39	2.29** ± 1.20 (+92%)	1.92* ± 0.46 (+62%)
Sult2a2	0.51 ± 0.22	2.90** ± 2.08 (+463%)	0.63 ± 0.35 NS (+22%)
Sultn	1.41 ± 0.56	5.93** ± 3.90 (+321%)	2.76** ± 1.18 (+96%)
Ugt1a1	1.08 ± 0.16	4.03** ± 0.80 (+273%)	2.36** ± 0.26 (+119%)
Ugt2b1	1.04 ± 0.25	2.84** ± 0.71 (+173%)	1.97** ± 0.53 (+90%)
Ugt2b5	1.30 ± 0.23	4.30** ± 2.16 (+231%)	2.36** ± 0.87 (+82%)

NS : Not statistically significant
* : Statistically different from the control group (p≤0.05)
** : Statistically different from the control group (p≤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 phenobarbital treated animals the induction of cyp3a was statistically significantly up-regulated (+413%, p≤0.01). The cyp2b transcript was only slightly up-regulated (+43%) and the effect was not statistically significant.

The transcripts of isoforms of sulfotransferase and udp glucoronosyltransferase known to inactivate T3 and T4 via glucoronide and sulfate derivatives were statistically significantly up-regulated in the liver of AE C656948 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 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.

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 phenobarbital 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:	[redacted]
Report Year:	2009
Report Title:	AE C656948 Definitive Mechanistic 4-day toxicity study in the male mouse (pharmacokinetic investigations of the clearance of intravenously administered ^{125}I-thyroxine)
Report No:	SA 08288
Document No:	M-328662-01-1
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
Acceptability/Reliability:	Yes

Executive Summary

AE C656948, a fungicide (batch number: Mix-batch: 08528/0002: light beige powder, 94.7% purity) was administered continuously 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 males 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 powder, 99.6% purity) 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 sulfonation. On Study Day 5 each animal received by intravenous injection via the tail 250 µl of diluted ^{125}I-Thyroxine solution in 0.9% sterile saline. Approximately 3 hours post-administration with ^{125}I-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 40 minutes, 1.5, 4 and 24 hours post ^{125}I-Thyroxine administration. The level of ^{125}I radioactivity in each sample was measured using a Cobra gamma scintillation counter. The rate of ^{125}I 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 5.

There were no clinical signs during the course of the study. One animal was sacrificed after intravenous injection of the radiolabeled Thyroxine due to a technical problem during the injection procedure. There was no statistically significant effect on body weight.

The results show that following an intravenous of ^{125}I-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 ^{125}I 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 ^{125}I 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 material:	AE C656948
Description	Light beige powder
Lot / Batch #:	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. Vehicle and / or positive control:	Vehicle for AE C656948: rodent diet Positive control: Phenobarbital Vehicle for Phenobarbital: methylcellulose 400
Positive control:	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
3. Test animals:	
Species:	Mouse - Male only
Strain:	C57BL/6J
Age:	8 weeks approximately at start of treatment
Weight at dosing:	20.3 to 23.5 g
Source:	[redacted]
Acclimation period:	At least 9 days
Diet:	certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
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: Start of treatment to final sacrifice for the subgroup 1 animals: 14 November – 19 November 2008

Start of treatment to final sacrifice for the subgroup 2 animals: 16 November – 21 November 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 28-day 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 toxicity 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 toxicity. Males only were used in this study as an increased incidence of thyroid follicular cell adenoma was observed in this sex only. In 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 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".

Table 5.5-116 Study design

Test group	Treatment	Dose level	Duration of treatment	Animals assigned
1	Control diet	0	4 days	8
2	AE C656948	2000 ppm	4 days	8
3	Phenobarbital	80 mg/kg/day	4 days	8

The stability of AE C656948 in the diet 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 97 to 98% 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 99% 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 5. On transfer to the radioactive suite on Day 5, animals were given certified rodent pelleted 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 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 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, 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 0, 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 PathTox System V4.2.2 (Module Enhanced Statistics).

Radioactivity measurements in the blood

For each animal, 2 replicated values were measured. Statistical analyses were conducted on the averaged values calculated per animal from the 2 replicate 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 2 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. Tests performed 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 control and the AE C656948 treated group

Statistical comparisons were performed per time 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 (p≤0.05), means were compared using the modified t-test (1-sided).

Comparison between the control and the Phenobarbital treated group

Statistical comparisons were performed per time 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 (p≤0.05), means were compared using the modified t-test (1-sided).

Group means were compared at the 5% and 1% levels of significance.

Statistical analyses were carried out 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 at least once during the acclimatization phase, then on Study Days 1 and 5.

3. Food consumption

Food consumption was not recorded.

4. Intravenous injection of ^{125}I-Thyroxine

^{125}I-Thyroxine solution (^{125}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 ^{125}I-Thyroxine is presented in Attachment 2 in the study report. Each animal received on Day 5 by intravenous injection via the tail 250 µl of the diluted 125I-Thyroxine solution corresponding to 1 µCi of ^{125}I-Thyroxine per animal. Approximately 3 hours after the intravenous injection of ^{125}I-Thyroxine, each animal received 0.1 mg of NaI in 250 µl of 0.9 % sterile saline by intraperitoneal injection. Dilution of the ^{125}I-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 collection.

A whole blood sample was collected from the retro-orbital venous 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 Isoflurane (Baxter, Maurepas, France). Samples were taken at 40 minutes, 1.5, 4 and 24 hours after the dosing with the ^{125}I-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 (^{125}I) was measured at the same time in all the blood samples generated both in subgroup 1 and subgroup 2 animals.

Radioativity (^{125}I) were measured using a Cobra gamma scintillation counter (Packard).

Radioactivity measurement was expressed as follows:

Whole blood radioactive content

Data from animals belonging to both subgroups 1 and 2 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 ^{125}I-Thyroxine solution.

2. Clinical signs

There were no clinical signs observed.

B. Body weight and body weight gain

After 4 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.

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 amount of radioactivity in 50 µl of blood of all the animals was approximately 40000 cpm.

Table 5.5-117 Whole blood radioactivity after a single administration of ^{125}I-Thyroxine Mean ± Standard deviation (cpm)

Timepoint	Control	AE C656948 (2000 ppm)	Phenobarbital (80 mg/kg/day)
Number of animals	**8**	**8**	**7**
Time point 40 min	19726 ± 1468	6163** ± 2025	10691** ± 1197
Time point 1h 30 min	16930 ± 1001	6388** ± 1982	10592** ± 1245
Time point 4h	13781 ± 1099	6111** ± 1304	9312** ± 1330
Time point 24h	3889 ± 561	2562 ** ± 482	2653** ± 547

** p≤ 0.01

The results show after an intravenous injection of ^{125}I-Thyroxine there was a statistically significantly lower level of radioactivity present in the blood of animals treated with AE C656948, at all time points, when compared with the controls. This decrease is considered to 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 animals treated with Phenobarbital, a reference compound known to induce an increase in Thyroxine clearance in the mouse through induction of Thyroxine glucuronidation.

Overall these results indicate that the Thyroxine clearance was increased in the AE C656948 treated animals when compared to control animals.

D. Deficiencies

No specific deficiencies were noted in this study

III. Conclusions

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 ^{125}I 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.

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, 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 ^{125}I 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.

Data Point:	KCA 5.5/15
Report Author:	[redacted]
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:	M-408352-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evaluated
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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. Phenobarbital (batch number: 061K0228: 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. Clinical 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 gall 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 mortalities and no treatment-related findings in term of clinical signs and body weight parameters during the course of the study.

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 -34%, $p<0.01$) whilst 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 pituitary gland.

In the Phenobarbital treated animals, after 3 days exposure at 80 mg/kg/day, mean T4 levels in the plasma were decreased (-38%, $p<0.01$), whilst mean TSH levels in the plasma were not changed when compared to the controls. An increase in the levels of Tsh transcript (+46%, $p<0.01$) was observed in the pituitary gland.

In conclusion, this study demonstrates that Fluopyram 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 levels were detected.

I. Materials and methods

A. Materials

1. **Test material:** Fluopyram AE C656948

Description	Beige powder
Lot / Batch #:	Mix-Batch:08528/0002
Purity:	94.7%
CAS #	658066-35-4

Stability of test compound:	Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration
2. Vehicle and / or positive control:	Vehicle for AE C656948: methylcellulose Positive control: Phenobarbital Vehicle for Phenobarbital: methylcellulose
Positive control:	Phenobarbital
Description:	White crystalline powder
Lot / Batch :	06100228
Purity:	99.6%
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
Age:	8 weeks approximately at start of treatment
Weight at dosing:	21.1 to 25.7 g
Source:	[redacted]
Acclimation period:	5 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
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: 27 September – 30 September 2010

2. Animal assignment and treatment

The dose levels for Fluopyram 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 300 mg/kg/day is equivalent to the top dose level (750 ppm) used in the mouse cancer bioassay in which thyroid tumors were observed in the males (SA 05094) and the dose level of 300 mg/kg/day is equivalent to the dose level used in previous mouse mechanistic studies conducted with Fluopyram (SA 07215, SA 08151 and SA 08288) where 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	Animal identity	
Males				Subgroup 1	Subgroup 2
1	Control	0	15	UT1M4180 to 4186	UT1M4187 to 4194
2	Fluopyram	100	15	UT2M4195 to 4201	UT2M4202 to 4209
3		300	15	UT3M4210 to 4216	UT3M4217 to 4224
4	Phenobarbital	80	15	UT4M4225 to 4231	UT4M4232 to 4239

3. Dosing preparation and analysis

Fluopyram and Phenobarbital were suspended in 0.5% aqueous solution of methylcellulose to provide the required concentration. There was one preparation of the test formulation for the entire study. When not in use the formulations were stored at approximately +4°C.

The stability of Fluopyram at 10 g/l and 30 g/l has been demonstrated in a previous study (SA 04060), 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:

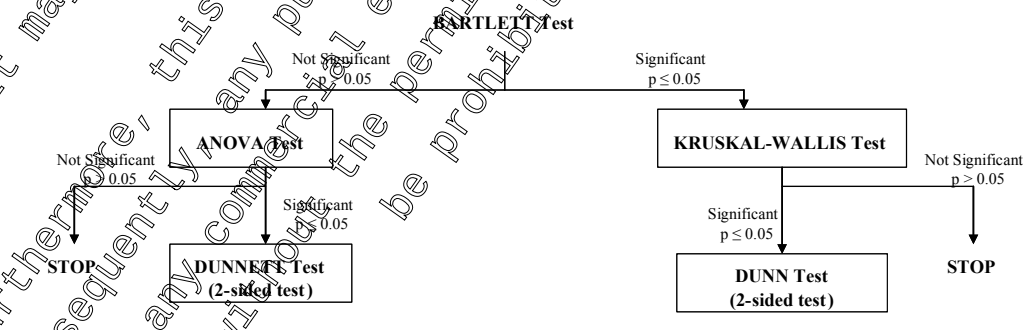
- Body weight parameters
- Hormonal parameters in the plasma
- Hormonal parameters in the bile

Statistical methods

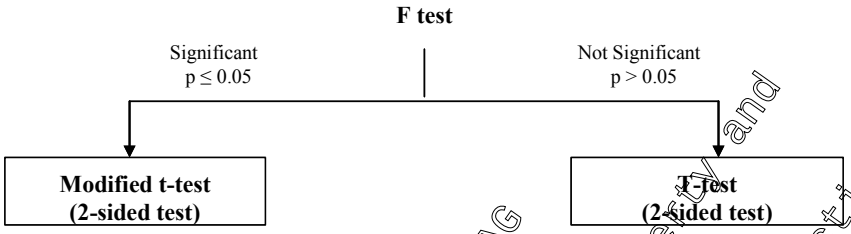
Mean and standard deviation were calculated for each group.

- Body weight change parameters,

Fluopyram: comparison between the treated groups and the vehicle control group



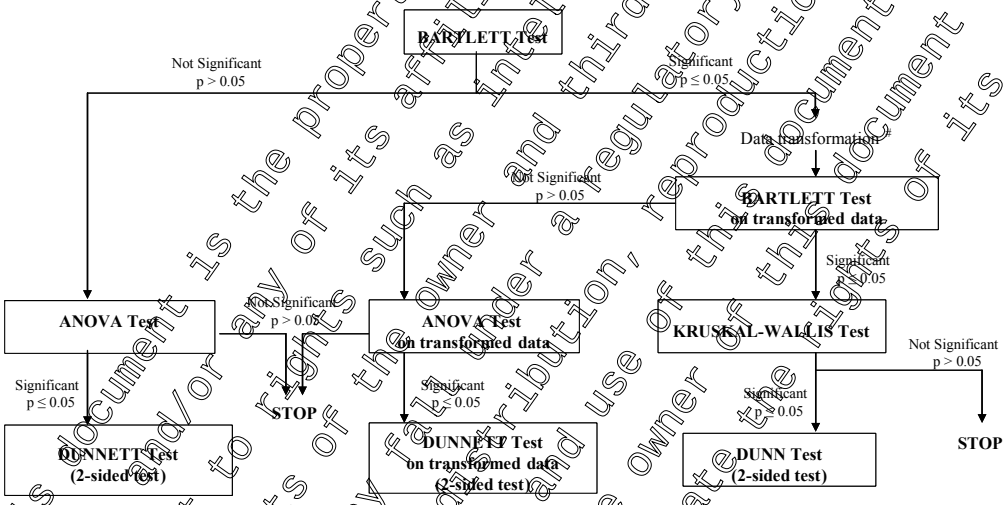
Phenobarbital: comparison between the treated group and the vehicle control group



– Body weight and hormonal parameters in the plasma

Mean and standard deviation were calculated for each group.

Fluopyram: comparison between the treated groups and the vehicle control group

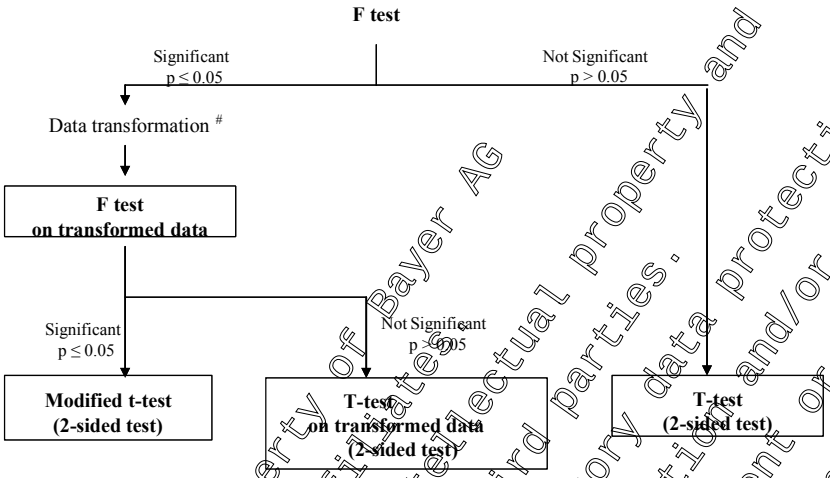


Data were transformed using the log transformation.

If one or more group variance(s) equal 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance.

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 log transformation.

- Hormonal parameters in the bile

T-tests were used for the comparison of T4 bile content in treated groups compared to control group.

Group means were compared at the 5% and 1% levels of significance.

Statistical analyses were carried out using PathTox System V4.2.2 (Module Enhanced Statistics) except the hormonal measurements in the bile which were performed using Microsoft Excel.

C. Methods

1. Observations

The animals were observed twice daily for morbundity 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

All animals were weighed at least once during the acclimatization phase. Each animal was weighed on the first day of test substance administration, then on Day 3.

3. Hormonal investigation

Blood sampling

On the day of sacrifice, 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 anesthetized 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 necropsied 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 Pathologist.

Tissue collection

A piece of the median and left liver lobes of 5 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 bladder 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 bile, were collected for T4 level measurements.

5. :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 ribosomal RNA electrophoretic profiles 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. Actin was selected as reference gene for the quantitative calculations of transcripts. The relative quantity (RQ) value of beta subunit Tsh transcript (Tsh b) was calculated using the following

formula:

$\Delta\Delta Ct = (Ct_{test} - Ct_{Actin})_{treated} - (Ct_{test} - Ct_{Actin})_{control}$

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. Each RQ value obtained for a given gene was normalized by dividing by the RQ value obtained for the control animal UT4M4180.

II. Results and discussion

A. Observations

1. Mortality

There was no mortality during the course of the study

2. Clinical signs

There were 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 statistical significance between treated and control values were as follows:

T4

A statistically significant dose-related decrease in mean T4 levels (-26% and -34% $p \leq 0.01$) was observed in the plasma of Fluopyram treated animals. A statistically significant decrease in mean T4 levels (-38%, $p \leq 0.01$) was observed in the plasma of Phenobarbital treated animals.

TSH

No statistically significant change in mean TSH levels was observed.

Table 5.5-119 Mean levels of hormones T4 and TSH hormone after exposure to Fluopyram or phenobarbital

Group	Mean plasma hormone values ± standard deviation (% change compared to control mean values)			
	Males			
	1 Control	2 Fluopyram 100 mg/kg/day	3 Fluopyram 300 mg/kg/day	4 Phenobarbital 80 mg/kg/day
T4 (nmol/l)	34.2 ± 8.7	25.4** ± 6.1 (-26%)	22.6** ± 4.6 (-34%)	21.3** ± 3.1 (-38%)
TSH (ng/ml)	3.45 ± 0.40	3.37 ± 0.24	3.54 ± 0.29	3.55 ± 0.37

**: The group mean is significantly different from the control at $p \leq 0.01$

In the bile, for the mean 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%, $p \leq 0.01$) was observed in the bile of phenobarbital treated animals. This difference was most likely due to some technical reasons and was considered not to be a treatment related effect.

Table 5.5-120 Mean levels of T4 in the bile after exposure to Fluopyram or phenobarbital

Group	Mean T4 values in the bile ± standard deviation (% change compared to control mean values)			
	Males			
	1 Control	2 Fluopyram 100 mg/kg/day	3 Fluopyram 300 mg/kg/day	4 Phenobarbital 80 mg/kg/day
T4 (nmol/l)	79.2 ± 7.9	79.0 ± 6.1	83.9 ± 8.5	64.5** ± 4.4 (-19%)

**: The group mean is significantly different from the control at $p \leq 0.01$

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 gland of Phenobarbital treated animals.

Table 5.5-121 Tsh b transcript levels after exposure to fluopyram or phenobarbital

Gene transcript	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)			
	Control	2 Fluopyram 100 mg/kg/day	3 Fluopyram 300 mg/kg/day	4 Phenobarbital 80 mg/kg/day
Tsh b #	0.994 ± 0.124	1.138 ± 0.230 (+21%)	1.494** ± 0.253 (+49%)	1.381** ± 0.198 (+46%)

Beta subunit tsh **: p≤0.01

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 -34%, p<0.01) whilst 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 pituitary gland.

In the phenobarbital treated animals, after 3 days exposure at 80 mg/kg/day, mean T4 levels in the plasma were decreased (-38%, p<0.01), whilst mean TSH levels in the plasma were not changed when compared to the controls. An increase in the levels of tsh transcript (+46%, p<0.01) was observed in the pituitary gland.

In conclusion, this study demonstrates that fluopyram 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 levels were detected.

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 fluopyram 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 levels were detected.

Data Point:	KCA 5.5/16
Report Author:	[redacted]
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:	M-426994-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evaluated
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The objective of this study was to investigate the mode of action by which fluopyram 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 thyroid tumors) on the plasma levels of thyroxine (T4) and thyroid stimulating hormone (TSH) were determined following daily oral gavage for 3 days. Four time points were evaluated 2h, 8h, 14h and 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 100 or 300 mg/kg body weight/day of fluopyram. T4 and TSH hormone levels were examined at four time points 2h, 8h, 14h 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% methylcellulose) 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 level measurements (T4 and TSH).

There were no mortalities and no treatment-related findings in term of clinical signs during the course of the study.

In the fluopyram treated animals, after 3 days of exposure at 100 or300 mg/kg/day, where animals were sacrificed 2h, 8h, 14h and 48h after the last dose, mean T4 levels in the plasma were statistically significantly ($p \leq 0.01$) decreased by between -18% 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 fluopyram administration to C57BL/6J male mice by oral gavage for three days at concentrations similar to or above 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 level was detected in this short term assay.

I. Materials and methods

A. Materials

1. **Test material:** Fluopyram (AE C656948)
   Description Beige powder
   Lot / Batch #: Mix-Batch:08528/0002
   Purity: 94.7%

CAS #	658066-35-4
Stability of test compound:	Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration
2. Vehicle and / or positive control:	0.5% aqueous solution of methylcellulose (vehicle)
3. Test animals:	
Species:	Mouse - Male only
Strain:	C57BL/6J
Age:	8 weeks approximately at start of treatment
Weight at dosing:	19.2 to 25.2 g
Source:	[redacted]
Acclimation period:	5 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
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: 15 January - 20 January 2011

2. Animal assignment and treatment

The dose levels for fluopyram 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 100 mg/kg/day is equivalent to the top dose level (750 ppm) used in the mouse cancer bioassay in which thyroid tumors were observed in the males (M-295688-01-1) 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 fluopyram (M-299522-01-1, M-308073-01-1 and M-328662-01-1), where thyroid hormone changes were detected.

Table 5.5-122 Study design

Group	Test substance	Time point	Dose level (mg/kg/day)	Number of animals per group	Animal identity
		Males			
1	Control	2h	0	15	UT1M5032 to 5046
2	Fluopyram	2h	100	15	UT2M5047 to 5061
3		2h	300	15	UT3M5062 to 5076
4	Control	8h	0	15	UT4M5077 to 5091
5	Fluopyram	8h	100	15	UT5M5092 to 5106
6		8h	300	15	UT6M5107 to 5121
7	Control	14h	0	15	UT7M5122 to 5136
8	Fluopyram	14h	100	15	UT8M5137 to 5151
9		14h	300	15	UT9M5152 to 5166

Group	Test substance	Time point	Dose level (mg/kg/day)	Number of animals per group	Animal identity
Males					
10	Control	48h	0	15	UTXM5167 to 5181
11	Fluopyram	48h	100	15	UTOM5182 to 5196
12		48h	300	15	UTDM5197 to 5211

3. Dosing preparation and analysis

Fluopyram was suspended in 0.5% aqueous solution of methylcellulose to provide the required concentrations. There was one preparation of the test formulations for the entire study. When not in use the formulations were stored at approximately +4°C.

The stability of Fluopyram at 10 g/l and 30 g/l has been demonstrated in a previous study (SA 04060), which covers the period of storage and usage for the current study.

4. Statistics

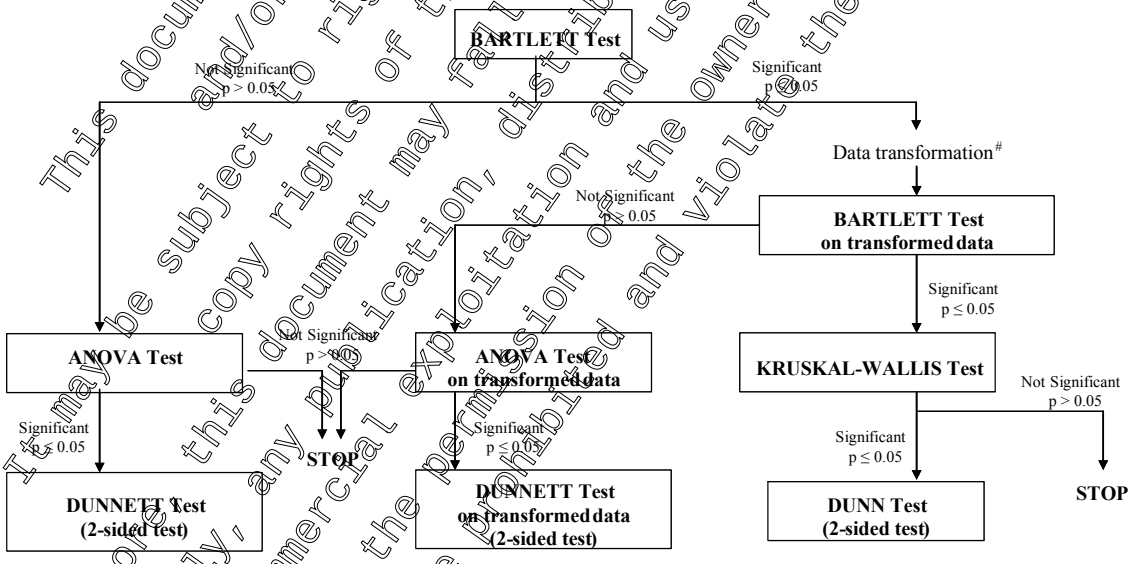
Variables analyzed

- Hormonal parameters in the plasma

Statistical methods

Mean and standard deviation were calculated for each group.

Fluopyram: comparison between the treated groups and the vehicle control group



Data were transformed using the log transformation.

If one or more group variances) equal 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 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

All animals were weighed at least once during the acclimatization phase. Each animal was weighed only on the first day of test substance administration.

3. Hormonal investigation

Blood sampling

On the day of sacrifice, 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 2h, 8h, 14h and 48h after the last dose. Animals were anesthetized 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 -74°C +10°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 -74°C +10°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.

II. Results and discussion

A. Observations

1. Mortality

There was no mortality during the course of the study

2. Clinical signs

There were no treatment-related clinical signs throughout the duration of the study.

B. Hormone analysis

The magnitude of the changes in the plasma hormone values relative to the controls and statistical significance between treated and control values were as follows:

T4

A statistically significant dose-related decrease in mean T4 levels (between -18% and -41%; $p \leq 0.01$) was observed in the plasma of fluopyram 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.

Table 5.5-123: Mean levels of hormones T4 and TSH hormone after exposure to fluopyram

Time Point	Hormone	Control	Fluopyram (100 mg/kg/day)	Fluopyram (300 mg/kg/day)
Males				
2h	T4 (nmol/l)	31.5 ± 9.1	22.8**± 6.1 (-28%)	24.0*± 6.3 (-24%)
	TSH (ng/ml)	2.7 ± 0.5	2.6 ± 0.5 (-1%)	2.7 ± 0.5 (+1%)
8h	T4 (nmol/l)	38.2 ± 7.0	28.8** ± 5.4 (-25%)	22.4** ± 4.1 (-41%)
	TSH (ng/ml)	2.8 ± 0.6	3.0 ± 0.5 (+7%)	3.1 ± 0.7 (+13%)
14h	T4 (nmol/l)	25.5 ± 5.3	20.9** ± 4.5 (-18%)	18.6** ± 4.3 (-27%)
	TSH (ng/ml)	3.1 ± 0.4	3.1 ± 0.7 (+2%)	3.1 ± 0.5 (+2%)
48h	T4 (nmol/l)	34.5 ± 8.5	25.4** ± 6.1 (-26%)	24.1** ± 4.4 (-30%)
	TSH (ng/ml)	3.1 ± 0.6	3.2 ± 0.5 (+3%)	3.2 ± 0.6 (+2%)

*: The group mean is significantly different from the control at p≤0.05
**: the group mean i s significantly different from the control at p≤0.01

C. Deficiencies

no specific deficiencies were noted in this study

III. Conclusions

In the fluopyram 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 T4 levels in the plasma were statistically significantly (p≤0.01) decreased by between -18% 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 fluopyram administration to C57BL/6J male mice by oral gavage for three days at concentrations similar to or above 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 level was detected in this short term assay.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements 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 similar to or above 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 level was detected in this short term assay.

Data Point:	KCA 5.5/17
Report Author:	[redacted]
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:	M-428031-02-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evalauated
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The objective of the present study was to investigate the mode of action by which fluopyram 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 Stimulating Hormone (TSH), the gene expression of Tsh in the pituitary gland and the hepatic UDP-glucuronosyltransferases (UDPGT) activity 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. Phenobarbital was administered by oral gavage and was used as a positive control for liver and thyroid 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: Mix-batch: 08528/0002: Beige powder , 94.7% purity), was administered to male C57Bl/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 animals dosed with 80 mg/kg/day phenobarbital (batch number: 09050075: White crystalline powder, 100% purity) 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 ppm fluopyram group and the phenobarbital group, where 15 additional males were fed untreated control diet 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. A detailed physical examination was performed once during the acclimatization phase and at least 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 necropsy and the liver was weighed. At 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 liquid nitrogen and stored at approximately -74°C + 10°C until used for Tsh transcript analyses by quantitative Polymerase Chain Reaction (qPCR). At both sacrifice times, 5 pools of 3 livers per group were homogenized for microsomal preparations in order to determine UDPGT specific enzyme activities using either bilirubin or T4 as substrate.

Dosing phase:

Fluopyram

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%; 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 (+11%; p≤0.01; +9%; p≤0.01 respectively) were recorded. Hepatic 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 bilirubin and +83%; p≤0.01 for T4 substrates), compared to controls. In addition, anincrease in the level of accumulation of Tsh transcript in the pituitary gland was recorded (+43%; p≤0.05).

At 750 ppm, a statistically significant decrease (-38%; p≤0.01) in mean T4 levels was recorded atterminal sacrifice. At necropsy, statistically significant increases in absolute mean liver weight andmean 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-bilirubin enzymatic activity (+48%; p≤0.01) together with a marginal increase of the UDPGT-T4 enzymatic (+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 g/l at a dosing volume of 5 ml /kg body weight) being administered during the first week of treatment. 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≤0.01) reduction in body weight and a statistically significant 42% (p≤0.01) reduction in cumulative body weight gain by Day 29. A statistically significant decrease (-23%; p≤0.01) in mean T4 levels was recorded at terminal sacrifice. At necropsy, statistically significant increases in absolute mean liver weight or mean liver weight relative to body weight (+10%; p≤0.01; +16%; p≤0.01 respectively) were recorded. Nonstatistically significant increases in the hepatic UDPGT-bilirubin enzymatic (+42%) and UDPGTT4 enzymatic (+32%) activities were observed. In addition, an increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded (+53%; p≤0.01).

Recovery phase:

Fluopyram:

In the males 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≤0.05) however, the observed increase was much lower than the one observed immediately following treatment with 750 ppm (+54%; p≤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 ($p \leq 0.05$). By the end of the recovery phase, body weight was marginally reduced by 3% the effect was not statistically 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. 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.

I. Materials and methods

A. Materials

1. Test material:	Fluopyram (AE C656948)
Description	Beige powder
Lot / Batch #:	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. Vehicle and / or positive control:	Vehicle for AE C656948: rodent diet Phenobarbital (positive control), vehicle methylcellulose
Positive control:	Phenobarbital
Description:	White crystalline powder
Lot / Batch :	Lot No. 0905007
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
Age:	8 weeks approximately at start of treatment
Weight at dosing:	18.1 to 24.7 g
Source:	[redacted]
Acclimation period:	13 to 15 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
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 at the appropriate dietaryconcentration (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 study represents the top dose level used in the mouse cancer bioassay in which thyroid tumors wereobserved in the males (SA 05094).

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 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 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 5.5-124 Study design

Group	Test substance	Dose level	Number of animals per group	Animal identity
		Males		
1	Control	0	15 + 15*	T1M1885 to 1914
2	Fluopyram	30 ppm (5 mg/kg/day)	15	T2M1915 to 1929
3		75 ppm (13 mg/kg/day)	15	T3M1930 to 1944
4		150 ppm (25 mg/kg/day)	15	T4M1945 to 1959
5		600 ppm (102 mg/kg/day)	15	T5M1960 to 1974
6		750 ppm (128 mg/kg/day)	15 + 15*	T6M1975 to 2004
7	Phenobarbital	80 mg/kg/day	15 + 15*	T7M2005 to 2034

• 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 polyethylene containers. There was one 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 was within a range of 89 and 98% of the nominal concentration. Therefore all values 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-085510-01-1), 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 preparation was documented in the study file. This suspension was stored in air-tight bottles at +5°C ± 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 g/l were verified. The sampling was done at two levels at the surface and at the bottom. The mean values 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 suspension in 0.5% methylcellulose 400 were between 93 and 101% 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 (SA 033 10), which covers the period of storage and usage for the current study.

4. Statistics

Variables analyzed

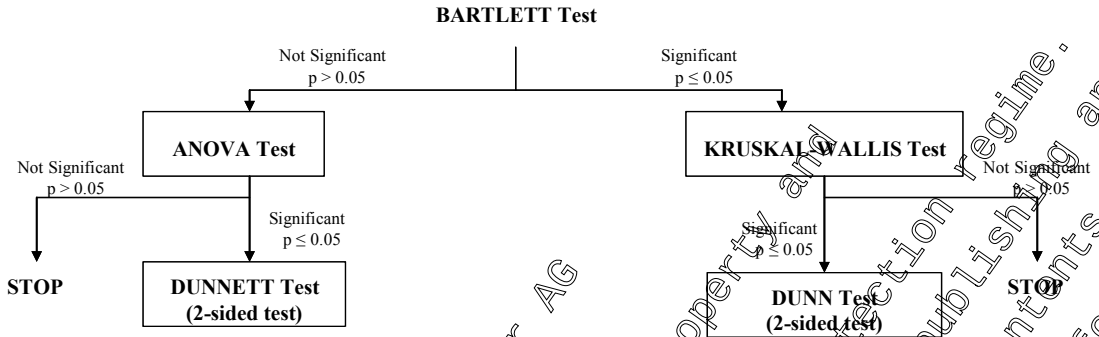
- Body weight parameters
- Body weight gain/day parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Hormonal parameters
- Terminal body weight, absolute and relative organ weights parameters - Liver enzyme activities
- qPCR determinations

Statistical methods

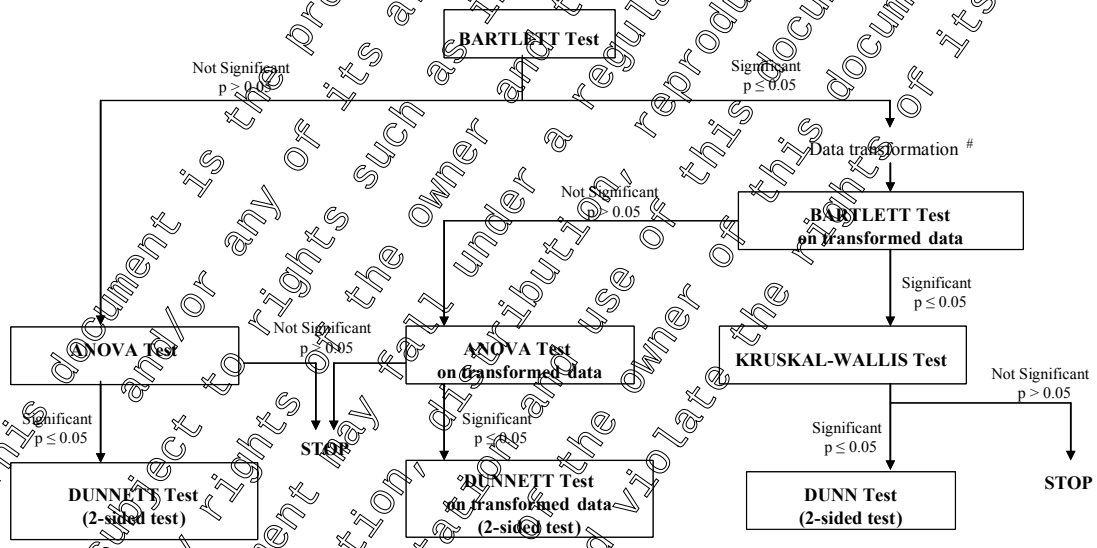
Mean and standard deviation were calculated for each group.

Fluopyram: comparison between the treated groups and the vehicle control group

- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,



- Body weight and average food consumption/day parameters
- Hormonal parameters
- Liver enzyme activities
- qPCR determinations



Data was transformed using the log transformation.

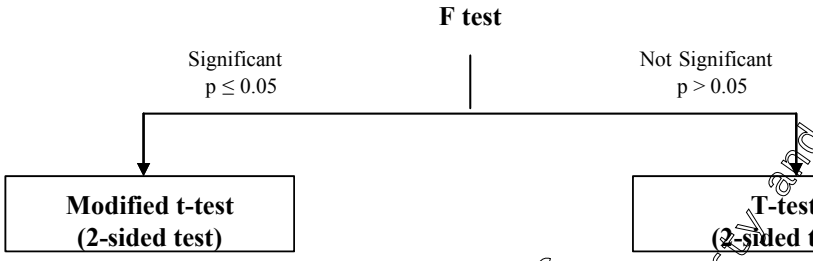
If one or more group variance(s) equal 0, means will be compared using non-parametric procedures.

Group means were compared at the 5% and 1% levels of significance.

Phenobarbital: comparison between the treated group and the vehicle control group

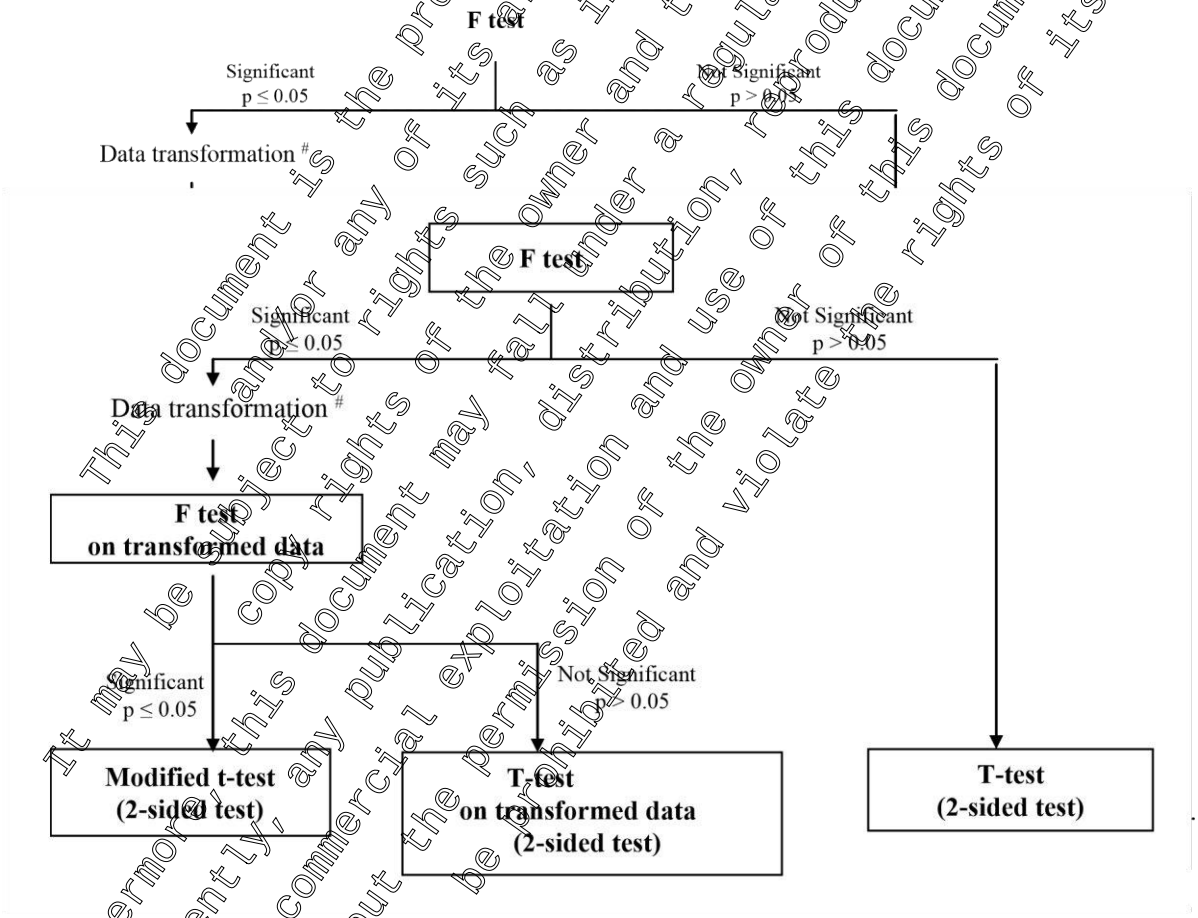
- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation was calculated for each group and per time period for body weight change parameters



- Body weight and average food consumption/day parameters
- Hormonal parameters
- Liver enzyme activities
- qPCR determinations

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.



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 at least once during the acclimatization phase, on study Day 1 and then 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 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. 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:

$$Test\ substance\ intake = \frac{Dose\ level\ (ppm) \times Group\ mean\ food\ consumption\ (g/day)}{Group\ mean\ bodyweight\ (g)\ at\ the\ end\ of\ the\ food\ consumption\ period}$$

4. Hormonal investigation

Blood sampling

On the day of sacrifice, between 8:30 a.m. and 11 a.m., blood samples were taken from all animals from the abdominal aorta. Animals were anesthetized by inhalation of Isoflurane (Baxter, Maurepas, France) prior to bleeding. Blood was collected on lithium heparin. 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 frozen at approximately -74°C + 10°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 " BioVetim, Vet Agro Sup, Campus Vétérinaire de Lyon, Aile 1 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 recovery phase. 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 necropsied. The necropsy 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 animals from each group as well as the pituitary gland of all the animals were sampled.

For each group, the pituitary gland was collected and flash frozen in liquid nitrogen. These samples were stored individually at approximately -74°C + 10°C until used for qPCR investigations.

6. Hepatotoxicity testing

At each sacrifice time, the livers were weighed, pooled by three in each treatment group and homogenized for microsomal 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 ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

Quantitative PCR

One 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 a beta subunit Tsh Taqmanassay (Assay on demand, Applied Biosystems, ref Mm03990915-g1), 1/25 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-microtubulin, 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) was calculated using the following formula:

$\Delta\Delta Ct$ = (CtTsh b – CtGusb) treated - (CtTsh b – CtGusb) 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 a given gene was normalized by dividing by the RQ value obtained for the control animal T1M1885 for the dosing phase and T1M1900 for the recovery phase.

UDPGT activities

UDPGT activity with bilirubin as substrate was determined using a spectrophotometry method consisting in the determination of conjugated bilepigments after their conversion into azo-pigment derivatives. Absorbance was measured at 530 nm. Three replicates from each sample were assayed.

The glucuronidation of the substrate T4 was subcontracted to CXR Biosciences Ltd, James Lindsay Place, Dundee Technopole, Dundee DD1 5JJ, Scotland, UK.

II. Results and discussion

A. Observations

1. Mortality

Fluopyram: There was no mortality in any groups throughout the study.

Phenobarbital: Animal T7M2020 was sacrificed for humane reasons on study day 2 and two animals (T7M2030 and T7M2033) 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 8 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 phase

Fluopyram: There were no treatment-related clinical signs.

Phenobarbital: All males displayed reduced motor activity during the dosing phase.

Recovery phase

Fluopyram: 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% ($p \leq 0.01$) reduction in body weight and a 42% ($p \leq 0.01$) 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 recovery period (-5%, $p \leq 0.05$), compared to the control group, but was only marginally lower than the control group by Day 29 (-3%; not statistically significant).

C. Food consumption

Dosing phase

Fluopyram: Food consumption was unaffected by treatment throughout the dosing phase.

Phenobarbital: Food consumption was considered to be comparable 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 phase.

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.

D. Hormone analysis

The magnitude of the changes in the plasma hormone values relative to the controls and statistical significance between treated and control values were as follows:

Dosing phase

Fluopyram:

T4

A statistically significant decrease in mean t4 levels ($p \leq 0.01$) was observed at all the dose levels in the plasma of fluopyram treated animals when compared to controls, though not in a clear dose-related manner.

TSH

No statistically significant change in mean TSH levels was observed.

Table 5.5-125 Mean levels of T4 and TSH hormones in the plasma after exposure to Fluopyram

Mean plasma hormone values ± standard deviation (% change compared to control mean values)						
Dose-level (ppm)	Fluopyram					
	0	30	75	150	600	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%)	16.3** ±2.9 (-38%)
TSH (ng/ml)	1.4 ±0.6	2.1 ±0.9 (+50%)	1.6 ±0.5 (+14%)	1.2 ±0.3 (-14%)	1.6 ±0.7 (+14%)	1.6 ±0.6 (+14%)

**: P≤0.01

Phenobarbital:

T4

A statistically significant decrease in mean T4 levels (-23%, p≤0.01) was observed in the plasma of phenobarbital treated animals.

TSH

No statistically significant change in mean TSH levels was observed.

Table 5.5-126 Mean levels of T4 and TSH hormones in plasma after exposure to Phenobarbital

Mean plasma hormone values ± standard deviation (% change compared to control mean values)		
	Phenobarbital	
Dose-level (ppm	0 ppm	80 mg/kg
T4 (nmol/l)	26.1 ±6.9	20.1**±2.7 (-23%)
TSH (ng/ml)	1.4 ±0.6	1.6±0.4 (+14%)

**: p≤0.01

Recovery Phase:

Fluopyram:

T4

No statistically significant change in mean T4 levels was observed.

TSH

No statistically significant change in mean TSH levels was observed.

Table 5.5-127 Mean levels of T4 and TSH hormones in plasma after exposure to Fluopyram followed by recovery period

Mean plasma hormone values ± standard deviation (% change compared to control mean values)		
	Fluopyram	
Dose-level (ppm)	**0**	**750**
T4 (nmol/l)	28.5 ±5.4	27.1±5.6 (-5%)
TSH (ng/ml)	1.5 ±0.3	1.4±0.4 (-7%)

Phenobarbital:

T4

No statistically significant change in mean T4 levels was observed.

TSH

No statistically significant change in mean TSH levels was observed.

Table 5.5-128 Mean levels of T4 and TSH hormones after exposure to Phenobarbital followed by recovery period

Mean plasma hormone values ± standard deviation (% change compared to control mean values)		
	Phenobarbital	
Dose-level (ppm)	**0 ppm**	**80 mg/kg**
T4 (nmol/l)	28.5±5.4	28.4 ±7.5 (-0.4%)
TSH (ng/ml)	1.5 ±0.3	1.6 ±0.5 (+7%)

E. QPCR analysis

Dosing phase

Fluopyram

A dose-related increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded at 600 and 750 ppm (+43%; p≤0.05; +54%; p≤0.01).

Table 5.5-129 Tsh b transcript levels after exposure to Fluopyram

Mean relative quantity ± standard deviation of Tsh b transcript (% change compared to control mean values)						
	Fluopyram					
Dose-level (ppm)	**0**	**30**	**75**	**150**	**600**	**750**
Tsh b	1.156 ±0.334	1.223 ±0.354 (+6%)	1.303 ±0.344 (+13%)	1.297 ±0.451 (+12%)	1.655* ±0.461 (+43%)	1.783** ±0.725 (+54%)

**: p≤0.01

Phenobarbital

An increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded in phenobarbital treated animals (+53%; p≤0.01).

Table 5.5-130 Tsh b transcript levels after exposure to Phenobarbital

Mean relative quantity ± standard deviation of Tsh b transcript (% change compared to control mean values)		
	Phenobarbital	
	0 ppm	80 mg/kg
Tsh b	1.156 ±0.334	1.764** ±0.586 (+53%)

**: p≤0.01

Recovery Phase:

Fluopyram:

A slight increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded in fluopyram treated animals after the recovery phase (+12%; p≤0.05).

Table 5.5-131 Tsh b transcript levels after exposure to Fluopyram followed by recovery phase

Mean relative quantity ± standard deviation of Tsh b transcript (% change compared to control mean values)		
	Fluopyram	
Dose-level (ppm)	**0**	**750**
Tsh b	1.046 ±0.087	1.169* ±0.184 (+12%)

*: p≤0.05

Phenobarbital

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 Tsh b transcript levels after exposure to Phenobarbital followed by recovery phase

Mean relative quantity ± standard deviation of Tsh b transcript (% change compared to control mean values)		
	Phenobarbital	
Dose-level (ppm)	**0 ppm**	**80 mg/kg**
Tsh b	1.046 ±0.087	1.090 ±0.139 (+4%)

F. Post mortem examinations

Terminal body weight and organ weight

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)						
	Fluopyram					
Dose-level (ppm)	**0**	**30**	**75**	**150**	**600**	**750**
Absolute liver weight (g)	1.31 ±0.08	1.37 ±0.13 (-5%)	1.40 ±0.09 (+7%)	1.45** ±0.13 (+11%)	1.67** ±0.12 (+27%)	1.78** ±0.12 (+36%)
Liver to body weight ratio (%)	5.338 ±0.310	5.570 ±0.306 (+4%)	5.667* ±0.278 (+6%)	5.825** ±0.275 (+9%)	6.786** ±0.468 (+27%)	7.088** ±0.341 (+33%)

*: p≤0.05; **: p≤0.01

Phenobarbital:

There was no change in mean terminal body weight in treated animals when compared to the controls. Mean absolute and relative liver weight were statistically significantly higher when compared to controls following phenobarbital treatment.

Table 5.5-134 Mean liver weight changes after exposure to Phenobarbital

Mean liver weight ±sd at scheduled sacrifice (% change when compared to controls)		
	Phenobarbital	
Dose-level (ppm)	**0 ppm**	**80 mg/kg**
Absolute liver weight (g)	1.31 ±0.08	1.44** ±0.12 (+10%)
Liver to body weight ratio (%)	5.338 ±0.310	6.190** ±0.291 (+16%)

**: p≤0.01

Recovery Phase:

Fluopyram:

There was no change in mean terminal body weight in treated animals when compared to the controls. There was no significant change in liver weight parameters in treated animals when compared to the controls.

Table 5.5-135 Mean liver weight changes after exposure to Fluopyram followed by recovery period

Mean liver weight ±sd at scheduled sacrifice (% change when compared to controls)		
	Fluopyram	
Dose-level (ppm)	**0**	**750**
Absolute liver weight (g)	1.32 ±0.10	1.37 ±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.

There was no significant change in liver weight parameters 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 (% change when compared to controls)		
Phenobarbital		
Dose-level (ppm)	**0 ppm**	**80 mg/kg**
Absolute liver weight (g)	1.32 ±0.10	1.33 ±0.09 (+1%)
Liver to body weight ratio (%)	4.984 ±0.296	5.085 ±0.282 (+2%)

G. UDPGT activities

Dosing phase

Fluopyram:

A dose-related increase in the UDPGT enzymatic activity using bilirubin as substrate was recorded at 600 and 750 ppm (+39%; p≤0.05; +48%, p≤0.01).

An increase in the UDPGT enzymatic activity using thyroxine as substrate was recorded at 600 ppm (+83%; p≤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 UDPGT activities after exposure to Fluopyram

Mean enzymatic UDPGT activities ± standard deviation (% change compared to control mean values)						
Fluopyram						
Dose-level (ppm)	**0**	**30**	**75**	**150**	**600**	**750**
UDPGT (Bilirubin)	1.985±0.577	2.217 ±0.375 (+12%)	2.394 ±0.327 (+21%)	2.624 ±0.198 (+32%)	2.758* ±0.324 (+39%)	2.947** ±0.422 (+48%)
UDPGT (Thyroxine)	0.770±0.144	0.768 ±0.190 (0%)	0.854 ±0.198 (+11%)	1.168 ±0.360 (+52%)	1.412** ±0.253 (+83%)	1.026 ±0.271 (+33%)

*: p≤0.05; **: p≤0.01

Phenobarbital:

Non-statistically significant increases of the hepatic UDPGT enzymatic activities (+42% and +32%) were recorded in the phenobarbital treated animals.

Table 5.5-138 Mean enzymatic UDPGT activities after exposure to Phenobarbital

Mean enzymatic UDPGT activities ± standard deviation (% change compared to control mean values)		
Phenobarbital		
Dose-level (ppm)	**0 ppm**	**80 mg/kg**
UDPGT (bilirubin)	1.985±0.577	2.821±0.676 (+42%)
UDPGT (Thyroxine)	0.770±0.144	1.020±0.210 (+32%)

Recovery phase

Fluopyram:

There was no significant change in the hepatic UDPGT enzymatic activities in the fluopyram treated animals after the recovery phase.

Table 5.5-139 Mean enzymatic UDPGT activities after exposure to Fluopyram followed by recovery period

Mean enzymatic UDPGT activities ± standard deviation (% change compared to control mean values)		
Fluopyram		
Dose-level (ppm)	**0**	**750**
UDPGT (bilirubin)	1.795±0.313	1.908±0.237 (+6%)
UDPGT (Thyroxine)	0.822±0.115	0.774±0.327 (-6%)

Phenobarbital:

There was no significant change in the hepatic UDPGT enzymatic activities in the phenobarbital treated animals after the recovery phase.

Table 5.5-140 Mean enzymatic UDPGT activities after exposure to Phenobarbital followed by recovery period

Mean enzymatic UDPGT activities ± standard deviation (% change compared to control mean values)		
Phenobarbital		
Dose-level (ppm)	**0 ppm**	**80 mg/kg**
UDPGT (bilirubin)	1.795±0.313	1.992±0.293 (+11%)
UDPGT (Thyroxine)	0.822±0.115	0.808±0.096 (-2%)

H. Deficiencies

No specific deficiencies were noted in this study

III. Conclusions

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. 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.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on fluopyram in the mouse.

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. 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

Data Point:	KCA 5.5/18
Report Author:	[redacted]
Report Year:	2012
Report Title:	Fluopyram: 28-day toxicity study for proliferation assessment in the C57BL/6J male mouse
Report No:	SA 11123
Document No:	M-428303-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evaluated.
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The objective of the present study was to investigate possible thyroid cell proliferation, caused by fluopyram 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% purity) 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 diet. 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 necropsied in the morning following twenty eight days of treatment; the thyroid and the liver were sampled and fixed. The thyroid was 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 on food consumption or body weight parameters for the group treated with fluopyram.

At necropsy, there was no change in mean terminal body weight when compared to control animals. Enlarged and dark livers were found in 14/15 and 5/15 animals, respectively. No microscopic changes in the thyroid gland were noted.

Assessment of cell proliferation in the thyroid revealed a 1.7 fold increase ($p \leq 0.01$) 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% ($p \leq 0.05$) and 4.8% ($p \leq 0.01$), throughout the study. Following an initial body weight loss between Study Days 1 and 8, overall cumulative body weight gain was reduced by 52% ($p \leq 0.01$) by Study Day 29, compared to the controls.

At necropsy, mean terminal body weight was lower (-4.4%, $p \leq 0.01$) 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 thyroid revealed 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 in 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 ($p \leq 0.01$) 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.

I. Materials and methods

A. Materials

1. Test material:	Fluopyram (AE C656948)
Description	Beige powder
Lot / Batch #:	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. Vehicle and / or positive control:	Vehicle for AE C656948: rodent diet Phenobarbital (positive control), 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
Age:	8 weeks approximately at start of treatment
Weight at dosing:	19.6 to 24.8 g
Source:	[redacted]
Acclimation period:	14 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
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: 08 June – 06 July 2011

2. Animal assignment and treatment

Three groups of male mice were dosed for at least 28 days with the appropriate compound by the appropriate route of administration. Each group consisted of 15 male mice.

Two groups were dosed by dietary 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 (M-408352-01-1) 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 (M-295688-01-1).

The third group 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 hormone level change was detected (M-299521-01-1).

Table 5.5-141 Study design

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	T2M2181 to 2195
3	Phenobarbital	80 mg/kg/day	15	T3M2196 to 2210

3. Diet preparation and analysis

Fluopyram was incorporated into the diet to provide the required dietary concentrations.

The test formulation was stored at room temperature and issued to the animal unit in polyethylene containers. There was one formulation used in the study and any unused diet 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 85-115% of the nominal concentration. The stability of fluopyram at 20 and 10000 ppm in the diet was demonstrated in an earlier study (M-085510-01-1), 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. This suspension was stored in air-tight bottles at +5°C ± 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 phenobarbital in aqueous 0.5% methylcellulose 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 90-110% of the nominal concentration. The stability of phenobarbital at 8 g/l has 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'-deoxyUridine), an analogue of thymidine (batch number: HMBB6206: an off white powder, 99% purity), supplied by Sigma-Aldrich; Germany, was used to evaluate cell proliferation in the study and was stored at 0 to -20 °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 bottles at room temperature when not in use. The unused solution was discarded at the end of the administration period.

The concentration data for BrdU 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 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 9.2) 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 period for body weight change parameters.

  The F test was performed to compare the homogeneity of group variances.

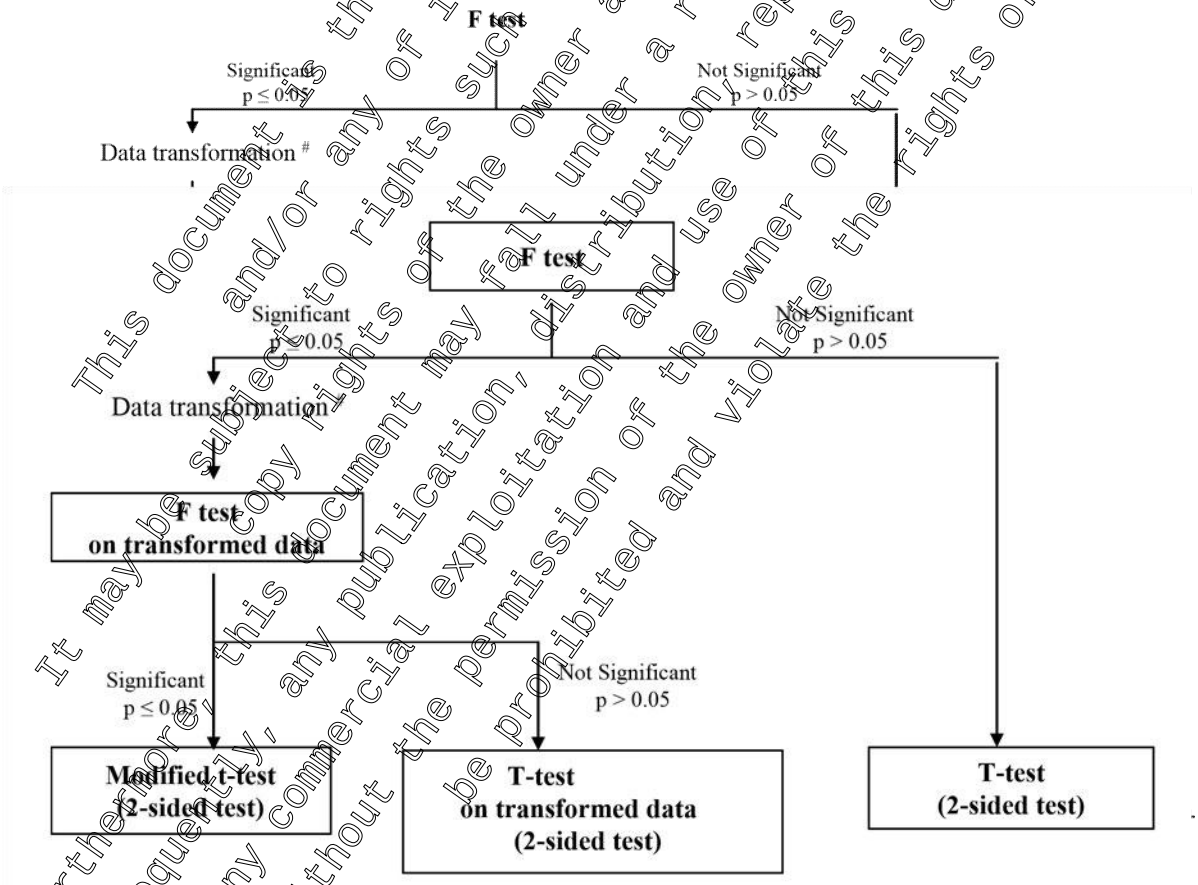
  If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

  If the F test was significant ($p\leq0.05$), 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

  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.



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 at least once during the acclimatization phase, on study Day 1 and then weekly during the treatment period. 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 1 to 4 was calculated for each sex using the following formula:

$$Test\ substance\ intake = \frac{Dose\ level\ (ppm) \times Group\ mean\ food\ consumption\ (g/day)}{Group\ mean\ bodyweight\ (g)\ at\ the\ end\ of\ the\ food\ consumption\ period}$$

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 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. Significant macroscopic abnormalities were recorded and sampled upon the decision of the Study Pathologist.

The following organs or tissues were sampled:

- Thyroid gland (with trachea)
- Liver
- 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.

Conventional histopathological examination

Histological samples containing 6 thyroid 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.

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 counter staining 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 slides was performed by 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 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 (slide scanner, Hamamatsu). 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 study.

2. Clinical signs

Fluopyram: There were no treatment-related clinical signs.

Phenobarbital: All mice displayed reduced motor activity form Study Day 2 onwards.

B. Body weight and bodyweight gain

Fluopyram: There was no effect on body weight parameters.

Phenobarbital: Mean body weight was reduced by between 3.3% and 4.8% throughout the treatment period, the effect being statistically significant on Study Days 8 ($p \leq 0.05$), 22 ($p \leq 0.05$) and 29 ($p \leq 0.01$). This effect resulted from a mean body weight loss of 0.07 g ($p \leq 0.01$), 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 2.0 g, compared to 2.1 g in the controls (-52%; $p \leq 0.01$), by Study Day 29.

C. Food consumption

Fluopyram: Food consumption was unaffected by treatment throughout the dosing phase.

Phenobarbital: Food consumption was unaffected by treatment throughout the dosing phase.

D. Water consumption

Fluopyram: Mean consumption of water containing BrdU was decreased by 7.9% (not statistically significant), compared to the control group.

Phenobarbital: Mean consumption of water containing BrdU was decreased by 7.9% ($p \leq 0.05$), 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 to the controls.

Phenobarbital: Terminal body weight was 4.4% lower than the controls and was statistically significant ($p \leq 0.01$).

F. Gross pathology

Fluopyram: Enlarged and dark livers were noted in 14/15 and 5/15 treated animals, respectively.

Phenobarbital: Enlarged and dark livers were noted in 3/15 and 4/15 animals, respectively.

G. Microscopic pathology

Only the thyroid glands were examined.

Fluopyram: No treatment-related microscopic change was noted.

Phenobarbital: No treatment-related microscopic change was noted.

H. Cell proliferation

Fluopyram: the mean brdu labeling index was found to be approximately 1.7 fold higher ($p \leq 0.01$) than the controls.

Phenobarbital: the mean brdu labeling index was similar to the controls.

Table 5.5-142 Mean brdu labeling index (proliferation rate/100 cells)

Group	Control	Fluopyram	Phenobarbital
N	13	15	14
Mean	12.45	21.09**	12.68
STD	6.17	5.28	3.71

**: $P \leq 0.01$

I. Deficiencies

No specific deficiencies were noted in this study.

III. Conclusions

In conclusion, a clear increase in thyroid cell proliferation, as evidenced by a 1.7 fold increase ($p \leq 0.01$) 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.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on fluopyram in the mouse.

In conclusion, a clear increase in thyroid cell proliferation, as evidenced by a 1.7 fold increase ($p \leq 0.01$) 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.

Data Point:	KCA 5.5/19
Report Author:	Blanck, O.
Report Year:	2013, Amended: 05-14-2013
Report Title:	Fluopyram: 28-day toxicity study for thyroid cell proliferation in the C57BL/6J male mouse
Report No:	SA 12058
Document No:	M-449821-03-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evalauated
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The objective of the present study was to characterize the thyroid effects caused by the administration of different dose levels of fluopyram to C57BL/6J 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 high 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% w/w purity), a fungicide of the pyridinyl-ethylbenzamide (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, 13, 25, 99, 124 and 247 mg/kg/day, respectively. Each group consisted of 15 male mice with the exception of the control group and the 1500 ppm fluopyram group, where 15 additional males were fed untreated 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 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 sacrificed either following at least 28 days of treatment or at the end of the recovery phase. All surviving animals were subjected to necropsy and the thyroid and the duodenum were sampled and fixed. The thyroid was 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.

Dosing phase

No treatment-related clinical signs, effects on body weight parameters, food or water consumption were observed at any dose level tested.

At necropsy, there was no change 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.

Assessment of follicular cell proliferation in the thyroid revealed a 1.21, 1.40, 1.61 and 2.33 fold increase in BrdU 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 1500 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 fluopyram 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 wash out period

I. Materials and methods

A. Materials

1. Test material:	Fluopyram (AE C656948)
Description	Beige powder
Lot / Batch #:	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. Vehicle and / or positive control:	Vehicle for AE C656948, rodent diet No positive control
3. Test animals:	
Species:	Mouse - Male only
Strain:	C57BL/6J
Age:	8 weeks approximately at start of treatment
Weight at dosing:	19.4 to 23.9 g
Source:	[redacted]
Acclimation period:	14 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages
Environmental conditions:	
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: 09 May – 07 June 2012

Recovery period for animals allocated to the recovery phase: 07 June – 04 July 2012

2. Animal assignment and treatment

Seven groups were dosed by dietary administration. One group consisted of control animals that received untreated 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 (M-408352-01-1) and (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 (M-295688-01-1).

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.

Each group consisted of 15 male mice with the exception of the control group and the 1500 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 recovery phase.

Table 5.5-143 Study design

Group	Test item	Dose level	Number of animals per group	Animal identity
		Males		
1	Control	0 ppm	15 + 15*	T1M0546 to 0575
2	Fluopyram	30 ppm (5 mg/kg/day)	15	T2M0576 to 0590
3	Fluopyram	75 ppm (13 mg/kg/day)	15	T3M0591 to 0605
4	Fluopyram	150 ppm (25 mg/kg/day)	15	T4M0606 to 0620
5	Fluopyram	600 ppm (99 mg/kg/day)	15	T5M0621 to 0635
6	Fluopyram	750 ppm (124 mg/kg/day)	15	T6M0636 to 0650
7	Fluopyram	1500 ppm (247 mg/kg/day)	15 + 15*	T7M0651 to 0680

*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 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 levels 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 values were within the in-house target range of 85115% of the nominal concentration, which the exception of one 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-085510-01-1), which covered the period of storage and usage for the present study.

BrdU (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 ± 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.

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 90-110% 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 to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Terminal body weight
- Thyroid cell proliferation parameters

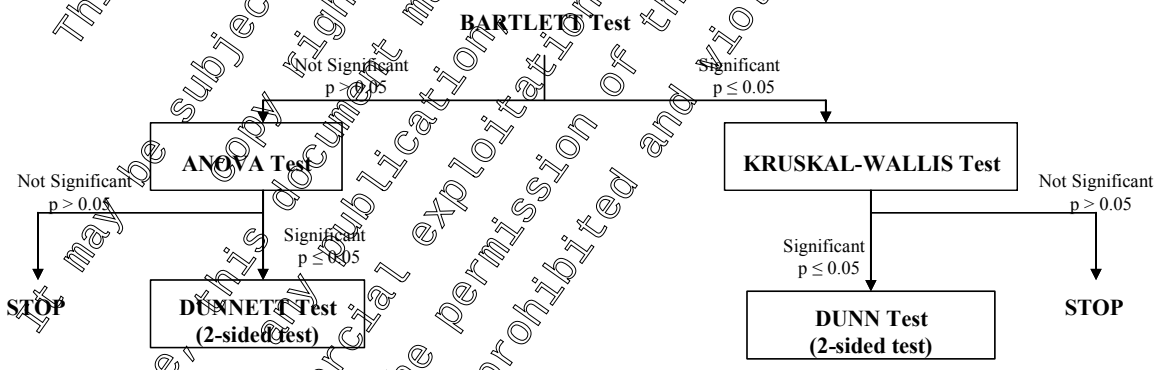
Statistical methods

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 9.2) were used for the cell proliferation data.

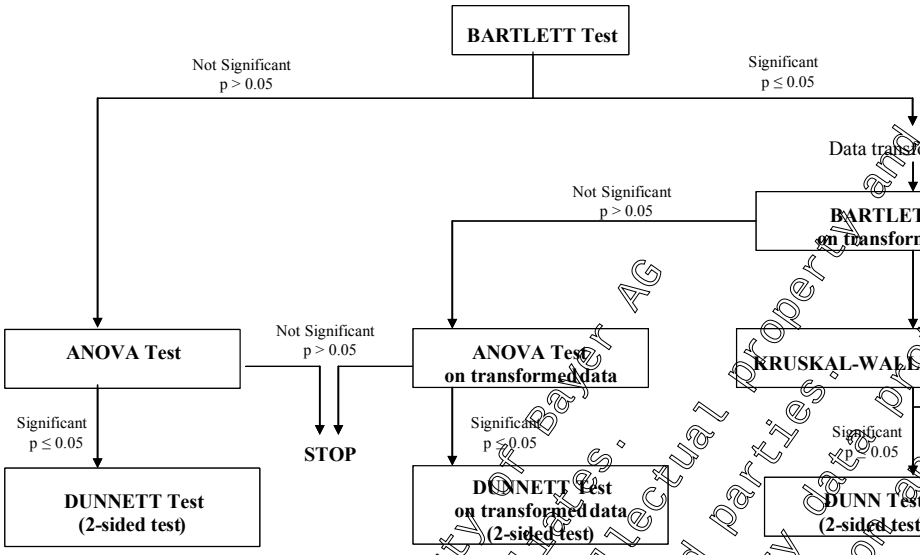
Dosing phase fluopyram: comparison between the treated groups and the vehicle control group

- Body weight change parameters
- Terminal body weight



- Body weight and average food consumption/day parameters

Data will be transformed using the log transformation for body weight parameters



– Thyroid follicular cell parameters

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 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≤0.05), means of the exposed groups were compared to 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 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 ANOVA test on log transformed data was not significant (p>0.05), STOP.

If the ANOVA test on log transformed data was significant (p≤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≤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.

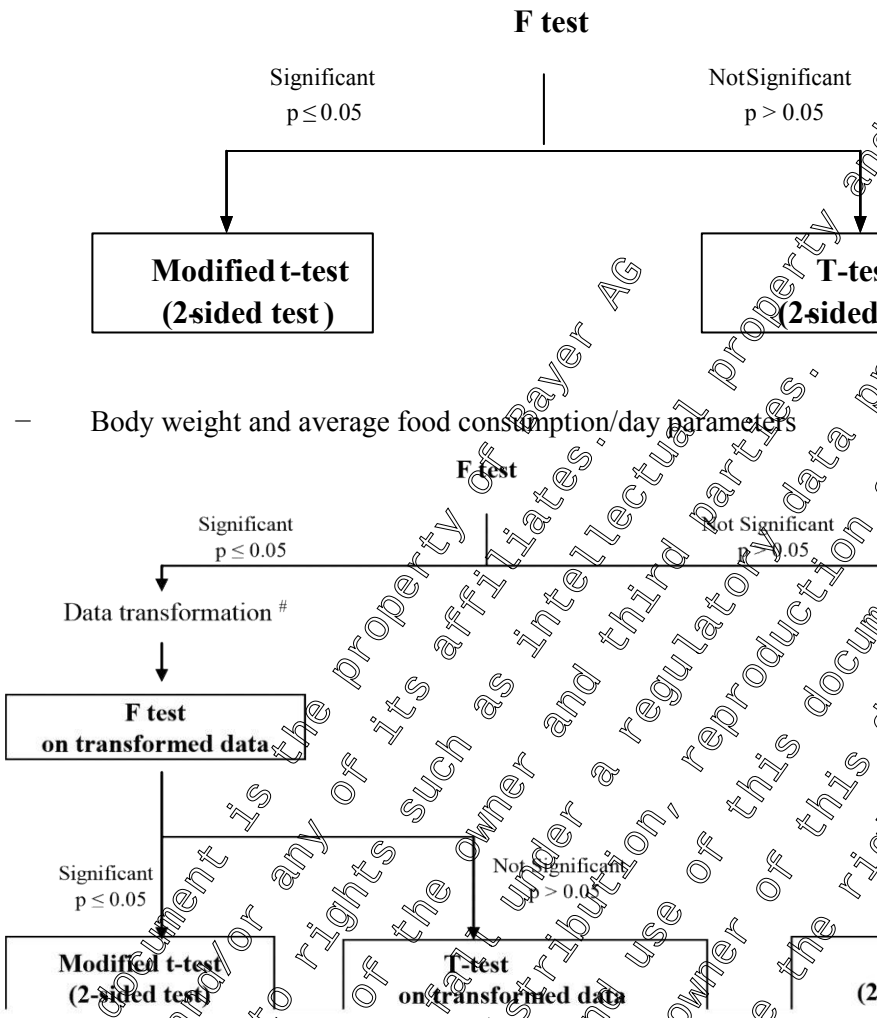
If the Kruskal-Wallis test was significant (p≤0.05), means of the exposed groups were compared to the mean of the control group using the Dunn test (1-sided).

If one or more group variance(s) equal 0, means will be compared using non-parametric procedures.

Recovery phase fluopyram: comparison between the treated groups and the vehicle control group

– Body weight change parameters

– Terminal body weight



– Body weight and average food consumption/day parameters

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 at least once during the acclimatization phase, on study Day 1 and then 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 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 1 to 4 was calculated for each sex using the following formula:

$$Test\ substance\ intake = \frac{Dose\ level\ (ppm) \times Group\ mean\ food\ consumption\ (g/day)}{Group\ mean\ bodyweight\ (g)\ at\ the\ end\ of\ the\ food\ consumption\ period}$$

4. Water consumption

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration (Study Day 23 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 procedure – Organ sampling

On Study Days 30 and 31 following treatment or on Study Day 29 following the recovery phase, all surviving animals were sacrificed by exsanguination 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 the external surfaces, all orifices and all major organs, tissues and body cavities. Significant macroscopic abnormalities were recorded and sampled upon the decision of the Study Pathologist.

The following organs or tissues were sampled:

- Thyroid gland (with trachea)
- 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 counter staining 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 slides was performed by 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 (slide scanner, Hamamatsu). The mean and standard deviation were calculated for each group.

II. Results and discussion

A. Observations

1. Mortality

There were no treatment-related mortalities during the course of this study.

2. Clinical signs

There were no treatment-related clinical signs.

B. Body weight and bodyweight gain

There was no treatment-related effect on body weight parameters.

C. Food consumption

Food consumption was unaffected by treatment.

D. Water consumption

Water consumption was unaffected by treatment.

E. Post mortem examinations

Terminal body weight

Mean terminal body weight was unaffected by treatment.

F. Gross pathology

No treatment-related macroscopic findings were observed.

G. Microscopic pathology

Only the thyroid 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 at the three highest dose levels.

Table 5.5-144 Mean BrdU labeling index (proliferation rate/100 cells)

Dose group	Control	30 ppm	75 ppm	150 ppm	600 ppm	750 ppm	1500 ppm
N	14	15	15	15	15	15	15
Mean	21.55	17.81	19.51	26.09	30.11**	34.78***	50.21***
STD	4.75	7.37	5.64	8.62	8.53	7.61	10.24

**: p≤0.01 ***: p≤0.001

Recovery phase

No change in proliferative index was noted at 1500 ppm when compared to controls.

Table 5.5-145 Mean BrdU labeling index (proliferation rate/100 cells)

Group	Control	1500 ppm
N	14	14
Mean	17.57	11.56
STD	5.18	4.78

I. Deficiencies

No specific deficiencies were noted in this study

III. Conclusions

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 fluopyram 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 wash out period.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on fluopyram in the mouse.

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 fluopyram 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 wash out period.

Data Point:	KCA 5.5/20
Report Author:	[redacted]
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 fluopyram to male mice (C57BL/6J and Pxr KO/Car KO)
Report No:	SA 12162
Document No:	M-449890-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evaluated
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Fluopyram (batch number: Mix-batch: 08528/0002, a beige powder, 94.7% purity), a fungicide of the pyridinyl-ethylbenzamide family, was administered continuously via the diet to groups of C57BL/6J or Pxr KO/Car KO mice (15/group/strain) for at least 28 days at concentrations of 0, 750 and 1500 ppm, corresponding to 125 and 256 mg/kg/day in C57BL/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 twice daily for moribundity and mortality except on weekends when they 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 aorta of each animal for possible further analysis. All animals were necropsied, 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 cell proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations. The pituitary gland was collected and flash frozen in liquid nitrogen and stored at approximately -74°C + 10°C for gene transcript analyses by quantitative Polymerase Chain Reaction (q-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 Biosciences for microsomal preparations in order to determine total cytochrome P-450 content, cytochrome P-450 isoenzyme and UDPGT specific enzyme activities.

C57BL/6J mice

No clinical signs were observed throughout the study. Body 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. Enlarged livers were noted in 14/15 animals, compared with 0/15 animals in the control group.

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≤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≤0.001) as were PROD (150 fold, P≤0.001), BQ (7.9 fold, P≤0.001), T4-GT (1.9 fold, P≤0.001) and BIL-GT (2.0 fold, P≤0.001) activities.

In the pituitary gland, *Tshb* gene transcripts were up-regulated (1.7 fold, P≤0.001) when compared to the controls.

At 750 ppm

At necropsy, mean absolute and relative liver weights were statistically significantly higher (+39% to +42%, P≤0.01) when compared to controls. Enlarged livers were noted in 7/15 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 thyroid revealed a 1.8 fold (P≤0.001) higher BrdU labeling index, when compared to the controls.

Regarding the hepatotoxicity testing, total cytochrome P-450 content was increased (3.6 fold, P≤0.001) as were PROD (70 fold, P≤0.001), BQ (5.5 fold, P≤0.001), T4-GT (1.8 fold, P≤0.001) and BIL-GT (1.8 fold, P≤0.001) activities.

In the pituitary gland, *Tshb* gene transcripts were up-regulated (1.6 fold, P≤0.01) when compared to the controls.

Pxr KO/Car KO mice

No treatment-related clinical signs were observed throughout the study. Body 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 slightly induced (1.4 fold, P≤0.01) whereas BQ and T4-GT activities were decreased (1.7 fold, P≤0.001 and 1.3 fold, P≤0.01; respectively).

In the pituitary gland, *Tshb* gene transcripts were slightly down-regulated (1.2 fold, P≤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.

Regarding the hepatotoxicity testing, PROD activity was slightly induced (1.4 fold, P≤0.001) whereas BQ activity was decreased (1.5, P≤0.001).

No were no changes in *Tshb* gene transcripts.

Conclusion

The clear 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 BQ activity in wild type mice and decrease in BQ 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 in *Tshb* gene transcripts and in an increase of thyroid follicular cell proliferation.

I. Materials and methods

A. Materials

1. Test material:	Fluopyram (AE C656948)
Description	Beige powder
Lot / Batch #:	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. Vehicle and / or positive control:	Vehicle for AE C656948: rodent diet No positive control
3. Test animals:	
Species:	Mouse - Male only
Strain:	C57BL/6J
Age:	10 weeks approximately at start of treatment
Weight at dosing:	22.1 to 26.9 g wildtype mice and 20.5 to 25.0 g Pxr KO/Car KO mice
Source:	[redacted] and Taconic Farms (Germantown, New York, 12526 USA, respectively, for wildtype and Pxr KO/Car KO mice
Acclimation period:	10 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages
Environmental conditions:	
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 September – 23 October 2012

2. Animal assignment and treatment

For both the wildtype and Pxr KO/Car KO mice, one group of control animals received untreated diet, whilst other two groups received fluopyram at the appropriate dietary concentration (750 ppm 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 (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 (M-428303-01-1).

An additional dose level (1500 ppm) in the current study was investigated to better elucidate the level 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 Study design

Group	Test item	Dose level	Number of animals per group		Animal identity			
					C57BL/6J		Pxr KO/Car KO	
			C57BL/6J	Pxr KO/Car KO	SG1	SG2	SG1	SG2
1	Control	0	15	15	T1M 2737 to 2743	T1M2752 to 2759	T1M2744 to 2751	T1M2760 to 2766
2	Fluopyram	750 ppm	15	15	T2M2767 to 2774	T2M2782 to 2788	T2M2775 to 2781	T2M2789 to 2796
3		1500 ppm	15	15	T3M2797 to 2803	T3M2812 to 2819	T3M2804 to 2811	T3M2820 to 2826

SG: Subgroup

3. Diet preparation and analysis

Fluopyram was incorporated into the diet to provide the required 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. All values 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-085510-01-1) which covered the period of storage and usage for the present study.

BrdU in filtered tap water from municipal 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 approximately 4°C when not 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

− 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.

P-450 content and enzymatic activities were not statistically analyzed in Pristima; only descriptive statistics are presented.

Group means were compared at least at the 5% levels of significance.

Statistical analyses were carried out using Pristima, version 6.3.2 build 17, Xybion Corp.

• - Total cytochrome P-450 content and liver enzyme activities

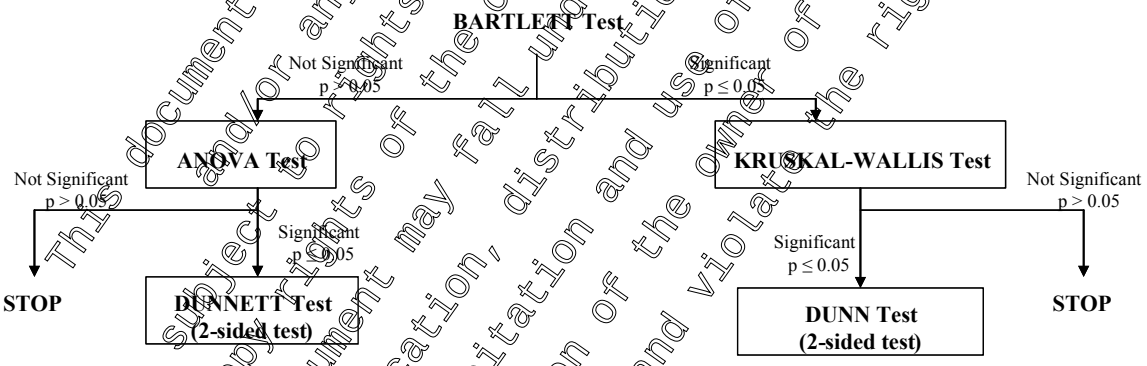
Statistical comparisons between fluopyram treated mice and their respective control groups were performed for all numerical data sets using a 2-tailed Student's t-test.

• - Body weight change parameters,

- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

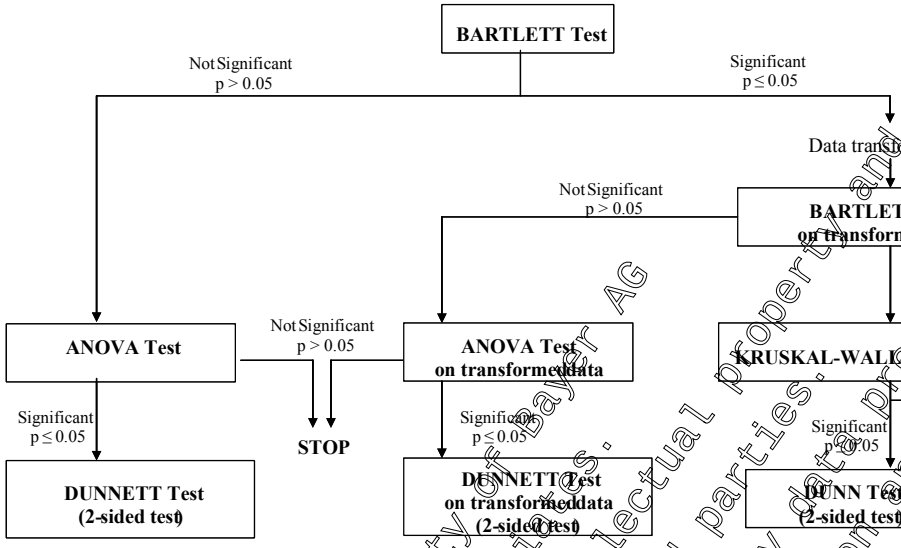
− Terminal body weight



• - Body weight and average food consumption/day parameters

- Quantity of gene transcripts

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.



Data were transformed using the log transformation for body weight, food consumption and gene transcript parameters.

− Thyroid follicular cell proliferation parameters

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 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≤0.05), means of the exposed groups were compared to 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 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 ANOVA test on log transformed data was not significant (p>0.05), STOP.

If the ANOVA test on log transformed data was significant (p≤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≤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 Kruskal-Wallis test was significant (p≤0.05), means of the exposed groups were compared to the mean of the control group using the Dunn test (1-sided).

If one or more group variance(s) equal 0, means will be compared using non-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 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 then 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 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 in mg/kg/day for each week and for Weeks 1 to 4 was calculated for each sex using the following formula:

$$Test\ substance\ intake = \frac{Dose\ level\ (ppm) \times Group\ mean\ food\ consumption\ (g/day)}{Group\ mean\ bodyweight\ (g)\ at\ the\ end\ of\ the\ food\ consumption\ period}$$

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. Clinical Chemistry

On Study Day 30, blood samples were taken from all animals in all groups by puncture of the abdominal aorta. Animals were anesthetized by inhalation of Isoflurane (Virbac, Carros, France). Blood was collected on heparin for possible further analysis.

6. Sacrifice and pathology

Necropsy procedure – Organ sampling

On Study Day 30, all surviving animals were sacrificed by exsanguination 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 following organs were weighed:

- Brain
- Liver

Tissue collection and histotechnology

The following organs were sampled:

- Duodenum
- Liver
- Pituitary gland
- Thyroid gland

For each animal, the thyroid gland, two central sections taken from the median and left lobes of the liver and a portion of the duodenum 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 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 hematoxylin.

For each group, one piece of both the median and left lobes of the liver plus the pituitary gland from each animal (both strains) were collected and flash frozen in liquid nitrogen. These samples were stored at approximately -74°C + 10°C until used for qPCR investigations.

The remaining portion 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 Biosciences for microsomal preparations.

Histopathology and cell proliferation assessment

Histopathological examinations were performed on thyroid gland and liver for all animals. Following the initial histopathological examination, a review of representative slides was performed by a second pathologist (Laëtitia ELIES) according to standard operating procedures.

A digital image of the whole slide containing the thyroid gland was acquired using the Nanozoomer 2.0 series (Hamamatsu). The labeling index, expressed as the number of BrdU-positive thyroid follicular cells per thousand, was measured on multiples fields by counting in total at least 1000 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 -70°C until required for total cytochrome P-450 content, specific cytochrome P-450 isoenzyme profile and glucuronidation assessments to check the hepatotoxic potential of the test item.

Total cytochrome p-450 content

Total cytochrome P-450 content of the liver microsomal fractions was determined according to LMS Spec-0002 (Omura and Sato, 1964).

Protein determination

The protein concentration of the liver microsomes was determined in aqueous solutions using a modification of the method of Lowry et al., (1951) and bovine serum albumin standards, according to LMS Spec-001. Results were maintained in the study file.

Cytochrome P-450 activity assays

Microsomal pentoxyresorufin-O-dependtylation (PROD) was used as a marker for Cyp2b activity, and was measured according to LMS Fluor-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 (T4) glucuronidation activity

Mouse liver microsomes were incubated with 125I-thyroxine and the formation of T4-glucuronide was determined by HPLC with radioflow detection.

Bilirubin glucuronidation 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 treated animals using RNeasy Mini kits (Qiagen). RNA quality controls were performed based on the ribosomal 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 Taqman 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:

Gene family	Isoform	Refset ID	Taqman assay ID (Applied Biosystems)
Thyroid Stimulating Hormone beta subunit	*Tshb*	NM_009432.1	Mm03990315-g1
Beta actin	Actb	NM_007393.1	Mm00607939_s1

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:

ΔΔCt = (Cttest – CtB2m) treated - (Cttest - CtB2m) control

RQ = 2-ΔΔCt

where Ct is the threshold cycle at which PCR 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 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 during 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 hind limbs on Study Days 29 and 30, whilst one mouse (T3M2825) from the 1500 ppm 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

C57BL/6J mice:

Body weight parameters were unaffected by treatment throughout the study.

Pxr KO/Car KO mice:

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 ($p \leq 0.05$), 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 the controls.

Pxr KO/Car KO mice:

At 750 ppm, mean food consumption was decreased by between -9% ($p \leq 0.01$; Study Day 15) and -4% (not statistically significant; Study Day 8) compared to the controls. In the absence of an effect on mean food consumption at the higher dose level of 1500 ppm, the decreased food consumption noted at 750 ppm was considered to be incidental.

Achieved dosages

The mean achieved dietary intake of fluopyram expressed in mg/kg/day received by the animals during the study was as follows:

Table 5.5-148 Mean achieved dietary intake of fluopyram

Mean achieved dietary intake of fluopyram (Weeks 1 – 4)		
Diet concentration (ppm)	**C57BL/6J (mg/kg/day)**	**Pxr KO/Car KO (mg/kg/day)**
750	125	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 wild-type (C57BL/6J) mouse groups or between control and Knock-Out (Pxr KO/Car KO) mouse groups.

In wild type animals, at 1500 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.

Table 5.5-149 Mean liver weight changes; Wild-Type Mice

Mean liver weight ±sd at scheduled sacrifice, wild-type groups (% change when compared to controls)			
Sex	**Males**		
Fluopyram dose level (ppm)	**0**	**750**	**1500**
Mean absolute liver weight (g)	1.277 ±0.06859	1.8035** ±0.15861 (+41%)	2.115*** ±0.19106 (+66%)
Mean liver to body weight ratio (%)	4.9285	6.8482**	7.9883***

Mean liver weight ±sd at scheduled sacrifice, wild-type groups (% change when compared to controls)			
Sex	**Males**		
Fluopyram dose level (ppm)	**0**	**750**	**1500**
	±0.18549	±0.44559 (+39%)	±0.48285 (+62%)
Mean liver to brain weight ratio (%)	288.9877 ±17.75730	409.6459** ±35.06164 (+42%)	480.1812*** ±43.73024 (+66%)

**P≤0.01; ***P≤0.001

In Knock-Out animals, at 1500 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.

Table 5.5-150 Mean liver weight changes; Knock-Out Mice

Mean liver weight ±sd at scheduled sacrifice, knock-out groups (% change when compared to controls)			
Sex	**Males**		
Fluopyram dose level (ppm)	**0**	**750**	**1500**
Mean absolute liver weight (g)	1.2945 ±0.08045	1.4008** ±0.07952 (+8%)	1.4307** ±0.07729 (+11%)
Mean liver to body weight ratio (%)	5.3119 ±0.24611	5.7075** ±0.27193 (+7%)	5.7970** ±0.23142 (+9%)
Mean liver to brain weight ratio (%)	297.0051 ±15.48663	315.5953* ±26.98886 (+6%)	336.8503** ±18.1761 (+13%)

*P≤0.05; **P≤0.01; ***P≤0.001

F. Gross pathology

C57BL/6J mice:

Enlarged livers were noted at 1500 and 750 ppm. These changes were associated with microscopic hepatocellular hypertrophy and were considered to be treatment-related.

Table 5.5-151 Macroscopic changes in the Liver; Wild-Type Mice

Incidence of macroscopic changes in the liver, scheduled sacrifice, wild-type groups			
Sex	Males		
Fluopyram dose level (ppm)	0	750	1500
Enlarged	0/15	7/15	14/15

Pxr KO/Car KO mice:

No macroscopic change was noted.

G. Microscopic pathology

C57BL/6J mice:

At 1500 ppm, hepatocellular hypertrophy, hepatocellular single cell necrosis, increased number of mitoses and interstitial mixed cell infiltrate were noted.

At 750 ppm, hepatocellular hypertrophy, hepatocellular single cell necrosis and interstitial mixed cell infiltrate were noted.

Table 5.5-152 Microscopic changes in the Liver; Wild-Type Mic

Incidence of microscopic changes in the liver, scheduled sacrifice, wild-type groups			
Sex	Males		
Fluopyram dose level (ppm)	0	750	1500
Number of examined animals	15	15	15
Hepatocellular hypertrophy: centrilobular to panlobular			
Minimal	0	0	2
Slight	0	14	6
Moderate	0	1	7
Total	0	15	15
Hepatocellular single cell necrosis: focal			
Minimal	0	5	9
Slight	0	0	1
Total	0	5	10
Increased number of mitoses			
Present	0	0	3
Interstitial mixed cell infiltrate: focal			
Minimal	5	9	11
Slight	1	0	0
Total	6	9	11

Pxr KO/Car KO mice

No relevant microscopic change was noted at 1500 or 750 ppm.

H. Cell proliferation

C57BL/6J mice:

Higher proliferative indexes were noted (2.6-fold at 1500 ppm and 1.8-fold at 750 ppm) when compared to controls.

Table 5.5-153 Mean BrdU labeling index (proliferation rate/100 cells); Wild-Type Mice

Thyroid gland proliferation index			
Fluopyram dose level (ppm)	**0**	**750**	**1500**
N	15	15	15
Mean	14.28	26.08***	36.61***
Std	3.96	7.16	10.27

***P≤0.001

Pxr KO/Car KO mice:

No change was noted between control and treated groups.

Table 5.5-154 Mean BrdU labeling index (proliferation rate/100 cells); Knock-Out Mice

Thyroid gland proliferation index			
Fluopyram dose Level (ppm)	**0**	**750**	**1500**
N	15	15	15
Mean	10.05	9.91	8.27
Std	3.88	4.06	3.38

I. Hepatotoxicity testing

C57BL/6J mice:

The total cytochrome P450 content of the liver microsomal fractions from male C57BL/6J 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/6J mice, respectively, over the concurrent control.

28 day exposure to fluopyram at 1500 ppm and 750 ppm increased microsomal BQ (a marker for Cyp3a11) activity by 7.9- and 5.5-fold concurrent controls in male C57BL/6J mice, respectively.

Following 28 days exposure to fluopyram at 1500 ppm and 750 ppm, microsomal thyroxine glucuronosyl transferase (T4-GT) activity was increased by 1.9- and 1.8-fold concurrent controls in male C57BL/6J mice, respectively.

Bilirubin glucuronidation in male C57BL/6J mice administered fluopyram 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 Results of Hepatotoxicity testing; Wild-Type Mice

Parameter	Control 0 ppm	Fluopyram 750 ppm	Fluopyram 1500 ppm
C57BL/6J			
Total P450 nmol/mg protein	0.34 ± 0.22 (100.0 ± 63.5)	1.24 ± 0.16*** a (363.3 ± 47.2)	1.25 ± 0.18*** (367.3 ± 51.3)
PROD pmols resorufin formed/min/mg protein	2.01 ± 0.20 (100.0 ± 10.0)	140.21 ± 15.11*** a (6991.0 ± 753.5)	302.14 ± 84.76*** (15065.3 ± 4226.4)
BQ nmols 7-OH quinoline formed /min/mg protein	2.77 ± 0.34 (100.0 ± 12.3)	15.20 ± 1.89*** a (549.3 68.3)	21.94 ± 1.83*** (792.8 ± 66.1)
T4-GT pmol T4-glucoronide formed/min/mg protein	0.58 ± 0.17 (100.0 ± 29.9)	1.06 ± 0.17*** a (183.8 ± 29.2)	1.09 ± 0.25*** (189.5 ± 43.6)
BIL-GT nmol bilirubin-glucuronide formed/min/mg protein	0.73 ± 0.11 (100.0 ± 14.4)	1.30 ± 0.22*** a (177.4 ± 30.0)	1.43 ± 0.42*** (195.6 ± 57.6)

a Values are Mean ± SD; Values in parenthesis are mean % control ± SD. n = 15 per group; a n = 14
A Students' t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; *** $p<0.001$

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-fold).

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.

Fluopyram had no effect on bilirubin glucuronidation in the Pxr KO/Car KO mice.

Table 5.5-156 Results of Hepatotoxicity testing; Knock-Out Mice

Parameter	Control 0 ppm	Fluopyram 750 ppm	Fluopyram 1500 ppm
Pxr KO/Car KO			
Total P450 nmol/mg protein	0.24 ± 0.09 (100.0 ± 36.5)	0.27 ± 0.12 (112.3 ± 49.7)	0.27 ± 0.12 (112.0 ± 47.5)
PROD pmols resorufin formed/min/mg protein	2.27 ± 0.30 (100.0 ± 13.0)	3.20 ± 0.81*** (141.3 ± 35.6)	3.24 ± 1.16** (142.9 ± 51.0)
BQ nmols 7-OH quinoline formed /min/mg protein	3.51 ± 0.39 (100.0 ± 11.0)	2.40 ± 0.40*** (68.5 ± 11.3)	2.09 ± 0.35*** (59.4 ± 10.1)
T4-GT pmol T4-glucoronide formed/min/mg protein	0.57 ± 0.19 (100.0 ± 34.0)	0.66 ± 0.17 (115.9 ± 29.9)	0.43 ± 0.15* (75.3 ± 26.7)
BIL-GT nmol bilirubin-glucuronide formed/min/mg protein	0.70 ± 0.24 [a] (100.0 ± 34.1)	0.66 ± 0.24 [a] (94.5 ± 34.1)	0.61 ± 0.28 (86.5 ± 39.4)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± 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 mice:

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.

Table 5.4-157 *Tshb* gene transcript levels; Wild-Type Mice

Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)		
	Control	Fluopyram 750 ppm	Fluopyram 1500 ppm
Tshb	1.23 ± 0.289	1.92** ± 0.306 (+56%)	2.05*** ± 0.586 (+67%)

**P≤0.01; ***P≤0.001

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 transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)		
	Control	Fluopyram 750 ppm	Fluopyram 1500 ppm
Tshb	1.25 ± 0.264	1.14 ± 0.177	1.04* ± 0.165 (-17%)

*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/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 BQ activity in wild type mice and decrease in BQ 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 in *Tshb* gene transcripts and in an increase of thyroid follicular cell proliferation.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013 as it provides mechanistic information on fluopyram in the mouse.

The clear 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 BQ activity in wild type mice and decrease in BQ 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 in *Tshb* gene transcripts and in an increase of thyroid follicular cell proliferation.

Data Point:	KCA 5.5/21
Report Author:	[redacted]
Report Year:	2013
Report Title:	Fluopyram: Assessment of pentoxyresorufin-o-dependentylation and benzyloxyquinoline-o-debenzylation in 50 liver microsomal samples
Report No:	CXR1284
Document No:	M-451628-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	Not previously evaluated
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

- Microsome samples (50 samples) were received, frozen, by CXR on 03-Aug-2011 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 France, where in-life phase of the study was conducted under the Study number SA 11005, Documentation No. M-428031-02-1, Rouquie D. (2012): Fluopyram – Mechanistic 28-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations).
- The activity of Cyp2b was measured in the mouse liver microsome preparations as the rate of dependentylation of pentoxyresorufin (PROD).
- 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 80mg/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 liver microsome preparations as the rate of debenzylation of benzyloxyquinoline (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 in BQ activity.
- Following a 28 day recovery period on control diet, BQ activity in both fluopyram and phenobarbital treated mice returned to control levels.

I. Materials and methods

A. Materials

1. **Test material:** Fluopyram (AE C656948)
   Description Beige powder
   Lot / Batch #: 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. **Vehicle and / or positive control:** Vehicle for AE C656948: rodent diet
   Phenobarbital (positive control)
   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
Age:	8 weeks approximately at start of treatment
Weight at dosing:	18.1 to 24.7 g
Source:	[redacted]
Acclimation period:	13 to 15 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages.
Environmental conditions:	
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 September – 23 October 2012

Recovery period: 22 June – 26 July 2011

2. Microsomal analysis dates: 14 March – 18 March 2013

3. 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 at the appropriate dietaryconcentration (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 cancer bioassay 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 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.

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
Males				
1	Control	0	15 + 15*	T1M1885 to 1914
2	Fluopyram	30 ppm (5 mg/kg/day)	15	T2M1915 to 1929
3		75 ppm (13 mg/kg/day)	15	T3M1930 to 1944
4		150 ppm (25 mg/kg/day)	15	T4M1945 to 1959
5		600 ppm (102 mg/kg/day)	15	T5M1960 to 1974
6		750 ppm (128 mg/kg/day)	15 + 15*	T6M1975 to 2004
7	Phenobarbital	80 mg/kg/day	15 + 15*	T7M2005 to 2034

4. Statistics

Statistical comparisons between treated microsomes and their control group were undertaken for all numerical data sets using a 2-tailed Student's t-test.

C. Methods

1. Microsomal samples

Microsome samples (50 samples) were received, frozen, by CXR on 03-Aug-2011 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, France, Study number SA 11105).

2. Pentoxyresorufin-*O*-depentylation (PROD)

The activity of Cyp2b in microsomal samples was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al.* (1985) Biochem. Pharmacol. 34, 18. 3337-3345, according to LMS Fluor-002.

3. Benzyloxyquinoline-*O*-debenzylation (BQ)

The activity of Cyp3a in microsomal samples was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline, as described by GENTEST HTS technical bulletin, according to LMS Fluor-005.

II. Results and discussion

A. Pentoxyresorufin-o-depentylation:

The activity of cyp2b was measured in the mouse liver microsome preparations as the rate of dependentylation of pentoxyresorufin (PROD).

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 80mg/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.

Table 5.5-160 Pentoxyresorufin-*O*-Depentylation

Treatment, 28 day exposure	PROD (pmol resorufin formed/min/mg protein)
Control	4.65 ± 1.12 (100.0 ± 24.2)[a]
Fluopyram 30ppm	67.22 ± 6.95*** (1447.1 ± 149.5)
Fluopyram 75ppm	156.89 ± 46.37*** (3377.5 ± 998.3)[a]
Fluopyram 150ppm	170.99 ± 29.09*** (3680.8 ± 626.2)
Fluopyram 600ppm	201.51 ± 45.02*** (4338.0 ± 969.1)
Fluopyram 750ppm	219.06 ± 31.31*** (4715.8 ± 674.0)
Phenobarbital 80mg/kg/day	151.87 ± 48.84*** (3269.3 ± 1051.4)
Treatment, 28 day recovery	
Control	4.63 ± 0.82 (100.0 ± 17.7)
Fluopyram 750ppm	5.27 ± 0.75 (113.9 ± 16.1)[a]
Phenobarbital 80mg/kg/day	5.33 ± 0.31 (115.1 ± 6.6)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 5 per group; [a] n =4 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<0.001.

Figure 5.5-12 Pentoxyresorufin-*O*-Depentylation, 28 Day Exposure

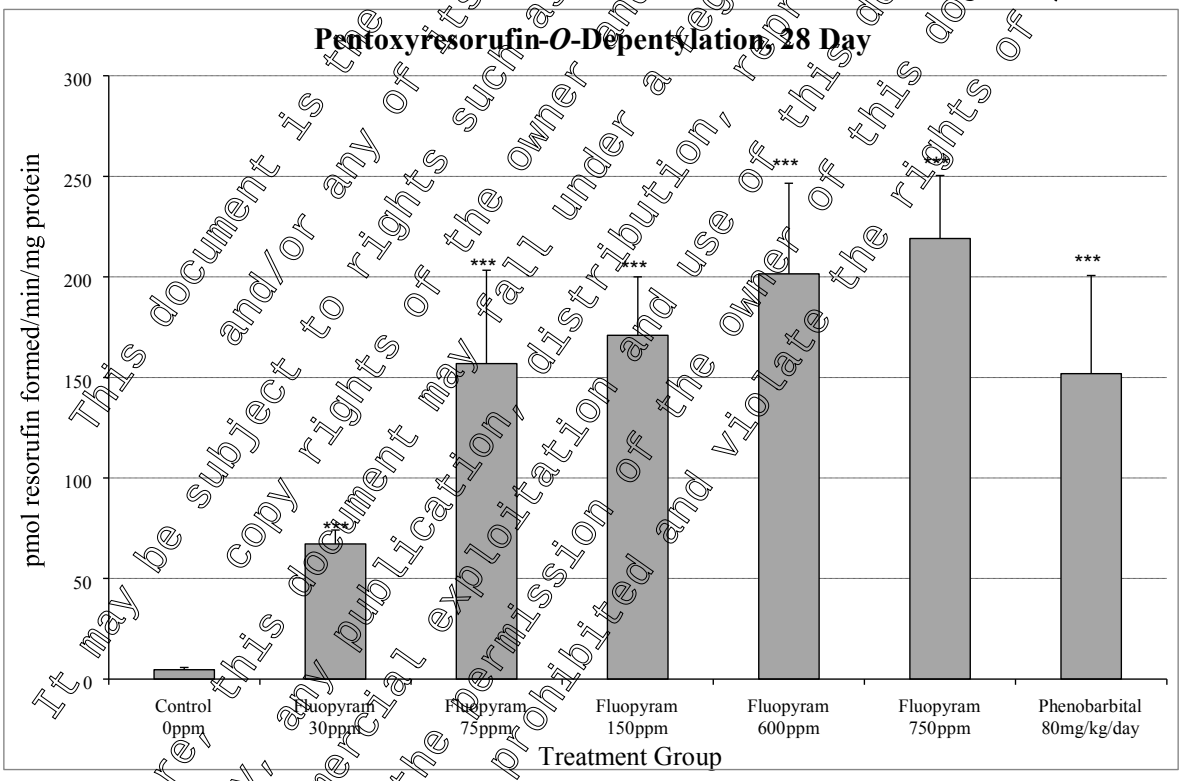
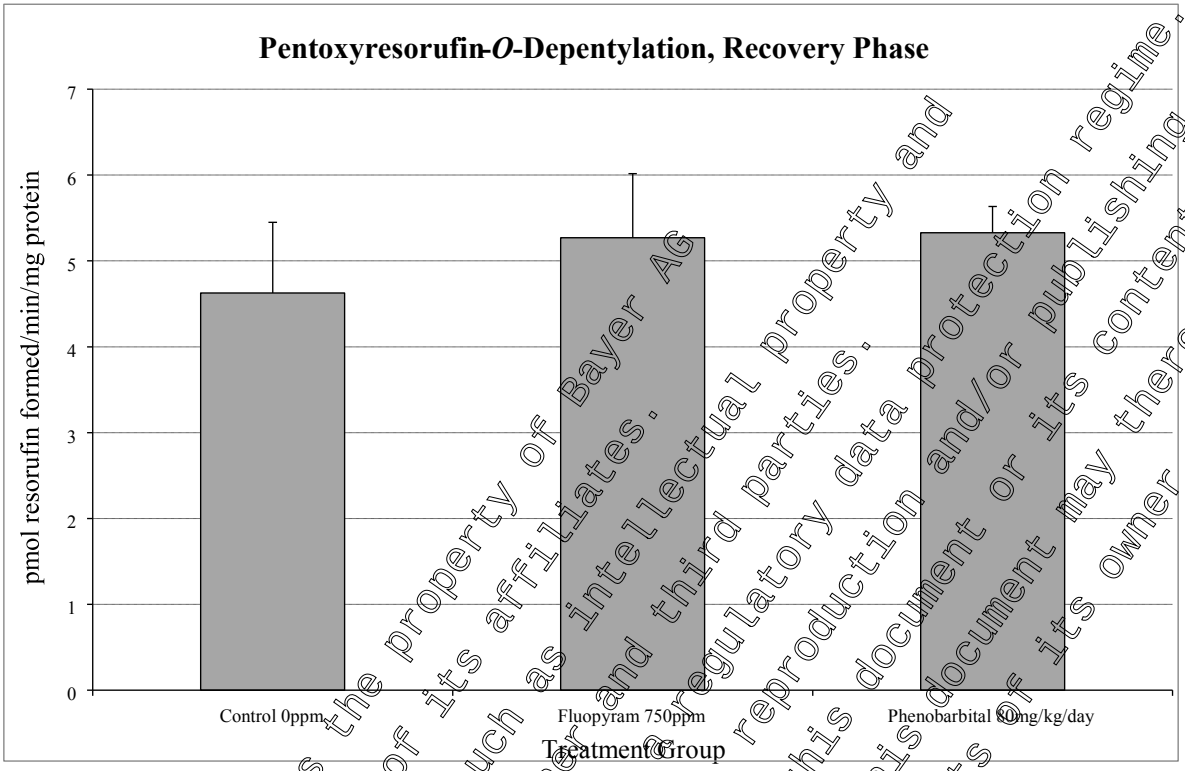


Figure 5.5-13 Pentoxyresorufin-*O*-Dependentylation, 28 Day Recovery



2. Benzyloxyquinoline-*O*-Debenzylation

The activity of CYP3A was measured in the mouse liver microsome preparations as the rate of debenzylation of benzyloxyquinoline (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 in bq activity. Following a 28 day recovery period on control diet, BQ activity in both fluopyram and phenobarbital treated mice returned to control levels.

Table 5.5-161 Benzyloxyquinoline-*O*-Debenzylation

Treatment, 28 day exposure	BQ (nmol 7-Hydroxyquinoline formed/min/mg protein)
Control	7.59 ± 0.67 (100.0 ± 8.8)[a]
Fluopyram 30ppm	10.68 ± 1.92* (140.7 ± 25.3)
Fluopyram 75ppm	16.71 ± 2.61*** (220.2 ± 34.4) [a]
Fluopyram 150ppm	21.98 ± 1.76*** (289.7 ± 23.2)
Fluopyram 600ppm	39.20 ± 7.49*** (516.6 ± 98.6)
Fluopyram 750ppm	47.24 ± 3.85*** (622.6 ± 50.8)
Phenobarbital 80mg/kg/day	23.00 ± 5.24*** (303.1 ± 69.1)
Treatment, 28 day recovery	
Control	6.93 ± 1.01 (100.0 ± 14.5)
Fluopyram 750ppm	6.68 ± 1.27 (96.4 ± 18.4) [a]
Phenobarbital 80mg/kg/day	6.45 ± 1.22 (93.1 ± 17.7)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 5 per group; [a] n =4 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<0.001.

Figure 5.5.4-14 Benzyloxyquinoline-*O*-Debenzylation, 28 Day Exposure

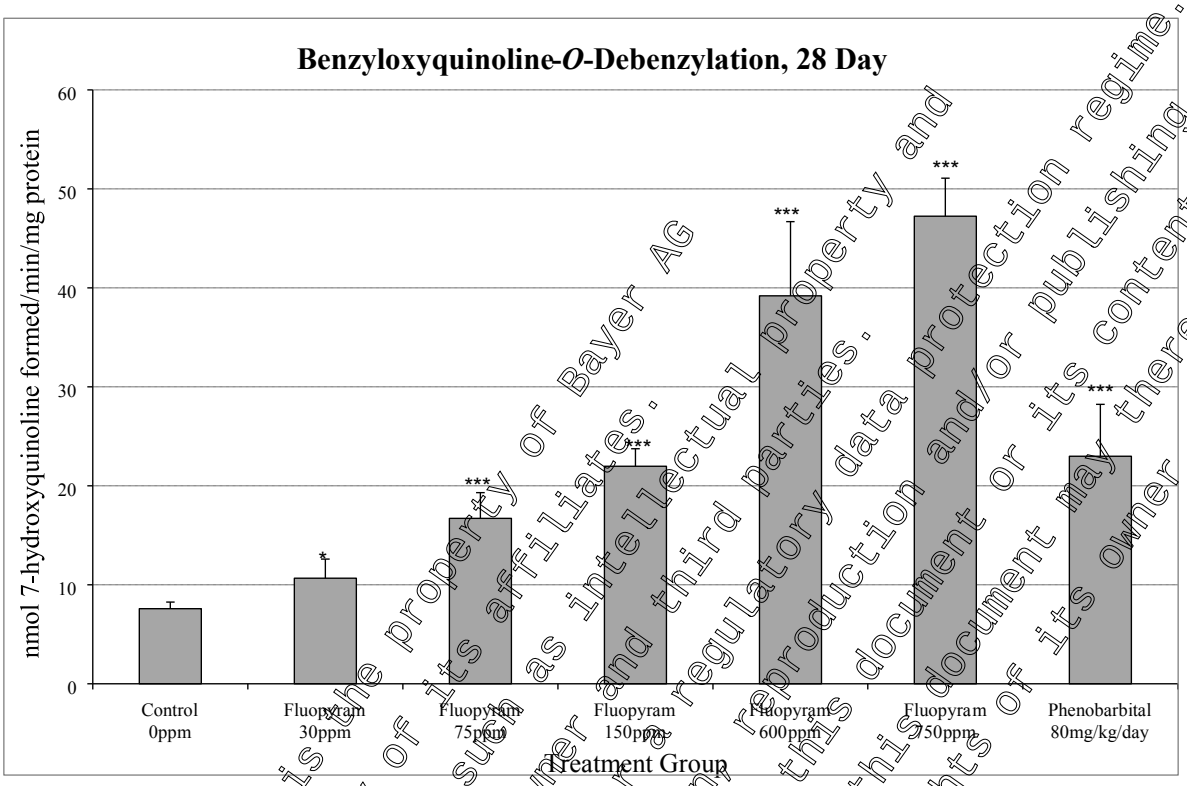
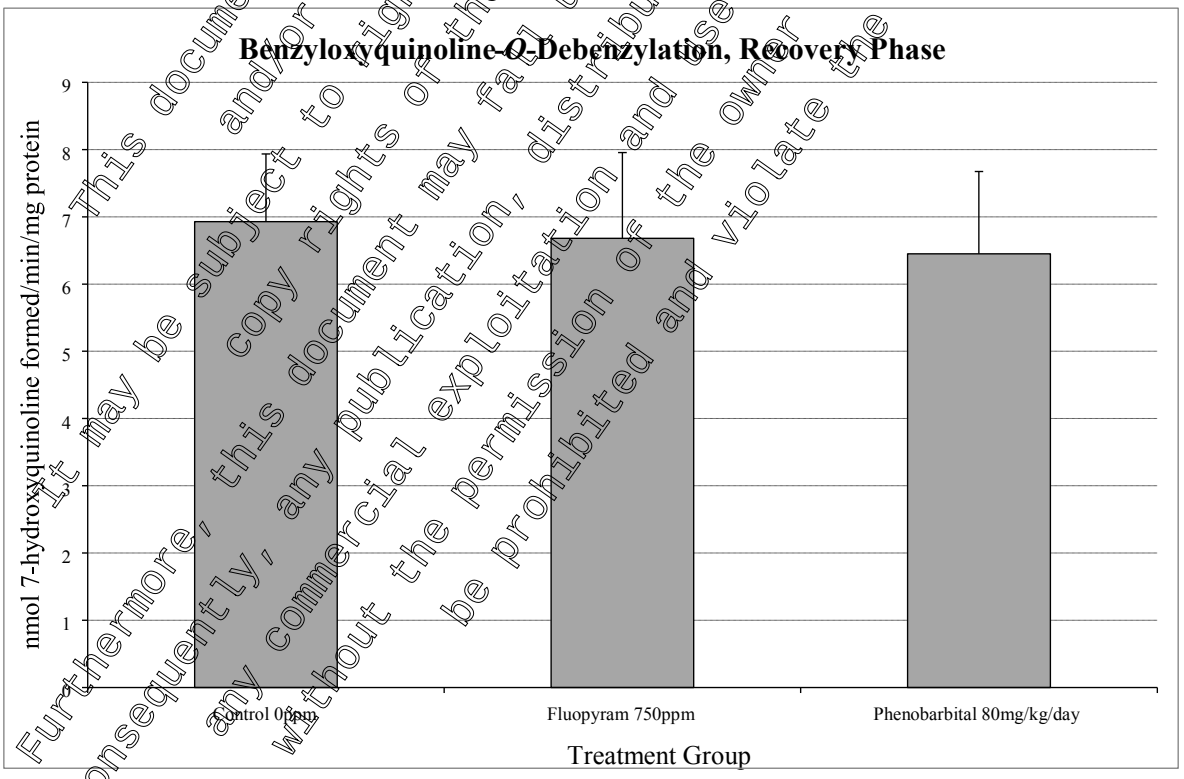


Figure 5.5-15 Benzyloxyquinoline-*O*-Debenzylation, 28 Day Recovery



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 80mg/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 liver microsome preparations as the rate of debenzylation of benzyloxyquinoline (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 in BQ activity. Following a 28 day recovery period on control diet, BQ activity in both fluopyram and phenobarbital treated mice returned to control levels.

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 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 phenobarbital at 80mg/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 liver microsome preparations as the rate of debenzylation of benzyloxyquinoline (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 in BQ activity. Following a 28 day recovery period on control diet, BQ activity in both fluopyram and phenobarbital treated mice returned to control levels.

Data Point:	KCA 5.5/22
Report Author:	[redacted]
Report Year:	2020
Report Title:	*In vitro* CYP and UGT induction in human and Wistar rat hepatocytes by Fluopyram
Report No:	KLC-BA20-06
Document No:	M-759019-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Primary cultures of cryopreserved plateable male human and Wistar rat hepatocytes 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 rat hepatocyte cultures treated daily for 3 days and 7 days with Fluopyram at 10, 30, 60 and 100 µM or with positive control inducers beta-Naphthoflavone (BNF), rifampicin (RIF) and phenobarbital (PB) for human hepatocytes and BNF, 5-pregnen-3β-ol-20-one 16α-carbonitrile (PCN) and PB for rat hepatocytes. RNA quantity established that Fluopyram at 10, 30 and 100 µM were suitable concentrations for mRNA expression and activity analysis.

In Wistar rat hepatocytes, reference inducers BNF (5 µM), PB (1000 µM) and PCN (6 µM) strongly induced CYP1A2, CYP2B1 and CYP3A1 expression, respectively and increased the related activities. PB and PCN also induced UGT2B1 and to a lesser extent UGT1A1 expression and BNF, PB and PCN increased UGT-T4 activity.

In human hepatocytes, reference inducers BNF (5 µM), PB (1000 µM) and RIF (15 µM) induced CYP1A2, CYP2B6 and CYP3A4 expression, respectively, and increased the related activities. PB and RIF induced UGT1A1 expression. BNF and RIF increased UGT-T4 activity.

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 Wistar rat hepatocytes. Fluopyram 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 CYP1A2, CYP2B6, CYP3A4 and UGT1A1 inducer in human hepatocytes but does not increase UGT-T4 activity in human hepatocytes.

I. Materials and methods

A. Materials

1. **Test material:** Fluopyram
   Description: Off-white powder
   Lot / Batch #: Mix-Batch: 08528/0002
   Purity: 94.5%
   CAS #: 658066-35-4
   Stability of test compound: Stable for a period covering the study duration (Expiry date: 19 March 2022)
2. **Control materials:**

Negative:	None (Culture medium was used as the negative control)
Solvent / final concentration:	DMSO
Positive:	Beta-Naphthoflavone (BNF) Phenobarbital (PB) sodium salt 5-pregnen-3β-ol-20-one-16α- carbonitrile (PCN) Rifampicin (RIF)
3. Test organisms:	male human and male Wistar rat hepatocytes
4. Test concentrations: Induction study-CYP/UGT mRNA expression and enzyme activity:	10, 30, 60 and 100 µM

B. Study design

1. In life dates: 01 September 2020 - 02 October 2020

2. Hepatocyte information

Cryopreserved male human and male Wistar rat hepatocytes provided by KALY-CELL (Plobsheim, France) and Bio-IVT were used. Cell yields and viabilities were determined using Trypan blue exclusion.

3. Solubility test

Preliminary solubility tests with Fluopyram confirmed by macroscopic observation that the compound was soluble up to 100 mM in DMSO. Fluopyram was considered soluble in treatment medium, without any presence of crystals up to 100 µM (from a 100 mM stock solution, 0.1% DMSO final in the medium). No crystals were observed up to 100 µM.

Stock solutions of BNF, RIF, PCN and Fluopyram 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 thereafter diluted in treatment medium containing a final concentration of DMSO of 0.1 %

4. Hepatocyte culture and treatment

Hepatocytes from 3 batches per species were seeded in 96-well plates at a density of 0.07×10^6 cells per well for human and at a density of 0.05×10^6 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 (HMM) supplemented with FFS (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) or 6 µM PCN (rat 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 (200 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.

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 crystals.

2. Measurement of RNA content and CYP and UGT mRNA expression of hepatocyte monolayers

On Day 3 and Day 7 of treatment, total RNA was extracted using the King FisherTM Flex Purification 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 mRNA expression was assessed in human hepatocytes for CYP1A2, CYP2B6, CYP3A4, UGT1A1, UGT1A6 and UGT2B7, and in Wistar rat hepatocytes for CYP1A2, CYP2B1, CYP3A1, UGT1A1, UGT1A5/6 and UGT2B1. The effect of BNF, PB, RIF (human hepatocytes only) and PCN (rat hepatocytes only) was assessed in parallel. After RNA extraction and quantification, cDNAs were synthesized from 0.1 µg total RNA using the iScript Reverse Transcription Supermix for RT-qPCR from Biorad (France) at 42°C for 30 min. cDNA samples were then diluted in water (1:5) and 5 µL of each sample was used for real-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, 1 min). In all cases, the quality of the PCR product was assessed by monitoring a fusion 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 used to compute an optimal Cq value. Cq values are inversely proportional to the amount of target nucleic acid in the sample (i.e. lowest Cq level corresponds to the greatest amount of target nucleic acid in the sample). Cq 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 cells versus controls (DMSO). It is described in the equation below, where E is the efficiency of the qPCR:

$$R = \frac{(Etarget)^{\Delta Cqtarget(vehicle\ control-sample)}}{(Eactin)^{\Delta Cqact(vehicle\ control-sample)}}$$

3. P450 Enzyme Activity (CYP1A, CYP2B and CYP3A) measurement

The effect of Fluopyram 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-Cell. The effect of the reference inducers was assessed in parallel: BNF, PB and RIF in human hepatocytes and BNF, PB and PCN in Wistar rat hepatocytes. Cells were first washed with PBS for 15 min before addition of the cocktail of substrates.

4. T4-glucuronidation measurement

The effect of Fluopyram at 10, 30 and 100 µM on T4-glucuronidation was assessed in human and Wistar rat hepatocytes on Day 3 and Day 7 after daily 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 µM.

5. Protein content measurement

On 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 PCN inducing CYP3A1 mRNA expression (mean 23.7-fold and 69.8-fold on Day 3 and mean 24.9-fold and 45.0-fold on Day 7, respectively).

Fluopyram strongly induced CYP3A1 mRNA expression at all tested concentrations, induction being maximal at the highest tested concentration of 100 µM, with the effects being more marked on Day 3 (mean 50.0-fold) compared to Day 7 (mean 11.4-fold). Fluopyram also induced CYP1A2 mRNA expression, induction being maximal from the lowest tested concentration of 10 µM up to 100 µM, with effects being more marked on Day 3 (mean 6.6- to 13.0-fold) compared to Day 7 (2.4- to 4.7-fold). The marginal increases in mRNA expression of CYP2B1 were minimal when compared to reference compound indicating no clear induction.

Table 5.5-162 CYP mRNA expression levels in Wistar rat hepatocytes cultures treated with Fluopyram (Mean±SD)

Treatment duration	Compound	Concentration (µM)	Isoform	CYP mRNA expression
Day 3	Fluopyram	10	CYP1A2	13.0±6.2
		30		9.7±3.2
		100		6.6±1.5
	BNF	5		64.7±24.1
	PCN	6		0.9±0.4
	PB	1000		5.1±3.3
	Fluopyram	10	CYP2B1	3.4±1.5
		30		3.5±0.9
		100		3.3±2.5
	BNF	5		3.6±1.2
	PCN	6		0.6±0.5
	PB	1000		39.2±40.4
	Fluopyram	10	CYP3A1	16.7±6.5
		30		43.8±5.1
		100		50.0±21.6
	BNF	5		3.5±1.1
	PCN	6		69.8±33.0
	PB	1000		23.7±10.0
Day 7	Fluopyram	10	CYP1A2	4.7±0.9
		30		3.3±0.5
		100		2.4±0.7
	BNF	5		14.3±4.0
	PCN	6		0.3±0.2
	PB	1000		2.6±0.3
	Fluopyram	10	CYP2B1	1.0±0.1
		30		0.8±0.4
		100		0.8±0.4
	BNF	5		0.5±0.3
	PCN	6		0.7±0.5
	PB	1000		7.1±1.5
	Fluopyram	10	CYP3A1	4.5±0.5
		30		4.5±1.9
		100		11.4±5.0
	BNF	5		1.2±0.2
	PCN	6		45.0±25.7
	PB	1000		24.9±7.1

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.

Fluopyram at all tested concentrations induced close to or over 2-fold UGT2B1 mRNA expression with a maximum attained from 10 µM up to 100 µM (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 5.5-163 UGT mRNA expression levels in Wistar rat hepatocytes cultures treated with Fluopyram (Mean±SD)

Treatment duration	Compound	Concentration (µM)	Isoform	UGT mRNA expression
Day 3	Fluopyram	10	UGT1A1	1.5±0.1
		30		1.8±0.1
		100		1.7±0.5
	BNF	5		0.5±0.1
	PCN	6		2.5±0.7
	PB	1000		1.9±0.3
	Fluopyram	10	UGT1A5/6	1.2±0.1
		30		1.4±0.2
		100		1.2±0.1
	BNF	5		0.8±0.4
	PCN	6		2.4±0.6
	PB	1000		1.7±0.3
	Fluopyram	10	UGT2B1	4.4±1.1
		30		7.3±3.2
		100		7.7±4.6
	BNF	5		1.6±0.5
	PCN	6		9.6±5.0
	PB	1000		8.4±5.3
Day 7	Fluopyram	10	UGT1A1	1.3±0.4
		30		1.1±0.5
		100		1.4±0.5
	BNF	5		0.5±0.0
	PCN	6		2.0±1.1
	PB	1000		2.1±0.2
	Fluopyram	10	UGT1A5/6	1.0±0.4
		30		0.8±0.3
		100		1.0±0.1
	BNF	5		0.3±0.1
	PCN	6		1.2±0.3
	PB	1000		1.3±0.3
	Fluopyram	10	UGT2B1	2.5±1.2
		30		1.9±1.4
		100		1.8±0.9
	BNF	5		1.2±0.1
	PCN	6		4.0±3.1
	PB	1000		5.1±1.8

B. CYP and UGT mRNA expression levels in human hepatocytes

1. CYP mRNA Expression levels

The reference inducers gave 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 24.8-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 µM up to 100 µM 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)

Treatment duration	Compound	Concentration (µM)	Isoform	CYP mRNA expression
Day 3	Fluopyram	10	CYP1A2	3.5±0.7
		30		4.7±1.0
		100		6.9±1.0
	BNF	5		13.9±8.6
	RIF	15		1.4±0.3
	PB	1000		1.3±0.3
	Fluopyram	10	CYP2B6	3.7±1.3
		30		3.7±1.1
		100		3.5±1.6
	BNF	5		1.1±0.2
	RIF	15		3.0±0.5
	PB	1000		4.5±1.2
	Fluopyram	10	CYP3A4	12.6±2.7
		30		14.6±3.8
		100		12.5±5.5
	BNF	5		0.5±0.2
	RIF	15		28.1±4.2
	PB	1000		24.8±2.9
Day 7	Fluopyram	10	CYP1A2	2.6±0.5
		30		3.5±0.9
		100		5.9±2.4
	BNF	5		8.4±2.3
	RIF	15		1.3±0.4
	PB	1000		1.1±0.2
	Fluopyram	10	CYP2B6	3.1±1.7
		30		2.1±0.9
		100		3.0±0.2
	BNF	5		0.8±0.4
	RIF	15		6.6±2.4
	PB	1000		4.1±1.8
	Fluopyram	10	CYP3A4	8.4±10.2
		30		5.9±6.7
		100		14.0±15.0
	BNF	5		0.4±0.2
	RIF	15		43.8±51.8
	PB	1000		25.6±28.8

2. UGT mRNA expression levels

Reference inducers RIF and PB induced about 2-fold UGT1A1 mRNA expression both on Day 3 and Day 7. UGT1A6 and UGT2B7 expression were not affected by reference inducers.

Fluopyram induced mean UGT1A1 mRNA expression both on Day 3 and Day 7 from 10 µM up to 100 µM, between 1.9- to 3.0-fold of control, and Fluopyram did not affect UGT1A6 and UGT2B7 mRNA expression.

Table 5.5-165 UGT mRNA expression levels in human hepatocytes cultures treated with Fluopyram (Mean±SD)

Treatment duration	Compound	Concentration (µM)	Isoform	UGT mRNA expression
Day 3	Fluopyram	10	UGT1A1	2.0±0.4
		30		2.0±0.4
		100		2.1±0.4
	BNF	5		1.5±0.5
	RIF	15		2.0±0.1
	PB	1000		2.1±0.2
	Fluopyram	10	UGT1A6	1.1±0.2
		30		1.0±0.2
		100		1.0±0.4
	BNF	5		1.0±0.2
	RIF	15		1.0±0.3
	PB	1000		1.1±0.3
	Fluopyram	10	UGT2B7	0.8±0.1
		30		0.8±0.1
		100		0.9±0.2
	BNF	5		1.1±0.2
	RIF	15		0.9±0.1
	PB	1000		0.9±0.1
Day 7	Fluopyram	10	UGT1A1	2.0±0.5
		30		1.9±0.5
		100		3.0±0.5
	BNF	5		2.0±0.3
	RIF	15		2.6±0.7
	PB	1000		2.6±1.0
	Fluopyram	10	UGT1A6	0.8±0.2
		30		0.8±0.2
		100		1.0±0.3
	BNF	5		0.9±0.2
	RIF	15		1.1±0.3
	PB	1000		1.1±0.4
	Fluopyram	10	UGT2B7	0.8±0.3
		30		1.0±0.3
		100		0.9±0.2
	BNF	5		0.9±0.4
	RIF	15		1.7±0.6
	PB	1000		1.5±0.6

C. CYP activities and T4-glucuronidation in wistar rat hepatocytes

1. CYP activities

The reference inducers gave 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 strongly increased CYP3A1 activity at all tested concentrations, increases being maximal at 30 µM on Day 3 (mean 54.1-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.

Table 5.5-166 CYP activities in Wistar rat hepatocytes cultures treated with Fluopyram (Mean±SD)

Treatment duration	Compound	Concentration (µM)	Isoform	CYP activity
Day 3	Fluopyram	10	CYP1A1/2	3.9±1.8
		30		13.6±6.1
		100		39.7±3.6
	BNF	5		42.1±16.8
	PCN	6		2.3±0.7
	PB	1000		4.5±3.1
	Fluopyram	10	CYP2B1	1.5±0.2
		30		1.7±0.1
		100		2.0±0.4
	BNF	5		1.1±0.2
	PCN	6		2.8±0.3
	PB	1000		3.7±2.7
	Fluopyram	10	CYP3A1	35.4±19.8
		30		54.1±11.8
		100		43.3±6.0
	BNF	5		1.1±0.3
	PCN	6		108.1±41.6
	PB	1000		30.2±5.8
Day 7	Fluopyram	10	CYP1A1/2	2.6±1.4
		30		3.8±1.7
		100		9.1±3.7
	BNF	5		45.1±18.4
	PCN	6		8.4±5.9
	PB	1000		10.8±6.9
	Fluopyram	10	CYP2B1	3.2±0.5
		30		2.6±0.4
		100		1.8±0.1
	BNF	5		0.7±0.2
	PCN	6		4.0±2.2
	PB	1000		6.7±0.9
	Fluopyram	10	CYP3A1	18.4±9.5
		30		32.0±24.8
		100		59.5±43.5
	BNF	5		1.0±0.0
	PCN	6		188.8±137.0
	PB	1000		68.0±52.3

2. T4-glucuronidation

Expected increases in UGT-T4 activity were seen on Day 7 with reference inducers BNF (mean 6.0-fold), PB (mean 2.2-fold) and PCN (mean 5.9-fold).

Fluopyram at all tested concentrations increased UGT-T4 activity over 2-fold on Day 7, increases being maximal at 30 and 100 µM (mean 3.4- to 3.8-fold).

Table 5.5-167 UGT-T4 activity in Wistar rat hepatocytes cultures treated with Fluopyram (Mean±SD)

Treatment duration	Compound	Concentration (µM)	Isoform	UGT-T4 activity
Day 3	Fluopyram	10	UGT-T4	1.3±0.5
		30		1.4±0.3
		100		1.4±0.4
	BNF	5		2.9±0.6
	PCN	6		1.9±0.3
	PB	1000		1.8±0.1
Day 7	Fluopyram	10	UGT-T4	2.2±0.7
		30		3.4±1.5
		100		3.8±1.1
	BNF	5		6.0±2.9
	PCN	6		5.9±1.6
	PB	1000		2.2±0.1

D. CYP activities and T4-glucuronidation in human hepatocytes

1. CYP activities

The reference inducers gave the expected responses with BNF increasing CYP1A1/2 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 21.8-fold and 12.6-fold on Day 7) and CYP3A4/5 activity (mean 4.7-fold and 4.3-fold on Day 3 and mean 11.3-fold and 11.1-fold on Day 7, respectively).

Fluopyram showed an over 2-fold increase in CYP1A1/2 and CYP2B6 activities at all tested concentrations on Day 3 and Day 7, with a maximum increase on Day 7 of CYP1A1/2 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). CYP3A4/5 activity was also increased over 2-fold on Day 7 from 10 µM up to 100 µM (mean 2.9- to 4.2- fold).

Table 5.5-168 CYP activities in human hepatocytes cultures treated with Fluopyram (Mean±SD)

Treatment duration	Compound	Concentration (µM)	Isoform	CYP activity
Day 3	Fluopyram	10	CYP1A1/2	2.6±1.5
		30		3.8±2.6
		100		4.9±4.0
	BNF	5		32.5±14.8
	RIF	15		0.9±0.3
	PB	1000		1.8±0.9
	Fluopyram	10	CYP2B6	3.0±2.1
		30		2.8±2.1
		100		3.0±1.6
	BNF	5		1.7±0.2
	RIF	15		2.9±1.1
	PB	1000		5.8±2.8
	Fluopyram	10	CYP3A4/5	1.7±0.4
		30		1.7±0.1
		100		1.6±0.6
	BNF	5		0.8±0.1
	RIF	15		4.3±0.5
	PB	1000		4.7±1.2
Day 7	Fluopyram	10	CYP1A1/2	8.9±5.7
		30		11.8±8.4
		100		23.3±18.9
	BNF	5		155.4±82.5
	RIF	15		2.4±0.5
	PB	1000		3.8±0.5
	Fluopyram	10	CYP2B6	11.6±11.2
		30		7.2±7.3
		100		12.1±9.5
	BNF	5		2.3±0.8
	RIF	15		11.6±3.0
	PB	1000		21.8±5.9
	Fluopyram	10	CYP3A4/5	3.7±1.7
		30		2.9±1.8
		100		4.2±3.8
	BNF	5		0.9±0.3
	RIF	15		11.1±8.8
	PB	1000		11.3±7.9

2. T4-glucuronidation

Slight increases in UGT-T4 activity were seen on Day 7 with reference inducers BNF (mean 2.3-fold) and RIF (mean 1.9-fold).

Fluopyram did not affect UGT-T4 activity on Day 3 nor on Day 7.

Table 5.5-169 UGT-T4 activity in human hepatocytes cultures treated with Fluopyram (Mean±SD)

Treatment duration	Compound	Concentration (µM)	Isoform	UGT-T4 activity
Day 3	Fluopyram	10	UGT-T4	0.9±0.1
		30		0.9±0.1
		100		0.8±0.2
	BNF	5		1.1±0.2
	RIF	15		1.1±0.1
	PB	1000		1.1±0.1
Day 7	Fluopyram	10	UGT-T4	1.3±0.4
		30		1.3±0.4
		100		1.2±0.4
	BNF	5		2.3±1.0
	RIF	15		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 Wistar rat hepatocytes. Fluopyram 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 CYP1A2, CYP2B6, CYP3A4 and UGT1A1 inducer but does not increase UGT-T4 activity in human hepatocytes.

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 inducible cytochrome 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 100 µM is a strong CYP3A 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 rat hepatocytes. Fluopyram at 10 µM, 30 µM and 100 µM is a CYP1A2, 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 LOAEL 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 (96.0 mg/kg/day) based on a 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 white blood cell and monocyte absolute cell (Ab) counts in the F1-generation, decreased hemoglobin 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. Thus, 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, (although mean body weight at attainment of sexual maturation was comparable to the controls 171 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 450 mg/kg/day, mean maternal body weight remained static between gestation days (GD) 6-8, compared to a gain of 6.8 g in the control group, with a reduced mean maternal body weight gain of 22% between GD 8-10 and 34% between GD 10-14, 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 centrilobular 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 findings 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 remnant present' and 'ureter convoluted and/or dilated', and skeletal variations 'at least one thoracic centrum split/split cartilage', 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 ([redacted], 2007, M-766189-01-1) 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 10-14, 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 examination of the liver, diffuse centrilobular hepatocellular hypertrophy was observed in 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 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 gain 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 clinical signs. At the high dose level of 75 mg/kg/day, mean body weight gain was reduced by between GD 14-18 (0.02 kg vs. 0.09 kg for controls) and between GD 18-22 (0.02 kg 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 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 controls. At necropsy, no treatment-related macroscopic findings were noted. Mean fetal body weight (combined and separate sexes) was 11% 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 treatment-related visceral or skeletal findings at this dose level.

There were no treatment-related maternal, litter or fetal findings at the mid dose of 25 mg/kg/day or the low dose of 10 mg/kg/day.

A dose level of 25 mg/kg/day AE C656948 was considered to be a NOEL both in the dam and in terms of fetal development in the New Zealand White rabbit.

Table 5.6-1 Fluopyram Summary of reproductive and developmental toxicity studies

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 generation dietary toxicity study				
Wistar rat 0, 40, 220, 1200 ppm [redacted] M-299334-01-1	14.5♂ 17.2♀	82.8♂ 96.0♀	Parents	Clinical pathology changes ↑ liver weight, protein droplet nephropathy (♂) and centrilobular nephropathy
	82.8♂ 93.1♀	>93 ♂♀	Reproduction	Clinical signs ↓ Body weight Delayed sexual development maturation secondary to decreased body weight
	17.0 ♂/♀	97.9 ♂♀	Offspring	↓ number of implantation sites, litter size and corpora lutea (secondary to severe systemic toxicity)
Developmental toxicity studies				
Doses mg/kg bw/day Reference	**NOAEL mg/kg bw/day**	**LOAEL mg/kg bw/day**	**Adverse effects / target organs**	
Sprague-Dawley Rat - 0, 30, 150, 450 [redacted] M-299438-01-2	30	150	dams	↓ body weight gain and food consumption, ↑ liver weight, and diffuse centrilobular hypertrophy in ♀
	150 ♂/♀	450 ♂/♀	fetus	↓ fetal body weight (5%), ↑ incidence of two minor variations at both the visceral and skeletal evaluation
New Zealand White Rabbit - ♀ 0, 10, 25, 60 [redacted] M-279723-01-1	25	75	dams	↓ body weight gain and food consumption
	25 ♂/♀	75 ♂/♀	fetus	↓ fetal body weight (-11%).

CA 5.6.1 Generational studies

Data Point:	KCA 5.6.1/01
Report Author:	[redacted]
Report Year:	2008
Report Title:	Technical Grade AE C656948: A Two Generation Reproductive Toxicity Study in the Wistar Rat
Report No:	201855
Document No:	M-299334-01-1
Guideline(s) followed in study:	OECD 416 (2001); US EPA Health Effects Test Guideline (OPPTS 870.3800; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines
Deviations from current test guideline:	Current guideline: OECD 416, 2001 Deviations: No deviations
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities.
Acceptability/Reliability:	Yes

Executive Summary

AE C656948 was administered continuously in the feed to the Wistar rat (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 animal were conducted weekly throughout the study, as well as an evaluation of multiple reproductive parameters, clinical chemistry and hematology. All animals placed on study were subject to a postmortem examination, which included (1) documenting and saving all gross lesions, (2) weighing designated organs and, (3) collecting representative tissue specimens for histopathologic evaluation.

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 lymphocytic infiltration. Increased liver weights were observed in both males and females and were associated with an increased incidence of centrilobular hypertrophy.

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 g in controls).

F1-generation 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 centrilobular hypertrophy in both genders and minimal to slight alveolar macrophages in the females.

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 gain 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 loss observed in these pups.

Reproductive Performance (P and F1): No test substance-related findings were observed.

At 220 ppm

P-generation Adults: No test substance-related findings were observed.

F1 -Offspring: No test substance-related findings were observed.

F1-generation Adults: No test substance-related findings were observed.

F2-Offspring: No test substance-related findings were observed.

Reproductive Performance (P and F1): No test substance-related findings were observed.

At 40 ppm

P-generation Adults: No test substance-related findings were observed.

F1 -Offspring: No test substance-related findings were observed.

F1-generation Adults: No test substance-related findings were observed.

F2-Offspring: No test substance-related findings were observed.

Reproductive Performance (P and F1): No test substance-related findings were observed.

The data from this study demonstrate that the parental systemic NOAEL was 220 ppm (14.5 mg/kg/day in males, 17.2 mg/kg/day in females) and that 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). 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 NOAEL 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 (97.9 mg/kg/day).

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Beige powder
Lot / Batch #:	08528/0002
Purity:	94.7% (Analyses dates of 5/4/05 and 3/30/07)
CAS #	658066-35-4
Stability of test compound:	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. Vehicle and / or positive control:	Acetone
3. Test animals:	
Species:	Rat

Strain:	Wistar Han Crl: WI(HAN)
Age:	(P) 8 weeks
Weight at dosing:	(P) Males: 224.9 – 279.1 g; Females: 145.6 – 203.3 g
Source:	[redacted]
Acclimation period:	6 days
Diet:	Purina Mills Rodent Lab Chow 5002 meal *ad libitum*
Water:	Tap water (Kansas City, MO), *ad libitum*
Housing:	Animals were housed individually (except during the mating phase and as noted below for the F1 and F2 pups) in suspended stainless steel cages and deotized cage board in the bedding trays. During gestation and lactation, individual dams (and litters) were housed in polycarbonate cages with corn-cob bedding.
Environmental conditions:	
Temperature:	18-26°C
Humidity:	30-70%
Air changes:	At least 10 changes per hour
Photoperiod:	12 hours light, 12 hours dark

B. Study design

1. In life dates: 09 March 2006 – 22 December 2006

2. Animal assignment and treatment

Following a minimum of six days of quarantine/acclimation, 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, UK). 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, Inc. Seaford, 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 cage. Pups born alive were identified by tattoo and pups found dead were identified with an indelible marking pen.

Study schedule: One hundred and twenty female and one hundred and twenty male rats were assigned to one of four treatment groups (30 animals/sex/group), nominal doses of 0, 40, 220 and 1200 ppm AE C656948 in the diet (see Table 5.6.1-1). Animals were exposed to the treated feed throughout the entire in-life phase of the study. In-life phases included: Premating: 10 weeks; Mating: 14 days; Gestation: approximately 22 days; and Lactation: weaning on Day 21. F1-pups were maintained after weaning for approximately six weeks prior to initiation of the second generation.

Table 5.6.1-1 Animal Assignment

Test group	Dose in Diet[a] (ppm)	Animals/group			
		P Males	P Females	F_1 Males	F_1 Females
Control	0	30	30	30	30
Low (LDT)	40	30	30	30	30
Mid (MDT)	220	30	30	30	30
High (HDT)	1200	30	30	30	30

[a] = Diets were administered from beginning of the study until sacrifice.
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. Females found to be inseminated were placed in a polycarbonate nesting cage. The day on which insemination 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 remaining females were placed in polycarbonate nesting cages, following the 14-day mating period.

3. Dose selection rationale

Doses were selected based upon the preliminary results which emerged in the rat over the course of a pilot reproductive toxicity testing study conducted with the test chemical at doses of 0, 30, 150, 750 and 1500 ppm AE C656948 (M-299533-01-1, 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 changes in both the 750- and 1500-ppm dose group. Changes in clinical chemistry and hematology parameters were also noted at these same dose levels in either the males or females. Based on these results, the doses selected for the two-generation 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 dietary concentration and no parental or reproductive effects at the lowest dietary concentration.

4. Dosage preparation and analysis

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 test substance in the feed for the females was adjusted down by 50% during the lactation period (Days 0-21) as follows, to avoid the large increase in dosage (mg/kg/day) that is otherwise associated with increased feed consumption that occurs during lactation. Thus, during lactation days 0-21, the dietary 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 5.6.1-2 Mean Daily Intake of the Test Substance

Phase of Study	40 ppm in mg/kg/day[a]	220 ppm in mg/kg/day[a]	1200 ppm in mg/kg/day[a]
Premating (P-gen) - Male	2.7	15.1	83.1
Premating (P-gen) – Female	3.2	17.6	96.3
Gestation (P-gen) – Female	3.0	15.5	90.3
Lactation (P-gen) – Female	3.1	15.9	92.5
Premating (F_1-gen) - Male	2.6	13.9	82.4
Premating (F_1-gen) – Female	3.1	16.8	95.6
Gestation (F_1-gen) – Female	2.8	14.4	95.9
Lactation (F_1-gen) – Female	3.3	18.1	105.2

[a] Individual values were based on the means for each particular phase 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 thereafter (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 (range 5.00-5.39 ppm; %RSD = 2.66) and 4975 ppm (range 4866-5115 ppm; %RSD = 1.79), respectively. Based on a %RSD ≤ 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 on Day 0) and 5229 ppm (5.153 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 rodent 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 94-109% for rodent ration spiked with 20, 40, 220 or 1200 ppm of AE C656948 (Moore and Neal, 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 groups, 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 $p \leq 0.01$.

Indices:

Reproductive indices: The following reproductive indices were calculated from breeding and parturition records of animals in the study:

$$Mating\ Index\ (\%) = \frac{\#inseminated\ females^{a}}{\#of\ females\ co-housed} \times 100$$

$$Fertility\ Index\ (\%) = \frac{\#\ of\ pregnant\ females^{b}}{\#\ of\ inseminated\ females} \times 100$$

$$Gestation\ Index (\%) = \frac{\#\ of\ females\ with\ live\ pups}{\#\ of\ pregnant\ females} \times 100$$

[a]Includes pregnant females not observed sperm positive or with an internal vaginal plug.

[b]Includes females which did not deliver but had implantation sites.

Offspring viability indices: The following viability indices were calculated from lactation records of litters in the study:

$$Birth\ Index (\%) = \frac{total\ \#\ of\ pups\ born/litter}{total\ \#\ of\ implantation\ sites/litter} \times 100$$

$$Livebirth\ Index\ (\%) = \frac{\#\ of\ live\ pups\ born/litter}{total\ \#\ of\ pups/litter} \times 100$$

$$Viability\ Index (\%) = \frac{\#\ of\ live\ pups/litter\ on\ day\ 4\ (pre-culling)}{\#\ of\ live\ pups\ born/litter} \times 100$$

$$Lactation\ Index (\%) = \frac{\#\ of\ live\ pups/litter\ on\ day\ 21}{\#\ of\ live\ pups/litter\ on\ day\ 4\ (post\ culling)} \times 100$$

C. Methods

1. Parental animals:

a. Mortality and clinical observations.

Mortality checks (cageside observations) were performed twice daily (AM and PM) during the workweek and once daily on weekends and holidays. Cageside observations characterized mortality, morbidity, behavioral changes, 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 removed 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 examination 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 measuring food consumption. During gestation, dam body weight was measured on Days 0, 6, 13, and 20, and fresh 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 F1-generation 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 at termination, sperm was collected from one testis and one epididymis for enumeration of homogenization-resistant spermatids and cauda epididymal 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 deferens. Sperm motility and counts was conducted using IVOS (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 Isoflurane 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 include:

Hematocrit (HCT)	Hemoglobin (HGB)
Total erythrocyte count (RBC)	Erythrocyte indices (MCV, MCH, MCHC)
Red cell distribution width (RDW)	Differential leukocyte count
Total leukocyte count (WBC)	RBC morphology
Total platelet count (PLT)	Hemoglobin Distribution Width (HDW)

Serum Chemistry parameters include:

Albumin (ALB)	Globulin (GLOB)
A/G ratio (A/G)	Alanine aminotransferase (ALT)
Aspartate aminotransferase (AST)	Alkaline phosphatase (ALK)
Total protein (TP)	Total bilirubin (T.BIL)
Glucose (GLUC)	Cholesterol (CHOL)
Triglyceride (TRIG)	Urea nitrogen (BUN)
Creatinine (CREAT)	Sodium (Na)
Potassium (K)	Chloride (Cl)

Calcium (Ca) Phosphorus (PO_4)

Creatine Phosphokinase (CPK)

2. Litter observations: The following litter observations (X) were made:

Table 5.6.1-3 F_1 / F_2 Litter Observations

Observation	Time of Observation (lactation day)						
	Day 0	Day 4[a]	Day 4[b]	Day 7	Day 14	Day 21	Days (0-21)
Number of live pups							X
Pup weight	X	X		X	X	X	
External alterations	X	X		X	X	X	
Number of dead pups							X
Sex of each pup (M/F)	X						
Preputial Separation	Performed post weaning						
Vaginal Patency	Performed post weaning						

[a] Before standardization (culling) b After standardization (culling)

The size of each litter was adjusted on lactation Day 4 to yield, as closely as possible, four males and four females per litter. If the number of male or female pups was less than four, a partial adjustment will be made (e.g., three females and five males). No adjustments were made for litters of fewer than eight pups. Adjustments were made by random selection of the pups using software provided by SAS. Culled pups were sacrificed by decapitation. Grossly abnormal pups underwent a gross internal and external examination, and all culled pups were discarded. The F1- and F2-pups not culled on lactation Day 4 were maintained with the dam until weaning on lactation Day 21. On lactation Day 21, a sufficient number of F1-pups/sex/litter 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 weighed. One pup/sex/litter for each generation had tissues collected and evaluated for any structural abnormalities or pathological 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 stillborn or found dead.

3. Post-mortem observations:

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 animals were subjected to postmortem examinations as follows.

Male rats were euthanized by carbon dioxide 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, sperm 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 evaluated. Sperm motility and counts were conducted using IVOS (Integrated Visual Operating System, 2005).

Each dam (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/or 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 uterine horns with 10% buffered formalin.

The following tissues were collected and weighed (XX). Micropathology was performed on those tissues designated with (XXX).

XX	Brain	XXX	Epididymis
XXX	Pituitary	XXX	Coagulating Gland
XXX	Liver	XXX	Ovary
XXX	Kidney	XXX	Oviduct
XXX	Spleen	XXX	Prostate
XX	Thyroid	XXX	Seminal Vesicle
XX	Thymus	XXX	Testis
XXX	Adrenal	XXX	Uterus
XXX	Cervix	XXX	Vagina

Animals found moribund while on study were sacrificed and a gross necropsy 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 F_1-offspring not selected as parental animals and all F_2-offspring were sacrificed at 21 days of age. These animals were subjected to postmortem examinations (macroscopic and/or microscopic examination) as follows.

The following tissues from 21-day weanlings were collected (X), collected and weighed (XX), and micropathology was performed (XXX):

XX	Brain	XXX	Uterus	XXX	Testis
XX	Spleen	XXX	Ovary	XXX	Epididymis
XX	Thymus	XXX	Vagina	XXX	Prostate
X	Gross Lesions	XXX	Cervix	XXX	Coagulating Gland
		XXX	Oviduct	XXX	Seminal Vesicle

Any gross lesion was documented and collected.

II. Results and discussion

A. Mortality

There were no test substance-related mortalities observed during the course of this study at any dietary level tested in either generation.

B. Observations

Clinical signs

There were no test substance-related clinical observations observed during the course of this study at any dietary level 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 in 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 (declined 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 food consumption from Day 56 to 63 on a g/animal/day was observed in the 1200 ppm dose group and was considered to be incidental.

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 10-week premating period at any dietary level tested. A slight decline in food consumption from Day 56 to 63 on a gram/animal/day was observed in the 1200 ppm dose group and is considered to be incidental.

Reported body weight (males – study duration, females – premating) and selected food consumption results (premating) are summarized in Table 5.6.1-4.

Table 5.6.1-4 Mean (S.E.) Body Weight and Food Consumption

Observations/study week	Dose Group			
	Control 0 ppm	LDT 40 ppm	MDT 220 ppm	HDT 1200 ppm
P Generation Males				
Mean body weight (g) - Week 15 S.E.	444.5 7.47	456.2 5.99	458.6 7.69	448.4 4.61
Mean weight gain (g) - Weeks 1-15	196.3	210.9	207.1	195.8
Mean food consumption (g/animal/day) - Weeks 1-10	23.3	23.8	23.5	23.5
Mean food consumption (g/kg/day) - Weeks 1-10	68.8	70.2	68.4	69.6
P Generation Females - Pre-mating				
Mean body weight (g) - Week 10 S.E.	244.9 2.81	237.6 3.52	241.0 2.72	234.2 2.69
Mean weight gain (g) - Weeks 1-10	72.9	64.1	66.8	58.3
Mean food consumption (g/animal/day) - Weeks 1-10	17.2	16.9	16.9	16.8
Mean food consumption (g/kg/day) - Weeks 1-10	80.8	80.5	80.0	80.7
F_1 Generation Males				
Mean body weight (g) - Week 14 S.E.	462.0 7.09	451.9 8.21	465.6 6.78	456.5 5.74
Mean weight gain (g) Weeks 1-14	193.7	186.5	191.5	189.2
Mean food consumption (g/animal/day) - Weeks 1-10	23.1	22.7	22.8	23.8
Mean food consumption (g/kg/day) - Weeks 1-10	64.2	64.4	62.2	66.2
F_1 Generation Females - Pre-mating				
Mean body weight (g) - Week 10 S.E.	237.5 3.51	244.1 3.97	244.5 3.63	230.6 3.14
Mean weight gain (g) - Weeks 1-10	62.4	66.7	61.3	56.3
Mean food consumption (g/animal/day) - Weeks 1-10	16.2	16.8	16.4	15.8
Mean food consumption (g/kg/day) - Weeks 1-10	77.1	78.6	75.4	76.8

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, body 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 F**1** generation females, respectively.

Table 5.6.1-5 Mean (S.E.) Body Weight and Food Consumption for P Generation Females During Gestation

P Generation Females - Gestation				
Observations/study week	**Dose Group**			
	Control 0 ppm	**LDT 40 ppm**	**MDT 220 ppm**	**HDT 1200 ppm**
Mean body weight (g) - Day 0 S.E.	247.4 3.28	239.3 3.95	243.1 2.46	232.7** 2.50
Mean body weight (g) - Day 6 S.E.	263.8 3.12	254.8 4.18	261.5 2.54	248.5** 3.51
Mean body weight (g) - Day 13 S.E.	286.0 3.20	276.9 4.61	283.8 2.85	272.6** 3.01
Mean body weight (g) - Day 20 S.E.	349.7 3.94	333.6 6.21	343.7 4.35	336.1 3.83
Mean weight gain (g) - Days 0-20 S.E.	102.3 2.73	94.4 4.28	100.6 3.20	103.4 2.90
Mean food consumption (g/animal/day) Days 0-20	20.0	20.1	20.0	20.0
Mean food consumption (g/kg/day) Days 0-20	75.3	78.0	75.8	79.6

** Statistically different from control, p≤0.01.

Table 5.6.1-6 Mean (S.E.) Body Weight and Food Consumption F_1 Generation Females During Gestation

F_1 Generation Females - Gestation				
Observations/study week	**Dose Group**			
	Control 0 ppm	**LDT 40 ppm**	**MDT 220 ppm**	**HDT 1200 ppm**
Mean body weight (g) - Day 0 S.E.	240.2 3.74	247.5 4.17	241.5 3.54	237.2 3.51
Mean body weight (g) - Day 6 S.E.	253.3 3.78	261.3 4.02	256.2 3.29	249.4 3.54
Mean body weight (g) - Day 13 S.E.	280.8 3.98	279.6 4.14	273.4 3.89	269.7 4.06
Mean body weight (g) - Day 20 S.E.	329.3 4.87	338.3 5.51	326.8 5.75	332.1 4.92
Mean weight gain (g) - Days 0-20 S.E.	89.1 2.84	90.8 3.04	85.3 4.16	100.9* 2.71
Mean food consumption (g/animal/day) Days 0-20	18.6	18.4	17.2	19.6
Mean food consumption (g/kg/day) Days 0-20	76.0	70.2	66.9	78.4

* Statistically different from control, $p \leq 0.05$.

d. Lactation

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 at any dietary level tested.

Reported body weight and selected food consumption results for lactation are summarized in Table 5.6.1-7.

Table 5.6.1-7 Mean (S.E.) Body Weight and Food Consumption - Lactation

P Generation Females - Lactation				
Observations/study week	**Dose Group**			
	Control 0 ppm	**LDT 40 ppm**	**MDT 220 ppm**	**HDT 1200 ppm**
Mean body weight (g) - Day 0 S.E.	267.9 3.34	264.2 3.94	270.3 2.79	255.8* 2.65
Mean body weight (g) - Day 4 S.E.	276.5 3.50	266.2 4.66	276.8 2.80	267.2 3.69
Mean body weight (g) - Day 7 S.E.	282.7 3.63	273.9 4.31	284.7 3.00	273.1 3.50
Mean body weight (g) - Day 14 S.E.	299.3 4.26	290.7 4.10	299.9 3.96	289.0 3.10
Mean body weight (g) - Day 21 S.E.	287.5 3.67	280.4 4.06	286.7 2.85	277.9 2.89
Mean food consumption (g/animal/day) Days 0-21	45.6	42.8	43.5	44.1
Mean food consumption (g/kg/day) Days 0-21	161.1	155.5	152.6	161.4
F_1 Generation Females - Lactation				
Observations/study week	**Dose Group**			
	Control 0 ppm	**LDT 40 ppm**	**MDT 220 ppm**	**HDT 1200 ppm**
Mean body weight (g) - Day 0 S.E.	257.7 3.25	266.8 4.37	260.6 4.56	258.5 3.95
Mean body weight (g) - Day 4 S.E.	263.0 4.31	274.4 4.45	271.4 4.63	263.0 3.70
Mean body weight (g) - Day 7 S.E.	273.3 4.14	282.6 4.62	279.9 4.63	271.3 4.18
Mean body weight (g) - Day 14 S.E.	293.5 3.85	297.5 5.19	296.8 4.14	291.3 3.91
Mean body weight (g) - Day 21 S.E.	286.4 4.24	290.6 4.45	293.3 3.75	284.5 4.59
Mean food consumption (g/animal/day) Days 0-21	45.8	46.3	45.1	46.2
Mean food consumption (g/kg/day) Days 0-21	166.5	163.4	161.5	168.7

* Statistically different from control, $p \leq 0.05$

D. Compound intake

Based on food consumption, body weight and dietary analyses results, the doses expressed as mean daily mg test substance/kg body weight during the pre-mating period (10 weeks) are presented in Table 5.6.1-8.

Calculation for test substance intake is:

Mean analytical concentration (ppm) specific for premating / 1000 X mean weekly food consumption (g/kg/body weight/day) during premating.

Table 5.6.1-8 Mean test substance intake during premating (mg/kg body weight/day)

Generation	Male			Female		
	LDT 40 ppm	MDT 220 ppm	HDT 1200 ppm	LDT 40 ppm	MDT 220 ppm	HDT 1200 ppm
P	2.7	15.1	83.1	3.2	17.6	96.1
F1	2.6	13.9	82.4	3.1	16.8	95.6

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 dietary level tested for either generation. These data are summarized in Table 5.6.1-9.

Table 5.6.1-9 Sperm Measures

Sperm Analysis		Dose Group (ppm)			
		Control 0 ppm	LDT 40 ppm	MDT 220 ppm	HDT 1200 ppm
P Generation Males					
Sperm Motility	% Motile	89.8	89.5	89.8	89.9
	% Progressive	64.0	62.7	64.6	63.3
Sperm Counts (sperm/gram)	Testis	38.2	N/A	N/A	34.92
	Epididymis	240.5	N/A	N/A	219.7
Sperm Morphology (mean total number)	Normal	198.7	N/A	N/A	197.3
	Abnormal	0.9	N/A	N/A	2.0
	Detached Head	0.3	N/A	N/A	0.8
F_{1_o} Generation Males					
Sperm Motility	% Motile	87.8	86.4	87.0	87.2
	% Progressive	60.4	61.1	61.8	61.9
Sperm Counts (sperm/gram)	Testis	28.3	N/A	N/A	29.1
	Epididymis	189.4	N/A	N/A	161.9
Sperm Morphology (mean total number)	Normal	197.0	N/A	N/A	195.3
	Abnormal	2.0	N/A	N/A	4.2
	Detached Head	1.0	N/A	N/A	0.5

N/A – Indicates 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 dietary level tested.

Results for both the P- and F1-generation animals are summarized in Table 5.6.1-10.

Table 5.6.1-10 Reproductive Performance

Observation	Dose Group (ppm)			
	Control 0 ppm	LDT 40 ppm	MDT 220 ppm	HDT 1200 ppm
P Generation – F_1 Offspring				
Number Cohoused	30	30	30	30
Number Mated	30	29	30	28
Number of Animals Delivered	30	25	28	26
Number of Animals with Implants	30	25	29	26
Mating Index	100.0	96.7	100.0	93.3
Fertility Index	100.0	86.2	96.7	92.9
Gestation Index	100.0	100.0	96.6	100.0
Days to Insemination\Mean (S.E.)	3.0 (0.43)	2.4 (0.22)	2.7 (0.43)	2.3 (0.20)
Gestation Length (days)\Mean (S.E.)	21.9 (0.12)	21.8 (0.10)	21.8 (0.09)	21.8 (0.09)
F_1 Generation – F_2 Offspring				
Number Cohoused	30	30	30	30
Number Mated	29	30	30	30
Number of Animals Delivered	27	27	27	28
Number of Animals with Implants	27	27	27	29
Mating Index	96.7	100.0	100.0	100.0
Fertility Index	93.1	90.0	90.0	96.7
Gestation Index	100.0	100.0	100.0	96.6
Days to Insemination\Mean (S.E.)	2.9 (0.41)	3.0 (0.43)	2.3 (0.23)	2.8 (0.32)
Gestation Length (days)\Mean (S.E.)	21.8 (0.11)	21.7 (0.09)	21.6 (0.11)	21.5 (0.10)

G. Clinical pathology (parental animals)

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 clinical 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**- Test substance-related hematology changes were limited to decreased hemoglobin and hematocrit in the 1200 ppm females.

***F1*-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. Terminal Body Weight and Organ weights:

P-Generation Adults: 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 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 liver 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 no corresponding micropathology or hematology findings were observed to support this finding.

F_1-Generation Adults: 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 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 (as compared to the controls) 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 (3) there were no statistically significant decreases in spleen weights (absolute and relative) at 220-ppm in the P-generation females.

The decrease in absolute spleen weights (absolute and/or relative) in 1200- and 220-ppm females was considered not to be biologically or 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 lymphocytic 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 F1-Generation Females:

None of the mean primordial (preantral) follicular, antral follicular, or corpora luteal counts for F1-generation 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 no 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.

Table 5.6.1-11 Litter parameters for F_1 and F_2 generations

Observation	Dose Group (ppm)			
	Control 0 ppm	LDT 40 ppm	MDT 220 ppm	HDT 1200 ppm
F_1 Generation				
Total Number of Implantation Sites (Mean)	377 (12.6)	274 (11.0)	323 (11.1)	304 (11.7)
Total Number born	354	287	303	295
Number stillborn	1	1	0	1
Sex Ratio Day 0 (% male)	53.2	54.0	46.0	44.6
Mean litter size	11.8	10.3	10.8	11.3
Birth index	93.9	91.8	89.6	96.8
Live birth index	99.7	99.7	100.0	99.7
Viability index	99.7	96.0	99.3	97.8
Lactation index	98.8	99.5	99.6	99.5
F_2 Generation				
Total Number of Implantation Sites (Mean)	301 (11.1)	311 (11.2)	297 (11.0)	323 (11.1)
Total Number born	287	299	289	303
Number stillborn	4	0	3	3
Sex Ratio Day 0 (% male)	47.4	49.7	49.1	44.1
Mean litter size	10.6	11.1	10.7	10.8
Birth index	95.0	96.3	97.1	91.0
Live birth index	98.7	100.0	99.7	99.2
Viability index	99.6	98.2	98.1	98.3
Lactation index	98.6	94.9	98.8	99.6

b Before standardization (culling)
c After standardization (culling)
d Does not include number stillborn

2. Litter and Pup Weights:

F_1-Pups: 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 declined 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.

F_2-Pups: Pup body weights at birth for all three treated groups were comparable to the control group. In the 1200 ppm dose 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 lactation was declined 8.6%, relative to control. There were no test substance-related effects on pup body weight observed at any 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.

Table 5.6.1-12 Mean (S.E.) Male/Female Combined Pup Weights (g)

F1 Generation					F2 Generation				
Lactation Day	**Control 0**	**LDT 40**	**MDT 220**	**HDT 1200**	**Lactation Day**	**Control 0**	**LDT 40**	**MDT 220**	**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 0.08
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 0.27	9.2 0.19
4[c] S.E.	9.7 0.21	9.3 0.22	9.8 0.20	9.5 0.22	**4[c] S.E.**	9.8 0.28	9.6 0.19	9.4 0.26	9.2 0.19
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	15.2 0.30	15.0 0.46	14.5 0.31
14 S.E.	32.1 0.46	30.9 0.72	31.8 0.50	30.6 0.53	**14 S.E.**	31.8 0.72	31.0 0.87	30.8 0.76	29.6 0.49
21 S.E.	49.1 0.74	47.2 0.96	48.8 0.75	46.8 0.80	**21 S.E.**	49.2 1.09	48.7 0.81	46.9 1.10	45.2* 0.80
GAIN	43.2	41.4	42.9	40.9	**GAIN**	43.2	42.9	41.1	39.5*

b: Before standardization (culling)
c: After standardization (culling)
* Statistically different from control, p≤0.05

Table 5.6.1-13 Mean (S.E.) Male Pup Weights (g)

F1 Generation					F2 Generation				
Lactation Day	**Control 0**	**LDT 40**	**MDT 220**	**HDT 1200**	**Lactation Day**	**Control 0**	**LDT 40**	**MDT 220**	**HDT 1200**
0 S.E	6.1 0.10	6.0 0.10	6.1 0.10	6.0 0.07	0 S.E	6.2 0.11	6.0 0.08	5.9 0.13	5.9 0.09
4[b] S.E.	9.9 0.23	9.6 0.23	10.0 0.22	9.7 0.23	4[b] S.E.	10.0 0.28	9.8 0.20	9.6 0.28	9.4 0.18
4[c] S.E.	10.0 0.22	9.6 0.23	10.1 0.22	9.7 0.23	4[c] S.E.	10.0 0.28	9.8 0.21	9.6 0.28	9.4 0.19
7 S.E.	16.0 0.29	15.4 0.37	15.9 0.30	15.5 0.37	7 S.E.	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 0.60	32.3 0.53	31.0 0.53	14 S.E.	32.3 0.69	31.4 0.88	31.1 0.79	30.1 0.51
21 S.E.	50.2 0.75	49.0 0.88	49.7 0.82	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 standardization (culling)
c: After standardization (culling)
** Statistically different from control, p≤0.01

Table 5.6.1-14 Mean (S.E.) Female Pup Weights (g)

F_1 Generation					F_2 Generation				
Lactation Day	Control 0	LDT 40	MDT 220	HDT 1200	Lactation Day	Control 0	LDT 40	MDT 220	HDT 1200
0 S.E	5.8 0.10	5.6 0.08	5.8 0.09	5.7 0.08	0 S.E	5.8 0.09	5.6 0.06	5.6 0.12	5.6 0.08
4b S.E.	9.5 0.21	9.0 0.19	9.7 0.20	9.4 0.23	4b S.E.	9.6 0.29	9.3 0.18	9.0 0.30	9.1 0.21
4c S.E.	9.5 0.22	9.0 0.19	9.7 0.21	9.3 0.23	4c S.E.	9.6 0.29	9.3 0.19	9.0 0.30	9.1 0.20
7 S.E.	15.3 0.30	14.4 0.36	15.2 0.30	14.9 0.35	7 S.E.	15.4 0.42	14.9 0.30	14.7 0.48	14.3 0.31
14 S.E.	31.5 0.46	30.2 0.66	31.4 0.49	30.2 0.54	14 S.E.	31.4 0.75	30.6 0.86	30.3 0.77	29.4 0.51
21 S.E.	48.0 0.77	46.0 0.84	48.2 0.75	45.9 0.86	21 S.E.	48.2 1.12	47.8 0.77	45.6 1.09	44.6* 0.84

b: Before standardization (culling)
c: After standardization (culling)
* Statistically different from control, $p \leq 0.05$

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 – 44.0) and is 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 ppm versus 173 g in controls). There were no findings 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 0 for the F2-pups with no effects on this measurement noted.

4. Offspring postmortem results:

<u>F_1-Juveniles</u>: The F_1-juveniles (after weaning PND 21) 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, relative to controls, upon passing criteria for balanopreputial separation and vaginal patency. Male body weights were declined 7.3% 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 tested.

<u>F_2-Juveniles</u>: There was no effect on body weight, relative to controls, upon passing criteria for balanopreputial separation and vaginal patency for the F_2-juveniles (after weaning PND 21) at any dietary level tested.

5. Anogenital distance (F2 pups)

As the effect on body weight and attainment of puberty landmarks was affected in the F_1 generation, measurement of anogenital distance on LD/PND 0 in the F_2 generation was added to the protocol. There was no treatment-related effect on anogenital distance measured at birth in either male or female F_2 offspring, as shown in the table below.

Table 5.6.1-15 Anogenital distance F2 generation at birth

Group (ppm in diet)	Number of litters	Mean (mm)
Males		
0	26	3.5
150	30	3.5
1000	28	3.7
4000	29	3.5
Females		
0	26	2.0
150	30	2.0
1000	28	2.0
4000	29	2.1

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 substance-related for one or more of the following reasons: the organ weight difference was associated with the statistically significant decrease in day 21 mean body weights, the change was not dose-related, and/or the change was relatively small.

2. Pathology

Macroscopic examination All gross lesions for F1 and F2 pups were considered to be incidental and not test substance-related.

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 LOAEL was 1200 ppm (82.8 mg/kg/day in males) based on increased clinical chemistry parameters (creatinine, total protein, albumin and urea nitrogen), increased kidney weight 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 (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 white blood cell and monocyte Ab counts in the F1-generation, decreased hemoglobin 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; [redacted].; 2009; M-345012-01-1 (Fluopyram - Evaluation of OECD joint review dossier - Questions addressed from German BfR - Metabolism, toxicology, dated February 23, 2009) and KIIA 5.6.1 /06; Yang, Y. G.; 2005; M-329297-01-1 (EPA DER for Oral (diet) developmental immunotoxicity study of TI 435 (clothianidin) in Crl: CD(SD) rats). The offspring NOAEL was 220 ppm (17.0 mg/kg/day).

Assessment and conclusion by applicant:

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

Based on this study the parental systemic NOAEL was 220 ppm (14.5 mg/kg/day in males, 17.2 mg/kg/day in females) based on Clinical pathology changes, increased liver weight, protein droplet nephropathy (males) and centrilobular nephropathy. 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). 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 NOAEL 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 (97.9 mg/kg/day).

CA 5.6.2 Developmental toxicity studies

Data Point:	KCA 5.6.2/01
Report Author:	[redacted]
Report Year:	2008
Report Title:	AE C656948 - Developmental toxicity study in the rat by gavage
Report No:	SA05276
Document No:	M-299438-01-2
Guideline(s) followed in study:	OECD 414 (2001); EPA Health Effects Test Guideline (OPPTS 870.3700; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
Deviations from current test guideline:	current guideline: OECD 414, 2018 Deviations: anogenital distance and thyroid hormones were not measured. These deviations do not impact the acceptability of the study
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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 l-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 stained according to a modification of the Tyl and Marr technique for skeletal examination of bone and cartilage.

Up to and including the highest dose level tested of 450 mg/kg/day, there were 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% lower 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 (37.6 g) than in the control group (59.4 g). 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 autopsy, 4/23 females had enlarged livers. At the histopathological examination of the liver, diffuse centrilobular hepatocellular hypertrophy was observed in all females.

At Cesarean section, mean fetal body weight was 5% lower for both the combined and separate sexes compared to the controls. No other litter parameters were affected.

At the external fetal examination, no treatment-related malformations or variations were observed and the incidence of runt fetuses 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 ‘ureter convoluted and/or dilated’ was higher at this dosage than in the controls at both the fetal and litter 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 ([redacted], 2007, M-766189-01-1).

At 150 mg/kg/day

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 change was less 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 skeletal fetal 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 consumption was 10% lower 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 caused significant maternal toxicity in terms of lower maternal body weight gain and food consumption, higher liver weight and diffuse centrilobular 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 No Observed Adverse Effect Level (NOAEL) was 30 mg/kg/day and the fetal No Observed Effect Level (NOEL) was 150 mg/kg/day.

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Beige Powder
Lot / Batch #:	Mix-Batch 08528/0002
Purity:	94.6 %
CAS #	658066-35-4
Stability of test compound:	Stable in suspension in the vehicle (aqueous solution of methylcellulose 400 at 0.5%) at concentrations of 0.0868 and 250 g/L for a period of 33 days under similar conditions to those of the current study.
2. Vehicle and / or positive control:	The vehicle was an aqueous solution of methylcellulose 400 at 0.5%
3. Test animals:	
Species:	Rat
Strain:	Crl:CD (SD) Sprague-Dawley
Age:	11 to 13 weeks approximately (at mating)
Weight at dosing:	246 to 301 g for the females
Source:	[redacted]
Acclimation period:	5 days prior to mating
Diet:	Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages
Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	55 ± 15%

Air changes: Approximately 10 to 15 changes per hou
Photoperiod: 12 hours dark / 12 hours light

B. Study design

1. In life dates: 04 January 2006 to 09 February 2006

2. Animal assignment and treatment

Adult virgin female rats were mated on a one-to-one basis with stock males of the same strain and supplier for each group. Each morning following pairing, rats 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 (w/v) in an aqueous solution of methylcellulose 400 (Fluka, Mulhouse, France) at 0.5% and stored at approximately 5°C (± 3°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 g/L). In addition, the intermediate concentration (15 g/L) of the first formulation (F1) and all concentrations of the second formulation (F2) were checked. Homogeneity and concentration 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.

Table 5.6.2-1 Study design and animal assignment

Test group	Test substance	Dose levels mg/kg/day	Concentrations g/L	Volume (mL/kg)	Number of animals
1	0	0	0	10	23
2	AE C656948	30	3	10	23
3		150	15	10	23
4		450	45	10	23

Dose levels were chosen based on the findings of a range-finding study ([redacted] 2004, M-246660-01-2), where groups of 8 sperm positive SD rats were dosed at 0, 50, 200 and 500 mg/kg/day.

Clear maternal 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, whilst mean liver weight was markedly increased by 43%. At 200 mg/kg/day, maternal corrected body weight change was 10% lower than controls. Mean food consumption was reduced by 14% between GD 6-8, whilst mean liver weight was moderately increased by 21%. No treatment-related changes were observed at 50 mg/kg/day.

Doses were administered 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 (0.5% aqueous methylcellulose).

C. Methods

1. Observations

All rats 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 GD: 1, 6, 8, 10, 12, 14, 16 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 uterine content. Autopsies were performed blind with regard to the animal study identification. Each 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. Runt fetuses were defined as live fetuses weighing less than 4 g at Cesarean section of the dam. Uterine horn(s) without visible implantations were immersed in a 10% solution of ammonium sulfide to visualise any sites which were not apparent. Intra-uterine 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.

5. Fetal examination

All data were recorded without knowledge of treatment group. All live 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 subsequently dissected for internal examination. The remaining half were eviscerated, fixed in absolute ethanol and stained according to a modification of the Tyl and Marr technique for skeletal examination of bone and cartilage.

Structural deviations were classified as follows:

Malformations:

A permanent structural change that is likely to adversely affect the survival or health

Variations:

A change that occurs within the normal population under investigation and is unlikely to adversely affect survival or health (this might include a delay in growth or morphogenesis that has otherwise followed a normal pattern 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 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, number of implantation sites, number of resorption (early 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 fetal body weight) or an arcsine root transformation (for pre- or 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. If the Bartlett test on transformed data was significant, 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 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 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 litter 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-Wallis tests were evaluated at the 5% level of significance. Group means were compared at the 5% and 1% levels of significance.

II. Results and discussion

A. Mortality

There were no mortalities during the course of the study.

B. Clinical observations

There were no treatment-related clinical signs.

C. Pregnancy rate

There was no effect on pregnancy rates, which were 96% in all treated group and in the controls.

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 weight gain of 6.8 g over the corresponding period in the control group, the effect being statistically significant ($p \leq 0.01$). Between GD 8 to 10 and 10 to 14, mean maternal body weight gain was 22% (not statistically significant) and 54% ($p \leq 0.01$) 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 maternal body weight gain ($p \leq 0.01$) compared with the controls.

At 150 mg/kg/day, 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 effect was statistically significant ($p \leq 0.05$ or $p \leq 0.01$) 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 ($p \leq 0.01$ at 450 mg/kg/day and $p \leq 0.05$ or $p \leq 0.01$ 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 ($p \leq 0.01$), 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 15% higher at 150 mg/kg/day, compared with the controls. The effect was statistically significant ($p \leq 0.01$) at both dose levels.

Mean maternal liver weights were similar to the controls at 30 mg/kg/day.

Table 5.6.2-2 Mean liver weight of pregnant females

Dose group (mg/kg/day)	0	30	150	450
Number	22	22	22	21
Mean liver weight (g) ± SD (% of controls)	13.95 ± 1.69 (-)	14.06 ± 1.61 (101)	16.05 ** ± 2.11 (115)	19.47 ** ± 2.33 (140)

** $p \leq 0.01$
SD: Standard deviation

At autopsy of the dams, enlarged liver was observed in 4/23 females at 450 mg/kg/day compared with 0/23 cases in the control group.

Histopathologic changes were observed in the liver at 450 and 150 mg/kg/day only.

Diffuse centrilobular hepatocellular hypertrophy was seen in a dose related manner at 450 and 150 mg/kg/day.

Table 5.6.2-3 Incidence of changes in the liver

Sex	Females			
Dose group (mg/kg/day)	**0**	**30**	**150**	**450**
Number of animals examined	23	23	23	23
Centrilobular hepatocellular hypertrophy, diffuse				
minimal	0	1	8	0
slight	0	0	10	1
moderate	0	0	2	21
marked	0	0	0	1
Total	**0**	**1**	**20**	**23**

G. Litter data

At 450 mg/kg/day, mean fetal body weight was 5% ($p \leq 0.05$) lower for both the combined and separate sexes.

At 150 and 30 mg/kg/day, mean fetal body weight for the combined sexes and the separate sexes was not statistically significantly different when compared to the control group.

Table 5.6.2-4 Mean fetal body weights

Dose group (mg/kg/day)	0	30	150	450
Number of litters	22	22	22	22
Mean litter body weight (g) for combined sexes ± SD (% of controls)	5.51 ± 0.39 (100)	5.48 ± 0.20 (100)	5.39 ± 0.35 (98)	5.26 * ± 0.36 (95)
Mean litter body weight (g) for males ± SD (% of controls)	5.67 ± 0.34 (100)	5.59 ± 0.26 (99)	5.54 ± 0.38 (98)	5.37 * ± 0.36 (95)
Mean litter body weight (g) for females ± SD (% of controls)	5.40 ± 0.42 (100)	5.38 ± 0.19 (100)	5.25 ± 0.37 (97)	5.13 * ± 0.37 (95)

* p≤0.05, SD: Standard Deviation

Other litter parameters including number of live fetuses, early or late resorptions and dead fetuses, were unaffected by treatment.

H. Fetal necropsy findings

1. Fetal evaluation: external observations

There were no malformations or treatment-related increase in variations observed at the external fetal observation.

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 in-house variability at all three dose levels, when data from the low dose from a recent in-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 chance.

There were two other malformations observed at the visceral examination, 'eventration of the diaphragm and caudate lung lobe absent' in 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 historical 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/day, the occurrence of these two variations was slightly higher than in the control group, but was considered to be incidental in view of the in-house historical control range.

Table 5.6.2-5 Visceral findings

Group	Dose group (mg/kg/day)				Historical Control Range	Dose group (mg/kg/day)				Historical Control Range
	0	30	150	450		0	30	150	450	
Observations	Fetal incidence (mean % of fetuses affected)*					Litter incidence (% of litters affected)				
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)	5/22 (22.7)	8/22 (36.4)	10/22 (45.5)	1/19-8/24 (5.3-33.3)
Ureter (unilateral /bilateral) convoluted and/or dilated	46/146 (33.2)	57/147 (36.9)	72/155 (46.2)	88/149 (58.6)	22/153-78/175 (20.5-45.1)	17/22 (77.3)	17/22 (77.3)	20/22 (90.9)	20/22 (90.9)	17/25-23/24 (68.0-95.8)

* mean % of litters affected defined as: sum of % of live fetuses affected per litter/no. of litters with live fetuses examined

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.

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: dumbbell and/or bipartite / normal cartilage', compared with the control group. The incidence was outside the in-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 150 mg/kg/day, the incidence of the variation 'at least one thoracic 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 Range	Dose group (mg/kg/day)				Historical Control Range
	0	30	150	450		0	30	150	450	
Observations	Fetal incidence (mean % of fetuses affected)*					Litter incidence (% 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 (0.0-0.6)	0/22 (0.0)	1/22 (4.5)	0/22 (0.0)	4/22 (18.2)	0/25-1/21 (0.0-4.8)
At least one thoracic centrum : dumbbell *or incomplete ossification* and/or bipartite/normal cartilage	3/157 (1.8)	9/160 (5.3)	12/167 (7.1)	29/159 (20.8)	1/144-12/176 (0.7-7.2)	2/22 (9.1)	7/22 (31.8)	9/22 (40.9)	14/22 (63.6)	1/19-9/23 (5.3-39.1)

* mean % of litters affected defined as sum of % of live fetuses affected per litter no. of litters with live fetuses examined *or incomplete ossification* : additional term included in the in-house historical control range.

With regards to 'At least one thoracic centrum: split/split cartilage' 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.

J. Deficiencies

The study has been carried out in line with the OECD 414 requirements in force at the time it was conducted. No measurements of the test material in the plasma, 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 centrilobular 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 NOAEL was 30 mg/kg/day and the fetal NOEL was 150 mg/kg/day.

Assessment and conclusion by applicant:

The study has been carried out in line 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 centrilobular 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 NOAEL was 30 mg/kg/day and the fetal NOEL was 150 mg/kg/day

Data Point:	KCA 5.6.2/02
Report Author:	[redacted]
Report Year:	2006
Report Title:	AE C656948 - Developmental toxicity study in the rabbit by gavage
Report No:	SA 05014
Document No:	M-279773-01-1
Guideline(s) followed in study:	OECD 414 (2001); EPA Health Effects Test Guideline (OPPTS 870.3700; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines.
Deviations from current test guideline:	Current guideline: OECD 414, 2018 Deviations: None, the 2018 update affects rat-specific requirements in the TG 414; thus applies to rats and not to rabbits.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Twenty-three time-mated female New Zealand White Rabbits per group were exposed to AE C656948, a fungicide of the pyramide family, (Mix-Batch 08528/0002, a beige powder, 94.6% purity) by gavage from Gestation Day (GD) 6 to 28. The doses 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, 14-16, 16-18, 18-20, 20-22, 22-24, 24-26, 26-28 and 28-29. Clinical observations were recorded daily. At scheduled sacrifice, on GD 29, a macroscopic 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 status of implantations (resorptions, dead and live fetuses). The liver was retained from all females and was weighed for pregnant females at terminal sacrifice. Live fetuses were removed from the uteri, counted, weighed and examined externally. The heads of fetuses from approximately half 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 sexed. Fetuses were eviscerated, skinned and fixed 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 hemorrhaging 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 (-0.17 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 macroscopic 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.

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 limited 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 epiphysal femoral disjunction. Consequently, this premature sacrifice was considered to be due to accidental trauma. At this dosage, no treatment-related maternal findings were noted.

There were no treatment-related effects on litter parameters.

There were no treatment-related external, visceral or skeletal findings at this dose level.

At 10 mg/kg/day

No treatment-related maternal findings were noted.

There were no treatment-related effects on litter parameters.

There were no treatment-related external, visceral, or skeletal fetal findings at this dose level.

In conclusion, a dose level of 75 mg/kg/day of AE C656948 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 sexes.

A dose level of 25 mg/kg/day of AE C656948 was considered to be a No Observed Effect Level (NOEL) both in the dam and in terms of fetal development in the New Zealand White rabbit.

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Beige Powder
Lot / Batch #:	Mix-Batch: 08528/0002
Purity:	94.6 %
CAS #	658066-35-4
Stability of test compound:	Stable in suspension in the vehicle (aqueous solution of methylcellulose 400 at 0.5%) at concentrations of 0.0868 and 250 g/L for a period of 33 days under similar conditions to those of the current study.
2. Vehicle and / or positive control:	The vehicle was an aqueous solution of methylcellulose 400 at 0.5%
3. Test animals:	
Species:	Rabbit
Strain:	New Zealand White Crl:KBL (NZW)
Age:	18 weeks approximately on arrival
Weight at dosing:	3.17 - 3.90 kg females
Source:	[redacted]
Acclimation period:	At least 5 days prior to dosing
Diet:	110 C-10 pelleted animal diet from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France) *ad libitum*
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*.

Housing:	Animals were caged individually in stainless steel wire mesh cages
Environmental conditions:	
Temperature:	19 ± 2°C
Humidity:	55 ± 15%
Air changes:	Approximately 10 to 15 changes per hour
Photoperiod:	12 hours dark / 12 hours light

B. Study design

1. In life dates: 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 study. Stock males 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 allocated 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 (methylcellulose).

The experimental groups were as follows:

Table 5.6.2-7 Study design and animal assignment

Test group	Test substance	Dose levels mg/kg/day	Concentrations g/L	Volume (mL/kg)	Number of animals
1	0	0	0	4	23
2	AE C656948	10	2.50	4	23
3		25	6.25	4	23
4		75	18.75	4	23

Dose levels were based on the results obtained in a range finding study in pregnant rabbits (KIIA 5.6.11 /02; [redacted] 2004; M-122897-01-1, AE C656948 - Range-finding study for developmental toxicity in the rabbit by gavage, DOC-056, SA 04051), where groups of 8 time-mated female NZW rabbits received 0, 50, 150, 450 and 750 mg/kg/day from GD 6 to 28. The highest two dose levels caused marked maternal toxicity, exceeding 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 150 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 GD 6 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 level) suspended (w/v) in an aqueous solution of methylcellulose 400 (Fluka, Mulhouse, France) at 0.5% and stored at approximately 5°C (± 3°C). Homogeneity of the suspensions was checked during the first 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 33 days under 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 daily for mortality (except once daily on weekends and public holidays).

Body weight

Body weights were measured on GD: 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29.

Food consumption:

Full feeder weights were measured on GD: 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 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 Dolethal® (Sanofi, Libourne, France) or found dead, were autopsied. A macroscopic examination of the visceral 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, uterine horn(s) were immersed in a 20% solution of ammonium sulfide according to the Salewski method (1964). The liver was taken and preserved in 10% neutral buffered formalin. Tissues and carcasses were then discarded.

On GD 29, surviving females 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 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, individual weights of live fetuses. Dead fetuses: were defined as 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 Staples and Schnell staining technique was used and a subsequent skeletal examination was performed.

4. 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 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, number of implantation sites, number of resorption (early 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 fetal body weight) or an arcsine root transformation (for pre- or 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. If the Bartlett test on transformed data was significant, 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 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 Fisher Exact test (2-sided) (for fetal death status parameter). Death status was analyzed both using the fetus as the statistical unit and using the litter as the statistical unit.

If one or more group variance(s) equaled 0, means were compared using non parametric procedures.

II. Results and discussion

A. Mortality

There was one mortality on GD 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 hemorrhaging in the lung of both females together with hemorrhaging and foam in the trachea of one female and a trachea filled with fluid for the other female. In addition, one female was killed for humane reasons 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 on GD 22 and 23. The macroscopic observation showed a severe fracture 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% in all groups.

E. Bodyweight

At 75 mg/kg/day, mean body weight gain was reduced between GD 14 and 18 (0.02 kg vs. 0.09 kg for controls, $p \leq 0.01$) and between GD 18 and 22 (0.02 kg vs. 0.07 kg for controls, $p \leq 0.01$), 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, 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 uterine weight) was more pronounced at 75 mg/kg/day (-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% ($p \leq 0.01$) for all intervals between GD 14 to 26, in comparison to controls.

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 ($p \leq 0.01$ for combined sexes and males, $p \leq 0.05$ 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 fetuses, early or late resorptions, fetal death status and percentage of male fetuses were unaffected by treatment 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 3.0% 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 findings.

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 C656948 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 sexes.

A dose level of 25 mg/kg/day of AE C656948 was considered to be a NOEL both in the dam 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 OECD 414 requirements in force at the time it was conducted.

At the a dose level of 75 mg/kg/day of AE C656948 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 sexes.

A dose level of 25 mg/kg/day of AE C656948 was considered to be a NOEL both in the dam and in terms of fetal development in the New Zealand White rabbit.

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 Wistar rats. Four dose groups (12 rats/sex/dose level) were administered the test substance at nominal doses of 0 (vehicle), 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-up 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 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.

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 triglyceride levels in males and/or females and decreased terminal body weight in females. Also, liver and kidney weights (absolute and relative) were increased in high-dose males and liver weight (absolute and relative) was increased in high-dose females. The only finding at the mid-dose was decreased food consumption in females, 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, 164.2 and 197.1 mg/kg for male and female rats, respectively).

Table 5.7-1 Fluopyram - Summary of neurotoxicity studies

Study Doses tested: ppm mg/kg bw/d	NOAEL ppm mg/kg bw/d	LOAEL ppm mg/kg bw/d	Main findings	Reference
Acute neurotoxicity in the rat (initial study) 0, 125, 500 and 2000 mg/kg bw	125 in males and < 125 in females	500 in males and < 125 in females	↓ motor and locomotor activity, clinical signs and ↓ body temperature	[redacted] M-289073-01-2
Acute neurotoxicity in the rat (follow-up study in females) 0, 25, 50 and 100 mg/kg bw	50	100	↓ motor and locomotor activity	[redacted] E M-289073-01-2
90-day neurotoxicity in the rat M-299110-01 0, 100, 500 and 2500 ppm 0, 6.69, 33.2, 164.2 / 0, 8.05, 41.2, 197.1, mg/kg bw/ day in M/F	164/197 (♂/♀)	>164/>197 (♂/♀)	None based on neurotoxicology endpoints	[redacted] M-299110-01-1

CA 5.7.1 Neurotoxicity studies in rodents

Data Point:	KCA 5.7.1/01
Report Author:	[redacted]
Report Year:	2007
Report Title:	An acute Oral Neurotoxicity Screening Study with Technical Grade AE C656948 in Wistar Rats
Report No:	201656
Document No:	M-289073-01-2
Guideline(s) followed in study:	OECD 424 (1997); EPA Health Effects Test Guideline (OPPTS 870.6200; 1998); M.A.F.F. in Japan notification 12 Nousan N°8147 (2000) guidelines
Deviations from current test guideline:	Current guideline: OECD 424, 1997 No deviations
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognized testing facilities:	Yes, conducted under GLP/Officially recognized testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Technical grade AE C656948 was administered by gavage in a single dose to nonfasted 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 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-up 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. The test substance was administered in 2% (v/v) Cremophor EL in deionized 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 micropathology. For the initial study, observations for moribundity and mortality were performed at least once daily (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 (FOB) and automated measurements of activity (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 weighed and skeletal muscle, peripheral nerves, eyes (with optic nerves) and tissues from the central nervous 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). Also, a functional observational battery (FOB) and automated measurements of activity (figure-eight maze) were conducted during the week prior to treatment and on 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 treated 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 males and females and for the follow-up study actual doses were 0, 25, 51, 100 mg/kg for females.

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. Females 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 males (51% and 49%, respectively) and females (53% and 58%, respectively) on day 0. Also, females had decreased body temperature. All signs of toxicity resolved by day 7.

125 mg/kg. Measures of motor and locomotor activity were statistically significantly 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 reduced (38%, each) on day 0.

50 mg/kg. There were no compound-related 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 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. 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 methods

A. Materials

1. Test material:	AE C656948
Description	Light beige powder
Lot / Batch #:	Mix-batch 08528/0002
Purity:	94.7% (May, 2005, certified through May, 2007)
CAS #	658066-35-4
Stability of test compound:	Stable in the dosing vehicle at the room temperature for 8 days
2. Vehicle and / or positive control:	2% (w/v) Cremophor EL in deionized water
3. Test animals:	
Species:	rat
Strain:	Wistar HAN CRL: WI (HAN)
Age:	At least 9 weeks
Weight at dosing:	Initial Study: 252.2 to 315.3 g for the males; 167.0 – 206.9 g for the females Follow-Up Study: 166.6 – 214.7 g for females
Source:	[redacted]
Acclimation period:	Initial Study – 8 days Follow-Up Study – 7 days

Diet:	Purina Mills Rodent Lab Chow 5002 in meal form provided for *ad libitum* 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 steel wire-mesh cages.
Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	50 ± 70%
Air changes:	Minimum daily average of 10.13 changes per hour
Photoperiod:	12 hours dark / 12 hours light

B. Study design

1. In life dates: 25 April 2006 – 18 August 2006

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, 125, 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 sacrifice. Animals were randomly assigned to the test groups.

Each rat was identified by cage card and tail 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.

The oral route of exposure was employed in accordance with the test guideline. The rat was selected due to its general acceptance and suitability as a rodent species for toxicological testing of this type, as well as the availability of a historical database on the Wistar strain. The study design for the initial study required a total of 96 rats (48 males and 48 females) and an additional 48 females were utilized for the follow-up study to establish a NOAEL for motor and locomotor activity. These animals were at least nine weeks of age when the treatment was administered. An additional 24 animals (8 males and 8 females, each for the initial study and 8 females for the follow-up study) were tested (FOB and motor activity) during the pre-treatment week and reserved for use in case they were needed to replace animals (e.g. if mis-dosed) that were assigned to the study.

For the initial study, four dose groups (12 rats/sex/dose 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 nominal doses of 0 (vehicle), 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 mL/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 FOB. Twelve rats/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 females/dose level were used in the same manner as animals from the initial study, except they were sacrificed 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 accommodate 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 Study design and animal assignment

Experimental Parameter	Initial Study - Dose Group (mg/kg bw)				Follow-Up Study - Dose Group (mg/kg bw) Females Only			
	Control	125	500	2000	Control	25	50	100
Total number of animals/sex/group	12	12	12	12	11-12[2]	12	12	12
Behavioral Testing (FOB, Motor Activity) [a]	12/sex	12/sex	12/sex	12/sex	11-12[2]	12	12	12
Neuropathology [b]	6/sex	0/sex	0/sex	6/sex	0	0	0	0

a *FOB and motor activity were assessed prior to dosing (both studies) and again during days 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 treatment-related neuropathology was noted at the 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 fasted female Wistar rats were administered an acute oral (gavage) dose of 2000 mg/kg as an aqueous suspension in 2% Cremophor EL in tap water, at a dosing volume of 10 mL/kg. Animals were observed for mortality and clinical signs for at least 14 days after treatment. The test substance produced no mortality and no clinical signs of toxicity. 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-labeled AE C656948 were examined to estimate the time of peak effect. In that study, adult male and female 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 doses selected 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 100 mg/kg dose was selected to either reproduce the findings in the initial study or possibly establish an overall NOAEL at a dose level slightly lower than the lowest dose tested in the initial study (i.e., 125 mg/kg). The remaining treated doses were selected to establish an overall NOAEL if 100 mg/kg was 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 suspending 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 results, the analytically-confirmed doses for males and females were 0, 126, 498 and 1840 mg/kg. For the **follow-up study**, doses of 0, 25, 50 and 100 mg/kg for females ranged from 100% to 102% of the nominal concentrations. Based on these results, the analytically-confirmed doses for females in the follow-up study were 0, 25, 51 and 100 mg/kg.

4. Statistics

Statistical evaluations were generally performed using software from either INSTEM Computer Systems or SAS. The level of significance was set at $p \leq 0.05$. Group means with equal variances were analyzed further 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 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 manner, using General Linear Modeling and Categorical Modeling (CATMOD) Procedures, with post-hoc comparisons using Dunnett's test and an Analysis of Contrasts, respectively.

Motor and locomotor activity (total session activity and activity for each 10-minute interval) was analyzed using ANOVA procedures. Session activity data were analyzed using 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 treatment 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 on which days there was a significant treatment by interval interaction. For those days, 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.

Continuous pathology data (e.g., brain 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 Dunnett's test for pair-wise comparisons. In the event of non-homogeneous variances, continuous data were analyzed using the nonparametric Kruskal-Wallis test followed by a Mann-Whitney U 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 $p \leq 0.05$, with the exception of Bartlett's test, which was tested at $p \leq 0.001$

C. Methods

1. Mortality and Clinical Observations:

Cage-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 14 days following treatment. For the follow-up study, females that were assigned to the study were tested using the FOB and motor activity on two occasions – one week prior to treatment and approximately 1 hour (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 testing 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 testing took 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 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. It should be noted that animals from the follow-up study were treated 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.

An additional 24 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 mis-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 foot splay 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 a historical control.

When applicable, observations were scored on intensity as follows: 1) slight (barely perceptible or infrequent) or 2) 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 (lacrimal, nasal, perianal, urine, oral), alopecia, emaciation, bite marks, exophthalmia, broken teeth/malocclusion, missing toe nail(s), 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 lighting, pupil response, righting reflex, grip 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 parameters (checked (X) parameters examined)

HOME CAGE OBSERVATIONS	**HANDLING OBSERVATIONS**	**OPEN FIELD OBSERVATIONS**
X Posture*	X Reactivity*	X Rearing+
X Piloerection	X Muscle tone*	X Piloerection*
X Involuntary motor movements e.g.:	X Palpebral closure*	X Respiratory abnormalities
X Repetitive chewing	X Lacrimation* chromodacryorrhea	X Posture*
X Convulsions*	X Salivation*	X Involuntary motor movements e.g.:
X Tremors*	X Nasal discharge	X Repetitive chewing
X Abnormal movements*	X Red/crusty deposits (stains)*	X Convulsions*
X Gate abnormalities	X Fur appearance	X Tremors*
X Vocalizations	X Emaciation	X Stereotypic behavior*
X Decreased activity	X Bite marks	X Bizarre behavior*
X Repetitive head bobbing	X Eye prominence*	X Abnormal movements *
X Increased reactivity	X Broken teeth/malocclusion	X Gait abnormalities* / Gait score*
	X Missing Toe Nail(s)	X Vocalizations
SENSORY OBSERVATONS	X Dehydration	X Arousal/ general activity level*
X Approach response+	X Cool-to-Touch	X Urination / defecation*
X Touch response+		
X Auditory response*	**PHYSIOLOGICAL OBSERVATIONS**	**NEUROMUSCULAR OBSERVATIONS**
X Pain response*	X Body weight*	X Forelimb grip strength*
X Pupil response*	X Body temperature+	X Hindlimb grip strength*
X Pupil size		X Landing foot splay*
X Air righting reflex+		

*Required parameters; +Recommended parameters

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 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 (Columbus, OH) 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 test to minimize acoustical variations during testing. The uniformity of light intensity (100±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 eliminating consecutive counts for a given beam. Thus, for locomotor activity, only one interruption of a given beam was counted until the rat 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 on 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 initial study. The necropsy involved an examination of all organs, body cavities, cut surfaces, external orifices and surfaces. For the initial study on day 14, a minimum of six males and six females at each dose 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% (w/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 neural 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. 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 a NOAEL 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 dorsal and ventral root fibers) from the cervical and lumbar swellings and gasserian ganglion were embedded in glycol methacrylate (GMA). Eyes, optic nerves and gastrocnemius muscle were embedded in paraffin and stained using H&E. Peripheral nerve tissues (sciatic, tibial and sural nerves) were embedded in GMA 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

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 SYSTEM
	BRAIN		**SCIATIC NERVE**
X	Olfactory bulbs	X	Sciatic nerve (bilateral)
X	Cerebral cortex		
X	Caudate-putamen/globus pallidus		
X	Hippocampus		
X	Thalamus		
X	Hypothalamus		**OTHER**
X	Midbrain (tectum, tegmentum, and cerebral peduncles)	X	Tibial nerve (bilateral)
X	Cerebellum	X	Sural nerve (bilateral)
X	Medulla oblongata		
	SPINAL CORD	X	Lumbar dorsal root ganglion
X	Cervical swelling	X	Lumbar dorsal root fibers
X	Thoracic swelling	X	Lumbar ventral root fibers
X	Lumbar swelling	X	Cervical dorsal root ganglion
X	Cauda equina	X	Cervical dorsal root fibers
	OTHER	X	Cervical ventral root fibers
X	Gasserian ganglion		
X	Optic nerve		
X	Eye		
X	Gastrocnemius muscle		

II. Results and discussion

A. Mortality

For the **initial and follow-up studies**, there were no compound-related 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, dilated kidney pelvis, multiple, bilateral, discolored zones on kidneys and abnormal contents in the urinary bladder (i.e. Thickened material).

B. Clinical observations

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 mid-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.

Table 5.7.1-2 Body weight – Initial Study (g ± s.d.)

Test Day	Dose Level (mg/kg bw)			
	Control	125	500	2000
Body weight-Males				
Day 0	279±17	288±13	283±10	282±14
Day 7	298±20	311±16	303±15	295±19
Day 14	314±25	329±20	320±19	311±22
Body weight-Females				
Day 0	190±13	187±8	186±8	190±6
Day 7	197±15	199±10	194±10	200±8
Day 14	208±14	209±8	205±8	216±34

Body weight was not statistically different from control (p≤0.05)
n=12

For the follow-up study, body weight was not measured beyond day 0 since a NOAEL for body weight had been established in the initial study. The day 0 body weights for the females in the follow-up study are summarized in the table below.

Table 5.7.1-3 Day 0 Body Weight – Follow-Up Study (g ± s.d.)

Test Day	Dose Level (mg/kg bw)			
	Control[1]	**25**	**50**	**100**
Body weight-Females				
Day 0	189±11	187±10	190±10	193±11

Body weight was not statistically different from control (p≤0.05) N=12 (unless otherwise noted). [1]: n=11

D. Neurobehavioral results

1. Functional observation battery (FOB)

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 treatment.

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 (3.1 vs. 1.0 for controls) and a statistical decrease in mean foot splay (66 vs. 84 for controls) for females 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

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.

Table 5.7.1-4 Functional observational battery results – Initial study

Males	Observation	Dose Level (mg/kg bw/day)			
		Control	125	500	200
Pretreatment	Urination - Number of Pools Mean ± S.D.	1.0±1.0	1.3±1.4	1.6±1.6	* 3.1±2.3
Day 0	No Findings	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)
Day 7	No Findings	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)
Day 14	Handling - Other: Not Observed Alopecia, Present	11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)
Females	**Observation**	**Dose Level (mg/kg bw/day)**			
		Control	**125**	**500**	**200**
Pretreatment Week	Handling - Other: Not Observed Alopecia, Present	12(100) 0(0)	12(100) 0(0)	10(83) 2(17)	12(100) 0(0)
	Handling - Other: Not Observed Scab, Present	12(100) 0(0)	12(100) 0(0)	11(92) 1(8)	12(100) 0(0)
	Reflex/Physiologic Observations – Foot Splay Mean ± S.D.	84±17	84±16	* **66±14**	81±15
Day 0	Handling – Ease of Removal: Minimal Resistance Minimal Resistance with Vocalizations	6(50) 6(50)	5(42) 7(58)	9(75) 3(25)	* 11(92) 1(8)
	Handling - Other: Not Observed Alopecia, Present	11(92) 1(8)	12(100) 0(0)	10(83) 2(17)	12(100) 0(0)
	Reflex/Physiologic Observations - Body Temperature (°C) Mean ± S.D.	37.9±0.4	37.8±0.3	* 37.4±0.5	* 36.9±0.5
Day 7	Handling - Other: Not Observed 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 14	Handling - Other: Not Observed Alopecia, Present	10(83) 2(17)	12(100) 0(0)	10(83) 2(17)	12(100) 0(0)

Values represent the number of animals and % incidence (in parentheses) with observation.
** Statistically different from control (p≤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 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% lower to 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 test substances 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% lower 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 normal variability in this laboratory for groups of 10-12 rats/sex/dose 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 & 5.7.1-7) 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% (locomotor), respectively and in females an average 53% and 72% (motor) and 58% and 77% (locomotor), repectively. 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-related effects 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 in females dosed with 100 mg/kg. There were no treatment-related differences from control in motor or locomotor activity in females at lower dose levels.

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 normal variability (approximately + 20%) on day 7 for high-dose males (22% lower 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 females at the same dose level. For the follow-up study, motor activity was slightly outside the range of normal variability on day 0 for low-dose females (22% lower than controls). This difference from control was not ascribed to treatment since the difference was small and not dose-related.

The initial study and follow-up study data for summary sessions for motor and locomotor activity are presented in the tables below.

Table 5.7.1-5 Summary Session Motor Activity Results – Initial Study (Percent Difference from Control) [a]

Test Day	Dose Level (mg/ kg bw)		
	125	500	2000
Males			
Pretreatment	17	-12	-6
Day 0	-5	-51*	-71*
Day 7	4	-14	-20
Day 14	6	-9	-9
Females			
Pretreatment	13	-7	22
Day 0	-26*	-53*	-72*
Day 7	1	-11	19
Day 14	-6	17	8

a Percent greater (+) or less (-) than concurrent control for N=12.
** Summary session motor activity was statistically different from control (p≤0.05; ANOVA).*

Table 5.7.1-6 Summary Session Motor Activity Results – Follow-Up Study (Percent Difference from Control) [a]

Test Day	Dose Level (mg/kg bw)		
	25	50	100
Females			
Pretreatment	-11	-9	-15
Day 0	-22	-20	-38

a Percent greater (+) or less (-) than concurrent control for N=12.
Summary session motor activity was not statistically different from control (p≤0.05; ANOVA).

Table 5.7.1-7 Summary Session Locomotor Activity Results – Initial Study (Percent Difference from Control) [a]

Test Day	Dose Level (mg/ kg bw)		
	125	500	2000
Males			
Pretreatment	28	-8	-10
Day 0	-4	-49*	-73*
Day 7	12	-9	-22
Day 14	23	-3	-15
Females			
Pretreatment	16	-12	22
Day 0	-31*	-58*	-77*
Day 7	7	-16	12
Day 14	7	7	11

a Percent greater (+) or less (-) than concurrent control for N=12.
** Summary session locomotor activity was statistically different from control (p≤0.05; ANOVA).*

Table 5.7.1-8 Summary Session Locomotor Activity Results – Follow-Up Study (Percent Difference from Control) [a]

Test Day	Dose Level (mg/kg bw)		
	25	50	100
Females			
Pretreatment	-11	-7	-20
Day 0	-14	-18	-38*

a Percent greater (+) or less (-) than concurrent control for N=12.
Summary session locomotor activity was not statistically different from control (p≤0.05; ANOVA).

For the initial study, motor and locomotor activity data were subjected to further analysis at each interval 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 85%, respectively, for motor and 33%, 78%, 91%, 87%, 94% and 89%, respectively, for locomotor). Also on day 0, the high-dose females exhibited statistically lower levels of motor and locomotor activity during intervals 1 through 6 (44%, 72%, 79%, 85%, 82% and 84%, respectively for motor and 46%, 79%, 88%, 95%, 90%, and 90%, respectively for locomotor). For mid-dose males, interval motor and locomotor activity was statistically lower than controls during intervals 2 through 5 or 6 (47%, 62%, 73%, 76% and 74%, respectively for motor and 47%, 65%, 76% and 79%, respectively for locomotor). 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%, 83% 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%, respectively). There were no differences ascribed to the test substance for males at the lowest dose 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 or 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 females, 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 summarized 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 (total activity counts for session)

Test Day	Dose Level (mg/kg bw)			
	Control	125	500	2000
Males				
Pretreatment	493±130	579±152	432±130	464±155
Day 0	480±130	454±148	237 * ±91	139 * ±55
Day 7	496±110	518±149	429±126	398±94
Day 14	433±82	457±140	395±120	393±91
Females				
Pretreatment	435±181	490±111	405±127	530±171
Day 0	521±132	385 * ±79	247 * ±68	148 * ±66
Day 7	480±115	484±98	426±89	573±114
Day 14	447±129	475±107	525±212	484±100

*Values represent mean ± s.d. for 1:00:00 (hh:mm:ss) Test Session n=12, * Summary session motor activity was statistically different from control (p≤0.05; ANOVA).*

Table 5.7.1-10 Motor Activity – Follow-Up Study (total activity counts for session)

Test Day	Dose Level (mg/kg bw)			
	Control[a]	25	50	100
Females				
Pretreatment	504±108	451±115	457±119	429±138
Day 0	539±92	423±185	430±69	335±93

Values represent mean ±s.d. for 1 hr Test Session (hh:mm:ss) n=12, unless otherwise noted.
a: n=11 Summary session motor activity was not statistically different from control (p≤0.05; ANOVA).

Table 5.7.1-11 Locomotor Activity – Initial Study (total activity counts for session)

Test Day	Dose Level (mg/kg bw)			
	Control	125	500	2000
Males				
Pretreatment	325±100	415±140	299±118	292±121
Day 0	335±112	322±120	171 * ±72	92 * ±33
Day 7	323±80	363±125	295±109	251±68
Day 14	287±65	323±108	277±90	243±76
Females				
Pretreatment	284±131	330±96	251±86	347±114
Day 0	362±108	251 * ±66	151 * ±57	82 * ±36
Day 7	311±87	333±77	261±148	348±88
Day 14	281±87	300±89	301±116	313±76

Values represent mean ±s.d. for 1:00:00 (hh:mm:ss) Test Session
*n=12, * Summary session locomotor activity was statistically different from control (p≤0.05; ANOVA).*

Table 5.7.1-12 Locomotor Activity – Follow-Up Study (total activity counts for session)

Test Day	Dose Level (mg/kg bw)			
	Control[a]	25	50	100
Females				
Pretreatment	322±89	286±76	300±86	256±97
Day 0	353±94	302±142	289±65	218±84

Values represent mean ± s.d. for 1 n=12 unless otherwise noted.
a: N=11 Summary session locomotor activity was not statistically different from control (p≤0.05, ANOVA).

E. Sacrifice and pathology

1. Gross pathology:

There were no compound-related gross lesions evident at terminal sacrifice in males 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 effect on 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.

Table 5.7.1-13 Absolute and Relative Brain Weights – Initial Study

Weights (g)	Dose Level (mg/kg bw)			
	Control	125	500	2000
Males				
Body wt [a]	312.6±23.5	328.4±19.8	316.1±17.4	307.3±21.1
Brain wt [b]	1.826±0.078	1.833±0.053	1.806±0.038	1.866±0.052
Brain/body wt [b]	0.577±0.037	0.547±0.019	0.565±0.023	0.619±0.046
Female				
Body wt [a]	209.1±15.2	207.7±10.5	204.7±7.6	209.2±7.9
Brain wt [b]	1.812±0.081	1.739±0.083	1.750±0.038	1.721±0.025
Brain/body wt [b]	0.878±.045	0.842±0.056	0.841±0.012	0.828±0.016

a N=12 Measurements were not statistically different from the control (p ≤ 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. Deficiencies

None

III. Conclusions

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 females 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. 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.

Data Point:	KCA 5.7.1/02
Report Author:	[redacted]
Report Year:	2008
Report Title:	A Subchronic Neurotoxicity Screening Study with Technical Grade AE C656948 in Wistar Rats
Report No:	201833
Document No:	M-299110-01-1
Guideline(s) followed in study:	OECD TG 424 (1997); US EPA Health Effects Test Guidelines OPPTS 870.6200; 1998); M.A.F.F. of Japan notification 12 Nousan N°8147 (2000) guidelines
Deviations from current test guideline:	Current guideline: OECD 424, 1997 No deviations
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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 0, 100, 500 and 2500 ppm for males and females. All test diets (including control) 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 C656948 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 daily. 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. Ophthalmologic 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 a gross necropsy. For selected animals, the brain was weighed in order to calculate the brain:body weight ratio 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 dietary concentrations of 100, 500 and 2500 ppm, respectively, was 6.69, 33.2 and 164.2 mg/kg/day for males 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 cholesterol and triglyceride levels in males and/or females and decreased terminal body weight in females. Also, liver and kidney weights (absolute 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 mid-dose was decreased food consumption in females, 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, 164.2 and 197.1 mg AE C656948/kg/day for male and female rats, respectively).

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Beige powder
Lot / Batch #:	Mix-batch 08528/0002
Purity:	94.7%
CAS #	658066-35-4
Stability of test 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 control:	Acetone served as a solvent in the diet preparation process and was allowed to evaporate prior to administration
3. Test animals:	
Species:	Rat
Strain:	Wistar HAN CRL: WI(HAN)
Age:	Approximately 8 weeks of age
Weight at dosing:	219.6 – 285.9 g range for males and 142.9 – 174.9 g range for females
Source:	[redacted]
Acclimation period:	10 days
Diet:	Purina Mills Rodent Lab Chow 5002 in meal form provided for *ad libitum* consumption during the acclimation period and throughout the study except during neurobehavioral testing.
Water:	Tap water, *ad libitum*
Housing:	Animals were caged individually in suspended stainless steel wire-mesh cages.
Environmental conditions:	
Temperature:	22 ± 4°C
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

Randomization procedures utilized software from INSTEM Computer Systems. Following acclimation, 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 tattoo 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, cage number and identified it with the study.

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, 33.2 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 observations 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 from control and high-dose groups. Hematologic and serum chemistry evaluations were performed on selected animals on the day of sacrifice and the liver, kidney and thyroid tissues from 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 5.7.1.-14 below.

Table 5.7.1-14 Study Design

Experimental Parameter	Dose Group ppm (mg/kg bw/day)			
	Control	100 (♂ 6.69) (♀ 8.05)	500 (♂ 33.2) (♀ 41.2)	2500 (♂ 164.2) (♀ 197.1)
Total number of animals/sex/group	12	12	12	12
Behavioral Testing (FOB, Motor Activity) [a]	10-12/sex	11-12/sex	10-12/sex	10-12/sex
Clinical Chemistry and Tissue Collection	4-6/sex	5-6/sex	5-6/sex	5-6/sex
Neuropathology [b]	6/sex	0/sex	0/sex	6/sex
Ophthalmic Examination [c]	11-12/sex	11-12/sex	11-12/sex	11-12/sex

a FOB and motor activity was assessed prior to dosing 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 dietary level.
c Ophthalmic exams were performed prior to dosing and at week 12.

The rationale for dose 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 rats (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 freezer (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 uneaten 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°C) exposure] and homogeneity of the test substance in the feed were established by analysis of samples at nominal concentrations of 5 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 those used in this study. These concentrations of 5 and 5000 ppm had percent relative standard deviations (%RSD) of 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 ppm, 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 ppm dietary 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 ppm.

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 $p \leq 0.001$, the level used to establish statistical significance was $p \leq 0.05$.

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 Dunnett's test and an Analysis of Contrasts, respectively.

Motor and locomotor 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 followed by a Mann-Whitney U Test for pair-wise comparisons.

Micropathology frequency data were analyzed using a Chi-Square Test followed by a one-tailed Fisher's Exact Test in cases of significant variation by the Chi-Square analysis. A probability value of $p \leq 0.05$ was accepted as significant for all statistical tests with the exception of Bartlett's Test in which a probability value of $p \leq 0.001$ was used.

C. Methods / observations

1. Mortality and Clinical Observations:

Cage-side observations were conducted twice 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 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.

3. Food Consumption:

Individual food consumption was measured weekly. Daily food consumption was averaged over the duration of the study, based on per kg body weight.

The average daily intake of the active ingredient (A.I.) (mg A.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 (ppm)/1,000] x [feed consumed (g/kg body wt/day)] = mg AI/kg body wt/day. Using this formula, the average consumption of A.I. for males and females that received diets containing analytically-determined nominal concentrations of 0, 100, 500 and 2500 ppm AE C656948 was as follows:

0, 6.69, 33.2 and 164.2 mg/kg/day, respectively for males and

0, 8.05, 41.2 and 197.1 mg/kg/day, respectively for females.

4. Neurobehavioral Assessment

All animals 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 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 prior to each test day, the appropriate 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 dose 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 room, 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 mazes cleaned during the ensuing interval to reduce the residual scent from the other sex.

a. Functional Observational Battery (FOB):

This FOB closely follows 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 foot splay and grip strength are based on established methods. The technicians 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 persons 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 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 (lacrimal, nasal, perianal, urine, oral), alopecia, emaciation, bite marks, exophthalmia, broken teeth/malocclusion, missing toe nail(s), 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 behavior), bizarre behavior, 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 lighting, pupil response, righting reflex, grip strength [Chatillon, Model DFIS-10 digital strain gauges (5.0 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)

FOB Parameters Evaluated (checked (X) parameters evaluated)

	HOME CAGE OBSERVATIONS		HANDLING OBSERVATIONS		OPEN FIELD OBSERVATIONS
X	Posture*	X	Reactivity*	X	Rearing+
X	Piloerection	X	Muscle tone*	X	Piloerection*
X	Involuntary motor movements e.g.:	X	Palpebral closure*	X	Respiratory abnormalities+
X	Repetitive chewing	X	Lacrimation* / chromodacryorrhea	X	Posture*
X	Convulsions*	X	Salivation*	X	Involuntary motor movements e.g.:
X	Tremors*	X	Nasal discharge	X	Repetitive chewing
X	Abnormal movements*	X	Red/crusty deposits (stains)*	X	Convulsions*
X	Gait abnormalities	X	Fur appearance	X	Tremors*
X	Vocalizations	X	Emaciation	X	Stereotypic behavior*
X	Decreased activity	X	Bite marks	X	Bizarre behavior*

X	Repetitive head bobbing	X	Eye prominence*	X	Abnormal movements*
X	Increased reactivity	X	Broken teeth/malocclusion	X	Gate abnormalities / Gait score*
		X	Missing Toe Nail(s)	X	Vocalizations
	SENSORY OBSERVATIONS	X	Dehydration	X	Arousal/ general activity level*
X	Approach response+	X	Cool-to-Touch	X	Urination / defecation*
X	Touch response+				
X	Auditory response*		**PHYSIOLOGICAL OBSERVATIONS**		**NEUROMUSCULAR OBSERVATIONS**
X	Pain response*				
X	Pupil response*	X	Body weight*	X	Forelimb grip strength*
X	Pupil size	X	Body temperature+	X	Hindlimb grip strength*
X	Air righting reflex+			X	Landing footsplay*

**Required parameters; +Recommended parameters*

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 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 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 (Columbus, OH) 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 uniformity 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, ten-minute intervals. Motor activity was measured as the number of beam interruptions that occurred during the test session. Locomotor activity was measured by eliminating consecutive counts for a given beam. Thus, for locomotor activity, only one interruption of a given beam was counted until the rat relocated in the maze and interrupted one of the other beams. Habituation was evaluated as a decrement in activity during the test session.

5. Ophthalmology

Pre-exposure and pre-terminal (week 12) ophthalmic 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 Binnoll (Welch Allyn, Inc. Skaneateles Falls, NY), and then a mydriatic agent was applied to each eye to dilate the pupil. After mydriasis, the conjunctiva, cornea and lens were examined with a slit lamp microscope (Kowa SL-15, Kowa Company, Ltd., 20001 So. Vermont Ave. Tollerance, CA 90502, USA), and the vitreous humor, retina, choroid, and optic disc were examined using an indirect ophthalmoscope [HEINE OMEGA (Heine USA, LTD, One Washington Center, Suite 555, One Washington Street, Dover, NH 03820, USA)].

6. Clinical Chemistry

Hematologic and serum chemistry evaluations were performed on all surviving **non-perfused** animals (four to six/sex/dietary 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 while 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% (w/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 neural 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 prior to study termination, were sacrificed by carbon dioxide asphyxiation and necropsied. For all **non-perfused** animals (four to six/sex/dietary level) that remained on study until termination, the liver, kidneys and thyroid were collected, weighed and preserved in 10% buffered formalin for possible histopathologic evaluation. Terminal body weights were performed immediately prior to necropsy to allow for calculation of organ to body weight ratios.

Micropathology examinations were conducted on a comprehensive selection of neural 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, thoracic, lumbar) and the cauda equina were embedded in paraffin and stained with hematoxylin and eosin (H&E). Dorsal root ganglia (including dorsal and ventral root fibers) from the cervical and lumbar swellings and gasserian ganglion were embedded in glycol methacrylate (GMA). Eyes, optic nerves and gastrocnemius muscle were embedded in paraffin and stained using H&E. Peripheral nerves (sciatic, tibial and sural) were embedded in GMA 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 cut in cross-section at approximately 2-3 µm and stained with a modified Lee's stain. In addition, histopathology was performed on any gross lesion collected at necropsy. Tissues from perfusion-fixed animals at the low- and 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 nervous system are summarized in below.

CNS and PNS Tissues Evaluated (checked (X) tissues evaluated)

	CENTRAL NERVOUS SYSTEM BRAIN		PERIPHERAL NERVOUS SYSTEM SCIATIC NERVE
X	Olfactory bulbs	X	Sciatic nerve (bilateral)
X	Cerebral cortex		
X	Caudate-putamen/globus pallidus		
X	Hippocampus		
X	Thalamus		
X	Hypothalamus		OTHER
X	Midbrain (tectum, tegmentum, and cerebral peduncles)	X	Tibial nerve (bilateral)
X	Cerebellum	X	Sural nerve (bilateral)
X	Medulla oblongata		
	SPINAL CORD		
X	Cervical swelling	X	Lumbar dorsal root ganglion
X	Thoracic swelling	X	Lumbar dorsal root fibers
X	Lumbar swelling	X	Lumbar ventral root fibers
X	Cauda equina		
	OTHER	X	Cervical dorsal root ganglion
X	Gasserian ganglion	X	Cervical dorsal root fibers
X	Optic nerve	X	Cervical ventral root fibers
X	Eye		

X 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 trimethyltin 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 or females at any dietary level.

B. Clinical observations

There were no treatment-related clinical observations seen at any dietary level in either sex. The findings summarized in Table 5.7.1-15 below were considered incidental and unrelated to treatment. These findings were malocclusion on days 74-88 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 Clinical Observations (Incidental Findings)

Observation	Dose Level ppm (mg/kg bw/day)			
	Control	100 (♂ 6.69)	500 (♂ 33.2)	2500 (♂ 164.2)
Males				
Hair, Alopecia				
Forelimb-Both	1/12	0/12	0/12	0/12
Thorax-Ventral	0/12	0/12	0/12	1/12
Females				
Observation	**Control**	**100 (♀ 8.05)**	**500 (♀ 41.2)**	**2500 (♀ 197.1)**
Lacrimal Stain, Red	0/11-12	0/11-12	0/11-12	1/11-12
Nasal Stain, Red	0/11-12	0/11-12	0/11-12	1/11-12
Malocclusion-Both Upper Incisors	0/11-12	0/11-12	0/11-12	1/11-12

Numbers represent the total number of animals exhibiting sign at least once/number of animals in each group

C. Bodyweight and bodyweight gain

For high-dose males, 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 data are summarized Tables 5.7.1-16 and 5.7.1-17 below for males and females, respectively.

Table 5.7.1-16 Body Weight and Body Weight Gain (Mean (g) ± s.d.) for Males

Day No.	Dose Level ppm (mg/kg bw/day)			
	Control	100 (6.69)	500 (33.2)	2500 (164.2)
Day 0	244.3±13.5 12	244.4±19.0 12	245.6±9.2 12	247.2±8.7 12
Day 21	325.7±23.6 12	325.3±34.8 12	327.3±12.9 12	309.8±13.5 12
Day 28	347.8±24.8 12	344.5±36.9 12	347.8±10.8 12	331.1±13.8 12
Day 35	365.5±25.9 12	360.7±38.1 12	366.8±14.8 12	344.9±14.7 12
Day 42	377.2±29.3 12	369.8±37.9 12	375.0±12.5 12	353.6±17.2 12
Day 49	388.9±30.4 12	380.6±40.1 12	387.6±16.3 12	360.9±17.3 12
Day 56	398.5±29.2 12	391.4±41.6 12	400.0±17.2 12	373.5±18.2 12
Day 63	405.9±30. 12	399.9±43.7 12	410.8±18.3 12	384.2±20.0 12
Day 70	414.6±32.5 12	408.8±42.9 12	420.4±19.2 12	394.7±19.9 12
Day 77	420.3±31.8 12	413.0±44.8 12	426.3±18.0 12	399.5±21.7 12
Day 84	426.6±32.2 12	421.9±47.1 12	431.1±19.0 12	408.0±21.0 12
Day 91	432.9±34.5 12	428.7±44.8 12	437.8±22.2 12	416.2±22.7 12
Total Body weight gain (Day 0–91) -Males	188.6±26.3 12	184.3±31.1 12	192.3±18.3 12	169.0±19.3 12

Values represent mean ± s.d., n.

Table 5.7.1-17 Body Weight and Body Weight Gain (Mean (g) ± s.d.) for Females

Day No.	Dose Level ppm (mg/kg bw/day)			
	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 12	155.8±7.7 12
Day 21	196.7±14.7 11	196.4±16.4 11	189.8±7.6 11	182.4±7.6* 11
Day 28	205.6±16.2 11	206.7±18.5 11	199.9±8.7 11	194.7±7.6* 11
Day 35	213.7±18.2 11	213.2±16.7 11	207.9±7.8 11	201.4±10.3* 11
Day 42	217.5±17.3 11	216.1±17.0 11	209.1±8.0 11	197.9±12.3* 11
Day 49	222.9±15.9 11	219.4±17.4 11	214.1±9.5 11	199.3±15.3* 11
Day 56	226.4±15.5 11	224.9±19.8 11	219.9±10.2 11	208.9±10.0* 11
Day 63	231.1±19.5 11	229.5±18.4 11	226.1±9.7 11	211.3±10.4* 11
Day 70	236.2±18.8 11	233.1±20.3 11	226.9±9.3 11	212.1±12.1* 11
Day 77	236.4±17.7 11	236.4±22.2 11	229.0±11.9 11	208.4±12.6* 11
Day 84	240.8±18.1 11	239.9±21.8 11	234.1±12.8 11	216.7±13.6* 11
Day 91	245.8±20.3 11	243.2±22.1 11	239.1±11.4 11	223.2±11.6* 11
Total Body weight gain (Day 0-91) --Females	89.3±13.4 11	85.1±12.7 11	86.8±8.3 11	66.5±10.2* 11

Values represent mean ± s.d., n.
*=p≤0.05, compared to control.

D. Food consumption

For high-dose males, food consumption was statistically significantly decreased (15%) 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 (7-12%) in mid-dose females beginning on day 21 through day 42, day 63 through day 70 and again on day 91. Food consumption was not affected by treatment in mid-dose males or in low-dose males or females. These data are summarized in Tables 5.7.1-18 and Table 5.7.1-19 below for males and females, respectively.

Table 5.7.1-18 Mean Food Consumption (g/animal/day ± s.d.) for Males

Day No.	Dose Level ppm (mg/kg bw/day)			
	Control	100 (6.69))	500 (33.2)	2500 (164.2)
Day 7	22.84±1.83 12	23.60±2.99 12	22.88±1.74 12	25.27±5.12 12
Day 21	23.69±1.67 12	24.21±2.53 12	23.20±1.64 12	20.12±2.34* 12
Day 35	24.07±1.59 12	24.06±2.74 12	23.89±1.88 12	22.38±1.47 12
Day 42	23.97±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

Values represent mean ± s.d., n
**=p≤0.05, compared to control.*

Table 5.7.1-19 Mean Food Consumption (g/animal/day ± s.d.) for Females

Dose Level ppm (mg/kg bw/day)			
Control	**100 (8.05)**	**500 (41.2)**	**2500 (197.1)**
18.19±4.45 12	16.08±1.55 12	16.09±1.34 12	20.88±5.65 12
18.19±2.20 11	16.72±1.35 11	16.29±1.04* 11	14.56±0.63* 11
17.61±1.65 11	16.48±0.93 11	16.12±0.61* 11	15.07±1.08* 11
18.22±1.72 11	17.09±1.15 11	16.86±1.05* 11	15.43±1.07* 11
18.53±1.71 11	17.10±1.38 11	16.54±1.07* 11	14.66±1.42* 11
17.96±2.55 11	16.45±1.41 11	16.40±1.23 11	14.32±1.67* 11
17.73±2.24 11	16.12±1.23 11	16.23±1.20 11	15.09±1.56* 11
18.99±3.13 11	17.07±1.00 11	16.79±1.31* 11	14.63±1.23* 11
18.35±2.25 11	16.85±1.15 11	16.23±1.19* 11	13.91±1.24* 11
17.68±1.82 11	17.37±1.43 11	16.36±1.36 11	13.98±2.33* 11
17.23±1.55 11	16.83±1.18 11	16.08±1.30 11	15.01±1.12* 11
17.86±2.31 11	16.60±1.37 11	16.01±1.20* 11	14.77±0.95* 11

*Values represent mean ± s.d., n *=p≤0.05 compared to control.*

E. Neurobehavioral results

1. Functional observation battery (FOB)

The data from Functional Observational revealed no 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.

Remaining observations considered incidental and unrelated to treatment included areas of hair loss in one control (all test weeks, 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 5.7.1-20.

Table 5.7.1-20 Functional Observational Battery Results

Males	**Observation**	**Dose Level ppm (mg/kg bw/day)**			
		Control	**100 (6.69)**	**500 (33.2)**	**2500 (164.2)**
Pretreatment	Handling – Alopecia (severity): Not Observed (0) Present (1)	11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)
Week 2	Handling – Alopecia (severity): Not Observed (0) Present (1)	11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)
Week 4	Handling – Alopecia (severity): Not Observed (0) Present (1)	11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	11(92) 1(8)
Week 8	Handling – Alopecia (severity): Not Observed (0) Present (1)	11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	11(92) 1(8)

Week 13	Handling – Alopecia (severity): Not Observed (0) Present (1)	11(92) 1(8)	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)
Females	**Observation**	**Dose Level ppm (mg/kg bw/day)**			
		Control	**100 (8.05)**	**500 (41.2)**	**2500 (197.1)**
Pretreatment	No Findings	12(100)	12(100)	12(100)	12(100)
Week 2	No Findings	12(100)	12(100)	12(100)	12(100)
Week 4	No Findings	11(100)	11(100)	11(100)	11(100)
Week 8	Body Weight (Mean ±S.D.)	224±18	220±20	215±10	203±8*
Week 13	Handling – Broken Teeth/Malocclusion (severity): Not Observed (0) Present (1)	11(100) 0(0)	11(100) 0(0)	11(100) 0(0)	10(91) 1(9)
	Body Weight (Mean ±S.D.)	240±19	238±24	233±13	216±12*

Values represent the number of animals and % incidence in parentheses) with observation
**=p≤0.05, compared with controls*
Severity: 0=Not Observed, 1=Slight, 2=Moderate to Severe

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 a 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 ± 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 a few occasions when measures of motor (**week 2**: 23% and 28% higher at the low- and high-dose, respectively) and locomotor (**week 2**: 28%, 21% and 25% higher at the low-, mid- and high-dose, respectively; **week 4**: 25% higher at the low-dose; **week 8**: 35% higher at the low-dose; **week 13**: 29% 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).

Table 5.7.1-21 Summary Session Motor Activity Results (Percent Difference from Control) [a]

Week No.	Dose Level ppm (mg/kg bw/day)		
	100 (♂ 6.69)	**500 (♂ 33.2)**	**2500 (♂ 164.2)**
Males[b]			
Pretreatment	+12	+18	+15
Week 2	+23	+19	+28
Week 4	+20	+5	+1
Week 8	+16	-1	+3
Week 13	+8	+9	+19
Females[c]			
Week No.	**100 (♀ 8.05)**	**500 (♀ 41.2)**	**2500 (♀ 197.1)**
Pretreatment[b]	+1	-13	+8
Week 2	-19	-8	+4
Week 4	-1	-9	+3
Week 8	0	-14	-13
Week 13	+0.5	-5	+3

a Percent greater (+) or less (-) than 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 animals and N=11 for remaining dietary levels.
Summary session motor activity was not statistically different from control ($p \leq 0.05$; ANOVA) at any time for any dietary level.

Table 5.7.1-22 Summary Session Locomotor Activity Results (Percent Difference from Control) [a]

Week No.	Dose Level ppm (mg/kg bw/day)		
	100 (♂ 6.69)	500 (♂ 33.2)	2500 (♂ 164.2)
Males[b]			
Pretreatment	+5	+14	+14
Week 2	+28	+21	+25
Week 4	+25	+13	-1
Week 8	+35	+11	+13
Week 13	+11	+20	+29
Females[c]			
Week No.	**100 (♀ 8.05)**	**500 (♀ 41.2)**	**2500 (♀ 197.1)**
Pretreatment[b]	+10	-12	+10
Week 2	-12	-8	-4
Week 4	-9	-17	-7
Week 8	+4	-16	-24
Week 13	+3	-5	-9

a Percent greater (+) or less (-) than 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 animals and N=11 for remaining dietary levels.
Summary session locomotor activity was not statistically different from control (p≤0.05; ANOVA) at any time for any dietary level.

Motor and locomotor activity data were also analyzed for differences at each 10-minute interval of each test session. For males and females, interval motor and locomotor activity were not affected by treatment at any dietary level in either sex.

Habituation was not affected by treatment with AE C656948 in males or females at any dietary level.

Table 5.7.1-23 Motor Activity (total activity counts for session)

Test Week	Dose Level ppm (mg/kg bw/day)			
	Control	100 (♂ 6.69)	500 (♂ 33.2)	2500 (♂ 164.2)
Males				
Pretreatment	542±215	605±207	640±165	626±136
Week 2	547±166	672±206	653±134	698±160
Week 4	558±103	670±319	586±167	552±165
Week 8	507±136	587±173	504±133	521±197
Week 13	414±147	447±133	452±142	491±234

Females[b]				
Test Week	**Control**	**100 (♀ 8.05)**	**500 (♀ 41.2)**	**2500 (♀ 197.1)**
Pretreatment[a]	824±309	830±270	716±275	891±260
Week 2	882±299	712±238	813±317	921±122
Week 4	759±233	704±205	692±200	782±187
Week 8	798±202	798±196	690±163	697±162
Week 13	641±185	644±260	674±332	662±168

Values represent mean ± 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=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.
Summary session motor activity was not statistically different from control (p≤0.05; ANOVA) at any time for any dietary level.

Table 5.7.1-24 Locomotor Activity (total activity counts for session)

Test Week	**Dose Level ppm (mg/kg bw/day)**			
	Control	**100 (♂ 6.69)**	**500 (♂ 33.2)**	**2500 (♂ 164.2)**
		Males[b]		
Pretreatment	332±137	348±142	378±108	377±88
Week 2	290±101	370±101	351±45	362±66
Week 4	276±62	344±144	312±93	274±97
Week 8	229±66	296±87	244±82	248±118
Week 13	177±72	197±55	212±62	229±127
		Females[b]		
Test Week	**Control**	**100 (♀ 8.05)**	**500 (♀ 41.2)**	**2500 (♀ 197.1)**
Pretreatment[a]	388±137	427±178	342±150	428±126
Week 2	419±153	369±138	387±202	404±63
Week 4	361±117	330±97	301±132	337±87
Week 8	360±95	373±96	302±88	275±73
Week 13	286±85	294±119	300±185	260±65

Values represent mean ± 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=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.
Summary session locomotor activity was not statistically different from control (p≤0.05; ANOVA) at any time for any dietary level.

F. Opthalmology

No treatment-related ophthalmologic findings were seen in either sex.

G. Clinical pathology

1. Clinical Chemistry

Cholesterol (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.

5.7.1-25 Clinical Chemistry

Clinical Chemistry Parameters	Dose Level ppm (mg/kg bw/day)			
	Control	100 (♂ 6.69)	500 (♂ 33.2)	2500 (♂ 164.2)
Males[a]				
Cholesterol	57±14	58±11	110±127	82±14$
Females[b]				
Weights (g)	**Control**	**100 (♀ 8.05)**	**500 (♀ 41.2)**	**2500 (♀ 197.1)**
Cholesterol	51±3	50±6	65±16	86±19*
Trigyceride	30±8	32±11	31±5	57±26*

Values represent mean ± s.d
a N = 6
b N = 4-5
** Statistically different from the control (p ≤0.05 Anova + Dunnett's tests).*
$ Statistically different from the control (p ≤0.05 Kruskal-Wallis Anova + Mann-Whitney U-tests).

2. Hematology

There were no compound-related hematology changes at any dietary level in either sex.

E. Sacrifice and pathology

1. Gross Pathology

There were no compound-related gross lesions or gross observations evident at terminal sacrifice in 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 non-perfused animals sacrificed at the end of the study are summarized in Table 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 body weights were not different from control in males at any dietary level or in low- or mid-dose females.

Compound-related organ weight changes, relative to control, included increased liver and kidney weights (absolute and relative) in high-dose males and increased liver weights (absolute and relative) in high-dose females.

Other mean organ weights for males and females 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 in either gender at any dietary level.

There were no significant differences in brain weights between control and treated perfused rats.

Table 5.7.1-26 Mean Body Weight and Absolute and Relative Organ Weights

Weights (g)	Dose Level ppm (mg/kg bw/day)			
	Control	100 (♂ 6.69, ♀ 8.05)	500 (♂ 33.2, ♀ 41.2)	2500 (♂ 164.2, ♀ 197.1)
Perfused Males				
Body Wt.[a]	419.7±39.2	413.4±49.3	440.3±20.9	418.1±23.3
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	0.427±0.041
Non-Perfused Males				
Body Wt.[b]	399.2±34.1	391.1±56.0	417.4±18.1	375.1±8.3
Liver Wt.[b]	14.937±0.755	15.971±2.618	18.183±3.361	21.337±1.080*
Liver/Body Wt[b].	3.752±0.162	4.079±0.703	4.351±0.722*	5.695±0.265*
Kidney Wt.[b]	2.817±0.170	2.841±0.427	3.573±1.171	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	0.033±0.006
Thyroid/Body Wt. b	0.0063±0.0011	0.0067±0.0016	0.0074±0.0019	0.0087±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]	1.894±0.101	1.781±0.075	1.632±0.379	1.769±0.124
Brain/Body Wt.[b]	0.762±0.046	0.726±0.067	0.674±0.159	0.775±0.063
Non-Perfused Females				
Body Wt.[d]	225.8±19.0	220.1±18.6	210.0±12.1	193.3±15.9*
Liver Wt.[d]	7.612±1.033	7.595±0.457	8.497±0.992	10.729±0.487*
Liver/Body Wt.[d]	3.371±0.358	3.460±0.202	4.043±0.402*	5.569±0.359*
Kidney Wt.[d]	1.736±0.152	1.781±0.116	1.686±0.157	1.656±0.066
Kidney/Body Wt.[d]	0.769±0.042	0.811±0.035	0.802±0.052	0.860±0.055
Thyroid Wt.[d]	0.020±0.005	0.020±0.005	0.019±0.003	0.023±0.004
Thyroid/Body Wt. d	0.0091±0.0029	0.0091±0.0015	0.0090±0.0011	0.0120±0.0023

Values represent mean ± sd
a N= 12; b N= 6
cN=10-11 d N= 4-5
** Statistically different from the control (p ≤0.05 Anova + Dunnett's tests).*
$ Statistically different from the control (p ≤0.05 Kruskal-Wallis Anova + Mann-Whitney U-tests).

3. Micropathology

There were no treatment-related findings in neural and/or non-neural tissues from perfusion-fixed high-dose males or females that were related to administration of the test substance. Tissues from animals at lower dose levels were, therefore, 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.

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 164.2 mg/kg/day in males and 197.1 mg/kg/day in females.

Assessment and conclusion by applicant:

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

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 triglyceride levels in males and/or females and decreased terminal body weight in females. Also, liver and kidney weights (absolute 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 mid-dose was decreased food consumption in females, 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, 164.2 and 197.1 mg AE C656948/kg/day for male and female rats, respectively).

CA 5.7.2 Delayed polyneuropathy studies

Fluopyram is not an organophosphate 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 metabolites

For the definition of residues for risk assessment of fluopyram it is required to assess the genotoxic potential of plant and livestock metabolites. Since not all the metabolites have genotoxicity data available an in silico assessment was conducted. The in silico assessment has been conducted using Derek Nexus, Leadscope and Toxtree software (expert and rules based predictive software respectively) (M-763978-01-1).
In addition to the in silico 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 genotoxicity 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 fluopyram 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 toxicity is currently ongoing. The completed studies, include an acute oral toxicity study in the rat, 14-day, 28-day and 90-day repeat dose studies in the rat. Ongoing studies include a developmental toxicity study in the rabbit and extended one generation reproduction 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 below

Table 5.8-1 Smmary of toxicology studies with Trifluoroacetic acid (TFA)

Study	Concentrations of [Substance] tested	Result	Reference
***In vitro* assay**			
Ames mutagenesis	Without and with S9: 1.6, 8, 40, 200, 1000 and 5000 µg/plate	Negative	[redacted] 2005 M-256628-01-1
Micronucleus assay using human lymphocyte cultures	Without and with S9: 340, 680 and 1360 µg/ml	Negative	[redacted] 2005 M-260807-01-1
Mutation at the Thymidine Kinase (tk) locus of mouse lymphoma L5178Y cells (MLA)	Without and with S9: 42.5, 85.1, 170, 340 680 and 1360 µg/ml	Negative	[redacted] 2005 M-260699-01-1
***In vivo* assay**			
Acute oral toxicity Wistar rat (5 females)	2000 mg/kw bw	LD50 > 2000 mg/kw bw	[redacted] M-444479-01-1
14-day dietary toxicity study in the Wistar rat (8/sex/ control and top dose group and 5/sex/ low and mid dose group)	0, 600, 1200 and 2400 ppm (42.63, 84.90 and 169.68 mg/kg bw/day ♂) (0, 45.41, 91.08, 189.59 mg/kg bw/day ♀)	Liver findings (increased organ weight in correlation with hepatocellular hypertrophy, increased cytochrome P-450, lauric acid hydroxylation activity, specific and total palmitoyl-CoA oxidation activities). NOAEL 600 ppm 42.63 mg/kg bw/day ♂ 45.41 mg/kg bw/day ♀	[redacted] ; 2001 M-202165-01-1
28-day dietary toxicity study in the Wistar rat (5/sex/ group)	0, 600, 1800, 5400 and 16000 ppm (0, 50, 149, 436 and 1315 mg/kg bw/day ♂) (0, 52, 157, 457and 1344 mg/kg bw/day ♀)	No adverse effects observed up to the top dose. Changes in few clinical chemistry parameters were not accompanied	[redacted] 2005 M-259106-01-1

		by changes in the correlated organs liver weight changes were not correlated with 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 study in the Wistar rat (10/sex/ group)	0, 160, 1600 and 16000 ppm (0, 9.9, 98, 1043 mg/kg bw/day ♂) (0, 12.2, 123, 1216 mg/kg bw/day ♀)	1600 ppm Changes in hematological and clinical chemistry parameters, organ weights and histopathological liver findings NOAEL 600 ppm 9.9 mg/kg bw/day ♂ 12.2 mg/kg bw/day ♀	[redacted] 2007 M-283994-01-1

Effect of TFA on reproductive and developmental toxicity

Information on potential effect of TFA on development is available from public literature and from GLP studies published on the ECHA website. a) In a 1996, a peer-review publication (M-765251-01-1) presents the results of a comparative study in hepatic and renal effects of the human anesthetic halothane and its main metabolite TFA were investigated.

Halothane or its oxidative metabolite trifluoroacetic acid (TFAA) were given to Sprague-Dawley rats on gestational days 10–20. Halothane 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 examined on postnatal days 3, 12 or 49 for hepatic and renal biochemistry and/or function through measurements of several serum and urinary parameters.

Neither halothane nor TFAA treatments had statistically significant effect on litter size, neonatal survival or postnatal growth.

Both prenatal 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 urinary excretion of b2-microglobulin. However, these hepatic and renal 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 neonatal 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 trifluoroacetic 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 (embryo-fetal).

Therefore, based on the available toxicological data on TFA there are no concerns for developmental toxicity studies in the rat.

However, The European Chemicals Agency (ECHA) had requested an extended 1-Generation study (EOGRTS) and a developmental toxicity study in the rabbits under the regulation of Registration, Evaluation, Authorization and Restriction of Chemicals (REACH)2. 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 phase 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.

The TFA toxicological database has been evaluated by EFSA in 2016, and the current acceptable daily intake (ADI) is 0.05 mg/kg bw/day based on the 90-day rat study (uncertainty 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; AE C656948-Pyridyl-Carboxylic-Acid (AE C657188) and AE C656948-Methyl Sulfoxide (AE 1344122) of fluopyram 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 407. bOTH

a. Toxicity studies on AE C656948-Pyridyl-Carboxylic-Acid (AE C657188)

AE C656948-Pyridyl-Carboxylic-Acid (AE C657188) was identified in plant metabolism studies (grapes, potatoes, beans, 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. 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 in the original fluopyram 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, it is also included in this dossier for AIR. These studies demonstrated that AE C657188 was not genotoxic and not toxic after an acute or subacute oral administration.

Data Point:	KCA 5.8.1/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	Rat acute oral toxicity AE C657188 (plant metabolite of AE C638206) Code: AE C657188 00 1B99 0002
Report No:	C008168
Document No:	Report includes Trial Nos.: TOX20044 M-197257-01-1
Guideline(s) followed in study:	EU (=EEC): 96/54/EECB1 tris; OECD: 423
Deviations from current test guideline:	current guideline: Current Guideline: OECD 423, 2001 No deviations
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an acute oral toxicity study, in a preliminary assay, one male and one female rat dosed at 4000 mg/kg bw died following treatment. Dose levels for the main study were based on these findings.

In the main study, two groups of three fasted, young adult Sprague Dawley rats (Hsd:Sprague-Dawley(CD))/sex were given a single oral dose of AE C657188 (batch number RAW 244045/1, 99.7% purity) in 1% aqueous methylcellulose at 500 or 2000 mg/kg bw and were observed for 14 days.

Both dose levels were tolerated without mortalities, minimal clinical signs and effects on weight gain, and no gross pathological findings.

The acute lethal oral dose of AE C657188 in rats was greater than 2000 mg/kg. Therefore, no labeling is required according to Commission Directive 93/21/EEC.

I. Materials and methods

A. Materials

1. **Test material:** AE C657188
   Description Off-white crystalline solid
   Lot / Batch #: RAW 244045/1
   Purity: 99.7 %
   CAS # Not reported
   Stability of test compound: Stable for the duration of the dosing phase
2. **Vehicle and / or positive control:** 1% aqueous methylcellulose
3. **Test animals:**
   Species: Rat
   Strain: Hsd:Sprague-Dawley(CD)
   Age: 8 - 11 weeks approximately
   Sex: Male/Female
   Weight at dosing: 202 to 232 g (main study)
   Source: [redacted]
   Acclimation period: At least 6 days (main study)
   Diet: Special Diet Services RM1 (E) SQC expanded pellets, *ad libitum*
   Water: Tap water, *ad libitum*

Housing:	Animals were housed in groups of the same sex within treatment groups, in metal stainless steel cages with grided floors (main study)
Environmental conditions:	
Temperature:	22 ± 3°C
Humidity:	30 - 70%
Air changes:	Not reported
Photoperiod:	Alternating 12-hour light and dark cycles

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 weight 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 female rats. The animals were assigned to their groups without conscious bias. Following overnight fasting, each group received a single dose of 500 or 2000 mg/kg of AE C657188 (99.7% purity) by gavage. The test substance was administered in 1% w/v aqueous methylcellulose at a volume of 20 mL/kg bw at 4000 and 500 mg/kg and 10 mL/kg bw at 2000 mg/kg. Clinical 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 15. On day 15 surviving animals were sacrificed and all animals were necropsied and examined for gross pathological changes.

3. Statistics

The data did not warrant statistical analysis.

II. Results and discussion

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 bw.

Details are provided in Table 5.8.1-1.

Table 5.8.1-1 Doses, mortality / animals treated

Dose (mg/kg bw)	Males
4000	1/1
500	0/3
2000	0/3
Dose (mg/kg bw)	**Females**
4000	1/1
500	0/3
2000	0/3

B. Clinical observations

In the preliminary study, a range of clinical signs was observed in both animals.

In the main 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 a range 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.

Assessment and conclusion by applicant:

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

The acute lethal oral dose of AE C657188 in rats was greater than 2000 mg/kg..

Data Point:	KCA 5.8.1/02
Report Author:	[redacted]
Report Year:	2000
Report Title:	Bacterial mutation assay AE C657188 (plant metabolite of AE C638206) Code: AE C657188 00 1B99 0002
Report No:	C008169
Document No:	Report includes Trial Nos.: Tox20045 M-197258-01-1
Guideline(s) followed in study:	EU (=EEC): 92/69/EEC B.13; B.14; IMAF: 4200; OECD: 471; USEPA (=EPA): OPPTS 870.5100
Deviations from current test guideline:	current guideline: OECD 471, 2020 Deviation: none
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In this *in vitro* assessment of the mutagenic potential of AE C657188 (Batch-No.: R001739, 99.7% purity), histidine dependent auxotrophic mutants of Salmonella typhimurium, strains TA1535, TA1537, TA98 and TA100, and tryptophan dependent mutant of Escherichia coli, strain WP2*uvr*A/pKM101 were exposed to AE C657188 up to 5000 µg/plate, diluted in dimethyl sulfoxide (DMSO). For each bacterial strain and dose level, triplicate plates were used in both the presence and absence of an Aroclor 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 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 well as in the pre-incubation modification of the Bacterial Mutation Assay.

I. Materials and methods

A. Materials

1. Test material:	AE C657188
Description	Off-white crystalline solid
Lot / Batch #:	R001739
Purity:	99.7 %
CAS #	Not reported
Stability of test compound:	Stable for the duration of the study
2. Control materials:	**Negative**: Culture medium **Solvent**: DMSO **Positive**: In the absence of S9 mix: Sodium azide (Sigma Chemical) for TA1535 and TA100 at 0.5 µg/plate, 9-Aminoacridine (Sigma Chemical) for TA1537 at 30 µg/plate, 2-Nitrofluorene (Aldrich Chemical Company) for TA98 at 1 µg/plate, 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) for WP2*uvr*A/pKM101 (CM891) at 0.05 g/plate. In the presence of S9 mix: 2-Aminoanthracene (Aldrich Chemical Company) for TA1535 at 2 µg/plate and WP2*uvr*A/pKM101 (CM891) at 10 µg/plate, Benzo[a]pyrene (Aldrich Chemical Company) for TA1537, TA98 and TA100 at 5 µg/plate.
3. Test organisms:	
Species:	Salmonella typhimurium and Escherichia coli
Strain:	Histidine-dependent auxotrophic mutants of Salmonella typhimurium:TA1535, TA100, TA1537 & TA98 Tryptophan dependent mutants of Escherichia coli: WP2*uvr*A/pKM101
Source:	Strains of S. typhimurium were obtained from Prof. Bruce Ames, University of California, USA. The strain of E. coli was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.
4. Test compound concentrations:	
Plate incorporation assay:	For all strains with or without S9 mix: 0, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate
Pre-incubation assay:	For all strains with or without S9 mix: 0, 50, 150, 500, 1500 and 5000 µg/plate
5. Metabolic activation:	The S9 fraction was isolated from the livers of Aroclor 1254 induced rats.

B. 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 chosen solvent, which provided a final concentration of 5000 µg/plate. This is the standard limit concentration recommended in the regulatory guidelines. The negative control was the chosen solvent, dimethyl 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 sodium phosphate buffer (pH 7.4) were placed in glass bottles. An aliquot of 100 µl of the test solution was added, followed immediately by 2 ml of molten agar containing 0.05 mM histine/biotin/tryptophan. The mixture 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* 72 hours. After this period the appearance of the background bacterial lawn was examined and revertant colonies counted using a Seescan automated colony counter.

Any toxic effects of the test substance would be detected by a substantial reduction 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 was obtained in the first test, a variation to the test procedure was used for the second. The variation used was the pre-incubation assay in which the bottles, which contained mixtures of bacteria, buffer or S9 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 again 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 non-toxic.

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 mutagenic 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 mutagenic 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.

II. Results and discussion

No signs of toxicity were observed towards the tester strains in either mutation test. No precipitation 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 activity of the liver preparations.

Results are presented in the following tables:

Table 5.8.1-2 Revertant colony counts obtained per plate using *S. typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537 and *E. coli* strain WP2*uvr*A/pKM101 – experiment 1.

Treatment	Concentration (µg/plate)	Metabolic activation +/- S9	Mean revertant colony counts in strains				
			TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/pKM101
AE C657188	5000	-	23	107	15	7	200
	1500	-	25	121	17	9	184
	500	-	25	97	13	11	197
	150	-	22	98	16	11	184
	50	-	27	99	13	11	200
	15	-	26	113	13	10	198
	5	-	27	114	13	11	187
Solvent control	0	-	21	105	11	11	197
AE C657188	5000	+	26	120	13	8	182
	1500	+	26	111	14	12	192
	500	+	25	112	16	12	179
	150	+	24	123	16	11	200
	50	+	27	108	14	10	185
	15	+	23	105	13	11	203
	5	+	27	112	16	9	202
Solvent control	0	+	26	107	15	13	192
Sodium azide	0.5	-	NA	558	197	NA	NA
AF-2	0.05	-	NA	NA	NA	NA	2134
9-Aminoacridine	30	-	NA	NA	NA	117	NA
2-nitrofluorene	1	-	400	NA	NA	NA	NA
Benzo[a]pyrene	5	+	257	804	NA	72	NA
2-aminoanthracene	2 – 10*	+	NA	NA	133	NA	1866

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide
NA : not applicable
*: 2 µg/plate for TA 1535, and 10 µg/plate for WP2*uvr*A

Table 5.8.1-3 **Revertant colony counts obtained per plate using *S. typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537 and E. coli strain WP2*uvr*A/pKM101 – Experiment 2.**

Treatment	Concentration (µg/plate)	Metabolic activation +/- S9	Mean revertant colony counts in strains				
			TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/pKM101
AE C657188	5000	-	22	105	14	8	183
	1500	-	23	98	10	8	225
	500	-	23	113	14	11	213
	150	-	21	98	12	12	201
	50	-	24	111	8	8	197
Solvent control	0	-	24	111	14	9	204
AE C657188	5000	+	23	94	13	7	198
	1500	+	28	104	11	12	213
	500	+	24	97	15	13	218
	150	+	23	94	16	13	218
	50	+	26	115	12	10	208
Solvent control	0	+	28	98	15	12	196
Sodium azide	0.5	-	NA	502	168	NA	NA
AF-2	0.05	-	NA	NA	NA	NA	2295
9-Aminoacridine	30	-	NA	NA	NA	110	NA
2-nitrofluorene	1	+	166	NA	NA	NA	NA
Benzo[a]pyrene	5	+	250	630	NA	85	NA
2-aminoanthracene	2 – 10*	+	NA	NA	140	NA	1939

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide
NA : not applicable
*: 2 µg/plate for TA 1535 and 10 µg/plate for WP2uvrA

III. Conclusions

It is concluded that AE C657188 showed no evidence of mutagenic activity in this *in vitro* bacterial system.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013.
It is concluded that AE C657188 showed no evidence of mutagenic activity in this *in vitro* bacterial system.

Data Point:	KCA 5.8.1/03
Report Author:	[redacted]
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 M-234744-01-1
Guideline(s) followed in study:	ICH: S 2 A; OECD: 473 (1997)
Deviations from current test guideline:	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.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In this *in vitro* assessment of the clastogenic potential of AE C657188 (batch OP2150091, purity 99.1%), the test compound was tested in an *in vitro* cytogenetics assay using duplicate 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 sulfoxide (DMSO) and the highest dose level used, 2256 µg/mL, was equivalent to 10 mM.

Treatment of cultures with AE C657188 in 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 (excluding gaps) in all treated cultures fell within historical negative control ranges.

No increases in the frequency of cells with numerical aberrations, 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 chromosome 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) or to its limit of cytotoxicity (following 20+0 hour treatment in the absence of S-9). AE C657188 was therefore considered not to be clastogenic for mammalian cells *in vitro*.

I. Materials and methods

A. Materials

1. **Test material:** AE C657188
   Description White powder
   Lot / Batch #: OP2150091
   Purity: 99.1 %
   CAS # Not reported
   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
Experiment 2:	without S9 mix (20 + 0 h) at 320.9, 377.5, 723.2 µg/mL with S9 mix (3 + 17 h) at 1001, 1385, 2256 µg/mL

B. Study design and methods

1. In life dates: 10 March - 29 April 2003

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 chromosome aberrations) in cells at their first post-treatment mitosis.

AE C657188 (batch OP2150091, purity 99.1%) was tested in an *in vitro* cytogenetics assay using duplicate 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 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 harvest (3+17). The S-9 used was prepared from a rat 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 AE C657188 on mitotic index. Chromosome aberrations were analyzed at three dose levels. The highest concentration chosen for analysis, 2256 µg/mL, induced approximately 34% and 37% mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9 respectively.

Table 5.8.1-4 Experiment 1 Treatment details

S-9	Treatment + recovery (hours)	Vehicle control	Concentration (µg/mL) AE C657188	Positive control
-	3+17	0[a]	739.2, 1444, 2256	NQO, 5.00 µg/mL
+	3+17	0[a]	378.5, 924.1, 2256	CPA, 6.25 µg/mL

[a] : Vehicle control was DMSO only; NQO: 4-Nitroquinoline 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 Experiment 2 Treatment details

S-9	Treatment + recovery (hours)	Vehicle control	Concentration (µg/mL) AE C657188	Positive control
-	20+0	0[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 compounds induced statistically significant increases in the proportion of cells with structural aberrations.

Treatment of cultures with AE C657188 in 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 (excluding gaps) in all treated cultures fell within historical negative control ranges.

No increases in the frequency of cells with numerical aberrations, which exceeded the historical negative control range, were observed in cultures treated with AE C657188 in the absence and presence of S-9.

Table 5.8.1-6: Mean mitotic indices and number of aberrant human lymphocytes, including and excluding gaps – Experiment 1

Treatment	Concentration (µg/mL)	Metabolic activation +/- S9	Treatment time (h)	Mitotix index	Aberrant cells Including gaps	Aberrant cells Excluding gaps
AE C657188	739.2	-	3	9.5	0	0
	1444	-	3	8.5	3	1
	2256	-	3	7.5	2	1
Solvent control	0	-	3	11.4	2	2
AE C657188	378.5	+	3	12.4	1	1
	924.1	+	3	11.1	0	0
	2256	+	3	9.3	3	2
Solvent control	0	+	3	14.8	2	1
NQO	5	-	3	-	27	19***
CPA	6.25	+	3	-	61	45***

*** p< 0.001 statistically significantly different from controls Fisher's test
NQO : 4-Nitroquinoline 1-oxide
CPA : Cyclophosphamide

Table 5.8.1-7 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 Including gaps	Aberrant cells Excluding gaps
AE C657188	320.9	-	20	7.6	0	0
	377.5	-	20	4.8	2	1
	723.2	-	20	4.0	2	1
Solvent control	0	-	20	7.5	1	1
AE C657188	1001	+	3	12.4	3	0
	1385	+	3	9.4	3	3
	2256	+	3	8.5	3	2
Solvent control	0	+	3	10.4	0	0
NQO	2.5	-	20	-	36	34***
CPA	3.125	+	3	-	41	36***

*** p< 0.001 statistically significantly different from controls Fisher's test
NQO : 4-Nitroquinoline 1-oxide
CPA : Cyclophosphamide

III. Conclusions

AE C657188 did not induce chromosome 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) or to its limit of cytotoxicity (following 20+0 hour treatment in the absence of S-9). AE C657188 was therefore considered not to be clastogenic for mammalian cells *in vitro*.

Assessment and conclusion by applicant:

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:	[redacted]
Report Year:	2019
Report Title:	AE C657188: Micronucleus test in human lymphocytes In vitro
Report No:	1969602
Document No:	M-673693-01-1
Guideline(s) followed in study:	OECD Test Guideline No. 487 (July 2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

AE C657188 was evaluated for its potential to induce micronuclei in human lymphocytes *in vitro*, in two independent experiments; Experiment I comprised a four hour exposure period in the presence and absence of metabolic activation (provided by S9) whilst experiment II provided a 20-hour exposure period in the absence of S9. Concentrations up to 2000 µg/mL were tested in duplicate and 1000 binucleated cells/culture were examined for cytogenetic damage.

In experiment I, no cytotoxicity or precipitation was observed (+/- 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 or absence of S9 mix. However, in experiment I in the absence of S9, a dose dependency, tested via trend test, was observed ($p = 0.039$). 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 gave the expected statistically significant increase in the number of micronucleated cells.

The test substance AE C657188 can be considered neither clastogenic nor aneugenic under the conditions of this *in vitro* micronucleus test, when tested up to the highest possible concentration, both in the presence and absence of metabolic activation.

I. Materials and methods

A. Materials

1. Test material

Test substance:	AEC657188
Purity:	99.9 % (w/w)
Batch no.:	BCOO 6709-1-1
Expiry date:	16th October 2020

2. Vehicle and/or positive control

Vehicle: DMSO

Positive controls:

-S9
Mitomycin C (MMC), 0.8 µg/mL (98% purity, dissolved in deionized water)
Demecolcine, 100 ng/mL (purity ≥98%, dissolved in deionized water)
+S9
Cyclophosphamide (CPA), 15 µg/ml (purity 97-103%, dissolved in saline)

3. Activation:

Metabolic activation was provided by S9-mix derived from phenobarbital/ß-naphthoflavone induced rat liver homogenate; each batch of prepared S9 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 $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation used for this study was 30.4 mg/mL.

4. Cell cultures and media:

Cells

Blood was drawn from healthy non-smoking donors, not receiving medication. Blood from a female donor (27 years old) and a male donor (22 years old) were used in experiments I and II, 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 peripheral blood lymphocytes.

Media

Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hours following collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1), supplemented with 200 mM GlutaMAX™. The medium was further supplemented with penicillin-streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (foetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).

All incubations were carried out at 37 °C with 5.5 % CO_2 in humidified 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, & 2000 µg/mL, both with and without S9 mix.

In the second experiment a continuous (20 hour) treatment was used in the absence of S9 mix at test concentrations of 0 (solvent control), 142, 249, 435, 762, 1333 & 2000 µg/mL, in the absence of S9 mix.

B. Test Performance

Experimental phase: 14th August 2019 to 17th September 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 √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 20 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 treatment with metabolic activation 50 µL S9 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 "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H2O, 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 10 % 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 item. After 20 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 "saline G". The washing 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 µg/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 S9-mix. In these experiments 19 mL instead of 20 mL culture medium and additionally 1 mL of S9-mix were 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.

Preparation of cells

Following the procedures above (approximately 40 hours from the start of treatment), the cultures were harvested by centrifugation. The cells were spun 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 centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °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 micronucleus assay is deemed 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 $25x10^{-6}$ cells
- The concurrent positive controls should produce a statistically significant increase in the micronucleus frequency and should be within the laboratory historical positive control 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’ 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 cells and concentrations
- The criteria for the selection of top concentrations should be met

Any mutant frequencies not meeting these criteria were excluded from the statistical analyses.

4. Assessment criteria

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative 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
- The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

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
- The increase is concentration-related 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 interval

When all of the criteria are met, 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 neither clearly 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. S9 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 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 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 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.

The results of both experiments, with and without metabolic activation, are summarised in the table below:

Table 5.8.1-8 Summary of results of experiment I and II

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	Historical control data 95% Ctrl limit	Historical control data Min - Max
Exposure period 4 hrs without S9 mix							
I	40 hrs	Solvent control[1]	1.80		0.20	0.01 – 1.20	0.00 – 1.55
		Positive control[2]	1.69	14.1	**11.30[S]**	2.66 – 22.74	3.95 – 28.60
		762	1.79	0.9	0.35		
		1333	1.78	2.6	0.35		
		2000	1.95	n.c	0.50		
Exposure period 20 hrs without S9 mix							
II	40 hrs	Solvent control[1]	1.83		0.30	0.00 – 1.14	0.05 – 1.60
		Positive control[3]	1.43	48.5	**4.20[S]**	1.15 – 6.44	1.95 – 8.80
		762	1.82	0.4	0.25		
		1333	1.75	9.7	0.20		
		2000	1.62	25.6	0.20		
Exposure period 4 hrs with S9 mix							
I	40 hrs	Solvent control[4]	1.72		0.30	0.00 – 1.24	0.10 – 1.30
		Positive control[3]	1.35	50.8	**3.75[S]**	1.01 – 7.34	1.80 – 8.85
		762	1.65	8.7	0.50		
		1333	1.57	21.0	0.40		
		2000	1.62	14.0	0.50		

* For the positive control groups and the test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
S The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c Not calculated as the CBPI is equal to or higher than the solvent control value
1 DMSO 0.5 % (v/v)
2 MMC 0.8 µg/mL
3 Demecolcine 100 ng/mL
4 CPA 15 µg/mL

In both independent experiments, no relevant increases in the numbers of micronucleated cells were observed following treatment with the test item either in the presence or absence of S9 mix. However, in experiment 1 in the absence of S9, a dose dependency tested via trend test was observed (p=0.039). Since none of the values exceeded the historical control data or were statistically significantly increased, this finding is considered to be biologically irrelevant.

Demecolcine (100 ng/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 of this assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 487 and is valid and acceptable to assess the clastogenicity of AE C657188 *in vitro*. AE C657188 is neither clastogenic nor aneugenic under the conditions of this study.

Data Point:	KCA 5.8.1/05
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C657188: V79/HPRT-test *in vitro* for the detection of induced forward mutations
Report No:	C034731
Document No:	Report includes Trial Nos.: A100551 M-237459-01-1
Guideline(s) followed in study:	EU (=EEC): 2000/32/EC; OECD: 476; USEPA (=EPA): OPPTS 870.5300
Deviations from current test guideline:	Current guideline: OECD 476, 2016 Deviation: None.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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-guanine phosphoribosyl transferase (HPRT) locus in V79 cells.

AE C657188 was tested at concentrations up to 5000 µg/mL with or without metabolic activation. Without and with S9 mix, AE C657188 induced no decreases in survival to treatment or in relative population growth. However, AE C657188 was tested up to its limit of solubility under culture conditions. Precipitation occurred in the culture medium at 4000 µg/mL and above, so that at 5000 µg/mL no further evaluation was possible. 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 mix.

Based on these results, AE C657188 was considered to be non-mutagenic in the V79/HPRT forward mutation assay, both with and without metabolic activation.

I. Materials and methods

A. Materials

1. Test material:	AE C657188
Description	Light grey powder
Lot / Batch #:	D0526
Purity:	97.7 to 99.1%
CAS #	80194-68-9
Stability of test compound:	Stable for the duration of the study
2. Control materials:	**Negative**: Culture medium [Eagle's minimal essential medium supplemented with 1% L-glutamine, 1% MEM-vitamins, 1% MEM NEAA, 1% penicillin/streptomycin and 10% fetal calf serum (FCS)] **Solvent**: DMSO for AE C657188 and Dimethylbenzanthracene not exceeding 1% (v/v) in the culture medium. No solvent needed for ethyl methanesulfonate as it is a liquid. **Positive**: Ethyl methanesulfonate (EMS), a directly alkylating agent, used at 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 S9 mix.
3. Test organisms:	
Cell line:	Chinese hamster V79 lung cells
Source:	Cells obtained from Prof. G. Speit, University of Ulm, Germany. These cells have since been recloned to maintain karyotypic stability. They have a modal chromosome number of 22 and a rapid population doubling time (10 to 14 hours)
Culture condition:	Incubation performed at 37°C in a humidified atmosphere with about 5% CO2.
4. Test compound concentrations:	AE C657188 was used at concentrations ranging from 1 to 5000 µg/mL in the clonal cytotoxicity assay and from 16 to 5000 µg/mL in the mutagenic assays.
5. Metabolic activation:	The S9 fraction was isolated from the livers of Aroclor 1254 induced male Sprague Dawley rats. The preparation dated from February 04, 2003 (protein content 26.4 mg/mL) and was kept frozen at -80°C. The batch was tested for contamination and cytotoxicity prior to use in the first study. Cofactors were freshly dissolved in sodium phosphate buffer (150 mM, pH 7.4)

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-thioguanine (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 flasks 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%). Thereafter, cell monolayers were washed with PBS, trypsinized and replated in 20 mL 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 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 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 Petri dishes (diameter of 100 mm) at 3x105 cells per dish (8 dishes per culture) in 20 mL culture medium 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.

Two trials were performed.

4. Parameters assessed:

The parameter “survival to treatment” in % was determined on the basis of the following calculation:

$$\frac{\text{Mean number of colonies (treated cultures)}\times 100}{\text{Mean number of colonies (vehicle control cultures)}}$$

The “absolute population growth” was calculated 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.

$$\frac{\text{Absolute population growth treated culture}\times 100}{\text{Absolute population growth of corresponding vehicle control culture}}$$

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 %.

$$\frac{\text{Mean number of colonies per dish}\times 100}{200}$$

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.

$$\frac{\text{Total number of mutant colonies} \times 100}{\text{Number of evaluated dishes} \times 3 \times 10^5 \times \text{C.E}}$$

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 10-6 cells.

- The mutant frequency of the two cultures of the vehicle 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 frequency of at least three times 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 range 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 criteria:

- Mutant frequencies were only used for assessment, if 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.

- 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 Dunnett 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 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 S9 mix was no longer interpretable at 5000 µg/mL. With S9 mix cells were exposed to concentrations of up to and including 5000 µg/mL. Substance precipitation occurred in the medium at the concentration of 5000 µg/mL. Therefore, the test with S9 mix was no longer interpretable at 5000 µg/mL.

Good cloning conditions were demonstrated by the absolute cloning efficiency for the vehicle controls ranging from 59.5% to 142.3% and from 54.0% to 87.3% without and with metabolic 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 after treatment with AE C657188 at any concentration (up to the highest dose of 5000 µg/mL) either with or without metabolic activation.

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
	1600	164.9	1.70
	500	158.5	2.25
	160	139.8	0.60
	50	96.6	1.75
	16	137.2	1.10
Negative control	0	77.9	0.85
Solvent control	0	100.0	0.65
EMS	900	43.9	601.25
AE C657188	4000	122.5	1.90
	2000	130.4	2.50
	1000	74.6	2.40
	500	91.4	1.80
	160	88.3	2.35
	50	79.3	1.35
	16	86.2	1.30
Negative control	0	96.2	2.05
Solvent control	0	100.0	1.15
EMS	900	14.8	329.2

ND : not determined
EMS : ethylmethanesulfonate

Table 5.8.1-10: Relative survival and mean mutation frequency (mutant colonies per 1 millions cells) –Experiment 2-with S9 mix

Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency
AE C657188	5000	1.0	ND
	1600	107.2	0.60
	500	103.1	0.70
	160	99.1	1.80
	50	84.65	1.85
	16	83.5	1.25
Negative control	0	103.4	1.40
Solvent control	0	100.0	0.90
DMBA	20	63.3	80.75
AE C657188	4000	128.85	1.15
	2000	129.65	1.65
	1000	95.1	1.15
	500	90.3	2.65
	160	93.5	2.15
	50	112.3	2.25
	16	96.35	3.35

Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency
Negative control	0	115.6	0.70
Solvent control	0	100.0	3.40
DMBA	20	47.6	71.00

ND : not determined
DMBA : dimethylbenzanthracene

III. Conclusions

AE C657188 was considered to be non mutagenic in this V79/HPRT forward mutation assay.

Assessment and conclusion by applicant:

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

AE C657188 was considered to be non mutagenic in this V79/HPRT forward mutation assay.

Data Point:	KCA 5.8.1/06
Report Author:	[redacted]
Report Year:	2001
Report Title:	AE C657188 (PCA) Preliminary 28-day toxicity study in the rat by dietary administration Version 2
Report No:	C034882
Document No:	M-204933-03-1
Guideline(s) followed in study:	Preliminary study based on OECD 407 (1995)
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
Acceptability/Reliability:	Yes

Executive Summary

AE C657188 (batch number D 0526, 99.1 % purity) was administered continuously via the diet to groups of Sprague-Dawley rats (3/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 signs. Body weight and food consumption were recorded once weekly. During the acclimatization phase all animals 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 findings occurred at the high dose of 20000 ppm and consisted of a slight decrease in food consumption in females of 9% on weeks 2 and 4, and 15% ($p \leq 0.05$) on week 3, and a slight decrease in inorganic phosphorus concentration of 12% ($p \leq 0.01$) 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

A. Materials

1. Test material:	AE C657188
Description	A white powder
Lot / Batch #:	D 0526
Purity:	99.1%
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:	
Species:	Rat
Strain:	Sprague Dawley (rl: CD(SD)IGS Br
Age:	6 to 7 weeks approximately
Weight at dosing:	220 to 243 g for the males – 161 to 198 g for the females
Source:	[redacted]
Acclimation period:	6 days
Diet:	Certified rodent powdered and irradiated diet A04C-10-P1 from U.A.R.. (Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge, France), *ad libitum*
Water:	Municipal tap water, ad libitum
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages
Environmental conditions:	
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: 14 June - 11 July 2001

2. Animal assignment and treatment

There were 5 animals of each sex per dose group. Animals were assigned to dose groups randomly by body weight. AE C657188 was administered in the diet for 28 days to Sprague Dawley rats at the following doses – 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 "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 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.

Table 5.8.1-11 Study design

Test group	Concentration in diet (ppm)	Dose per animal (study averages)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	5	5
2	20	1.5	1.63	5	5
3	200	15.0	15.9	5	5
4	2000	149	162	5	5
5	20000	1574	1581	5	5

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% levels 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 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.

An ophthalmic examination was performed on all animals during the acclimatization phase and on control and high dose group animals at the end of Week 3. After instillation 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 acclimatization phase, on 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

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. 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 inhalation of Isoflurane. Blood was collected on EDTA for hematology, on lithium heparin for plasma and clot activator for serum for clinical chemistry and on sodium citrate for coagulation parameters.

The following hematology parameters were assayed using a Technicon H1 (Bayer Diagnostics, Puteaux, France): red blood cell count, 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 Hitachi 911 (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 Clinitek 200+ and Ames Multistix dipsticks (Bayer Diagnostics, Puteaux, France). Urinary refractive index was measured using an Atago clinical refractometer (Bioblock Scientific, Illkirch, France). Glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen were assayed using a Clinitek 200+ and Ames Multistix 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 necropsy was performed on all surviving animals. Animals were deeply anesthetized by Isoflurane inhalation, then exsanguinated 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 examined microscopically. Adrenal gland, brain, kidney, liver, ovary, spleen, testis, thyroid gland (with parathyroid gland) 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 marrow (sternum), brain, epididymis, oesophagus, exorbital (lachrymal) gland, eye and optic nerve, Harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, 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, 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 adrenal gland, brain, epididymis, heart, kidney, liver, lung, ovary, pituitary gland, prostate, spleen, seminal vesicles, testis, thymus, thyroid gland, parathyroid gland, uterus and vagina from all 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 mortality

1. Clinical signs of toxicity

There were no treatment-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 level in either sex.

C. Food consumption and compound intake

At 20000 ppm in females, food intake was reduced by 9% on weeks 2 and 4, but the effect was not statistically significant, and by 15% ($p \leq 0.05$) on week 3 compared to control values. There was no impact on food consumption in males at any dose level or in females at 2000, 200 or 20 ppm.

Compound intake details are presented in Table 5.8-10.

D. Hematology, clinical chemistry, and urinalysis

1. Hematology

Hematological examination showed no treatment-related findings.

2. Clinical Chemistry

At 20000 ppm in males, clinical chemistry changes consisted of a 12% ($p \leq 0.01$) reduction in inorganic phosphorus concentration, when compared to controls.

No relevant changes were observed at 2000, 200 or 20 ppm in males or at any dose level in females.

3. Urinalysis

Urinalysis revealed no treatment-related findings.

E. Sacrifice and pathology

There were no changes in terminal body weights or organ weights at any dose in either sex.

There were no treatment-related macroscopic at any dose in either sex or microscopic changes at 20000 ppm in either sex.

F. Deficiencies

No specific deficiencies were noted in this study.

III. Conclusions

The NOAEL was considered to be 20000 ppm (equivalent to 1574 and 1581 mg/kg/day in males and females, respectively).

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).

b. Toxicity studies on AE C656948-Methyl Sulfoxide (AE 1344122)

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 in 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 fluopicolide to upgrade the genotoxicity package, this study has been included in the fluopyram dossier for ADR. 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:	[redacted]
Report Year:	2003
Report Title:	Acute toxicity in the rat after oral admnisitration AE 1344122 Project AE C638206
Report No:	C034663
Document No:	Report includes Trial Nos.: AT00486 M-235328-01-1
Guideline(s) followed in study:	EU (=EEC): 67/548/EEC , 27.06.1967; OECD: No. 423, 17.12.2001; USEPA (=EPA): OPPTS 870.1100, August 1998
Deviations from current test guideline:	current guideline: Current Guideline: OECD 423, 2001 No deviations
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an acute oral toxicity study, two groups of three fasted, young adult Wistar female rats (HsdCpb:Wu) were given a single oral dose of AE 1344122 (batch number YG3228 , 98.8% purity) in demineralized water with the aid of 2% Cremophor EL at 2000 mg/kg bw and were observed for 14 days.

This dose level was tolerated without mortalities, clinical signs and effects on weight gain, and no gross pathological findings.

According to OECD guideline 423, the LD50 cut off of AE 1344122 is higher than 5000 mg/kg (category 5/unclassified in the Globally Harmonized Classification System).

I. Materials and methods

A. Materials

1. Test material:	AE 1344122
Description	White powder
Lot/ Batch #:	YG3228
Purity:	98.8 %
CAS #	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
Strain:	HsdCpb:Wu
Age:	9 to 10 weeks approximately
Weight at dosing:	148 to 166 g (main study)
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Provimi Kliba 3883.0.15 Maus/Ratte Haltung, Kaiseraugst, Switzerland, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were group housed conventionally in polycarbonate cages
Environmental conditions:	
Temperature:	20 - 24°C
Humidity:	50 - 60%
Air changes:	Approximately 10 changes per hour
Photoperiod:	Alternating 12-hour light and dark cycles

B. Study design and methods

1. In life dates: 04 April - 24 April 2003

2. Animal assignment and treatment

The test substance was tested using a stepwise procedure, each step using three animals of the same sex (females). The test substance was initially at 2000 mg/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, each group received a 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 10 mL/kg bw. Clinical 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 15. On day 15, surviving animals were sacrificed and all animals were necropsied and examined for gross pathological changes.

3. Statistics

The data did not warrant statistical analysis.

II. Results and discussion

A. Mortality

In the main study, no mortalities occurred at 500 or 2000 mg/kg bw.

Details are provided in Table 5.8.1-12.

Table 5.8.1-12 Doses, mortality / animals treated

Dose (mg/kg bw)	Females
2000 (1st step)	0/3
2000 (2nd step)	0/3

B. Clinical observations

No clinical 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 1344122 is higher than 5000 mg/kg.

Assessment and conclusion by applicant:

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

According to OECD guideline 423, the LD50 cut off of AE 1344122 is higher than 5000 mg/kg.

Data Point:	KCA 5.8.1/08
Report Author:	[redacted]
Report Year:	2003
Report Title:	Salmonella/microsome test - Plate incorporation and preincubation method Code: AE 1344122
Report No:	C035150
Document No:	Report includes Trial Nos: AT00599 M-218257-01-1
Guideline(s) followed in study:	EU (=EEC): 2000/32/EC B.13/14; OECD: 471; USEPA (=EPA): OPPTS 870.5100
Deviations from current test guideline:	Current guideline: OECD 471, 1997 Deviation: none
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In this *in vitro* assessment of the mutagenic potential of AE 1344122 (Batch-No.: YG3228, 98.8% purity), histidine dependent auxotrophic mutants of Salmonella typhimurium, strains TA 1535, TA 1537, TA 98, TA 100 and TA 102 were exposed to AE 1344122 up to 5000 µg/plate, diluted in dimethyl sulfoxide (DMSO). For each bacterial strain and dose level, triplicate plates were used in both the presence and absence of an Aroclor 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°C, the numbers of revertant 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 µg/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 µg/plate for assessment purposes.

AE 1344122 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; [redacted].; 2009; M-344996-01-1 (Check of S9 metabolizing capacity S9 fraction, batch of February 4, 2003).

Therefore, AE 1344122 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.

I. Materials and methods

A. Materials

1. Test material:	AE 1344122
Description	Fine white powder
Lot / Batch #:	YG3228
Purity:	98.8 %
CAS #	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
Positive:	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 µg/plate only in plate 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 S9 mix in all strains at 3 µg/plate.
3. Test organisms:	
Species	Salmonella typhimurium LT2 mutants
Strain:	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102
Source	Strains obtained from Prof. Bruce Ames in 1997 and stored in the laboratory since then
4. Metabolic activation:	The S9 fraction was isolated from the livers of Aroclor 1254 induced male Sprague Dawley rats.
5. Test concentrations:	
Preliminary cytotoxicity assay (+/-S9) and plate incorporation:	For all strains with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 µg/plate
Mutation assay – pre-incubation:	For all strains with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 µg/plate

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 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.

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 minutes. 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, TA 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

There was no indication of a bacteriotoxic effect of AE 1344122 at any dose up to and including 158 µg/plate. Higher 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 µg/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 demonstrating the sensitivity of the system.

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	Concentration (µg/plate)	Metabolic activation +/- S9	Mean revertant colony counts in strains				
			TA 98	TA 100	TA 1535	TA 1537	TA 102
AE 1344122	5000	-	20	152	16	6	234
	1581	-	22	173	16	6	204
	500	-	16	165	16	8	224
	158	-	18	163	20	8	199
	50	-	17	159	15	7	191
	16	-	13	158	13	6	194
Solvent control	0	-	22	165	18	7	205
AE 1344122	5000	+	23	145	11	9	262
	1581	+	26	197	9	9	297
	500	+	35	198	7	7	244
	158	+	30	214	10	10	239
	50	+	29	183	14	7	274
	16	+	26	194	12	9	260
Solvent control	0	+	31	209	10	9	264
Sodium azide	10	-	NA	NA	755	NA	NA
4-NPDA	0.5 – 10*	-	172	NA	NA	109	NA
2-nitrofluorene	0.2	-	NA	387	NA	NA	NA
MMC	0.2	-	NA	NA	NA	NA	592
2-aminoanthracene	3	+	1272	1375	185	354	694

* 4-NPDA : 4-Nitro-1,2-phenylene diamine - 0.5 µg/plate for TA 98 and 10 µg/plate for TA 1537
MMC : Mitomycin C

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.**

Treatment	Concentration (µg/plate)	Metabolic activation +/- S9	Mean revertant colony counts in strains				
			TA 98	TA 100	TA 1535	TA 1537	TA 102
AE 1344122	5000	-	5	70	6	7	107
	1581	-	23	143	18	10	262
	500	-	25	136	20	7	279
	158	-	27	131	19	6	270
	50	-	22	133	19	10	286
	16	-	28	141	18	8	292
Solvent control	0	-	22	140	15	7	268
AE 1344122	5000	+	14	106	9	6	113
	1581	+	39	150	13	11	303
	500	+	30	155	16	9	309
	158	+	37	162	16	9	301
	50	+	45	158	14	10	314
	16	+	50	162	12	14	282
Solvent control	0	+	44	168	13	11	304
Sodium azide	10	-	NA	NA	735	NA	NA
4-NPDA	0.5 - 10*	-	171	NA	NA	129	NA
Nitrofurantoin	0.2	-	NA	433	NA	NA	NA
Cumene hydroperoxide	50	-	NA	NA	NA	NA	493
2-aminoanthracene	3	+	1171	1707	227	570	641

* 4-NPDA : 4-Nitro-1,2-phenylene diamine - 0.5 µg/plate for TA 98 and 10 µg/plate for TA 1537

A. Deficiencies

No deficiencies were identified.

III. Conclusions

It is concluded that, AE 1344122 showed no evidence of mutagenic activity in this *in vitro* bacterial system.

Assessment and conclusion by applicant:

It is concluded that, AE 1344122 showed no evidence of mutagenic activity in this *in vitro* bacterial system.

Data Point:	KCA 5.8.1/09
Report Author:	[redacted]
Report Year:	2003
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 M-234746-01-1
Guideline(s) followed in study:	ICH: S 2 A; OECD: 473 (1997)
Deviations from current test guideline:	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.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In this *in vitro* assessment of the clastogenic potential of AE 1344122 (batch YG3228, purity 98.8%), the test compound was tested in an *in vitro* cytogenetics assay using duplicate 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 sulfoxide (DMSO) and the highest dose level used, 2532 µg/mL, was equivalent to 10 mM.

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 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 µg/mL). At 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 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) or to its limit of cytotoxicity (following 20+0 hour treatment in the absence of S-9). AE 1344122 was therefore considered not to be clastogenic for mammalian cells *in vitro*.

I. Materials and methods

A. Materials

1. **Test material:** AE 1344122
   Description: Fine white powder
   Lot / Batch #: YG3228
   Purity: 98.8 %
   CAS #: 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
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 concentrations:**

Experiment 1:	without S9 mix (3 + 17 h) at 1296, 2026, 2532 µg/mL with S9 mix (3 + 17 h) at 1620, 2026, 2532 µg/mL
Experiment 2:	without S9 mix (20 + 0 h) at 306.1, 498.5, 1123 µg/mL with S9 mix (3 + 17 h) at 1829, 2152, 2532 µg/mL

B. Test performance

1. In life dates: 05 March 2003 - 01 May 2003

The experimental phase of the study was performed from March 5th to May 1st, 2003 at Covance Laboratories, Harrogate, North Yorks, UK.

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 chromosome aberrations) in cells at their first post-treatment mitosis.

AE 1344122 (batch YG3228, purity 98.8 %) was tested in an *in vitro* cytogenetics assay using duplicate 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 sulfoxide (DMSO) and the highest dose level used, 2532 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 harvest (3+17). The S-9 used was prepared from a rat 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 AE 1344122 on mitotic index. Chromosome aberrations were analyzed at three dose levels (see Table 5.8.1-15). The highest concentration chosen for analysis, 2532 µg/mL, induced approximately 27% and 20% mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9 respectively.

Table 5.8-15 Experiment 1 Treatment details

S-9	Treatment + recovery (hours)	Vehicle control	Concentration (µg/mL) AE 1344122 (metabolite of AE C638206)	Positive control	
-	3+17	0[a]	1296, 2026, 2532	NQO,	5.00 µg/mL
+	3+17	0[a]	1620, 2026, 2532	CPA,	6.25 µg/mL

[a] : Vehicle control was DMSO only; NQO: 4-Nitroquinoline 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 (see Table 5.8.-16) and the highest concentrations chosen for analysis, 1123 and 2532 µg/mL, induced approximately 48% and 44% mitotic inhibition in the absence and presence of S-9, respectively.

Table 5.8.1-16 Experiment 2 Treatment details

S-9	Treatment + recovery (hours)	Vehicle control	Concentration (µg/mL) AE 1344122 (metabolite of AE C638206)	Positive control
-	20+0	0[a]	306.1, 498.5, 1123	NQO, 5.00 µg/mL
+	3+17	0[a]	1829, 2152, 2532	CPA, 6.25 µg/mL

[a] : Vehicle control was DMSO only; 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 were sampled in each experiment, 20 hours after the start of treatment; both compounds induced statistically significant increases in the proportion of cells with structural 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 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 µg/ml). At 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.

Table 5.8.1-17 Mean mitotic indices and number of aberrant human lymphocytes, including and excluding gaps – Experiment 1

Treatment	Concentration (µg/mL)	Metabolic activation +/- S9	Treatment time (h)	Mitotic index	Aberrant cells Including gaps	Aberrant cells Excluding gaps
AE 1344122	1296	-	3	11.2	3	2
	2026	-	3	9.4	6	6
	2532	-	3	9.6	3	3
Solvent control	0	-	3	13.1	3	2
AE 1344122	1620	+	3	9.8	0	0
	2026	+	3	8.6	3	1
	2532	+	3	7.8	5	3
Solvent control	0	+	3	9.8	3	3
NQO	5	-	3	-	51	49***
CPA	6.25	+	3	-	66	63***

*** p< 0.001 statistically significantly different from controls Fisher'stest
NQO : 4-Nitroquinoline 1-oxide
CPA : Cyclophosphamide

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 Including gaps	Aberrant cells Excluding gaps
AE 1344122	306.1	-	20	8.0	10	4
	498.5	-	20	5.8	7	4
	1123	-	20	4.6	9	8
Solvent control	0	-	20	8.8	4	2
AE 1344122	1829	+	3	11.7	4	4
	2152	+	3	11.2	3	2
	2532	+	3	6.9	5	2
Solvent control	0	+	3	12.4	4	4
NQO	2.5	-	20	-	45	37***
CPA	3.125	+	3	-	66	58***

*** p< 0.001 statistically significantly different from controls Fisher's test
NQO : 4-Nitroquinoline 1-oxide
CPA : 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 C638206) in the absence and presence of S-9.

A. Deficiencies

No deficiencies were identified.

III. Conclusions

AE 1344122 did not induce chromosome 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) or to its limit of cytotoxicity (following 20+0 hour treatment in the absence of S-9). AE 1344122 was therefore considered not to be clastogenic for mammalian cells *in vitro*.

Assessment and conclusion by applicant:

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

AE 1344122 did not induce chromosome aberrations in cultured human peripheral blood lymphocytes, and was therefore considered not to be clastogenic for mammalian cells *in vitro*.

Data Point:	KCA 5.8.1/10
Report Author:	[redacted]
Report Year:	2019
Report Title:	AE 1344122: Micronucleus test in human lymphocytes In vitro
Report No:	1969603
Document No:	M-673685-01-1
Guideline(s) followed in study:	OECD Test Guideline No. 487 (July 2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

AE 1344122 was evaluated for its potential to induce micronuclei in human lymphocytes *in vitro*, in two independent experiments; Experiment I comprised a four hour exposure period in the presence and absence of metabolic activation (provided by S9) whilst experiment II provided a 20-hour exposure period in the absence of S9. Concentrations up to 2024 µg/mL were tested in duplicate and 1000 binucleated cells/culture were examined for cytogenetic damage.

In experiment I, no cytotoxicity or precipitation was observed (+/- 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 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 or absence of S9 mix in either experiment.

Appropriate reference substances gave the expected statistically significant increase in the number of micronucleated cells.

The test substance can be considered neither clastogenic nor aneugenic under the conditions of this *in vitro* micronucleus test, when tested up to the highest possible concentration, both in the presence and absence of metabolic activation.

I. Materials and methods

A. Materials

1. Test material

Test substance: AE 1344122 in the report
Purity: 98.8 % (w/w)
Batch no.: BCS-BA50602
Expiry date: 18th February 2026

2. Vehicle and/or positive control	Vehicle: DMSO Positive controls: -S9 Mitomycin C (MMC), 0.8 µg/mL (98% purity, dissolved in deionized water) Demecolcine, 75 ng/mL (purity ≥98%, dissolved in deionized water) +S9 Cyclophosphamide (CPA), 17.5 µg/ml (purity 97-103%, dissolved in saline)

3. Activation:

Metabolic activation was provided by S9-mix derived from phenobarbital/ß-naphthoflavone induced rat liver homogenate; each batch of prepared S9 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 $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation used for this study was 30.9 mg/mL.

4. Cell cultures and media:

Cells

Blood was drawn from healthy non-smoking donors, not receiving medication. Blood from a female donor (29 years old) and a male donor (29 years old) were used in experiments I and II, 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 peripheral blood lymphocytes.

Media

Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hours following collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1), supplemented with 200 mM GlutaMAX™. The medium was further supplemented with penicillin/streptomycin (100 I./mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (foetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).

All incubations were carried out at 37 °C with 5.5 % CO_2 in humidified air.

5. Test compound concentrations used:

Test concentrations for the first experiment (4-hour pulse treatment) were 0 (solvent control), 15.3, 26.8, 47, 82.2, 144, 252, 441, 771, 1349 & 2024 ng/mL, both with and without S9 mix.

In the second experiment a continuous (20 hour) treatment was used at test concentrations of 0 (solvent control), 144, 252, 441, 771, 1349 & 2024 µg/mL, in the absence of S9 mix.

B. Test Performance

Experimental phase: 21st August 2019 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 √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 20 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 treatment with metabolic activation 50 µL S9 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 "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H2O, 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 10 % 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 item. After 20 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 "saline G". The washing 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 µg/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 S9-mix. In these experiments 19 mL instead of 20 mL culture medium and additionally 1 mL of S9-mix were 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.

Preparation of cells

Following the procedures above (approximately 40 hours from the start of treatment), the cultures were harvested by centrifugation. The cells were spun 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 centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °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 micronucleus assay is deemed 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 $25x10^{-6}$ cells
- The concurrent positive controls should produce a statistically significant increase in the micronucleus frequency and should be within the laboratory historical positive control 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" 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 cells and concentrations
- The criteria for the selection of top concentrations should be met

Any mutant frequencies not meeting these criteria were excluded from the statistical analyses.

4. Assessment criteria

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative 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
- The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

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
- The increase is concentration-related 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 interval)

When all of the criteria are met, 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 neither clearly 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. S9 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 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 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 pH was observed. Similarly, in experiment II, no precipitation or cytotoxicity was observed up to the highest applied concentration. The highest concentration applied (2024µg/ml) was therefore the limit concentration.

The results of both experiments, with and without metabolic activation, are summarised in the table below:

Table 5.8.1-19 Summary of results of experiment I and II

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	Historical control data	
						95% Ctrl limit	Min - Max
Exposure period 4 hrs without S9 mix							
I	40 hrs	Solvent control[1]	1.73		0.25	0.01 – 1.20	0.00 – 1.55
		Positive control[2]	1.76	n.c	**6.45**[S]	2.66 – 22.74	3.95 – 28.60
		771	1.78	n.c	0.30		
		1349	1.79	n.c	0.45		
		2024	1.79	n.c	0.45		
Exposure period 20 hrs without S9 mix							
II	40 hrs	Solvent control[1]	1.74		0.20	0.00 – 1.14	0.05 – 1.60
		Positive control[3]	1.82	n.c	**2.85**[S]	1.15 – 6.44	1.95 – 8.80
		771	1.65	12.2	0.30		
		1349	1.71	4.8	0.30		
		2024	1.74	0.5	0.20		
Exposure period 4 hrs with S9 mix							
I	40 hrs	Solvent control[1]	1.92		0.30	0.00 – 1.24	0.10 – 1.30
		Positive control[4]	1.77	15.5	**2.85**[S]	1.01 – 7.34	1.80 – 8.85
		771	1.85	0.9	0.40		
		1349	1.84	8.4	0.15		
		2024	1.84	8.8	0.35		

* For the positive control groups and the test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
S The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c Not calculated as the CBPI is equal to or higher than the solvent control value
1 DMSO 0.5 % (v/v)
2 MMC 0.8 µg/mL
3 Demecolcine 75 ng/mL
4 CPA 17.5 µg/mL

In both independent experiments, neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells were observed following treatment with the test item either in the presence or absence of S9 mix.

Demecolcine (100 ng/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 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 this assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 487 and is valid and acceptable to assess the clastogenicity of AE 1344122 *in vitro* AE 1344122 is neither clastogenic nor aneugenic under the conditions of this study.

Data Point:	KCA 5.8.1/11
Report Author:	[redacted]
Report Year:	2003
Report Title:	V79/HPRT-test *in vitro* for the detection of induced forward mutations Code: AE 1344122 (metabolite of AE C628206)
Report No:	C035061
Document No:	Report includes Trial Nos.: AT00591 M-218169-01-1
Guideline(s) followed in study:	EU (=EEC): 2000/32/EC; OECD: 476; USEPA (=EPA): OPPTS 870.5300
Deviations from current test guideline:	Current guideline: OECD 476, 2016 Deviation: None.
Previous evaluation:	Yes, evaluated and accepted in the DAR (2011)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The purpose of the study was to assess the point mutagenic potential of AE 1344122 (batch YG3228, purity 98.8%) at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus in V79 cells.

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, AE 1344122 was tested up to its limit of solubility under culture conditions. 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 mix.

Based on these results, AE 1344122 was considered to be non-mutagenic in the V79/HPRT forward mutation assay, both with and without metabolic activation.

I. Materials and methods

A. Materials

1. **Test material**: AE 1344122
   Description White powder

Lot / Batch #:	YG3228
Purity:	98.8%
CAS #	Not reported
Stability of test compound:	Stable for the duration of the study

2. Control materials:

Negative: Culture medium [Eagle's minimal essential medium supplemented with 1% L-glutamine, 1% MEM-vitamins, 1% MEM NEAA, 1% penicillin/streptomycin and 10% foetal calf serum (FCS)]

Solvent: DMSO for AE 1344122 and Dimethylbenzanthracene not exceeding 1% (v/v) in the culture medium, No solvent needed for ethyl methanesulfonate as it is a liquid.

Positive: Ethyl methanesulfonate (EMS), a directly alkylating agent, used at 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 S9 mix.

3. Test organisms:

Cell line: Chinese hamster V79 lung cells

Source: Cells obtained from Prof. G. Speit, University of Ulm, Germany. These cells have since been recloned to maintain karyotypic stability. They have a modal chromosome number of 22 and a rapid population doubling time (10 to 14 hours)

Culture condition: Incubation performed at 37°C in a humidified atmosphere with about 5% CO_2.

4. Test compound concentrations: AE 1344122 was used at concentrations ranging from 1 to 2500 µg/mL in the clonal cytotoxicity assay and from 75 to 2400 µg/mL in the mutagenic assays.

5. Metabolic activation: The S9 fraction was isolated from the livers of Aroclor 1254 induced male Sprague Dawley rats. The preparation dated from February 04, 2003 (protein content 26.4 mg/mL) and was kept frozen at -80°C. The batch was tested for contamination and cytotoxicity prior to use in the first study. Cofactors were freshly dissolved in sodium phosphate buffer (150 mM, pH 7.4)

B. Study design and methods

1. In life dates: 20 May - 03 July 2003

The selection of V79 forward mutations is based on the resistance of induced mutants to the purine analogue 6-thioguanine (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 hours 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). 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::

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 monolayers were washed with PBS, trypsinized and replated in 20 mL 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 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 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 Petri dishes (diameter of 100 mm) at 3x105 cells per dish (8 dishes per culture) in 20 mL culture medium 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.

Two trials were performed.

4. Treatment protocol 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.

5. Parameters assessed:

The parameter "survival to treatment" in % was determined on the basis of the following calculation:

$$\frac{\text{Mean number of colonies (treated cultures)}\times 100}{\text{Mean number of colonies (vehicle control cultures)}}$$

The "absolute population growth" was calculated 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.

$$\frac{\text{Absolute population growth treated culture}\times 100}{\text{Absolute population growth of corresponding vehicle control culture}}$$

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 %.

$$\frac{\text{Mean number of colonies per dish}\times 100}{200}$$

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.

$$\frac{\text{Total number of mutant colonies} \times 100}{\text{Number of evaluated dishes} \times 3 \times 10^5 \times \text{C.E}}$$

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 10-6 cells.

- The mutant frequency of the two cultures of the vehicle 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 frequency of at least three times 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 range 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 criteria:

- Mutant frequencies were only used for assessment, if 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.

- 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 Dunnett 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 cloning efficiency for the vehicle controls ranging from 54.5% to 75.3% and from 52.5% to 74.9% without and with metabolic 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 after treatment with AE 1344122 at any concentration (up to and including a dose level of 2400 µg/mL) either with or without metabolic activation.

Table 5.8.1-20 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 1344122	2400	97.5	2.00
	1200	100.6	2.40
	600	82.4	2.65
	300	91.6	2.15
	150	81.2	4.20
	75	105.2	1.95
Negative control	0	94.8	1.20
Solvent control	0	100.0	5.10
AE 1344122	2400	95.9	2.70
	1200	89.4	0.60
	600	95.6	4.20
	300	89.3	2.70
	150	92.0	3.95
	75	104.7	3.70
Negative control	0	115.5	3.40
Solvent control	0	100.0	3.80
EMS	900	13.3	745.15

EMS : ethylmethanesulfonate

Table 5.8.1-21 Relative survival and mean mutation frequency (mutant colonies per 1 millions cells) –Experiment 2-with S9 mix

Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency
AE 1344122	2400	147.3	2.05
	1200	119.3	1.35
	600	107.8	2.00
	300	129.8	0.30
	150	137.6	2.85
	75	100.1	3.00
Negative control	0	115.4	0.75
Solvent control	0	100.0	2.75
AE 1344122	2400	102.4	0.70
	1200	116.6	4.85
	600	109.7	1.10
	300	108.1	3.20
	150	96.1	0.95
	75	118.3	0.00
Negative control	0	115.0	2.45
Solvent control	0	100.0	4.00
DMBA	20	70.0	87.70

N : not cloned due to cytotoxicity
DMBA : dimethylbenzanthracene

III. Conclusions

AE 1344122 was considered to be non mutagenic in this V79/HPRT forward mutation assay, both with and without metabolic activation.

Assessment and conclusion by applicant:

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

AE 1344122 was considered to be non mutagenic in this V79/HPRT forward mutation assay, both with and without metabolic activation.

Data Point:	KCA 5.8.1/12
Report Author:	[redacted]
Report Year:	2003
Report Title:	28-day toxicity study in the rat by dietary administration Code: AE 1344122
Report No:	C037198
Document No:	M-222343-01-1
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 endocrine measurements incorporated into current guideline. These deviations do not impact the acceptability of the study given it is intended to investigate the repeat 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 recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

AE 1344122 (batch number YG3228, 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 examinations 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 animals in the control 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 tissues were taken, fixed and examined microscopically. Toxicological findings were confined to the highest dose level of 20000 ppm and consisted of scabs around the nose/head region, soiling around the eye and chromodacryorrhea in males and anogenital soiling in females. 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 week one 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 granular 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 kidneys of 8/10 females. This change was correlated with the coarsely granular casts observed in the urine.

Conclusion:

The NOAEL AE 1344122 in this 28-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).

I. Materials and methods

A. Materials

1. **Test material:** 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:	
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN)
Age:	7 weeks approximately
Weight at dosing:	236 to 273 g for the males – 169 to 204 g for the females
Source:	R. Janvier, Le Genest St Isle, France
Acclimation period:	12 days
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from 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 suspended stainless steel wire mesh cages
Environmental conditions:	
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: 19 February - 03 April 2003

2. Animal assignment and treatment

There were 10 animals of each sex per dose group. Animals were assigned to dose groups randomly by body weight. AE 1344122 was administered in the diet for 28 days to Wistar rats at the following doses – 0, 20, 200, 2000 and 20000 ppm (equating to 1.5, 14.9, 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 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 1344122 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, 200 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 of 85-115% of nominal concentrations.

Table 5.8.1-22 Study design

Test group	Concentration in diet (ppm)	Dose per animal (study averages)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	10	10
2	20	1.5	1.7	10	10
3	200	14.9	16.8	10	10
4	2000	152	167	10	10
5	20000	1495	1616	10	10

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 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 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 Dunn test (2-sided), if the Kruskal-Wallis test 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% levels of significance. Statistical analyses were carried out using the 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 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 (Mydriaticum, 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 intervals throughout the treatment periods. Diet-fasted animals were weighed before necropsy.

3. Food consumption

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. 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 lithium heparin for plasma and clot activator for serum for clinical chemistry and on sodium citrate for coagulation parameters.

The following hematology parameters were assayed using an Advia 120 (Bayer Diagnostics, Puteaux, 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's 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 phosphorus, total cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase activities, total protein and albumin concentrations were assayed on serum samples using an Hitachi 911 (Roche Diagnostics, Meylan, France).

On study days 29, 30, 31 or 32, overnight urine samples were collected from all animals for urinalysis. Feed and water were not accessible during urine collection.

Additional urine samples were collected overnight from all animals once per week 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 dipsticks (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 urobilinogen were assayed using a Clinitek 200+ and Ames Multistix 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 days 29, 30, 31 or 32, a complete necropsy was performed on all surviving animals. Animals were deeply anesthetized by Isoflurane inhalation, then exsanguinated 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 examined microscopically. Adrenal gland, brain, epididymidis, 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 tissues were sampled: adrenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, oesophagus, exorbital (lachrymal) gland, eye and optic nerve, Harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, 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, 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 ppm, the only finding was scabs around the nose/head region in 2/10 males and nasal soiling 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 lower dose levels in either sex.

2. Mortality

There was no mortality in any group.

3. Neurotoxicity assessment:

No findings were observed at the neurotoxicity assessment at any dose level.

There were no treatment-related clinical signs in any group.

4. Ophthalmology examination

At 20000 ppm, chromodacryorrhea was observed in two males.

No ocular abnormalities were observed at lower dose levels.

B. Body weight and body weight gain

At 20000 ppm, absolute body weight in males was reduced by 7% ($p \leq 0.01$) 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 28, whilst overall 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 weight gain (g) in male and female rats

Week	Dose level (ppm)									
	Males					Females				
	0	20	200	2000	20000	0	20	200	2000	20000
Overall body weight gain between week 1 (study day 1) and week 4 (% control)	141 (-)	135 (96)	137 (97)	141 (100)	116 (82)	53 (-)	49 (92)	57 (108)	54 (102)	44 (83)

No relevant changes in mean body weight or mean body weight gain were observed at 2000 ppm or below.

C. Food consumption and compound intake

Food consumption was reduced by 13% ($p \leq 0.05$) 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% ($p \leq 0.01$) by Day 28 in males at 20000 ppm.

No relevant changes were observed at 2000, 200 or 20 ppm in males or 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 urinary volume was higher than control levels for both sexes.

Table 5.8.1-24 Mean urinary pH and volumes in male and female rats

Week	Dose level (ppm)									
	Males					Females				
	0	20	200	2000	20000	0	20	200	2000	20000
pH values	6.8	7.0	6.9	6.6	6.2*	6.2	6.3	6.2	6.2	5.8
Mean urinary volume (ml)	4.2	4.5	4.7	3.4	5.9	2.1	1.8	2.3	2.3	4.2**

* $p \leq 0.05$; ** $p \leq 0.01$ significantly different from the control using the Kruskal-Wallis or 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% ($p \leq 0.01$) lower in males 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 of no toxicological significance.

No relevant changes were observed at 2000, 200 or 20 ppm

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 lesions 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 change was correlated with the coarsely granular casts observed in the urine.

Table 5.8.1-25 Incidence of treatment-related lesions in the kidney

Sex	Male					Female				
Dosage level (ppm)	**0**	**20**	**200**	**2000**	**20000**	**0**	**20**	**200**	**2000**	**20000**
Number examined	10	10	10	10	10	10	10	10	10	10
Tubular degeneration/regeneration										
Minimal	0	0	0	0	0	0	0	0	0	5
Slight	0	0	0	0	0	0	0	0	0	3
Moderate	0	0	0	0	0	0	0	0	0	0
Total	0	0	0	0	0	0	0	0	0	8
Single cell necrosis										
Minimal	0	0	0	0	0	0	0	0	0	2
Slight	0	0	0	0	0	0	0	0	0	5
Moderate	0	0	0	0	0	0	0	0	0	1
Total	0	0	0	0	0	0	0	0	0	8

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 noted in this study

III. Conclusions

The NOAEL AE 1344122 in this 28-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).

Assessment and conclusion by applicant:

Study meets the current 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 in this 28-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).

Groundwater metabolites

Trifluoroacetic acid (TFA), is a fluopyram 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 toxicity is currently ongoing. The currently available studies; 3 *in 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 sunmitted when available.

Data Point:	KCA 5.8.1/49
Report Author:	[redacted]
Report Year:	2013
Report Title:	Sodium Trifluoroacetate - Acute oral toxicity study in rats
Report No:	12/333-001P
Document No:	M-444479-01-1
Guideline(s) followed in study:	OECD 425; Commission Regulation (EC) No 440/2008; B.1.TRIS; US-EPA 712-C-98-190 , OPPTS 870.1100;
Deviations from current test guideline:	Current Guideline: OECD 425, 2008 No deviations
Previous evaluation:	Not evaluated
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The single-dose oral toxicity study with Sodium Trifluoroacetate was performed according to the acute toxic class method (OECD TG 425, adopted at 3rd October 2008, Commission Regulation (EC) No 440/2008; B.1.TRIS; US-EPA 712-C-98-190 , OPPTS 870.1100).

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 additional animals survived, the LD_{50} was defined to be greater than the limit dose and the test was terminated according to Test Guidelines OECD 425/OPTTS 870.1100.

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 a concentration of 200 mg/ml at a dosing volume of 10 mL/kg bw. Clinical observations were performed at 30 minutes, 1, 2, 3, 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.

There was no mortality at a dose level of 2000 mg/kg bw. Treatment with Sodium Trifluoroacetate at the dose level of 2000 mg/kg bw did not cause any test 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 treatment-related effect.There was no evidence of observations at a dose level of 2000 mg/kg bw at necropsy.

Under the conditions of this study, the acute oral LD50 value of the test item Sodium Trifluoroacetate was found to be above 2000 mg/kg bw in female CRL:(WI) rats.

Sodium Trifluoroacetate is non-toxic after acute oral administration at a dose l evel of 2000 mg/kg bw.

The study result triggers the following classification/labelling:

- EU Directive 1999/45/EC (as amended): none
- Regulation (EC) No 1272/2008 (CLP): none
- GHS (rev. 4) 2011: unclassified

I. Materials and methods

A. Materials

1. Test material:	Sodium Trifluoroacetate
Description:	solid white
Lot/Batch no:	SES 11755-1-1
Purity:	95.1%
Stability of test compound:	guaranteed for study duration; expiry date: 2013-01-24
2. Vehicle and / or positive control:	Distilled water
3. Test animals:	
Species:	Wistar rat
Strain:	CRL:(WI)
Age:	8 - 9 weeks
Weight at dosing:	190 g - 220 g
Source:	[redacted]
Acclimatisation period:	at least 6 days
Diet:	ssniff® SM R/M "Autoclavable complete diet for rats and mice - breeding and maintenance" (ssniff Spezialdiaeten GmbH, Soest, Germany) *ad libitum*
Water:	tap water *ad libitum*
Housing:	individually in Type II polypropylene/polycarbonate cages; Lignocel Bedding for Laboratory Animals

B. Study design and methods

1. In life dates: The study was carried out at [redacted] from 3 October 2012 to 30 October 2012

2. Animal assignment and treatment

Dose:	2000 mg/kg bw
Application route:	oral
Application volume:	10 mL/kg bw
Fasting time:	before administration: overnight after administration: 3 hours
Group size:	5 females
Post-treatment observation period:	14 days
Observations:	mortality, clinical signs, body weight, gross necropsy

II. Results and discussion

A. Mortality

Sodium trifluoroacetate did not cause mortality at the limit dose level of 2000 mg/kg bw.

Details are provided in Table 5.8.1-26.

Table 5.8.1-26 Doses, mortality / animals treated

Dose (mg/kg bw)	Toxicological findings*	Duration of signs	Onset of death after (days)	LD_{50} (mg/kg bw)
Group 1 2000	0/0/5	--	--	LD_{50} > 2000

*number of dead animals/number of animals with clinical signs/number of animals tested.

B. Clinical observations

No clinical signs were observed in females dosed at 2000 mg/kg.

C. Body weight

Body weight and body weight gain of sodium trifluoroacetate treated animals showed no indication of a treatment-related effect.

D. Necropsy

No abnormalities were observed at gross necropsy.

E. Deficiencies

No deficiencies are noted.

III. Conclusions

Sodium trifluoroacetate is non-toxic after acute oral administration with an LD_{50} value above 2000 mg/kg bw in female rats.

Assessment and conclusion by applicant:
The study was performed under GLP conditions in accordance with the current OECD guideline 425 (2008) without any deviations. The study is therefore considered valid and acceptable for assessment.

The acute oral LD_{50} of Sodium trifluoroacetate is > 2000 mg/kg bw. According to the classification criteria of the CLP Regulation (EU) No. 1272/2008, no classification is required.

Data Point:	KCA 5.8.1/50
Report Author:	[redacted]
Report Year:	2005
Report Title:	Trifluoroacetate (TFA): reverse mutation in five histidine-requiring strains of Salmonella typhimurium
Report No:	2014/82
Document No:	M-256628-01-1
Guideline(s) followed in study:	OECD 471(1997) ; EEC Annex V, B13/14; UKEMS Guidelines; Japanese MOHW; JMAFF; ICH Harmonised Tripartite Guideline; US-EPA OPPTS 870.5100 (1998);
Deviations from current test guideline:	current guideline: OECD 471, 2020 Deviation: none
Previous evaluation:	Not evaluated
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Trifluoroacetate (TFA) was assayed 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 Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9), in two separate experiments.

An initial toxicity Range-Finder Experiment was carried out in strain TA100 only, in the absence and presence of S-9, using final concentrations of TFA at 1.6, 8, 40, 200, 1000 and 5000 µg/plate, plus negative (solvent) and positive controls. No 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 treatments.

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 colonies 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.

1. Materials and methods

A. Materials

1. Test material:	Trifluoroacetate (Sodium Trifluoroacetate)
Description	White powder
Lot / Batch #:	016911/1
Purity:	99.1 %
CAS #	2923-18-4
Stability of test compound:	Stable for the duration of the study
2. Control materials:	**Negative**: Culture medium
	Solvent: Sterilised water
	Positive:

Chemical	Final concentration (µg/plate)	Use: Strain(s)	Use: S-9
2-Nitrofluorene (2NF)	5.0	TA98	–
Sodium azide (NaN_3)	2.0	TA100, TA1535	–
9-Aminoacridine (AAC)	50.0	TA1537	–
Mitomycin C (MMC)	0.2	TA102	–
Benzo[a]pyrene (B[a]P)	10.0	TA98	+
2-Aminoanthracene (AAN)	5.0	TA100, TA1535, TA1537	+
	20.0	TA102	+

Activation
The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. S9 mix was constitued of S9 fraction, sodium phosphate buffer pH 7.4, glucose-6-phosphate (disodium), NADP (disodium), magnesium chloride, potassium chloride, L-histidine HCl (in 250 mM $MgCl_2$), d-biotin and water

Test organisms:
Five bacterial strains of *Salmonella typhimurium* (TA98, 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 TA102 was originally obtained from Glaxo Group Research Limited. For all assays, bacteria were cultured for 10 hours at 37±1°C in nutrient broth (containing ampicillin for strains TA98 and TA100 and ampicillin and tetracycline for strain TA102). Incubation was carried out in a shaking incubator. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics of histidine dependence, *uvrB* 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 assay: 156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate.

B. Study design and methods
1. In-life dates: The study was carried out at Covance Laboratories Ltd, Otley Road, Harrogate, North Yorkshire (UK) from 11 March 2005 and 10 May 2005.

2. Preliminary cytotoxicity/mutation assay

TFA was tested in strain TA100, at 1.6, 8, 40, 200, 1000 and 5000 µg/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 mixing 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±1°C, with shaking, before the addition of 2.5 mL molten agar at 46±1°C. Volumes for positive control treatments were reduced to 0.05 mL. Plating of these treatments then proceeded as for the normal plate-incorporation procedure. Colonies were counted electronically using a Seescan Colony Counter (Seescan Plc) or manually where confounding factors such as split agar affected the accuracy of the automated counter. The background bacterial lawn was inspected for signs of toxicity.

Statistics

The m-statistic was calculated to check that the data were Poisson-distributed, and Dunnett'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.

4. Acceptance criteria:

The mean The assay was considered valid if the following criteria were met:

1. the negative control counts 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-9 preparation
3. no more than 5% of the plates were lost through contamination or 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 ($p \leq 0.01$) and the data set(s) showed a significant dose correlation

the positive responses described above were reproducible

II. Results and discussion

Preliminary cytotoxicity assay

No evidence of toxicity 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 analyses demonstrated achieved concentrations within 100±10% of the nominal test article concentrations for all treatment concentrations in each of the main mutation experiments

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.
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.

Table 5.8.1-27 Summary of mean revertant colonies

Dose (µg/plate)	S-9 mix (-/+)	*Salmonella typhimurium* strains TA98	TA100	TA1535	TA1537	TA102
		mean ± SD				
Experiment 1						
Solvent control	–	27 ± 6	105 ± 14	15 ± 6	19 ± 1	221 ± 8
TFA 1.6	–	27 ± 8	103 ± 15	14 ± 2	15 ± 8	214 ± 20
8	–	25 ± 7	101 ± 7	17 ± 6	18 ± 3	213 ± 27
40	–	28 ± 4	91 ± 26	11 ± 3	22 ± 1	219 ± 16
200	–	36 ± 3	101 ± 1	13 ± 3	16 ± 6	216 ± 10
1000	–	34 ± 4	103 ± 6	12 ± 5	20 ± 1	223 ± 6
5000	–	25 ± 5	104 ± 5	15 ± 2	17 ± 7	211 ± 27
Positive controls						
2NF: 5.0	–	1192 ± 121				
NaN_3: 2.0	–		666 ± 26	643 ± 17		
AAC: 50.0	+				204 ± 30	
MMC: 0.2	+					643 ± 34
Experiment 2						
Solvent control	–	20 ± 4	97 ± 5	14 ± 3	19 ± 2	249 ±17
TFA 156.25	–	28 ± 7	103 ± 6	15 ± 4	16 ± 4	271 ± 29
312.5	–	25 ± 3	104 ± 9	16 ± 4	16 ± 7	234 ± 33
625	–	33 ± 8*	103 ± 2	18 ± 4	23 ± 4	208 ± 27
1250	–	23 ± 3	95 ± 10	13 ± 5	17 ± 4	219 ± 37
2500	–	21 ± 3	99 ± 2	10 ± 3	20 ± 1	248 ± 40
5000	–	19 ± 2	88 ± 9	14 ± 4	18 ± 2	232 ± 27
Positive controls						
2NF: 5.0	–	577 ± 20				
NaN_3: 2.0	–		438 ± 30	438 ± 30		
AAC: 50.0	+				75 ± 12	
MMC: 0.2	–					620 ± 8

Dose (µg/plate)	S-9 mix (-/+)	*Salmonella typhimurium* strains TA98	TA100	TA1535	TA1537	TA102
		mean ± SD				
Experiment 1						
Solvent control	+	30 ± 7	107 ± 16	17 ± 3	20 ± 5	202 ± 30
TFA 1.6	+	36 ± 8	92 ± 12	13 ± 3	17 ± 3	193 ± 16
8	+	36 ± 10	99 ± 19	14± 7	20 ± 1	162 ± 10
40	+	49 ± 7	99 ± 5	19 ± 3	13 ± 3	180 ± 14
200	+	33 ± 8	107 ± 6	16 ± 2	18 ± 4	185 ± 20
1000	+	31 ± 2	88 ± 8	20 ± 2	17 ± 8	189 ± 7
5000	+	36 ± 15	93 ± 8	15 ± 3	22 ± 8	177 ± 45
Positive controls						
B[a]P: 10.0	+	245 ± 32				
AAN: 5.0	+		977 ± 35	196 ± 24	97 ± 3	
AAN: 20.0	+					492 ± 13
Experiment 2						
Solvent control	+	41 ± 5	112 ± 12	17 ± 3	22 ± 2	196 ± 34
TFA 156.25	+	32 ± 10	76 ± 5	12 ± 1	24 ± 7	176 ± 29
312.5	+	29 ± 6	94 ± 5	20 ± 3	23 ± 1	208 ± 3
625	+	38 ± 10	69 ± 5	21 ± 6	18 ± 3	178 ± 29
1250	+	41 ± 6	75 ± 6	11 ± 3	20 ± 4	212 ± 35
2500	+	26 ± 5	93 ± 9	10 ± 3	21 ± 2	246 ± 6
5000	+	34 ± 3	90 ± 4	15 ± 2	20 ± 4	232 ± 18
Positive controls						
B[a]P: 10.0	+	399 ± 62				
AAN: 5.0	+		1058 ± 107	290 ± 72	124 ± 16	
AAN: 20.0	+					796 ± 246

* Dunnett's test, significant at 1% level

TFA = trifluoro acetate; 2NF = 2-Nitrofluorene (2NF); NaN_3= sodium azide; AAC = 9-Aminoacridine; MMC = mitomycin C; B[a]P = Benzo[a]pyrene; AAN = 2-Aminoanthracene

III. Conclusions

Trifluoroacetate (TFA) did not induce mutation in five histidine-requiring strains of Salmonella typhimurium (TA98, TA100, TA1535, TA1537, TA 102) when tested under the conditions of this study.

Assessment and conclusion by applicant:

Under the conditions of the test, trifluoroacetate (TFA) did not induce mutation in five histidine-requiring strains of Salmonella typhimurium (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.

Data Point:	KCA 5.8.1/51
Report Author:	[redacted]
Report Year:	2005
Report Title:	Trifluoroacetate (TFA) - Induction of chromosome aberrations in cultured human peripheral blood lymphocytes
Report No:	2014/83-D6172
Document No:	M-260807-01-1
Guideline(s) followed in study:	OECD 473 (1997), OPPTS 870.5375 (1998), EC directive 2000/32/EC (2000), JMAFF No. 8147 (2000)
Deviations from current test guideline:	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 such as statistical significance or cell proliferation criteria
Previous evaluation:	Not evaluated
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

TFA was tested in an *in vitro* cytogenetics assay using duplicate human lymphocyte cultures prepared from the pooled blood of three male 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 water for injection (purified water) and the highest dose level used in the main experiments, 1360 µg/mL (equivalent to 10 mM) was determined following a preliminary cytotoxicity range-finding experiment.

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 harvest (3+17). The S-9 used was prepared from a rat 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 TFA on mitotic index. Chromosome aberrations were analysed at 340.0, 680.0, 1360 µg/mL. The highest concentration chosen for analysis, 1360 µg/mL, induced approximately 16% and 0% mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9 respectively.

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 analysed at 85.00, 170.0, 340.0, 1360 µg/mL (- S9) or 170.0, 340.0, 1360 µg/mL (+S9) and the highest concentration chosen 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 absence or presence of S-9, that were considered to be of any biological relevance.

1. Materials and methods

A. Materials

1. Test material:	Trifluoroacetate (Sodium Trifluoroacetate)
Description	White powder
Lot / Batch #:	016911/1
Purity:	99.1%
CAS #	2923-18-4
Stability of test compound:	Stable for the duration of the study
2. Control materials:	
Negative:	Culture medium tissue
Vehicle/Solvent	Sterile water
Positive:	non- activation (-S9 mix): 4-Nitroquinoline 1-oxide in DMSO, final concentration 2.50 µg/mL with activation (+S9 mix): Cyclophosphamide in DMSO, final concentration 6 µg/mL

Activation:
The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. Glucose-6-phosphate (180 mg/mL), NADP (25 mg/mL), 150 mM KCl and rat liver S-9 were mixed in the ratio 1:1:1.2. An aliquot 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 homogenate in the test system was 2%. Cultures treated in the absence of S-9 received an equal volume of 150 mM KCl.

Test cells:
Blood from three healthy, non-smoking male 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 initiation. 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 mL Hepes-buffered RPMI medium containing 20% (v/v) foetal calf serum and 50 µg/mL gentamycin. Phytohaemagglutinin (PHA, reagent grade) was 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°C and rocked continuously. All cultures were then incubated at 37ºC.

Test compounds concentration used (mM):

	Preliminary test	Main test
Non activated conditions – 3 h + 17h	0.6641, 1.328, 2.656, 5.313, 10.63, 21.25, 42.50, 85.00, 170.0, 340.0, 680.0, 1360 µg/mL	85.00, 170.0, 340.0, 680.0, 1360 µg/mL
Non activated conditions – 20 h	0.6641, 1.328, 2.656, 5.313, 10.63, 21.25, 42.50, 85.00, 170.0, 340.0, 680.0, 1360 µg/mL	85.00, 170.0, 340.0, 680.0, 1360 µg/mL
Activated conditions – 3h+17h	0.6641, 1.328, 2.656, 5.313, 10.63, 21.25, 42.50, 85.00, 170.0, 340.0, 680.0, 1360 µg/mL	85.00, 170.0, 340.0, 680.0, 1360 µg/mL

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 were 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 37°C. Treatment details are provided in the scheme below.

Table 5.8.1-28 Treatment details

Treatment	S-9	Number of cultures	
		3+17*	20+0*
Cytotoxicity range-finding experiment			
Negative control	-	2	2
	+	2	
Test article (all doses)	-	1	1
	+	1	
Experiment 1			
Negative control	-	4	
	+	4	
Test article (all doses)	-	2	
	+	2	
Positive controls (all doses)	-	2	
	+	2	
Experiment 2			
Negative control	-		4
	+	4	
Test article (all doses)	-		2
	+	2	
Positive controls (all doses)	-		2
	+	2	

* Hours treatment + hours recovery

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 S-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 pellets were clear.

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 resuspended 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 chromosome 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% (v/v) filtered Giemsa stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried and mounted with coverslips.

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 polyploid, endoreduplicated and hyperdiploid cells, observed during this search was noted 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 fragment. Observations were recorded on raw data sheets with the microscope stage co-ordinates of any aberrant cell.

Evaluation criteria
The data were evaluated as to whether exposure to the test article was associated with:

1. The proportion of cells with structural aberrations at one or more concentrations exceeds the historical vehicle control range in both replicate cultures.
2. A statistically significant increase 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 aberrations excluding gaps
3. polyploid, endoreduplicated 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 ≤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 ≤0.05 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 20-hour exposure to TFA in the absence of S9 in Experiment 2. 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 chromosome damage. The aberrations included two chromosome exchanges in one cell. However, the percentages of cells with aberrations fell within the historical vehicle control frequencies. Also, exposure at 1360 µg/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.

Table 5.8.1-29 Summary of cells with structural aberrations

Substance Dose (µg/mL)	+/- S9	Cells scored	Cells with aberrations Including gaps	 Excluding gaps	Mitotic Index (mean)
Experiment 1 (3 hour treatment + 17 hour recovery, +/-S9)					
Solvent	+	200	5	1	7.1
TFA 340	+	200	0	0	7.6
680	+	200	3	1	7.6
1360	+	200	4	4	7.9
CPA 6.25	+	168	53	49[a]	
Solvent	–	200	2	1	8.3
TFA 340	–	200	3	2	8.2
680	–	200	6	3	7.2
1360	–	200	0	0	7.0
NQO 2.50	–	186	45	44[a]	
Experiment 2 (3 hour treatment + 17 hour recovery, +S9)					
Solvent	+	200	1	1	7.6
TFA 340	+	200	3	1	7.7
680	+	200	1	0	6.1
1360	+	200	1	1	6.2
CPA 6.25	+	97	48	40[a]	
Experiment 2 (20 hour treatment + 0 hour recovery, -S9)					
Solvent	–	200	1	0	6.1
TFA 85	–	200	0	0	5.3
170	–	200	0	0	3.8
340	–	200	1	1	3.7
1360	–	200	6	4	2.4
NQO 2.50	–	102	40	34[a]	

[a] statistical significance p ≤ 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 of 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.

Table 5.8.1-30 Summary of numbers and types of numerical aberrations

Substance Dose (µg/mL)	+/- S9	Cells scored	Numerical aberrations: hyperdiploid	endoreduplicated	polyploid	Total abs	% with num abs
Experiment 1 (3 hour treatment + 17 hour recovery, + S9)							
Solvent	+	200	0	0	0	0	0
TFA → 340	+	203	0	1	2	3	1.5
→ 680	+	200	0	0	0	0	0
→ 1360	+	202	0	0	2	2	1.0
CPA → 6.25	+	168	0	0	0	0	0
Solvent	-	200	0	0	0	0	0
TFA → 340	-	200	0	0	0	0	0
→ 680	-	202	2	0	0	2	1.0
→ 1360	-	200	0	0	0	0	0
NQO → 2.5	-	186	0	0	0	0	0
Experiment 2 (3 hour treatment + 17 hour recovery, + S9)							
Solvent	+	200	0	0	0	0	0
TFA → 340	+	202	0	0	2	2	1.0
→ 680	+	200	0	0	0	0	0
→ 1360	+	201	0	0	1	1	0.5
CPA → 6.25	+	97	0	0	0	0	0
Experiment 2 (20 hour treatment + 0 hour recovery, - S9)							
Solvent	-	201	0	0	1	1	1.0
TFA → 85	-	200	0	0	0	0	0
→ 170	-	201	0	1	0	1	0.5
→ 340	-	203	2	0	1	3	1.5
→ 1360	-	200	0	0	0	0	0
NQO → 2.5	-	102	0	0	0	0	0

abs = aberrations, num = numerical

III. Conclusion

Trifluoroacetate (TFA) did not induce chromosome aberrations in cultured human peripheral blood lymphocytes when tested up to 1360 µg/mL in either the absence or the presence of a rat liver metabolic activation system (S9).

Assessment and conclusion by applicant:

Under the conditions of the test, trifluoroacetate (TFA) did not induce chromosome aberrations in cultured human peripheral blood lymphocytes.
The study was performed under GLP 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 were investigated, 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 laboratory 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:	[redacted]
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 study:	OECD 476 (1997), OPPTS 870.5300 (1998), UKEMS (1990)
Deviations from current test guideline:	Current guideline: OECD 490, 2016 The study was carried out in compliance with OECD 476 (1997) and therefore a few deviations occurred compared to in OECD TG 490 (2016). cytotoxicity was not calculated by using the latest formulae for calculating Relative Suspension Growth (RSG) ,and mutation frequency. However, the acceptance criteria were in line with what currently required.
Previous evaluation:	Not evaluated
N	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

TFA was assayed for its ability to induce mutation at the *tk* locus (Trifluorothymidine resistance) in mouse lymphoma cells using a fluctuation protocol. The study consisted of a cytotoxicity range-finding experiment followed by two independent experiments, each conducted in the absence and presence of metabolic activation by an Aroclor 1254 induced rat liver post-mitochondrial fraction (S-9).

A 3 hour treatment incubation period was 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-hour treatment incubation and Experiment 2 was performed using a 24 hour treatment incubation.

In the cytotoxicity range-finding experiment, 3 hour treatment, doses were tested in the absence and presence of S-9 ranging from 42.5 to 1360 µg/mL (10 mM). Cells survived all doses of TFA. The highest dose tested (1360 µg/mL) yielded 142% 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, doses were tested in the absence of S-9 ranging from 5.313 to 1360 µg/mL (10 mM). Cells survived all doses of TFA. The highest dose tested (1360 µg/mL) yielded 49% RTG.

Accordingly, for the first experiment (3 hour treatment) doses were chosen in the absence and presence of S-9 ranging from 360 to 1360 µg/mL. The highest dose tested (1360 µg/mL) yielded 112% and 93% RTG in the absence and presence of S-9 respectively. 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 a repeat experiment.

In the second experiment doses were tested in the absence of S-9 (24 hour treatment) and in the presence of S-9 (3 hour treatment) ranging from 360 to 1360 µg/mL. The highest dose tested (1360 µg/mL) yielded 76% and 104% RTG in the absence and presence of S-9 respectively.

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-nitroquinoline 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 provided 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

A. Materials

1. Test material:	Trifluoroacetate (Sodium Trifluoroacetate)
Description	White powder
Lot / Batch #:	016911/1
Purity:	99.1%
CAS #	2923-18-4
Stability of test compound:	Stable under the test conditions
2. Control materials:	
Negative:	Tissue culture medium
Vehicle/Solvent	Culture medium/Sterile water
Positive:	non- activation (-S9 mix): 4-Nitroquinoline 1-oxide, final concentration 0.05 – 0.20 µg/mL with activation (+S9 mix): benzo(a)pyrene (BP), final concentration 2-3 µg/mL

Activation:
The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats, induced with Aroclor 1254. The S-9 was obtained from Molecular Toxicology Incorporated, USA. Glucose-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 (19 mL) to give a total of 20 mL. Cultures treated in the absence of S-9 received 1 mL 150mM KCl (3-hour treatments only).

Test cells:
L5178Y TK $^{+/-}$ mouse lymphoma cells were stored as frozen stocks in liquid nitrogen, the original cultures were obtained from Dr Donald Clive, Burroughs Wellcome Co. Each batch of frozen cells was purged of TK^{-} mutants, checked for spontaneous mutant frequency and that it was mycoplasma free.

Culture medium:
For each experiment, the cells diluted in RPMI 10 media and incubated in a humidified atmosphere of 5% v/v CO_2 in air.

Three types of RPMI 1640 medium were prepared as follows:

	RPMI A	RPMI 10	RPMI 20
Horse serum (heat inactivated)	0% v/v	10% v/v	20% v/v
Penicillin / streptomycin	100 units/mL 100 µg/mL	100 units/mL 100 µg/mL	100 units/mL 100 µg/mL
Amphotericin B	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL
Pluronic	0.5 mg/mL	0.5 mg/mL	-

Test compounds concentration used (µg/ml):

	Preliminary toxicity	Test 1	Test 2
Non activated conditions	42.50, 85.00, 170.0, 340.0, 680.0, 1360	250, 500, 1000, 1200, 1400, 1500, 1600	250, 500, 1000, 1200, 1400, 1500, 1600
Activated conditions	42.50, 85.00, 170.0, 340.0, 680.0, 1360	250, 500, 1000, 1200, 1400, 1500, 1600	250, 500, 1000, 1200, 1400, 1500, 1600

B. Study design and methods

1. In life dates: 14 March 2005 – 25 May 2005

The study was carried out at [redacted] from 14 March 2005 and 25 May 2005.

Cell treatment

Treatment of cell cultures for the cytotoxicity range-finding experiment was carried out using single cultures only and positive controls were not included.

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, cultures were centrifuged (200 x g) for 5 minutes, washed and resuspended in 20 mL RPMI 10. Cultures were incubated at 37°C for 1 day, recounted and where possible diluted to 2×10^5 cells/mL. 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 microtitre plate for determination of relative total growth. The plates were incubated at 37ºC in a humidified incubator gassed with 5% v/v CO_2 in air for 6 to 7 days. Wells containing viable clones were identified by eye using background illumination and counted.

For Experiment 1 in the absence and presence of S-9 (and Experiment 1 repeat in the absence of S-9) and in Experiment 2 in the presence of S-9 (3 hour treatments) at least 10^7 cells in a volume of 17 mL tissue culture medium (cells in RPMI 10 diluted with RPMI A [no serum] to give a final concentration of 5% serum) were used. For Experiment 2 in the absence of S-9 (24 hour treatment) at least 4×10^6 cells in a volume of 18 mL RPMI 10 were used. 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).

3 hour treatment: After 3 hours incubation at 37±1°C 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±1°C for 24 hours, cultures were centrifuged (200 x g) for 5 minutes, washed and resuspended in fresh RPMI 10 medium (20 mL). Cell densities were determined using a Coulter counter and, where sufficient cells survived, adjusted to 2×10^5 cells/mL.

Cells were transferred to tissue 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.

Expression period

Cultures were maintained in flasks for a period of 2 days during which the TK^- mutation would be expressed. Sub-culturing was performed as required with the aim of not exceeding 1×10^6 cells per mL and, where possible, retaining at least 1×10^7 cells/flask. From observations on recovery and growth of the cultures.

Plating for viability

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 10^4/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 37ºC in a humidified incubator gassed with 5% v/v CO_2 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 1 x 10^5/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 10^3 cells per well). Plates were incubated at 37ºC in a humidified incubator gassed with 5% v/v CO_2 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 (LMF) was compared with the LMF from each treatment dose based on Dunnett'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.

Acceptance criteria
The assay was considered valid if all the following criteria were met:

1. the mean mutant frequencies in the negative (solvent) control cultures fell within the normal range (50 to 170 mutants per 10^6 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 negative control mutant frequencies was greater than half the historical mean value)
3. the mean plating 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 range 8 to 32 following 3 hour treatments
5. There was no excessive heterogeneity between replicate cultures.

Evaluation criteria
The test article was considered to be mutagenic if all the following criteria were met:

1. the assay was valid according to the acceptance criteria
2. the mutant frequency at one or more doses was significantly greater than that of the negative control ($p \leq 0.05$)
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 µg/mL (10 mM). Cells survived all doses of TFA. The highest dose tested (1360 µg/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 5.313 to 1360 µg/mL (10 mM). Cells survived all doses of TFA. The highest dose tested (1360 µg/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 360 to 1360 µg/mL. All doses tested in the absence and presence of S-9 were selected to determine viability and 5-trifluorothymidine (TFT) resistance 2 days after treatment. The highest dose tested (1360 µg/mL) yielded 76% and 104% RTG in the absence and presence of S-9, respectively.

Mutation Assay

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, provided 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.
In addition, for the negative and positive controls and dose of test article 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 repeat, and ranged from 26% to 58% in Experiment 2. Marked increases in the number of both small and large colony mutants were observed following treatment with the positive control chemicals NQO and BP.

Table 5.8.1-31 Summary of results

Dose (µg/mL)		-S9		+S9	
		% rel. total growth	mutant frequency§	% rel. total growth	mutant frequency§
Experiment 1 (3 hour treatment +/-S9)					
TFA	0	100	58.86	100	61.07
	360	88	45.14	94	91.37
	560	100	45.25	90	91.29
	760	119	44.31	78	94.48
	960	122	51.85	83	101.77*
	1160	120	59.43	103	75.57
	1360	112	55.90	93	85.10
NQO	0.15	57	314.40		
	3	42	435.99		
BP	2			46	648.58
	3			30	975.59
Experiment 2 (24 hour treatment - S9, 3 hour treatment + S9)					
TFA	0	100	56.49	100	50.34
	360	81	44.44	100	74.18
	560	82	58.47	106	63.84
	760	82	41.65	81	58.07
	960	93	52.71	83	57.83
	1160	90	47.63	81	79.88
	1360	76	51.89	104	56.79
NQO	0.05	34	294.33		
NQO	0.1	14	398.07		
BP	2			63	270.86
BP	3			25	542.41

§ 5-TFT (5-trifluorothymidine) resistant mutants 10^6 viable cells 2 days after treatment
* Comparison of each treatment with control: Dunnett's test (one-sided), significant at 5% level

III. Conclusion

Trifluoroacetate (TFA) did not induce mutation at the *tk* locus of L5178Y mouse lymphoma cells in the absence and presence of a rat liver metabolic activation system.

Assessment and conclusion by applicant:

Trifluoroacetate (TFA) did not induce mutation at the *tk* locus of L5178Y mouse lymphoma cells in the absence and presence of a rat liver metabolic activation system.

The study was carried out in compliance with OECD 475 (1997) and therefore a few deviations occurred compared to an OECD TG 490 (2016): cytotoxicity was not calculated by using the latest formulae for calculating Relative Suspension Growth (RSG) ,and mutation frequency. However, the acceptance criteria were in line with what currently required.

Therefore the study is considered to be acceptable and reliable.

Data Point:	KCA 5.8.1/53
Report Author:	[redacted]
Report Year:	2001
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 study:	None
Deviations from current test guideline:	Not applicable
Previous evaluation:	Not evaluated
GLP/Officially recognised testing facilities:	No, as it was mechanistic study and not subjected to specific Quality Assurance inspections. The study was performed according to SOPs which were previously accepted and periodically inspected by the Quality Assurance Unit YES
Acceptability/Reliability:	Yes

Executive summary

The potential systemic toxicity of sodium trifluoroacetate, (TFA, batch number 129I3458: 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 untreated diet and acted as a control. A further group of 5 rats per sex received clofibric 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 interim sacrifice after 3 days of treatment to assess liver weight, hepatic cellular proliferation and liver histopathology only.
Animals were observed daily for mortality and clinical 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 final necropsy for haematology and clinical chemistry determinations. At study termination, brain, kidneys, liver, ovaries, spleen, testes and thyroid were weighed; duodenum, kidneys, liver, ovaries, spleen, testes and thyroid gland were collected from the five remaining males and females in each group (Day 15). Hepatic cellular proliferation was assessed using a specific immunostaining technique. A liver 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.
TFA did not provoke mortalities or clinical signs during the study. Body weight evolution and food consumption were not affected by TFA administration. At 2 400 ppm, no meaningful changes were observed in haematology or plasma chemistry parameters, except a slight decrease in total white blood cell count in females (-30%, relative to controls). Mean liver weights were found statistically significantly higher in males at terminal sacrifice, in correlation with a slight diffuse centrilobular hepatocellular hypertrophy in 1/5 males. An increased number of hepatocellular mitoses and a higher hepatocellular labelling index were found in males 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 palmitoyl-CoA oxidation activities were increased in males reaching 119, 259, 184 and 492% of control, respectively. Only a slight dose-related increase in total cytochrome P- 450 content was found in females, 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 hepatocellular hypertrophy in 2/5 males. At this dose level, hepatic total cytochrome P-450 content, lauric acid hydroxylation 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 dose 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 in the literature.

I. Materials and methods

A. Materials

1. Test material:	Sodium Trifluoroacetate
Description:	white powder
Lot/Batch no:	129H3458
Purity:	98.7%
Stability of test compound:	stable under the test conditions
2. Vehicle and / or positive control:	None
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj: WI (TOPS HAN)
Age:	8 weeks approximately
Weight at dosing:	Males 304-355 g; females 212-231g
Source:	R. Janvier, Le Genest St Isle, France
Acclimatisation period:	at least 6 days
Diet:	Certified and irradiated rodent powder diet A04C-10 PI from U.A.R. (Usine d'Alimentation Rationnelle, Villemoisson-sur-ORGE, France) *ad libitum*
Water:	Tap water (filtered and softened), *ad libitum*
Housing:	Animals were housed individually in suspended stainless steel wire mesh cages
Environmental conditions:	
Temperature:	22 + 2°C
Humidity:	55 + 5%
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: The study was carried out at [redacted]. from 4 April 2001 to 29 August 2001

2. Animal assignment and treatment

TFA was administered by the oral route via the diet up to the day before sacrifice. Groups of 10 male and 10 female rats were given the appropriate diet mixture for 14 days. The treatment groups were as follows:

Table 5.8.1-32 Animal assignment (number of animals/sex)

Test group	Test substance	Concentration in diet (ppm)	Sacrifice Day 4	Sacrifice day 15
1	Vehicle	0	3	5
2		600	0	5
3	Trifluoroacetate	1200	0	5
4		2400	3	5
5	Clofibric acid	5000	0	5

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 in use, the diet formulations were stored at below -15°C.

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 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 faeces.

2. Body weight

Each animal was weighed once during the acclimatization period, 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

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 was calculated.

4. Clinical pathology

On study Day 15, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus. Animals were fasted overnight prior to bleeding and anesthetized by inhalation of isoflurane. Blood was collected on EDTA for haematology (0.5 mL) and lithium heparin for plasma chemistry parameters (2.5 mL).

The following haematology parameters were measured red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation, and platelet count. A blood smear was prepared and stained with Wright stain.

The following clinical chemistry parameters were measured: total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase, total protein, albumin, globulin and albumin/globulin ratio.

5. Sacrifice and pathology

On study 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 microscopically.
At interim sacrifice, only the liver and the brain from three rats/sex in both the control and TFA high dose groups were weighed; the brain was then discarded. Liver and duodenum were sampled and fixed in 10% buffered formalin for histological and immunohistochemical assessment. At final sacrifice, 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 buffered 10% formalin with the exception of testes fixed in Davidson's fixative. Histological sections of the kidneys, liver, ovaries, spleen, testes and thyroid glands were prepared for all animals in all groups and stained with haematoxylin and eosin.
Histopathological examinations were performed on the liver in all animals from the control and TFA high dose groups sacrificed on study Day 4. The liver, kidneys, spleen, ovaries, testes and thyroid glands were examined in TFA high dose and control animals sacrificed 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 cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile to check the hepatotoxic potential of the test substance.
Microsomal preparations were not performed from one male and one female from the clofibric acid treated group due to technical limitations, and from animals sacrificed at the interim sacrifice date.
Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. One quantification was performed for each sample.

Specific cytochrome P-450 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)-7-methoxycoumarin of 12-hydroxy-lauric acid (lauric acid used as substrate). Ethoxyresorufin is a highly selective substrate for the isoform 1A, the isoform IIB metabolizes preferentially the O-dealkylation of pentoxyresorufin, while the benzoxyresorufin O-debenzylation is mainly metabolized by the isoform III A. Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes 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

An immunohistochemical staining was used to evidence the proliferating cell nuclear antigen (PCNA) in order to assess cell cycling. A monoclonal antibody raised against PCNA (Dako, Trappes, France) was applied to formalin fixed paraffin-embedded, deparaffinized liver sections.
The immunological 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 PCNA positive 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, Surrey SMS 4DS, U.K. who performed the enzyme activity determination.

9. 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 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).

II. Results and discussion

A. Mortality

There were no treatment-related mortalities during the study.

B. Clinical observations

There were no treatment-related clinical signs during the study.

C. Body weight

Body weight and body weight gain of sodium trifluoroacetate treated animals showed no indication of a treatment-related effect.

Positive control: During the first treatment week the rats lost weight (males: -14 g; females: -1 g). Lower body weight gain resulted in lower body weights (males: -19 %; females: -10%, $p < 0.01$), when compared with control mean values on Day 14 of the study.

Table 5.8.1-33: Summary of mean body weights

Dose (ppm)	Trifluoroacetate 0	600	1200	2400	Pos. Contr.	Trifluoroacetate 0	600	1200	2400	Pos. Contr.
Body weight (g)	males					females				
Day 1	336	335	341	336	336	221	224	223	223	223
Day 7	376	373	383	368	322*	239	240	237	235	222*
Day 14	406	410	421	408	327*	251	249	248	246	227*

Pos. = positive; Contr. = control

* statistically different from control $p \leq 0.01$

Table 5.8.1-34: Summary of mean body weight gain

Dose (ppm)	Trifluoroacetate 0	600	1200	2400	Pos. Contr.	Trifluoroacetate 0	600	1200	2400	Pos. Contr.
	males					females				
Body weight gain (g)										
Day 7	35	38	43	36	-14*	17	16	14	12	-1*
Day 14	66	75	80	76	-9*	29	25	26	23	4*

Pos. = positive; Contr. = control
* statistically different from control $p \leq 0.01$

D. Food consumption

Trifluoroacetate: Food consumption was not affected.
Positive control: Mean food consumption was significantly decreased, more pronounced in males than in females (-26 and -36% in males and -13 and -23% in females on weeks 1 and 2, respectively). Lower food consumption correlated with the observed body weight loss in both sexes.

Table 5.8.1-35: Summary of mean food consumption

Dose (ppm)	Trifluoroacetate 0	600	1200	2400	Pos. Contr.	Trifluoroacetate 0	600	1200	2400	Pos. Contr.
Food consumption (g)	males					females				
Day 7	27.3	27.9	27.9	26.6	20.1*	19.5	18.5	18.2	18.5	16.9
Day 14	27.4	27.6	28.9	28.2	17.6*	19.8	18.5	18.6	19.5	15.3*

* statistically different from control $p \leq 0.01$

E. Haematology

Trifluoroacetate: A tendency towards lower total white blood cell counts was noted in females at 2400 ppm (-30% compared to controls, statistically significant $p \leq 0.05$). This slight change was associated with lower mean absolute lymphocyte count (-38% compared to controls, statistically significant $p \leq 0.01$). In the absence of relevant change in absolute neutrophil count, the statistically significant change in neutrophil percentage observed in females at 2400 ppm was considered not to be toxicologically relevant.
Positive control: No toxicologically relevant changes observed.

Table 5.8.1-36: Summary of haematology

Dose (ppm)	Trifluoroacetate 0	600	1200	2400	Pos. Contr.	Trifluoroacetate 0	600	1200	2400	Pos. Contr.
Parameter (unit)	males					females				
White blood cell count (10^9/L)	15.1	12.8	13.9	14.4	17.3	11.9	10.9	9.9	8.3*	11.7
Neutrophil count (10^9/L)	3.0	2.8	3.3	3.7	3.2	1.9	2.0	2.3	2.0	2.0
Neutrophils (%)	20	20	23	26	19	15	18	23	24*	17
Lymphocyte count (10^9/L)	11.4	9.7	9.9	9.9	13.1	9.3	8.3	7.1	5.8**	9.0

* statistically different from control $p \leq 0.05$ ** statistically different from control $p \leq 0.01$

F. Clinical chemistry

Trifluoroacetate: No treatment-related variation was observed.

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.

Table 5.8.1-37: Summary of clinical chemistry

	Trifluoroacetate				Pos.	Trifluoroacetate				Pos.
Dose (ppm)	**0**	**600**	**1200**	**2400**	**Contr.**	**0**	**600**	**1200**	**2400**	**Contr.**
Parameter (unit)	males					females				
Aspartate amino transferase (IU/L)	50	55	57	57	97	53	57	58	58	64*
Alkaline phosphatase (IU/L)	99	112	109	113	214*	63	60	77	63	67
Urea (mmol/L)	4.71	4.63	4.69	5.09	7.09**	5.62	5.74	5.06	5.20	4.84
Protein (g/L)	63	63	64	63	58**	63	62	62	65	60
Cholesterol (mmol/L)	1.89	1.26	1.46	1.44	0.95**	1.69	1.79	1.55	1.74	1.62

* statistically different from control $p \leq 0.05$ ** statistically different from control $p \leq 0.01$

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.
Positive control: The increase in total cytochrome P-450 content was pronounced after clofibric acid administration, especially in the males (35% increase compared to control mean).

Enzymatic activities

Trifluoroacetate: No significant changes occurred in BROD, EROD and PROD activities, whereas a significant dose-related increase in lauric acid hydroxylation was observed in males reaching 159% increase at 2400 ppm, when compared to controls. In 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: BROD, EROD and PROD activities were not affected by the clofibric acid administration, whereas a significant increase in lauric acid hydroxylation was observed in males and females (+363% and +118%, respectively).

Cell cycling assessment

Trifluoroacetate: After 3 days of treatment, the labelling index was higher in males and females at 2400 ppm, when compared to controls. At terminal sacrifice, no effect of treatment on hepatocellular proliferation was noted at 2400 ppm.
Positive control: At terminal sacrifice, the labelling index was higher in comparison to control groups in males and females.

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 ≥ 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: Summary of hepatotoxicity assessment

Dose (ppm)	Trifluoroacetate 0	600	1200	2400	Pos. Contr.	Trifluoroacetate 0	600	1200	2400	Pos. Contr.
Parameter (unit)	males					females				
Cytochrome P-450 activity										
Cytochrome P-450 (nmol)	1.40	1.51	1.64	1.66	1.89	0.95	1.06	1.03	1.08	1.12
Enzymatic activities										
BROD (pmol/min/mg protein)	14.71	20.20	22.58	17.51	42.86	2.99	3.65	4.35	4.45	13.31
EROD (pmol/min/mg protein)	54.75	71.24	23.90	24.98	11.34	55.95	38.14	41.34	29.63	29.99
PROD (pmol/min/mg protein)	8.31	8.47	7.85	5.94	12.32	4.74	3.72	3.77	4.32	5.26
Lauric acid hydroxylation (nmol/min/mg protein)	3.20	5.85	7.20	8.28	14.82	2.56	2.10	2.05	2.26	5.59
Cell cycling										
PCNA positive cells /1000 (day 3)	9.2	--	--	20.8	--	8.4	--	--	17.4	--
PCNA positive cells /1000 (day 14)	2.8	--	--	3.7	7.7	3.2	--	--	3.3	5.8
Palmitoyl-CoA oxidation activity										
Whole protein content (mg/protein/g liver)	234	238	247 *	246	261 *	224	244 **	236	237	250 ***
Palmitoyl-CoA oxidation (nmol/min/mg homogenate protein)	4.38	5.39	6.37 **	8.06 **	45.05 ***	4.50	4.18	4.50	4.24	22.64 ***
Palmitoyl-CoA oxidation (µmol/min/g liver)	1.03	1.29	1.57 **	1.98 **	11.78 ***	1.00	1.02	1.06	1.01	5.64 ***

*, **, *** 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 liver weights were statistically significantly increased in male rat ≥ 1200 ppm. There was no 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 associated 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

weight was not considered treatment-related since it was not associated with any histopathological finding and the absolute weight was not increased.

Table 5.8.1-39: Summary of organ weights

Dose (ppm)	Trifluoroacetate 0	600	1200	2400	Pos. Contr.	Trifluoroacetate 0	600	1200	2400	Pos. Contr.
Parameter (unit)	males					females				
Interim sacrifice day 3										
Body weight (g)	306	--	--	315	--	203	--	--	196	
Liver weight (g) - abs.	9.2	--	--	10.2	--	5.9	--	--	5.7	
Liver weight (g) - rel.	3.0	--	--	3.2		2.9	--	--	2.9	
Terminal sacrifice day 14										
Body weight (g)	373	374	381	367	304**	231	228	226	223	213**
Liver weight (g) - abs.	9.9	10.7	11.7	11.7	14.7**	6.3	6.1	6.2	6.4	8.6**
Liver weight (g) - rel.	2.6	2.9	3.1**	3.2**	4.8**	2.7	2.7	2.7	2.9	4.1**
Thyroid weight (g) - abs.	0.016	0.019	0.016	0.020	0.019	0.015	0.012	0.014	0.013	0.015
Thyroid weight (g) - rel.	0.004	0.005	0.004	0.005	0.006**	0.006	0.005	0.006	0.006	0.007

Pos. Comtr. = positive control
** statistically different from control $p \leq 0.01$ -- no data

I. Gross necropsy

Trifluoroacetate: Only few gross pathology changes were noted and considered as incidental findings.
Positive control: At terminal sacrifice larger livers were observed in 2/5 males.

J. Micropathology

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 2/5 males at 2400 and 1200 ppm, respectively.

K. All other changes were considered to be incidental in origin and unrelated to the treatment.

III. Conclusions

The NOAEL is 600 ppm (43 /45 mg/kg bw/day males/ females) based on liver findings (increased organ weight in correlation with hepatocellular hypertrophy, increased cytochrome P-450, lauric acid hydroxylation activity, specific and total palmitoyl-CoA oxidation activities) in male rats.
Trifluoroacetate is a very weak peroxisome proliferator in male rats at doses ≥ 1200 ppm (85 mg/kg bw/day).

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 clofibric 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 carried out to meet regulatory purposes but allowed identification of TFA target organs in rat and provided indication for dose section of further short-term/subchronic regulatory studies.

Data Point:	KCA 5.8.1 / 54
Report Author:	[redacted]
Report Year:	2005
Report Title:	Sodium trifloroacetate (TFA) 28-day toxicity study in the rat by dietary administration
Report No:	SA 05054
Document No:	M-259106-01-1
Guideline(s) followed in study:	OECD 407 (1995); Directive 96/54/EC, Method B.7
Deviations from current test guideline: (OECD 407, 2008)	The study lacks the new requirement that were not included in the previous OECD 407 (1995) in force when the study was carried out. In particular Estrous cyclicity, circulating levels of T3, T4, TSH were not measured.
Previous evaluation:	Not evaluated
GLP/Officially recognised testing facilities:	Yes, yes
Acceptability/Reliability:	Yes, yes

Executive summary

Sodium trifluoroacetate (TFA) was administered continuously via the diet to groups of Wistar rats (5/sex/group) for 28 days at concentrations of 600, 1800, 5400 and 16000 ppm (equating approximately to 50, 149, 436 and 1315 mg/kg/day in males and 52, 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 tissues 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 23 % in females. When compared to the controls, total cholesterol concentration was decreased by 30 % in males and blood glucose concentrations were decreased by 29 % in males and by 30 % in females. Urine analysis revealed higher ketone levels in both sexes. At necropsy, mean absolute and relative liver weights were increased by between 24 to 33 % in males and 15 to 24 % in females. In addition enlarged livers were observed in 4/5 males and 4/5 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 no effect 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 ketone 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 at all 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 Level (NOAEL) of Sodium Trifluoroacetate (TFA) was 16000 ppm in both sexes over a 28-day administration period (equivalent to 1315 mg/kg body weight/day in males and 1344 mg/kg body weight/day in females).

I. Materials and methods

A. Materials

1. Test material:	Sodium Trifluoroacetate
Description:	white crystals
Lot/Batch no:	01691171
Purity:	99.1%
Stability of test compound:	stable under the test conditions
2. Vehicle and / or positive control:	None
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj: WI (TOPS HAN)
Age:	6 weeks approximately
Weight at dosing:	192 to 223 g Males; 161 to 186 g, females
Source:	[redacted]
Acclimatisation period:	at least 6 days
Diet:	Certified and irradiated rodent powder diet A04C-10 PI from U.A.R. (Usine d'Alimentation Rationnelle, Villemoisson-sur-ORGE, France) *ad libitum*
Water:	Tap water (filtered and softened), *ad libitum*
Housing:	Animals were housed individually in suspended stainless steel wire mesh cages
Environmental conditions:	
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: The study was carried out at Bayer CropScience, Sophia Antipolis, France. from 2 March 2005 to 16 June 2005

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 Animal assignment

Test group	Concentration in diet (ppm)	Animals assigned	
		Male	Female
1	0	5	5
2	600	5	5
3	1800	5	5
4	5400	5	5
5	16000	5	5

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 levels of the test substance were verified on the study preparation. Homogeneity and concentration checks of TFA in 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 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 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

Each animal was weighed twice 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.

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. Opthalmic 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 in all groups by puncture 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 bleeding and anesthetized by inhalation of isoflurane. Blood was collected on EDTA for hematology (0.5 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).

The following hematology parameters were measured 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, platelet count, prothrombin time. A blood smear was prepared and stained with Wright stain**.**

The following clinical chemistry parameters were measured, total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase, total protein, albumin, globulin and albumin/globulin ratio.

6. Urinalysis

On study Day 25, in the morning, prior to sacrifice, overnight urine samples were collected from all surviving animals in all groups. An approximately equal number of animals 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 urinary volume. The following parameters were analysed: pH, urinary refractive index, glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen. 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.

7. Sacrifice and pathology

On study Days 29 or 30, all surviving animals from all groups were sacrificed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of 60 mg/kg body weight). Animals were fasted overnight prior 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-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, esophagus, exorbital (lachrymal) gland, eye and optic nerve, Gall bladder, Harderian gland, heart, intestine (duodenum, jejunum, ileum, cecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, 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, 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, mammary 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 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).

II. Results and discussion

A. Mortality
No mortalities were noted during the study.

B. Clinical observations
No clinical signs were observed during the study in either sex.

C. Body weight
Body weight and body weight gain were not affected by treatment.

D. Food consumption
No effect on mean food consumption was noted in either sex.

E. Ophthalmology
There were no treatment related ophthalmological findings during the study in either sex.

F. Hematology
No treatment related effects.

G. Clinical chemistry
Slightly higher alanine aminotransferase activity (ALAT) was observed at 16000 ppm in both sexes (+37% in males and +23% in females). Decreased lower cholesterol concentration (CHOL) was noted in males ≥5400 ppm (-30% 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 chemistry

Parameter means (unit)	Dose group (ppm) males					Dose group (ppm) females				
	0	600	1800	5400	16000	0	600	1800	5400	16000
ALAT (IU/L)	38	44	45	43	52**	35	36	41	40	43*
CHOL (mmol/L)	2.14	1.69	1.65	1.51*	1.50*	1.75	1.84	1.60	1.86	2.00
GLUC (mmol/L)	5.77	4.01**	3.70**	4.25**	4.09**	6.17	4.32**	5.19	4.18**	4.32**

*, ** statistically different from control $p \leq 0.05$, $p \leq 0.01$

H. Urinalysis
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.

Table 5.8.1-42 Urinalysis summary

Parameter means (unit)	Dose group (ppm) males					Dose group (ppm) females				
	0	600	1800	5400	16000	0	600	1800	5400	16000
Ketones										
0.0 g/L	1	0	0	0	0	0	1	0	0	0
0.05 g/L	3	0	0	0	0	0	2	1	2	0
0.15 g/L	1	1	0	0	0	0	2	3	2	3
0.04 g/L	0	1	0	0	0	0	0	0	1	1
≥ 0.8 g/L	0	3	5	5	5	0	0	0	0	0
Volume mL	7.1	9.9	9.6	8.5	11.7*	2.0	4.4	2.5	3.2	5.3

* statistically different from control $p \leq 0.05$

I. Organ weight

At 16000 ppm, mean absolute and relative liver weights were higher and statistically different in both sexes, when compared to controls. At 5400 ppm in both sexes and at 1800 ppm in males, 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)	Male				Female			
	600	1800	5400	16000	600	1800	5400	16000
Mean absolute liver weight	NC	+6% NS	+9% NS	+24% p≤0.05	NC	NC	+7% NS	+15% p≤0.05
Mean liver to body weight ratio	NC	+15% p≤0.01	+19% p≤0.01	+33% p≤0.01	NC	+7% NS	+13% p≤0.05	+18% p≤0.01
Mean liver to brain weight ratio	NC	NC	+12% NS	+27% p≤0.01	NC	+10% NS	+12% NS	+24% p≤0.01

NC: no relevant change NS: not statistically significant

The other organ weight differences, even 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 changes were considered as incidental and not treatment related.

K. Micropathology

There were no treatment related histopathological changes. All histopathological findings encountered were considered to have arisen spontaneously.

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.

Assessment and conclusion by applicant:
This study was designed to provide information of the effects of Sodium trifluoroacetate (TFA) following dietary administration to the rats for 28-days. In this study, the NOAEL was the highest dose tested of 16000 ppm equivalent to 1315 mg/kg body weight/day in males and 1344 mg/kg body weight/day in females, because the few findings observed were not consistently correlated with other findings (like decreased blood glucose and increased level of urine ketones were not correlated with urinalysis changes and appropriate histopathological organs).
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 to be adverse in the absence of histopathological findings or other clinical chemistry parameters changes.
The study is valid and reliable, although the thyroid hormones and few ED parameters which are currently required by OECD TG 407 (2008) a have not been investigated.

Data Point:	KCA 5.8.1 / 55
Report Author:	[redacted]
Report Year:	2007
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 study:	O.E.C.D. guideline 408 (September 1998) E.E.C. Directive 2001/59/EC Method B.26 (August 2001) U.S. E.P.A., OPPTS Series 870, Health Effects Testing Guidelines, N°870.3100 (August 1998) M.A.F.F. in Japan 12 Nousan N°8147 (November 2000)
Deviations from current test guideline: (OECD 408, 2018)	The study lacks the new requirement that were not included in the previous OECD 408 (1998) in force when the study was carried out. The following parameters were not included: HDL, LDL, blood urea nitrogen, bile acids, total T4, T3 and TSH; Peyer's patches & coagulating glands; no vaginal smears prepared and examined, no sperm analysis performed.
Previous evaluation:	Not evaluated
GLP/Officially recognised testing facilities:	Yes /yes
Acceptability/Reliability:	Yes/yes

Executive summary

Sodium trifluoroacetate (TFA, batch number KTS 10279-1-1, white solid, 99% purity), was administered continuously via dietary administration to separate groups of Wistar rats (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, 1216 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 diet 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 during Week 14. Urine samples were collected overnight on the week before necropsy from all surviving animals. Before necropsy a blood sample was collected from the retro-orbital venous plexus of each surviving animal for hematology and clinical chemistry determinations. All animals were necropsied, 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 males 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 and 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 cytoplasm. 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 treatment. Clinical pathology determinations revealed lower mean hemoglobin concentration and mean corpuscular hemoglobin 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 aminotransferase and alanine aminotransferase activities of some individual values in males were higher and were also considered to be treatment related. At urinalysis, higher ketone levels were noted in both sexes.
At necropsy, mean absolute and relative liver weights were increased by 8 to 14% 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 hepatocellular 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 the response and the association with clinical pathology changes.
There were no adverse effects at 160 ppm and this 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 Level (NOAEL) in both sexes.

I. Materials and methods

A. Materials

1. Test material:	Sodium trifluoroacetate (TFA)
Description:	white solid
Lot/Batch no:	KTS 10279-1-1
Purity:	99%
Stability of test compound:	guaranteed for study duration; expiry date: 2006-10-05
2. Vehicle:	none
3. Test animals	
Species:	Wistar rat
Strain:	Rj:WI (IOPS HAN)
Age:	7 weeks
Weight at dosing:	males: 225 g - 259 g, females: 165 g - 200 g
Source:	[redacted]
Acclimatisation period:	at least 12 days

Diet:	certified rodent powdered and irradiated diet A04CP1 10 (S.A.F.E. (Scientific Animal Food and Engineering, Augy, France))
Water:	tap water
Housing:	suspended, stainless steel, wire-mesh cages

4. Environmental conditions

Temperature:	20 – 24 °C
Humidity:	40 - 70%
Lighting:	12-hour light, 12-hour dark cycles (7 am - 7pm)
Air changes:	Approximately 10 - 15 changes per hour

B. Study design

1. In life dates: The study was carried out at [redacted]. from 5 April 2006 to 13 February 2007. In-life dates from 12 April 2006 to 28 July 2006

2. Animal assignment and treatment

TFA was administered to Wistar rats by the oral route via a diet mixture up to the day before sacrifice. The dose levels were set after taking into account the results from a preliminary 14-day rat toxicity study (KCA 5.8.1/17), in which sodium trifluoroacetate was found to be a weak peroxisome proliferator. In addition, in a preliminary 28-day rat toxicity study (KCA 5.8.1/18), 16000 ppm was considered to be a No Observed Adverse Effect Level (NOAEL), on the basis of higher plasma alanine aminotransferase activity, lower total cholesterol and glucose concentrations, higher urinary ketone levels, and higher mean absolute and relative liver weights in both sexes, and in the absence of associated histopathological findings.

Groups of 10 male and 10 female rats were given control diet or the appropriate diet mixture.

Table 5.8.1-44: Animal assignment

Test group	Concentration in diet (ppm)	Animals assigned	
		Male	Female
1	0	10	10
2	160	10	10
3	1600	10	10
4	16000	10	10

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 test substance formulations were prepared approximately every four weeks. There were three preparations. When not in use the diet formulations were stored at room temperature. 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. Homogeneity and concentration checks of TFA in the diet were within 86 to 108% of the nominal concentration, with the exception of only one analysis out of eight at 16000 ppm (129%) which was outside the in-house target range of 85 to 115% of the nominal concentration. In isolation, this result was considered acceptable for the study. Therefore, homogeneity and concentration checks of TFA at 16000 ppm were considered to be acceptable. At 1600 and 160 ppm, concentrations and/or homogeneity were within the in-house target range and were therefore considered to be acceptable.

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 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. 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 out:

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 or tonic movements, stereotypic behaviour (e.g. excessive grooming, repetitive circling), bizarre behaviour (e.g. self-mutilation, walking backward) and other neurological-related changes were recorded for all surviving animals.

c. *Sensory reactivity*

The following reflexes and responses were recorded:

- Pupillary reflex (by covering the eyes of the animal for a few seconds and then observing pupillary constriction by focusing a narrow beam of light in the eyes).
- Surface righting reflex (by putting the animal on its back and measuring its ability/rapidity to reassume a normal standing position).
- Corneal reflex (by touching the medial canthus with a fine object and observing the quick and complete closure of the eyelids).
- Flexor reflex (by pinching the toes and measuring the presence/strength of the flexor response of each hindlimb).
- Auditory startle response (by measuring the animal response to an auditory stimuli).
- Tail pinch response (by pinching the tail with a forceps and measuring the animal reaction).

d. Grip strength

The fore- and hindlimb grip strength of all animals were measured quantitatively using a grip strength tester equipped with one pull of one push strain 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 twice 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 formula:

$$\text{Test substance intake (mg/kg/day)} = \frac{\text{Dose level (ppm) x Group mean food consumption (g/day)}}{\text{Group mean body weight (g) at the end of the food consumption period}}$$

5. Ophthalmic 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 14 of the treatment period, animals from control 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 plexus. An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were fasted overnight prior to bleeding and anesthetized by inhalation of isoflurane. Blood was collected on EDTA for hematology (0.5 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).

The following hematology parameters were measured 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, platelet count, prothrombin time. A blood smear was prepared and stained with Wright stain.

The following clinical chemistry parameters were measured: total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase, total protein, albumin, globulin and albumin/globulin ratio.

7. Urinalysis

On study Day 87 or 88, in the morning, prior to sacrifice, overnight urine samples were collected from all surviving animals in all groups. An approximately equal number of animals 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 urinary volume.
The following parameters were analysed: pH, urinary refractive index, glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen. 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.

8. Sacrifice and pathology

On study Days 93, 94 or 95, all surviving animals from all groups were sacrificed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of 60 mg/kg body weight). Animals were fasted overnight prior 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-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, esophagus, exorbital (lachrymal) gland, eye and optic nerve, Gall bladder, Harderian gland, heart, intestine (duodenum, jejunum, ileum, cecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, 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, 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, mammary 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.

9. 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 one or more group variance(s) equalled 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 PathTox System V4.2.2. (Module Enhanced Statistics).

II. Results and discussion

A. Test substance analysis

See section 'Diet preparation and analysis'.

B. Mortality

One male from the 1600 ppm group was found dead on study day 15. At necropsy, this animal was noted to have 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.

C. In-life observations

No treatment-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 limited 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 level 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 fortuitous and due to a particularly high mean value in the control group. Furthermore, the mean value observed in the high dose females for this parameter was within the in-house historical control range

E. Ophthalmology

There was no evidence of treatment-related effects 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 iris of the left eye.

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 reduction in mean body weight gain of 17% on day 92, when compared to controls. The effect was statistically significant at most time points (p≤0.01 or 0.05). 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. The effect was statistically significant on a number of occasions for cumulative body weight gain (p≤0.01 or 0.05).
Body weight parameters were not affected in either sex at 1600 ppm and at 160 ppm.

Table 5.8.1-45: Summary of mean body weights (g)

Dose (ppm)	Males Mean body weight (g) on study day 1	8	15	22	29	36	43	50	57	64	71	78	85	92
0	245	299	348	384	412	442	466	485	503	516	524	535	543	550
160	246	298	348	384	410	438	461	480	498	509	516	530	536	544
1600	244	294	342	376	401	431	449	470	483	496	499	514	522	529
16000	243	291	332*	359+	378+	404+	421+	439+	450+	465+	471+	482+	490+	496+
Dose (ppm)	**Females Mean body weight (g) on study day 1**	**8**	**15**	**22**	**29**	**36**	**43**	**50**	**57**	**64**	**71**	**78**	**85**	**92**
0	182	204	220	228	239	249	260	264	269	271	274	278	280	282
160	181	203	222	230	238	248	256	264	271	276	276	277	280	283
1600	183	200	217	228	237	244	251	257	262	268	271	275	277	284
16000	183	199	214	223	231	239	245	253	255	258	260	264	267	270

* Statistically significant different from control ($p \leq 0.05$)
+ Statistically significant different from control ($p < 0.01$)

In males, there was a dose-related trend towards lower terminal body weight when compared to controls, the effect being statistically significant at 16000 ppm (-11 %, p≤0.01). In females, the mean terminal body weight was slightly lower at 16000 ppm (not statistically significant).

G. Food consumption and compound intake

Up to the highest dose level tested food consumption was not affected in either sex.

Table 5.8.1-46: Achieved intake (mg/kg bw/day)

Diet concentration (ppm)	160	1600	16000	160	1600	16000
	Males			Females		
Weeks 1 to 13	9.9	98	1043	12.2	123	1216

H. Haematology

Treatment-related changes were noted only in females at 16000 and 1600 ppm.

When compared to the controls, lower mean haemoglobin concentration (−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 haemoglobin (−7%, p≤0.01) and haematocrit (−6%, p≤0.01).

At 1600 ppm, lower mean haemoglobin 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.

No treatment-related change was noted in males at any dose level and in females at 160 ppm.

The few other statistically significant differences were considered to be incidental in view of their occurrence at the lowest dose and/or their low magnitude.

Table 5.8.1-47 - Summary of haematology parameter changes in females

Parameter / Dose (ppm)	Mean ± SD (% change when compared to control) Hb (g/dL)	MCV (fl)	Hct (L/L)	MCH (pg)
0	15.6 ± 0.7 (--)	52 ± 1 (--)	0.462 ± 0.019 (--)	17.4 ± 0.4 (--)
160	15.6 ± 0.4 (±0%)	51 ± 2 (–2%)	0.467 ± 0.010 (+1%)	17.1 ± 0.5 (–2%)
1600	14.9 ± 0.6* (–4%)	50 ± 1 (–4%)	0.448 ± 0.018 (–3%)	16.8 ± 0.3** (-3%)
16000	14.4 ± 0.4** (–8%)	49 ± 1** (–6%)	0.435 ± 0.010** (–6%)	16.2 ± 0.3** (–7%)

Hb = haemoglobin concentration; MCV = mean corpuscular volume; Hct = haematocrit;
MVH = mean corpuscular haemoglobin
* = statistically significant different from control (p≤0.05); ** = statistically significant different from control (p≤0.01)

I. Clinical chemistry

Treatment-related changes were observed at 16000 and 1600 ppm in both sexes. Mean total bilirubin and glucose concentrations were lower in both sexes and mean alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase activities were higher in males only.
The slightly lower mean total bilirubin concentration noted at 160 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 5.8.1-48: Summary of clinical chemistry parameter changes in males and females

Parameter / Dose (ppm)	Mean ± SD (% change when compared to control) Bili (mmol/L)	Gluc (mmol/L)	ALP (IU/L)	AST (IU/L)	ALT (IU/L)
males					
0	1.6 ± 0.4 (--)	5.87 ± 0.53 (--)	80 ± 9 (--)	89 ± 37 (--)	47 ± 25 (--)
160	1.1 ± 0.2 (–31%)	5.40 ± 0.64 (–8%)	68 ± 11 (–15%)	83 ± 21 (–7%)	47 ± 20 (±0%)
1600	0.5 ± 0.1** (–69%)	4.21 ± 0.44** (–28%)	106 ± 18 (+33%)	146 ± 118 (+64%)	87 ± 84 (+85%)
16000	0.3 ± 0.2** (–81%)	4.14 ± 0.84** (–29%)	156 ± 39** (+95%)	111 ± 24 (+25%)	65 ± 19* (+38%)
females					
0	2.1 ± 0.5 (--)	5.57 ± 0.86 (--)	50 ± 12 (--)	73 ± 12 (--)	38 ± 9 (--)
160	1.8 ± 0.4 (–14%)	5.13 ± 0.56 (–8%)	45 ± 10 (–10%)	82 ± 17 (+12%)	40 ± 10 (+5%)
1600	1.0 ± 0.6** (–52%)	4.19 ± 0.45** (–25%)	53 ± 15 (+6%)	87 ± 16 (+19%)	47 ± 17 (+26%)
16000	0.5 ± 0.3** (–76%)	4.62 ± 1.11** (–17%)	50 ± 12 (±0%)	85 ± 12 (+16%)	45 ± 5 (+18%)

Bili = total bilirubin; Gluc = glucose; ALP = alkaline phosphatase; AST = aspartate amino transferase
ALP = alanine amino transferase
* = statistically significant different from control (p≤0.05); ** = statistically significant different from control (p≤0.01)

Several males from all treated and control groups had elevated aspartate aminotransferase and alanine aminotransferase 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.

Table 5.8.1-49: Semi-quantitative urinalysis- incidence summary table

Dose (ppm)		0	160	1600	16000	0	160	1600	16000
		males				females			
n° samples examined		10	10	8	10	9	10	10	10
Glucose	0	10	10	8	10	9	10	10	10
	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0
Bilirubin	0	10	10	8	10	9	10	10	10
	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0
Ketones	0	0	0	0	0	6	10	3	0
	1	1	1	0	0	3	0	1	0
	2	9	7	0	0	0	0	6	3
	3	1	2	0	0	0	0	0	4
	4	0	0	8	10	0	0	0	5
Occult blood	0	10	10	8	9	9	10	10	10
	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	0	0	0	1	0	0	0	0
	4	0	0	0	0	0	0	0	0
Protein	0	0	0	0	0	9	10	10	10
	1	0	1	0	0	0	0	0	0
	2	9	8	8	8	0	0	0	0
	3	1	0	0	2	0	0	0	0
	4	0	1	0	0	0	0	0	0
Urobilinogen	0	10	10	8	10	9	10	10	10
	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0

K. Organ weight

Mean absolute and relative liver weight were statistically significantly higher in male and female rats at 16000 and 1600 ppm when compared to controls. These changes were dose- and treatment related and associated with hepatocellular hypertrophy.

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: Summary of liver weight data at terminal sacrifice

Parameter	Mean (% change when compared to control)		
	Absolute liver weight (g)	Liver to body weight ratio	Liver to brain weight ratio
Dose (ppm)	males		
0	12.15 (--)	2.327 (--)	566.930 (--)
160	11.61 (–4%)	2.258 (–3%)	546.177 (–4%)
1600	13.25* (+9%)	2.657** (+14%)	613.081 (+8%)
16000	14.48 (+19%)	3.102** (+33%)	701.329** (+24%)
Dose (ppm)	females		
0	5.96 (--)	2.243 (--)	307.108 (--)
160	6.25 (+5%)	2.343 (+4%)	316.173 (+3%)
1600	6.71* (+13%)	2.520** (+12%)	334.508 (+9%)
16000	7.36** (+23%)	2.880** (+28%)	382.160** (+24%)

* = statistically significant different from control ($p \le 0.05$); ** = statistically significant different from control ($p \le 0.01$)

L. Gross necropsy findings

a. *Unscheduled death*

One male was found dead on study day 15. This animal was noted to have torsion and a dark content within the ileum and jejunum. This intestinal torsion was considered to be the cause of death and was therefore incidental. All other macroscopic findings were related to agonal changes found at the histopathology examination and were considered not to be treatment-related.

b. *Terminal sacrifice*

With the exception of the higher incidence of foci (red or white) within the liver observed in males at 16000 ppm, all the other changes were considered to be incidental and not treatment-related

M. Histopathology

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 strain 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 sacrifice*

Treatment-related histopathological changes were observed in the liver.

In all male and most females at 16000 ppm, as well as in a proportion of males at 1600 ppm, a minimal to moderate diffuse centrilobular to panlobular hepatocellular hypertrophy with ground-glass appearance of the hepatocellular cytoplasm was observed. This latter observation is usually induced by peroxisome proliferators. This change was associated with a loss of the periportal hepatocellular vacuolation observed at 16000 ppm in both sexes and at 1600 ppm in males. The effect was dose-related and correlated with the higher mean liver weight noted in these groups.

There was also a higher incidence 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 incidence 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 under laboratory conditions.

Table5.8.1-51: Incidence and severity of microscopic changes in the liver, all animals, terminal sacrifice

Dose (ppm)	**0**	**160**	**1600**	**16000**	**0**	**160**	**1600**	**16000**
Sex	**Males**				**Females**			
Number of animals examined	10	10	9	10	10	10	10	10
Centrilobular to panlobular hepatocellular hypertrophy, diffuse								
Minimal	1	0	3	1	0	0	0	5
Slight	0	0	2	6	0	0	0	4
Moderate	0	0	0	3	0	0	0	0
Total	1	0	5	10	0	0	0	9
Periportal hepatocellular vacuolation, diffuse								
Minimal	4	3	0	0	5	6	7	0
Total	4	3	0	0	5	6	7	0
Hepatocellular necrotic focus (i), focal/multifocal								
Minimal	1	0	1	5	1	0	1	1
Slight	1	1	1	1	0	1	0	0
Moderate	1	1	1	1	0	0	0	0
Total	3	2	3	7	1	1	1	1

III. Conclusion

Based on the study results (changes in haematological and clinical chemistry parameters, organ weights and histopathological liver findings) the NOAEL of the present study was established at 160 ppm in both sexes after 90-day exposure to sodium trifluoroacetate (TFA) which is equivalent to 10 / 12 mg/kg bw/day in males and females.

Assessment and conclusion by applicant:
This study was designed to provide information of the effects of Sodium trifluoroacetate (TFA) following dietary administration to the rats 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 valid and reliable, although the ED parameters which are currently required by OECD TG 408 (2018) and other few parameters have not been investigated.

CA 5.8.2 Supplementary studies on the active substance

Data Point:	KCA 5.8.2/01
Report Author:	[redacted]
Report Year:	2010
Report Title:	Fluopyram: 28-day immunotoxicity study in the female Wistar rat by dietary administration
Report No:	SA 09357
Document No:	M-387119-01-1
Guideline(s) followed in study:	EPA OPPTS 870.7800 (1998)
Deviations from current test guideline:	Current guideline: not applicable
Previous evaluation:	No, not previously submitted.
GLP/Officially recognised testing facilities:	Yes (certified laboratory)
Acceptability/Reliability:	Yes

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, 17.2, 53.6, 156.3 mg/kg body weight/day). An additional group of 10 female Wistar rats were administered cyclophosphamide (CPS) daily by gavage 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 with an intravenous injection of sheep red blood cells (sRBC) on study day 24. All animals were euthanized on study day 30.

Animals were observed daily for mortality and clinical signs. Body weight and food consumption were recorded once weekly. A detailed physical 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 animal on day 30 (just before necropsy) for specific anti-SRBC immunoglobulin M (IgM) analysis. All animals were necropsied, gross pathology observations were performed and selected organs (spleen and thymus) weighed.

All animals survived the scheduled necropsy. 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 administration.

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 of CPS 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-observed-effect-level (NOAEL) of 1800 ppm (equivalent to 156 mg/kg bodyweight/day) for the immunological parameters investigated.

I. Materials and methods

A. Materials

1. Test material:	AE C656948
Description	Light beige solid
Lot / Batch #:	08528/0002
Purity:	94.7% w/w
CAS #	Not stated
Stability of test compound:	Stable in rodent diet for a period covering the study duration (Expiry date: 24 February 2011)
2. Vehicle and / or positive control:	Basal diet / cyclophosphamide monohydrate (CPS)
3. Test animals:	
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN)
Age:	7 weeks approximately at start of dosing
Weight at dosing:	170-209g (females)
Source:	[redacted]
Acclimation period:	12 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Rats were housed individually in suspended stainless-steel 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: 27 January 2010 – 24 June 2010

2. Animal assignment and treatment

There were 10 female animals per dose group. Animals were assigned to dose groups randomly by body weight. AE 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-085510-01-1) 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 results were within 98 and 101% of the nominal concentration. All these results were therefore within the acceptable in-house target ranges.

Table 5.8.2-1 Study design

Test group	Concentration in diet (ppm)	Dose per animal (study averages)	Animals assigned
1	0	0	10
2	200	10.2	10
3	600	33.6	10
4	1800	156.3	10
Test group (CPS)	**Concentration (mg/kg/day)**	**Dose per animal (study averages)**	**Animals assigned**
5	3.5	3.5	10

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 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 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 nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

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 cyclophosphamide) using the following formula:

$$\text{Achieved dosage intake (mg/kg bodyweight/day)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day) per week}}{\text{Group mean body weight (g) at end of week}}$$

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 in PBS (Phosphate Buffered Saline), counted using a cell counting instrument (Siemens Advia 120) and diluted in PBS in order to obtain a 5 x 10^8 cells/mL preparation. On day 26, all animals in all groups were immunized by intravenous injection in the tail 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 retro-orbital venous plexus 4 days after SRBC immunization prior to terminal sacrifice. Animals were not diet fasted. Blood (0.5 ml) was placed into tubes with clot activator (for serum 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 30, a complete necropsy was performed on all surviving animals. Animals were deeply anesthetized by Isoflurane inhalation, then exsanguinated 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.

II. Results and discussion

A. Clinical signs and mortality

1. Clinical signs of toxicity

There were no clinical signs evident in any group.

2. Mortality

There was no mortality in any group.

B. Body weight and body weight gain

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 significant. No treatment-related effects on body weight parameters were observed at 200 and 600 ppm. Body weight parameters in females were unaffected by treatment with cyclophosphamide.

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 (CPS)
Sex	Females				
BW Day 1	187±11	189±8	188±9	189±8	187±10
BW Day 8	208±11	209±10	212±12	207±9	205±10
BW Day 15	226±13	224±17	229±11	228±8	221±16
BW Day 22	242±15	241±17	250±15	238±9	239±18
BW Day 29	253±18	251±16	258±9	246±11	248±15
Body weight gain					
BWG Day 8	2.9±0.9	2.8±0.7	3.3±1.0	2.6±0.6	2.7±0.6
BWG Day 15	2.5±0.5	2.2±1.2	2.4±0.7	2.1±0.5	2.4±1.3
BWG Day 22	2.4±0.7	2.4±0.7	3.1±1.5	2.3±0.7	2.6±0.7
BWG Day 29	1.5±0.9	1.5±0.7	1.2±0.8	1.1±0.6	1.2±0.8

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-related effects on food consumption were observed at 200 and 600 ppm. At 3.5 mg/kg body weight/day, mean 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 weight/day.

D. Ophthalmoscopic examination

Ophthalmoscopic examination was not conducted in this study.

E. SRBC-specific IgM 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 high mean anti-SRBC IgM concentration observed in the control group confirmed the sensitization of the animals. No relevant treatment-related change in SRBC-specific IgM concentration was observed after fluopyram administration.

In the positive control group (CPS), at 3.5 mg/kg/day mean anti-SRBC IgM concentration was markedly lower (-89%) when compared to the control group.

Table 5.8.2-3 Mean T-cell dependent antibody responses in the 28-day immunotoxicity study with AE C656948 (mean ± SD)

Parameter	AE C656948 Dosage level (ppm)				CPS (3.5 mg/kg/day)
	0	200	600	1800	
IgMa (u/mL)	8706 ± 7820	7192 ± 4510	11918 ± 11218	8097 ± 3737	933 ± 399*

*: p≤0.05
CPS - cyclophosphamide

F. Sacrifice and pathology

1. Terminal body weights and organ weight

There were no changes in terminal body weights at any dose. At 1800 ppm, there were no treatment-related 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). However, 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.

Table 5.8.2-4 Organ weight changes in the 28-day immunotoxicity study with AE C656948 (mean ± SD)

Parameter	AE C656948 Dosage level (ppm)				CPS (3.5 mg/kg/day)
	0	200	600	1800	
Females					
Spleen weight Absolute (g)	0.741 ± 0.089	0.731 ± 0.109 (-1%)	0.742 ± 0.117 (0%)	0.674 ± 0.096 (-9%)	0.584 ± 0.079** (-21%)
Bodyweight-relative	0.2862 ±0.0290	0.2894 ± 0.0384 (+1%)	0.2857 ± 0.0431 (0%)	0.2696 ± 0.0446 (-6%)	0.2321 ±0.0291** (-19%)
Thymus weight Absolute (g)	0.572 ± 0.113	0.598 ± 0.134	0.597 ± 0.108	0.582 ± 0.115	0.434 ± 0.103* (-24%)
Bodyweight-relative	0.2214 ±0.0442	0.2352 ± 0.0419	0.2303 ± 0.0413	0.2320 ± 0.0448	0.1731 ± 0.0452* (-22%)

*: p≤0.05; **: p≤0.01; Figures in parentheses are % differences from control

G. Deficiencies

No specific deficiencies were noted in the study.

III. Conclusions

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 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 immunotoxicological information on the effects of AE C656948 in the rat.

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 ppm (corresponding to approximately 156 mg/kg body weight/day) is considered to be a NOAEL for the immunotoxicological parameters investigated

CA 5.8.3 Endocrine disrupting properties

a. Human estrogen receptor (ERalpha) binding assay

Data Point:	KCA 5.8.3/01
Report Author:	[redacted]
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:	M-632695-01-1
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 recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The purpose of this study was to test Fluopyram Technical Estrogen ERalpha binding and functional assays.

(The functional assays are described under point d. Estrogen receptor transactivation (OECD TG 457) - ERalpha - agonist and antagonist functional assay (KCA 5.8.3/04))

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 estrogen receptor (ERalpha).

Under the conditions of the test used, Fluopyram Technical shows no indication of binding to the alpha estrogen receptor (ERalpha).

I. Materials and methods

A. Materials

1. **Test material:** Fluopyram Technical
   Description Powder
   Lot / Batch #: Mix-batch 08528/0002
   Purity: 94.5%
   CAS # 658066-35-4
   Stability of test compound: Stable until 20 April 2020 (when stored at +10 to +30°C)
2. **Vehicle:** Dimethylsulfoxide

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 Eurofins. The experiment is accepted in accordance with Eurofins validation Standard Operating Procedure.

4. Experimental conditions:
Binding Assay

Receptor:	Estrogen ER alpha (h) (agonist radioligand)
Source:	Human recombinant (sf9 cells)
Ligand:	[3H] Estradiol
Concentration:	0.5 nM
Kd:	0.20 nM
Non Specific:	Diethylstilbestrol (1µM)
Incubation:	120 min RT
Detection method:	Scintillation counting
Assay volume and format:	200 µL in 96-well plates
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

Experiemental dates: 13 June 2018- 20 June 2018

B1 Binding Assay - Estrogen ER alpha (h) (agonist radioligand)

1. Purpose: Evaluation of the affinity of compounds for the humanestrogen receptor (ER alpha) expressed in transfected Sf9 cells, determined in a radioligand binding assay.

2. Experimental protocol:

Full length receptor (10 ng) is incubated for 120 min at 22°C with 0.5 nM [3H] Estradiol in the absence or presence of the test compound in a buffer containing 10 mM Tris/HCl (pH 7.4), 10 % glycerol, 1mM DTT and 0.1% BSA.

Nonspecific binding is determined in the presence of 1µM diethylstilbestrol.

Following incubation, the samples are filtred rapidly under vacuum through glass fiber filters (GFB, 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 cocktail (Microscint 0, Packard).

The results are expressed as a percent inhibition of the control radioligand specific binding.

The standard reference compound is diethylstilbestrol, which is tested in each experiment at several concentrations to obtain a competition curve from which its IC_{50} is calculated.

Reference: Obourn JD et al (1993), Hormone- and DNA-binding mechanisms of the recombinant human estrogen receptor. Biochemistry 32(24): 6229 - 6236.

3. Analysis 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 inhibition of control specific binding) and Hill coefficients (nH) were determined by non-linear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting

$$Y = D + [\frac{A-D}{1+(C/C_{50})^{nH}}]$$

where Y = specific binding, A = left asymptote of the curve, D = right asymptote of the curve, C = compound concentration, C_{50} = IC_{50}, and n_H = 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.).

The inhibition constants (K_i) were calculated using the Cheng Prusoff equation

$$Ki = \frac{IC_{50}}{(1 + L/K_D)}$$

where L = concentration of radioligand in the assay, and KD = affinity of the radioligand for the receptor. A scatchard plot is used to determine the KD.

4. Results interpretation guide:

Results showing an inhibition (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 IC_{50} or EC_{50} values from concentration-response curves) that the laboratory recommends.

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 level.

Low to moderate negative values have no real meaning and are attributable to variability of the signal around the control level. High negative values (≤ 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

Binding Assays

Table 5.8.3-1 Test compound results

Substance/Concentration			% Inhibition of Control Specific Binding		
Compound I.D.	Client Compound I.D.	Test Concentration	1st	2nd	Mean
Estrogen ER alpha (h) (agonist radioligand					
100044628-1	Fluopyram Technical	1.0E-05 M	-14.9	-36.3	-25.6
100044628-1	Fluopyram Technical	1.0E-04 M	-11.0	37.1	-24.1

A. Deficiencies

None

III. Conclusions

Under the conditions of the test used Fluopyram Technical shows no indication of binding to the alpha estrogen receptor (ERalpha).

Assessment and conclusion by applicant:

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

Fluopyram Technical shows no indication of binding to the alpha estrogen receptor (ERalpha).

b. Estrogen (ERbeta) Binding Assay

Data Point:	IIA 5.8.3/02
Report Author:	[redacted]
Report Year:	2018
Report Title:	*In vitro*: Effect of Compound Fluopyram Technical on Estrogen ERbeta Binding Assay
Report No:	Study No: ER095-0005801 Client Activity Id: YXGM0123
Document No:	M-632859-01-1
Guideline(s) followed in study:	No specific guideline but based on OECD TG 493
Deviations from current test guideline:	None
Previous evaluation:	Not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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 µM.

Compound binding was calculated as a % inhibition of the binding of a radioactively labeled estradiol, ligand specific for the beta estrogen receptor (ERbeta).

Under the conditions of the test used, Fluopyram Technical shows no indication of binding to the beta estrogen receptor (ERbeta).

I. Materials and methods

A. Materials

1. Test material:	Fluopyram Technical
Description	Powder
Lot / Batch #:	Mix-batch 08528/0002
Purity:	94.5%
CAS #	658066-35-4
Stability of test compound:	Stable until 20 April 2020 (when stored at +10 to +30°C)
2. Vehicle:	Dimethylsulfoxide
3. Reference substance:	Diethylstilbestrol
4. Experimental conditions:	
Source:	Human recombinant insect Sf9 cells
Vehicle:	1.00% DMSO
Incubation Time/Temp	2 hours @ 25°C
Incubation Buffer	10 mM Tris-HCl, pH 7.4 10% Glycerol, 1 mM DTT 0.1% BSA
Kd	0.13nM*
Ligand	0.50 nM [3H] Estradiol
Non Specific Ligand:	1.0 µM Diethylstilbestrol
Specific Binding:	90%*
Quantitation Method:	Radioligand Binding
Significance Criteria:	≥50% of max inhibition
Bmax:	3000 pmole/mg Protein *

B. Study design and methods

1. Experimental dates: 12 June 2018- 21 June 2018

2. Purpose: The objective of this study is to investigate the effect of compound Fluopyram Technical on Estrogen ERbeta Binding Assay.

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 DMSO to 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 aliquot 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)}×100

a: average cpm of non-specific binding

b: average cpm of total binding

c: cpm in the presence of test or reference substance

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 ERbeta Binding Assay

Cat#	Assay Name	Batch*	Spec.	Rep.	Conc.	% Inh.	IC_{50}*	Ki	n_H	R
Compound: Fluopyram, PT # 1219310										
226050	Estrogen ERβ	423211	hum	2	100µM	14		-	-	-
			hum	2	10µM	10		-	-	-

No significant responses (≥ 50% inhibition or stimulation for Biochemical assays) were noted.

A. Deficiencies

None

III. Conclusions

Under the conditions of the test used, Fluopyram Technical shows no indication of binding to the beta estrogen receptor (ERbeta).

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013. Fluopyram Technical shows no indication of binding to the beta estrogen receptor (ERbeta).

c. Androgen receptor (AR) Binding Assay

Data Point:	KCA 5.8.3/03
Report Author:	[redacted]
Report Year:	2018
Report Title:	*In vitro* Pharmacology: Human AR (h) (agonist radioligand) Receptor Binding Assay Study of Fluopyram Technical BAYER SAS Study Number: TXGM0123
Report No:	100044626_TXGM0123
Document No:	M-632697-01-1
Guideline(s) followed in study:	No specific guideline but based on OECD TG 493
Deviations from current test guideline:	None
Previous evaluation:	Not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The purpose of this study was to test Fluopyram Technical in the AR (h)(agonist radioligand) assay.

Fluopyram Technical was tested at 1.0E-05 M and 1.0E-04 M (equivalent to 10 µM and 100 µM).

Compound binding was calculated as a % inhibition of the binding of a radioactively labeled ligand specific for the androgen receptor (AR).

Under the conditions of test used, Fluopyram Technical shows no indication of binding to the androgen receptor.

I. Materials and methods

A. Materials

1. **Test material:** Fluopyram Technical
   Description Powder
   Lot / Batch #: Mix-batch 08528/0002
   Purity: 94.5%
   CAS # 658066-35-4
   Stability of test compound: Stable until 20 April 2020 (when stored at +10 to +30°C)
2. **Vehicle:** Dimethylsulfoxide
3. **Reference substance:** Testosterone
4. **Experimental conditions:**
   Receptors: AR (h)(agonist radioligand)
   Source: LNCaP cells (cytosol)
   Ligand: [^{3}H]methyltrienolone
   Concentration: 1 nM
   Kd: 0.8 nM
   Non Specific: Testosterone (1 µM)
   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 LNCaP cells determined in a radioligand binding assay.

3. Experimental protocol:

Fractions of cell cytosol (10_6 cell/point) are incubated for 24 h at 4°C with 1 nM [$_3$H]methyltrienolone 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 Na_2MoO_4, 2 mM DTT, 5 µM triamcinolone acetonide and 10% glycerol.

Nonspecific binding is determined in the presence of 1 µM testosterone.

Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/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 (Microscint 0, Packard).

The results are expressed as a percent inhibition of the control radioligand 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 IC_{50} is calculated.

Reference: *Zava DT et al (1979), Androgen receptor assay with [^{3}H]Methyltrienolone (R1881) in the presence of progesterone receptor, Endocrinology 104: 1007.*

4. Analysis 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

$$100 - \left(\frac{measured\ specific\ binding}{control\ specific\ binding} \times 100\right)$$

obtained in the presence of Fluopyram Technical.

The IC50 values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (nH) were determined by non-linear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting

$$Y = D + \left[\frac{A-D}{1+(C/C_{50})^{nH}}\right]$$

where Y = specific binding, A = left asymptote of the curve, D = right asymptote of the curve, C = compound concentration, $C_{50} = IC_{50}$, and n_H = 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.).

The inhibition constants (K_i) were calculated using the Cheng Prusoff equation

$$Ki = \frac{IC_{50}}{(1 + L/K_D)}$$

where L = concentration of radioligand in the assay, and KD = affinity of the radioligand for the receptor. 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 50% are considered to represent significant effects of the test compounds. 50% is the most common cut-off value for further investigation (determination of IC_{50} or EC_{50} values from concentration-response curves) 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 level.

Low to moderate negative values have no real meaning and are attributable to variability of the signal around the control level. High negative values (≥ 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: Fluopyram Technical

Compound I.D.	Client Compound I.D.	Test Concentration	% Inhibition of Control Specific Binding		
			1st	2nd	Mean
AR (h) (agonist radioligand)					
100044626-1	Fluopyram Technical	1.0E-05 M	-2.0	-2.8	-2.4
		1.0E-04 M	-2.2	-2.2	-1.4

Table 5.8.3-4 Results of Human AR (h) (agonist radioligand) Receptor Binding Assay: Testosterone

Compound I.D.	IC50 (M)	Ki (M)	nH
AR (h) (agonist radioligand)			
Testosterone	4.6E-09 M	2.0E-09 M	1.0

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 current guidance and the requirements in 283/2013. Fluopyram Technical shows no indication of binding to the androgen receptor.

d. Estrogen ERalpha agonist and antagonist functional assay

Data Point:	KCA 5.8.3/04
Report Author:	[redacted]
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:	M-632695-01-1
Guideline(s) followed in study:	No specific guideline but based on OECD TG 493 and 457
Deviations from current test guideline:	None
Previous evaluation:	Not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The purpose of this study was to test Fluopyram Technical Estrogen ERalpha binding and functional assays.

(The Estrogen ERalpha binding assay is described under point a. Estrogen or androgen receptor binding affinity (OECD TG 493) - Human estrogen receptor (Eralpha) binding assay (KCA 5.8.3/01)

Fluopyram Technical was tested at 1.0E-05 M and 1.0E-04 M (equivalent to 10µM and 100 µM).

Cellular agonist effect was calculated as a % of control response to a known reference agonist for the specified target and cellular antagonist effect was calculated as a % inhibition of control reference agonist response for the specified target.

Under the conditions of the test used, the functional assays showed that Fluopyram Technical had neither an agonist or antagonist effect on the alpha estrogen receptor.

I. Materials and methods

A. Materials

1. Test material:	Fluopyram Technical
Description	Powder
Lot / Batch #:	Mix-batch 08528/0002
Purity:	94.5%
CAS #	658066-35-4
Stability of test compound:	Stable until 20 April 2020 (when stored at +10 to +30°C)
2. Vehicle:	Dimethylsulfoxide
3. Reference substance:	ERalpha *(h)* (agonist effect) - Estriol ERalpha *(h)* (antagonist effect) - 4-OH tamoxifen In each experiment and if applicable, the respective reference compound was tested concurrently with Fluopyram Technical, and the data was compared with historical values determined at Eurofins. The experiment is accepted in accordance with Eurofins validation Standard Operating Procedure.

4. Experimental conditions:

Cellular and Nuclear Receptor Functional Assays

Agonist effect

Receptors:	ERalpha (h) (agonist effect)
Source:	Human recombinant
Stimulus:	none (1µM Estriol for control)
Incubation:	RT
Measured component:	Coactivator recruitment
Detection method:	AlphaScreen

Antagonist effect:

Receptors:	ERalpha (h) (antagonist effect)
Source:	Human recombinant
Stimulus:	Estradiol (30 nM)
Incubation:	RT
Measured Component:	Coactivator recruitment
Detection Method:	AlphaScreen

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

Experiemental dates: 13 June 2018- 20 June 2018

B2 Cellular and Nuclear Receptor Functional Assays - ERalpha (h) (agonist effect)

1. Purpose: Evaluation of the agonist 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 protein) is mixed with 200 nM biotin-tagged-SRC1-4 coactivator and 0.4 µg fluorescence acceptor (anti-GST antibody coupled-beads) in a buffer containing 20 mM Hepes/NaOH (pH 7.4), 80 mM Nacl, 0.08 % Tween 20, 0.08% BSA and 0.8 mM DTT.

The mixture is then incubated for 30 min at 22°C in the presence of one of the following: incubation buffer (basal control), the reference agonist estriol at 1 µM (EC_{100}, stimulated control) or at various concentrations (EC_{50}-determination), or the test compounds.

Thereafter, the fluorescence donor (streptavidin coupled-beads) is added at a final concentration of 0.4 µg.

Following 120 min at 22°C, the signal is measured at λex=680 nm and λem=520 and 620 nm using a microplate reader (EnVision, Perkin Elmer).

The results are expressed as a percent of the control response to 1 µM estriol.

The standard reference agonist is estriol, which is tested in each experiment at several concentrations to generate a concentration-response curve from which its EC_{50} value is calculated.

Reference: *Lau J et al (2003), A Homogeneous in vitro Functional Assay for Estrogen Receptors: Coactivator Recruitment, Mol.Endocrinol., 17:346.*

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

$$100 - (\frac{measured\ response}{control\ response} \times 100)$$

obtained in the presence of Fluopyram Technical.

The EC50 values (concentration producing a half-maximal response) and IC50 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 values using Hill equation curve fitting

$$Y = D + [\frac{A-D}{1+(C/C_{50})^{nH}}]$$

where Y = response, A = left asymptote of the curve, D = right asymptote of the curve, C = compound concentration, and C50= EC50 or IC50, 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.).

For the antagonists, the apparent dissociation constants (KB) were calculated using the modified Cheng Prusoff equation

$$K_B = \frac{IC_{50}}{(1 + A/EC_{50A})}$$

where A = concentration of reference agonist in the assay, and EC50A = EC50 value of the reference agonist.

5. Results interpretation guide:

Results showing an inhibition (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 IC_{50} or EC_{50} values from concentration-response curves) that the laboratory recommends.

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 level).

Low to moderate negative values have no real meaning and are attributable to variability of the signal around the control level. High negative values (≥ 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.

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 5 min at 22° C 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 buffer (basal and stimulated control), the reference antagonist (IC_{50} determination), or the test compounds.

Thereafter, 30 nM estriol, 200 nM biotin-tagged-SRC1-4 coactivator and 0.4 µg fluorescence acceptor (anti-GST antibody coupled-beads) are added and the mixture is incubated for 30 min at 22° C.

For control basal measurements, estriol is omitted from the reaction mixture. Fluorescence donor (streptavidin coupled-beads) is then added at a final concentration of 0.4 µg.

Following 120 min at 22° C, the signal is measured at λex=680 nm and λem=520 and 620 nm using a microplate reader (EnVision, Perkin Elmer).

The results are expressed as a percent of the control response to 30 nM estriol.

The standard reference agonist is 4-OH Tamoxifen, which is tested in each experiment at several concentrations to generate a concentration-response curve from which its IC_{50} value is calculated.

Rereference: *Liu J et al (2003), A Homogeneous in vitro Functional Assay for Estrogen Receptors: Coactivator Recruitment., Mol.Endocrinol., 17:246.*

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:

As described for B2 above

5. Results interpretation guide:

As described for B2 above.

II. Results and discussion

Cellular and Nuclear Receptor Functional Assays

Table 5.8.3-5 Agonist Effect: Fluopyram

Compound I.D.	Client Compound I.D.	Test Concentration	% of Control Agonist Response		
			1st	2nd	Mean
ERalpha (h) (agonist effect)					
100044628-1-0	Fluopyram Technical	1.0E-05 M	-0.5	-0.2	-0.3
		1.0E-04 M	3.1	-0.5	1.3

Table 5.8.3-6 Agonist Effect: Estriol

Compound I.D.	IC50 (M)	nH
ERalpha (h) (agonist effect)		
Estriol	3.4E-09 M	n/a

Table 5.8.3-6 Antagonist Effect: Fluopyram

Compound I.D.	Client Compound I.D.	Test Concentration	% Inhibition of Control Agonist Response		
			1st	2nd	Mean
ERalpha (h) (antagonist effect)					
100044628-1	Fluopyram Technical	1.0E-05 M	6.3	-2.9	1.7
		1.0E-04 M	-4.0	-7.3	-5.7

Table 5.8.3-7 Antagonist Effect: 4-OH tamoxifen

Compound I.D.	IC50 (M)	nH
ERalpha (h) (antagonist effect)		
4-OH tamoxifen	1.2E-09 M	2.0E-10 M

A. Deficiencies

None

III. Conclusions

Under the conditions of the test used, the functional assays showed that Fluopyram Technical had neither an agonist or antagonist effect on the alpha estrogen receptor.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2013. Fluopyram Technical had neither an agonist or antagonist effect on the alpha estrogen receptor.

e. Aromatase assay (US EPA OCSPP 890.1200)

Data Point:	KCA 5.8.3/05
Report Author:	Brock, C.
Report Year:	2018
Report Title:	*In vitro* Pharmacology: Human Aromatase Assay_Bayer SAS Assay. Study of Fluopyram Technical BAYER SAS Study Number: TXGM0123
Report No:	100044627_TXGM0123
Document No:	M-632696-01-1
Guideline(s) followed in study:	No specific guideline but based on US EPA OCSPP 890.1200
Deviations from current test guideline:	None
Previous evaluation:	Not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The purpose of this study was to test Fluopyram Technical in Human Aromatase Assay_Bayer SAS Assay.

Fluopyram Technical 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.

I. Materials and methods

A. Materials

1. Test material:	Fluopyram Technical
Description	Powder
Lot / Batch #:	Mix-batch 08528/0002
Purity:	94.5%
CAS #	658066-35-4
Stability of test compound:	Stable until 20 April 2020 (when stored at +10 to +30°C)
2. Vehicle:	Dimethylsulfoxide
3. Control:	Water
4. Reference substance:	Letrozole In each experiment and if applicable, the respective reference compound is tested concurrently with Fluopyram Technical, and the data is compared with historical values determined at Eurofins. The experiment is accepted in accordance with Eurofins validation Standard Operating Procedure
5. Experimental conditions:	
Source:	Human recombinant (insect cells)
Substrate/Stimulus/Tracer:	Testosterone (10 nM)
Incubation:	5 min; 37 °C
Measured Component:	Estradiol
Detection Method:	HTRF
Assay volume and format:	10 µl in 384-well plate
Compound addition:	[100x] 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.

B. Study design and methods

1. Experimental dates: 11 June 2018- 20 June 2018

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 purified 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 potassium 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 5 min at 37°C.

For basal control measurements, the enzyme 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 λex= 337 nm, λem= 620 nm and λem= 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

$$\frac{measured\ specific\ activity}{control\ specific\ activity} \times 100$$

and as a percent inhibition of control specific activity

$$100 - (\frac{measured\ specific\ activity}{control\ specific\ activity} \times 100)$$

obtained in the presence of Fluopyram Technical.

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 concentration-response curves generated with mean replicate values using Hill equation curve fitting:

$$Y = D + [\frac{A-D}{1+(C/C_{50})^{nH}}]$$

where Y = specific activity, A = left asymptote of the curve, D = right asymptote of the curve, C = compound concentration, C50 = IC50 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 inhibition (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) 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 level.

Low to moderate negative values have no real meaning and are attributable to variability of the signal around the control level. High negative values (≥ 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 5.8.3-8 Results of Human Aromatase Assay: Fluopyram

Compound I.D.	Client Compound I.D.	Test Concentration	% Inhibition of Control Specific Binding		
			1st	2nd	Mean
Aromatase Assay_Bayer SAS					
100044627-1	Fluopyram Technical	1.0E-05 M	4.6	4.7	-0.1
100044627-1	Fluopyram Technical	1.0E-04 M	24.6	2.8	13.7

Table 5.8.3-9 Results of Human Aromatase Assay: Letrozole

Compound I.D.	IC50 (M)	nH
Aromatase Assay_Bayer SAS		
Letrozole	2.8E-10 M	-1.2

A. Deficiencies

None

III. Conclusions

Under the conditions of the test, the results show that Fluopyram Technical does not cause enzyme inhibition in this Human Aromatase Assay.

Assessment and conclusion by applicant:

Study meets the current guidance and the requirements in 283/2012. Fluopyram Technical does not cause enzyme inhibition in this Human Aromatase Assay.

f. Thyroperoxidase (TPO) inhibition

Data Point:	KCA 5.8.3/06
Report Author:	[redacted]
Report Year:	2008
Report Title:	AE C656948 (Fluopyram) *In vitro* studies on the potential interactions with thyroid peroxidase-catalyzed reactions
Report No:	AT04481
Document No:	M-399276-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test 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
Acceptability/Reliability:	Yes

Executive Summary

To investigate a potential effect of AE C656948 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 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 µM, the highest concentration tested. Similarly, TPO-catalyzed iodine formation was not affected by 300 µM AE C656948.

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
Description	Light beige powder
Lot / Batch #:	Mix-batch 08528/0002
Purity:	94.7%
CAS #	658066-35-4
Stability of test compound:	Stable for a period covering the study duration.
2. Vehicle and /or positive control:	Dimethylsulfoxide
3. Positive control substances:	Amitrole (3-amino-1,2,4-triazole) from Sigma (Lot number 083K0649) and ETU (ethylenethiourea, 2-imidazolidinethione) from Riedel-de-Haën (Lot number 3223X)
4. Bilogical 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 dates: 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.

C. Methods

1. Determination of TPO-catalyzed guaiacol oxidation :

Guaiacol oxidation was used as a measure for peroxidative activity. Incubations were carried out at room temperature in 0.1 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 3.5 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 (ΔE/min) of the absorption at 470 nm was used to calculate the peroxidase activity.

2. Determination of TPO-catalyzed iodine formation: :

Incubations were carried out as described above, however, guaiacol was replaced by potassium iodide (100 µL of 100 mM solution in water, final concentration 10 mM).

The following final concentrations were used:

AE C656948: 3.0 – 30 – 300 µM

Amitrole: 0.1 µM

ETU: 5 µM

The initial linear increase (ΔE/min) of the absorption at 350 nm was used to calculate the enzymatic activity.

II. Results and discussion

The results of the TPO-catalyzed oxidation of guaiacol are summarized below. Amitrole, the positive control, at a concentration of 1 µM 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 µM did not affect this reaction.

Table 5.8.3-10 Effect of AE C656948 on TPO-catalyzed guaiacol reaction

Compound	Concentration (µM)	ΔE/min ± SD	% of control
Vehicle	-	0.121 ± 0.006	100
AE C656948	3	0.122 ± 0.002	100.8
	30	0.123 ± 0.005	101.6
	300	0.124 ± 0.001	102.5
Amitrole		0.054 ± 0.003	44.6

The results of the TPO-catalyzed iodine formation are summarized below. Up to 300 µM AE C656948 did not affect TPO-catalyzed iodine formation. Neither 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, whilst Amitrole at a concentration of 0.1 µM inhibited the initial rate of this reaction by 50%.

Table 5.8.3-11 Effect of AE C656948 on TPO-catalyzed iodine formation

Compound	Concentration (µM)	ΔE/min ± SD	% of control
Vehicle	-	0.259 ± 0.012	100
AE C656948	3	0.269 ± 0.012	103.9
	30	0.246 ± 0.005	95.0
	300	0.260 ± 0.012	100.4
Amitrole	0.1	0.131 ± 0.013	50.6

A. Deficiencies

None

III. Conclusions

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 conclusion by applicant:
Study meets the current guidance and the requirements in 283/2013.
AE C656948 does not affect thyroid hormone synthesis at the level of TPO.

Data Point:	KCA 5.8.3/07
Report Author:	[redacted]
Report Year:	2020
Report Title:	*In vitro* CYP and UGT induction in human and Wistar rat hepatocytes by Fluopyram
Report No:	KLC-BA20-06
Document No:	M-759019-01-1
Guideline(s) followed in study:	No specific guideline
Deviations from current test guideline:	Current guideline: none
Previous evaluation:	No, not previously submitted.
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Primary cultures of cryopreserved plateable male human and Wistar rat hepatocytes 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 rat hepatocyte cultures treated daily for 3 days and 7 days with Fluopyram at 10, 30, 60 and 100 µM or with positive control inducers beta-Naphthoflavone (BNF), rifampicin (RIF) and phenobarbital (PB) for human hepatocytes and BNF, 5-pregnen-3β-ol-20-one 16α-carbonitrile (PCN) and PB for rat hepatocytes. RNA quantity established that Fluopyram at 10, 30 and 100 µM were suitable concentrations for mRNA expression and activity analysis.

In Wistar rat hepatocytes, reference inducers BNF (5 µM), PB (1000 µM) and PCN (6 µM) strongly induced CYP1A2, CYP2B1 and CYP3A1 expression, respectively and increased the related activities. PB and PCN also induced UGT2B1 and to a lesser extent UGT1A1 expression and BNF, PB and PCN increased UGT-T4 activity.

In human hepatocytes, reference inducers BNF (5 µM), PB (1000 µM) and RIF (15 µM) induced CYP1A2, CYP2B6 and CYP3A4 expression, respectively, and increased the related activities. PB and RIF induced UGT1A1 expression, BNF and RIF increased UGT-T4 activity.

In conclusion

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 Wistar rat hepatocytes. Fluopyram 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 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 potential of Fluopyram to induce human and rat inducible cytochrome 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 100 µM is a strong CYP3A 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 rat hepatocytes. Fluopyram at 10 µM, 30 µM and 100 µM is a CYP1A2, CYP2B6, CYP3A4 and UGT1A1 inducer but does not increase UGT-T4 activity in human hepatocytes

Endocrine disrupting (ED) properties

In addition to the evaluation of the endocrine disrupting potential activity 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/ECHA (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 5.8.3/08
Report Author:	[redacted]
Report Year:	2021
Report Title:	Appendix I - Assessment of the endocrine disrupting properties of the active substance fluopyram in accordance with Commission Regulation (EU) 2018/605
Report No:	M-764022-01-1
Document No:	M-764022-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	Not applicable
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Not applicable
Acceptability/Reliability:	Yes

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. 1107/2009. A literature search did not find any relevant studies conducted in the last 10 years published in the open literature. At the time of preparation, there was no *in vitro* mechanistic data available on the US EPA CompTox Chemicals Dashboard. However, *in vitro* and *in vivo* mechanistic studies have been conducted to investigate EATS-mediated endocrine activity.

Data from relevant studies were added to the Excel template provided as Appendix E1 to the EFSA/ECHA 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 (LoE) spreadsheets of the Excel template. Appendix E1 is provided as a supplement to this document.

A summary of all the studies considered relevant for the mammalian toxicology review is outlined in Table 5.8.3-12.

Table 5.8.3-12: Outline of dataset considered for mammalian toxicology ED assessment

Type of toxicity	Species	Duration/Type	Used for T/EAS modality	Reference	Matrix ID
Repeat Dose Studies in mammals	Rat	28 days	T & EAS	M-085510-01-1	1
		28 days (dermal)	T & EAS	M-293833-01-1	13
		90 days	T & EAS	M-250946-01-1	4
		90 days (neurotoxicity)	T & EAS	M-299110-01-1	14
		104 weeks chronic/carcinogenicity	T & EAS	M-298339-01-1	8
		2-generation reproduction	T & EAS	M-299334-01-1	12
		1-generation reproduction dose range finding	T & EAS	M-299533-01-1	11
		Prenatal developmental toxicity	T & EAS	M-299438-01-2	9
	Rabbit	Prenatal developmental toxicity	T & EAS	M-279773-01-1	10
	Mouse	28 days	T & EAS	M-088486-01-1	
		90 days	T & EAS	M-251136-01-1	5
		78 weeks carcinogenicity	T & EAS	M-295688-01-1	7
	Dog	28 days	T & EAS	M-242097-01-1	3
		90 days	T & EAS	M-276047-01-1	6
		52 weeks	T & EAS	M-284279-01-1	15

Table 5.8.3-12 (cont'd)

Type of toxicity	Species	Duration/Type	Used for T/EAS modality	Reference	Matrix ID
In vivo mechanistic studies	Rat	7 days	T	M-299274-01-1	21
		3 & 7 days	T	M-408029-01-1	22
		28 day	T	M-427431-01-1	23
	Mouse	3 days	T	M-426994-01-1	30
		3 days	T	M-308073-01-1	32
		3 days	T	M-408352-01-1	33
		3 days	T	M-426994-01-1	34
		4 days	T	M-328662-01-1	31
		3 & 14 days	T	M-299522-01-1	24
		28 days	T	M-428031-02-1	25
		28 days	T	M-428303-01-1	26
		28 days	T	M-449821-03-1	27
		28 days (wild type v Pxr KO/Car KO)	T	M-449890-01-1	28
		28 days (liver micrsome samples were provided from 28 day study M428031-02-1)	T	M-451628-01-1	29

In vitro mechanistic studies	Rat	Phase I enzyme activity assessment hepatocyte cultures and cellular proliferation (96 hours)	T	M-450157-01-1	36
	Human	Phase I enzyme activity assessment hepatocyte cultures and cellular proliferation (96 hours)	T	M-450156-01-1	35
	Rat & Human	Phase I & II gene transcript & enzyme activity assessment hepatocyte cultures (3 & 7 days)	T	M-759019-01-1	37
	Domestic pig	inhibition of thyroperoxidase (TPO)	T	M-298376-01-1	20
	Human	Stably transfected Human ERα Transcriptional Activation assay	T	M-632695-01-1	16
	Human	AR binding assay	EAS	M-632697-01-1	18
	Human	ER binding assays	EAS	M-632695-01-1 & M-632859-01-1	16, 17
	Human	Aromatase assay (OCSPP 890.1200)	EAS	M-632696-01-1	19

Overall conclusion for humans:

EAS and T modalities have been sufficiently investigated to allow the ED assessment of fluopyram with respect to humans.

In guideline studies with fluopyram, 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 kidney was a target organ in the rat only.

EAS adversity and endocrine activity have been sufficiently investigated. Investigation of EAS-mediated endocrine activity *in vitro* indicated fluopyram 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 treatment-related effect on sexual maturation in females in the 2-generation reproduction study in F1 offspring. The slight delay in preputial separation in F1 males, which was well within the HCD range of the conducting laboratory, was considered to be secondary 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, male 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 E, A, and S modalities can be considered to have been sufficiently investigated, and it can be concluded that the ED 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* enhanced 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 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 in the T modality, the ED criteria are not met for fluopyram with respect to human health.

CA 5.9 Medical data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Information provided on medical surveillance on manufacturing is confidential and included in (M-763420-01-1, M-763418-01-1 and M-763422-01-1) and document JCA.

CA 5.9.2 Data collected on humans

There are no publications on human poisoning cases with Fluopyram.

CA 5.9.3 Direct observations

4 cases with skin irritation, in one case with eye 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 irritants.
A child eating a soybean treated with Fluopyram only remained asymptomatic.

CA 5.9.4 Epidemiological studies

No epidemiological studies have been published concerning 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.

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

First Aid:

- Remove patient from exposure/terminate exposure

- Thorough skin decontamination with copious amounts of water and soap, if available with polyethylenglykol 300 followed by water.
  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 toxicity
-

Treatment:

- Gastric lavage does not seem to be required in regard of the low toxicity.
- The application of activated charcoal and sodium sulphate (or other carthartic) might be considered in significant ingestions.
- As there is no antidote, treatment has to be symptomatic and supportive.

CA 5.9.7 Expected effects of poisoning

No delayed or persisting effects are to be expected.

Appendix 1: Summary tables for harmonised classification and labelling (fluopyram)

1. Acute oral toxicity

a) Animal studies on acute oral toxicity

One animal study of acute oral toxicity is available and is summarised in Table 1, below.

Table 1: Summary table of animal studies on acute oral toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels, duration of exposure	Value LD_{50}	Reference
Acute oral toxicity (rat) OECD 423 (2001); EEC B.1; EPA OPPTS 870.1100 (1998); JMAFF (2000) No deviations	Rat (Wistar): ♀ 3/group	Fluopyram (94.7% purity) Batch No.: 08528/0002	2000 mg/kg bw Single dose	>2000 mg/kg bw	[redacted] (2005) M-259398-01-1

b) Human data on acute oral toxicity

No human data are available.

c) Other studies relevant for acute oral toxicity

Studies of acute oral neurotoxicity in the rat performed at dose levels of up to 2000 mg/kg bw did not report any mortality.

d) Summary of acute oral toxicity and conclusion on classification

A GLP- and guideline-compliant (OECD 423) study of acute oral toxicity in the rat performed with fluopyram reports an LD_{50} value of >2000 mg/kg bw. Studies 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 LD_{50} exceeds 2000 mg/kg bw, classification for acute oral 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 oral toxicity was required.

2. Acute dermal toxicity

a) Animal studies on acute dermal toxicity

One animal study of acute dermal toxicity is available and is summarised in Table 2, below.

Table 2: Summary table of animal studies on acute dermal toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels, duration of exposure	Value LD_{50}	Reference
OECD 402 (1987); EEC B.3; EPA OPPTS 870.1200 (1998). No deviations	Rat (Wistar): ♂,♀ 5/group	Fluopyram (94.7% purity) Batch No.: 08528/0002	2000 mg/kg bw Single dose	>2000 mg/kg bw	[redacted] (2005) M-259275-01-1

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 LD_{50} value of >2000 mg/kg bw. No human data are available and no additional relevant studies are available. As the acute dermal LD_{50} 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) on 4th December 2014. The CLH opinion adopted at RAC-31 concluded that no classification for acute dermal toxicity was required.

3) Acute inhalation toxicity

a) Animal studies on acute inhalation toxicity

One animal study of acute inhalation toxicity is available and is summarised in Table 3, below.

Table 3: Summary table of animal studies on acute inhalation toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, form and particle size (MMAD)	Dose levels, duration of exposure	Value LC_{50}	Reference
OECD 403 (1981); EEC (1992); EPA OPPTS 870.1300 (1998); JMAFF (2000) No deviations	Rat (Wistar): ♂,♀ 5/group	Fluopyram (94.7% purity) Batch No.: 08528/0002 Solid aerosol (dust) MMAD 5.6 µm	5.1125 mg/L 4 hours, nose only	5.1125 mg/L	[redacted] (2006) M-283420-01-1

b) Human data on acute inhalation toxicity

No human data are available.

c) Other studies relevant for acute inhalation toxicity

No other relevant studies are available.

d) Summary of acute inhalation toxicity and conclusion on classification

A GLP- and guideline-compliant (OECD 403) study of acute inhalation toxicity in the rat performed with fluopyram reports an LC_{50} value (4-hour, nose-only) of 5.1125 mg/L. Although the MMAD attained in this study (5.6 µm) 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 LC_{50} exceeds 5 mg/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

a) Animal studies on skin corrosion/irritation

One animal study of skin corrosion/irritation is available and is summarised in Table 4, below.

Table 4: Summary table of animal studies on skin corrosion/irritation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels, duration of exposure	Results - Observations and time point of onset - Mean scores/animal - Reversibility	Reference
OECD 404 (2002); EEC B.4. (1967); EPAOPPTS 870.2500 (1998); JMAFF (2000) No deviations	Rabbit (NZW); 3♀	Fluopyram (94.7% purity) Batch No.: 08528/0002	0.5 g (4 hours)	No signs of irritation in any animal Mean (24-72 hour) scores for erythema: 0.00, 0.00, 0.00 Mean (24-72 hour) scores for oedema: 0.00, 0.00, 0.00 Reversibility: NA	[redacted] (2005) M-263302-01-1

b) Human data on skin corrosion/irritation

No human data are available.

c) Other studies relevant for skin corrosion/irritation

A 28-day dermal repeated dose toxicity study in the rat is available (see Section 9), and is summarised in Table 5, below.

Table 5: Summary table of other studies relevant for skin corrosion/irritation

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
28-day dermal OECD 410 (1981) No deviations Rat (Wistar) 10/sex/group	Fluopyram (94.6% purity) Batch 082528/0002 0, 100, 300, 1000 mg/kg bw/d 4 weeks (5 applications/week)	NOAEL (local effects): ♂/♀ 1000 mg/kg bw/d LOAEL (local effects): ♂/♀ No evidence of local effects at the application site	[redacted] (2007) M-293833-01-1

d) Summary of skin corrosion/irritation

A GLP- and guideline-compliant (OECD 404) study of skin corrosion/irritation in the rabbit performed with fluopyram 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) on 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

a) Animal studies on serious eye damage/serious eye irritation

One animal study of serious eye damage/serious eye irritation is available and is summarised in Table 6 below.

Table 6: Summary table of animal studies on serious eye damage/serious eye irritation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results - Observations and time point of onset - Mean scores/animal - Reversibility	Reference
OECD 405 (2002); EEC B.5. (1967); EPAOPPTS 870.2400 (1998); JMAFF (2000) No deviations	Rabbit (NZW); 3♀	Fluopyram (94.7% purity) Batch No.: 08528/0002	0.1 g	Signs of irritation limited to conjunctival erythema (Grade 1) in two rabbits at 24 hours only Mean (24-72 hour) scores for corneal opacity: 0.00, 0.00, 0.00 Mean (24-72 hour) scores for iritis: 0.00, 0.00, 0.00 Mean (24-72 hour) scores for conjunctival erythema: 0.66, 0.00, 0.00 Mean (24-72 hour) scores for chemosis: 0.00, 0.00, 0.00 All signs reversible by 48 hours	[redacted] (2005) M-263277-01-1

b) Human data on serious eye damage/serious eye irritation

No human data are available.

c) Other studies relevant for serious eye damage/serious eye irritation

No other relevant studies are available.

d) Summary of serious eye damage/eye irritation and conclusion on classification

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, iritis, conjunctival erythema 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.

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 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 fluopyram for respiratory sensitisation (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

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
OECD 429 (2002) No significant deviations	Mouse (CBA/J): 5♀/group	Fluopyram (94.7% purity) Batch No.: 08528/0002	0%, 0.5%, 1.0%, 2.5%, 5.0% (25 µL in DMF); three consecutive daily dermal exposures	SI values: 1.0 (0%), 0.9 (0.5%), 1.0 (1.0%), 1.1 (2.5%), 0.9 (5.0%) EC_3 not calculated	[redacted] (2006) M-281845-01-1

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 sensitisation 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 1) 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 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.

Table 8: Summary table of animal studies on STOT SE

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
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/<125 mg/kg bw LOAEL ♂/♀: 500/125 mg/kg bw/d ↓motor activity ♂ (≥500 mg/kg bw); ♀ (≥125 mg/kg bw) ↓locomotor activity ♂ (≥500 mg/kg bw); ♀ (≥125 mg/kg bw) ↓body temperature ♀ (≥500 mg/kg bw) No evidence of neuropathology	[redacted] (2007) M-289073-01-2
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, 25, 50, 100 mg/kg bw	NOAEL ♀: 50 mg/kg bw LOAEL ♀: 100 mg/kg bw/d ↓motor activity ♀ (-38%; 100 mg/kg bw) ↓locomotor activity ♀ (-38%; 100 mg/kg bw)	[redacted] (2007) M-289073-01-2

b) Human data on STOT SE

No human data are available.

c) Other studies relevant for STOT SE

No other relevant studies are available

d) Summary of STOT SE and conclusion on classification

Fluopyram is shown to be of low toxicity following single exposure by all routes investigated. In the acute oral toxicity study in the rat (Section 1), no signs of systemic toxicity observed at the limit dose of 2000 mg/kg bw. In the acute dermal toxicity study in the rat (Section 2), no signs of systemic toxicity observed at the limit dose of 2000 mg/kg bw. 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, ungroomed hair coat and limpness. Measurements of reflexes made on the first day post exposure day were normal in all 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 treatment. All signs were fully reversible within five days. In the initial acute oral neurotoxicity 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 reduction in body temperature was also noted for females at 500 and 1000 mg/kg bw. In the follow-up 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 (≤300 mg/kg bw and >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 any study. The acute inhalation toxicity study (Section 3) reports some findings including bradypnoea/dyspnoea and reduced rectal temperature that may be consistent with the response to a respiratory irritant. 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 (RAC-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 exposure)

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, mouse 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, below.

Table 9: Summary table of animal studies for STOT RE

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
28-day oral (dietary) Non-guideline Rat (Wistar) 5/sex/group	Fluopyram (98.6% purity) Batch No.: FLD 999 0, 50, 400, 3200 ppm 28 days	NOAEL (400 ppm): ♂/♀: 31.0/36.1 mg/kg bw/d LOAEL (3200 ppm): ♂/♀: 254/263 mg/kg bw/d target organs: liver, kidney, thyroid ↓body weight gain (m/f) ↑platelet count (♂) ↑prothrombin time (♂) ↑cholesterol, triglycerides (♂,♀) ↓ast, alp activity (♂,♀) ↑liver weight (m,f) ↑thyroid weight (♂) hepatocyte hypertrophy (m/f) thyroid follicular cell hypertrophy (♂) kidney hyaline droplet nephropathy (♂)	[redacted] (2004) M-085510-01-1

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
28-day oral (dietary) Non-guideline Mouse (C57BL/6J) 5/sex/group	Fluopyram (99.4% purity) Batch No.: FLH 1046 0, 150, 1000, 5000 ppm 28 days	NOAEL (1000 ppm) ♂/♀: 24.7/31.1 mg/kg bw/d LOAEL (3200 ppm) ♂/♀: 162/197 mg/kg bw/d Target organ: liver Mortality (5000 ppm) (♂,♀) ↓body weight gain (M ↑ALT activity (♂) ↑liver weight (M/F) Hepatocyte centrilobular hypertrophy, single cell and focal necrosis, eosinophilia and bile duct/oval cell hyperplasia (M/F)	[redacted] (2004) M-088486-01-1
28-day oral (gavage) Non-guideline Dog (Beagle) 2/sex/group	Fluopyram (99.0% purity) Batch No.: PFI 0304 0, 30, 150, 750 mg/kg bw/d 28 days	NOAEL ♂/♀: 150/150 mg/kg bw/d LOAEL ♂/♀: 750/750 mg/kg bw/d Target organs: liver, bone marrow ↓erythrocyte count, haemoglobin concentration, haematocrit (♂), ↑ALP (♂,♀) ↑GGT (♀) ↓albumin (♂,♀) ↑triglycerides (♀) ↑liver weight (M,F) Hepatocyte hypertrophy (♂,♀)	[redacted] (2004) M-242097-01-1
90-day oral (dietary) OECD 408; Directive 2001/59/EC, Annex V, Method B.26; US-EPA, OPPTS 870.3100; JMAFF (2000) No deviations Rat (Wistar) 10/sex/group	Fluopyram (99.0% purity) Batch No.: PFI 0304 0, 50, 200, 1000, 3200 ppm 90 days	NOAEL: ♂/♀ (200 ppm): 12.5/14.6 mg/kg bw/d LOAEL: ♂/♀ (1000 ppm): 60.5/70.1 mg/kg bw/d Target organs: liver, thyroid, kidneys ↓body weight, food consumption (♂,♀) ↑haemoglobin concentration (♂,♀), haematocrit (♂) ↓cholesterol ↑GGT ↕TSH, T3, T4 ↑liver weight, centrilobular hypertrophy, periportal/ mid-zonal hepatocyte vacuolation (♂,♀) ↑thyroid weight, follicular cell hypertrophy (♂,♀) ↑kidney weight: hyaline droplet nephropathy (♂)	[redacted] (2005) M-250946-01-1

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
90-day oral (dietary) OECD 408; EC B.26; EPA 870.3100; JMAFF No deviations Mouse (C57BL/6J) 10/sex/group	Fluopyram (99.0% purity) Batch No.: PFI 0304 0, 30, 150, 1000 ppm 90 days	NOAEL: ♂/♀ (150 ppm): 25.6/32.0 mg/kg bw/d LOAEL: ♂/♀ (1000 ppm): 188/216 mg/kg bw/d Target organs: liver, adrenals ↑ALT (♂,♀) ↓albumin (♂,♀) ↓cholesterol (♂) ↑ALP (♂) ↑liver weight (M, F; 34-45%) ↑adrenal weight (♂) 87-92% Liver: centrilobular hypertrophy, focal necrosis (♂,♀) Adrenals: ↓ceroid pigment (♂), ↑cortical vacuolation (♀)	[redacted] (2011) M-251136-01-1
90-day oral (dietary) OECD 409 (1998); EEC B.27 (2001); EPA OPPTS 870.3150 (1998); JMAFF (2000) No deviations Dog (Beagle) 4/sex/group	Fluopyram (94.6% purity) Batch 082528/0002 0, 800, 5000, 20000/10000 ppm 90 days	NOAEL: ♂/♀ (800 ppm): 28.5/32.9 mg/kg bw/d LOAEL: ♂/♀ (5000 ppm): 171/184 mg/kg bw/d Target organ: liver ↓body weight gain ↓food consumption ↑ALP (♂,♀) ↑GGT (♂,♀) ↑ALT (♂,♀) ↑AST (♂,♀) ↓albumin (♂,♀) ↓bilirubin (♂,♀) ↑liver weight (♂,♀) Liver: hepatocyte hypertrophy, intracytoplasmic eosinophilic droplets, single cell necrosis (♂,♀)	[redacted] (2006) M-276047-01-1
12-month oral (dietary) OECD 452; EEC B.30 (1992); EPA OPPTS 870.; JMAFF (2000) No deviations Dog (Beagle) 4/sex/group	Fluopyram (94.6% purity) Batch 082528/0002 0, 100, 400, 2000 ppm 12 months	NOAEL: ♂/♀ (400 ppm): 13.2/14.4 mg/kg bw/d LOAEL: ♂/♀ (2000 ppm): 67.6/66.1 mg/kg bw/d Target organ: liver ↓body weight (♂,♀) ↓food consumption (♂,♀) ↑ALP (♂,♀) Liver: centrilobular hypertrophy (♂,♀)	[redacted] (2007) M-294279-01-1
28-day dermal OECD 410 (1981) No deviations Rat (Wistar) 10/sex/group	Fluopyram (94.6% purity) Batch 082528/0002 0, 100, 300, 1000 mg/kg bw/d 4 weeks (5 applications/week)	NOAEL: ♂/♀ 300 mg/kg bw/d LOAEL: ♂/♀ 1000 mg/kg bw/d Target organ: liver ↑cholesterol (♀) ↑prothrombin time (♂) ↑liver weight (♂,♀) Hepatocyte hypertrophy (♂,♀)	[redacted] (2007) M-293833-01-1

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	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
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: ♂♀ (2500 ppm) 164.2/197.1 mg/kg bw/d LOAEL: -/♂/♀ (-) No evidence of neurotoxicity or neuropathology	[redacted] (2008) M-299110-01-1

d) Summary of STOT RE and conclusion on classification

STOT-RE is assigned on the basis of findings of 'significant' or 'severe' toxicity, where effects are seen below guidance values of ≤10 mg/kg bw/d (for classification in Category 1) or >10-100 mg/kg bw/d (for classification in Category 2). The guidance values refer to effects seen in a 90-day rat study, and can be used as a basis to extrapolate equivalent guidance values for toxicity studies of greater or lesser duration.

For fluopyram, none of the relevant repeated dose toxicity studies report LOAEL values below the Guidance Values (GVs) for classification in STOT RE Category 1 (i.e. ≤30 mg/kg bw/d for 28-day oral toxicity studies; ≤10 mg/kg bw/d for 90-day oral toxicity studies; ≤2.5 mg/kg bw/d for 12-month oral toxicity studies; ≤60 mg/kg bw/d for 28-day dermal toxicity studies). Consequently, classification for STOT RE (Category 1) is not considered further.

The 28-day oral toxicity studies in the rat and mouse report LOAELs below the Guidance Value (GV) for classification in STOT RE Category 2 (≤300 mg/kg bw/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 (≤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 reports a LOAEL below the Guidance Value (GV) for classification in 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 LOAELs 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 further.

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.

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.

Table 11: Comparison of repeated dose toxicity study LOAELs and CLP Guidance Values

Study	LOAEL	Guidance Value (GV)	Reference
Rat 28-day oral (dietary)	3200 ppm: ♂ 254 mg/kg bw/d ♀ 263 mg/kg bw/d	**Cat 1 ≤30 mg/kg bw/d** Cat 2 ≤300 mg/kg bw/d	[redacted] (2004) M-085510-01-1
Mouse 28-day oral (dietary)	3200 ppm ♂ 162 mg/kg bw/d ♀ 197 mg/kg bw/d	**Cat 1 ≤30 mg/kg bw/d** Cat 2 ≤300 mg/kg bw/d [Rat GVs]	[redacted] (2004) M-088486-01-1
Dog 28-day oral (gavage)	♂ 750 mg/kg bw/d ♀ 750 mg/kg bw/d	**Cat 1 ≤30 mg/kg bw/d** Cat 2 ≤300 mg/kg bw/d [Rat GVs]	[redacted] (2004) M-242097-01-1
Rat 90-day oral (dietary)	1000 ppm: ♂ 60.5 mg/kg bw/d ♀ 70.1 mg/kg bw/d	**Cat 1 ≤10 mg/kg bw/d** Cat 2 ≤100 mg/kg bw/d	[redacted] (2005) M-250946-01-1
Mouse 90-day oral (dietary)	1000 ppm; ♂ 188 mg/kg bw/d ♀ 216 mg/kg bw/d	**Cat 1 ≤10 mg/kg bw/d** Cat 2 ≤100 mg/kg bw/d [Rat GVs]	[redacted] (2011) M-251136-01-1
Dog 90-day oral (dietary)	♂ 171 mg/kg bw/d ♀ 184 mg/kg bw/d	**Cat 1 ≤10 mg/kg bw/d** Cat 2 ≤100 mg/kg bw/d [Rat GVs]	[redacted] (2006) M-276047-01-1
Dog 12-month oral (dietary)	2000 ppm ♂ 65.6 mg/kg bw/d ♀ 66.1 mg/kg bw/d	**Cat 1 ≤2.5 mg/kg bw/d** Cat 2 ≤25 mg/kg bw/d [Rat GV, extrapolated]	[redacted] (2007) M-294279-01-1
Rat 28-day dermal	♂ 1000 mg/kg bw/d ♀ 1000 mg/kg bw/d	**Cat 1 ≤60 mg/kg bw/d** Cat 2 ≤600 mg/kg bw/d	[redacted] (2007) M-293833-01-1
Rat 90-day neurotoxicity (oral, dietary)	♂ >164.2 mg/kg bw/d ♀ >197.1 mg/kg bw/d	**Cat 1 ≤10 mg/kg bw/d** Cat 2 ≤100 mg/kg bw/d	[redacted] 2008 M-299110-01-1

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 12, below.

Table 12: 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	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
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: ♂/♀ (2500 ppm):164.2/197.1 mg/kg bw/d LOAEL: ♂/♀ (-) No evidence of neurotoxicity or neuropathology	[redacted] (2008) M-299170-01-1

The 28-day oral toxicity study in the rat identifies effects on the liver (increased organ weight, hepatocyte hypertrophy) and thyroid (increased organ weight, follicular hypertrophy) in both sexes, and additionally on the kidney (hyaline droplet nephropathy) in male rats at the LOAEL (equivalent to 254 and 263 mg/kg bw/d in males 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 (increased organ weight, hepatocyte centrilobular hypertrophy, single cell and focal necrosis, eosinophilia and bile duct/oval cell hyperplasia) at the LOAEL (equivalent to 162 and 197 mg/kg bw/d in males and females, respectively). These effects are not regarded as of sufficient severity for classification and represent a rodent-specific mechanism (Section 11).

The 90-day oral toxicity study in the rat reports a LOAEL of 1000 ppm (equivalent to 60.5 and 70.1 mg/kg bw/d in males and females 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 toxicity. Reductions in haemoglobin concentration (by 4% in both sexes) and haematocrit (by 4% in males) were also seen; other erythrocyte parameters were not affected by treatment at this dose level. Findings at 1000 ppm are not of sufficient magnitude (<10%) to be considered as 'significant' or 'severe' toxicity, and are therefore not relevant for STOT RE classification. Bilirubin 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 cholesterol concentrations were significantly increased in males (by 45%) and females (by 65%). Absolute liver weights were 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 females. Necropsy 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 histopathological 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 do not represent 'significant' or 'severe' toxicity, are shown to be due to an MoA not of relevance to humans (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, histopathology, changes in clinical chemistry and urinalysis parameters) are attributable to a male rat-specific toxicity (α2μ-globulin nephropathy), and are not relevant to STOT RE classification.

In conclusion, therefore, although there are effects of treatment in some studies at dose levels 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 severity to trigger classification. Effects seen at higher dose levels exceed the Guidance Values and are not therefore 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. Germ cell mutagenicity

a) Genotoxicity tests *in vitro*

The results of *in vitro* genotoxicity studies with fluopyram are reported in Table 13, below.

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
Ames test OECD 471 (1997); EEC B13/14 (2000); EPA OPPTS 870.5100 (1998); JMAFF (2000) No deviations	Fluopyram (94.7% purity) Batch 08528/0002	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA102 All strains treated up to 5000 µg/plate (+/-S9)	No cytotoxicity at any concentration (+/-S9) Precipitation: 1581 and 5000 µg/plate (+/-S9) Negative result in all strains tested (+/-S9)	[redacted] (2006) M-269978-01-1
Ames test OECD 471 (1997); EEC B13/14 (2000); EPA OPPTS 870.5100 (1998);	Fluopyram (95.7% purity) Batch 2007-010986	*S. typhimurium* 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)	[redacted] (2008) M-298529-01-1

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 µg/mL (4/18h) +-S9 120, 180, 240 µg/mL (4/30h) +-S9 60, 20, 180 µg/mL (18/18 h) -S9	Cytotoxicity at ≥120 µg/mL (-S9) Cytotoxicity at ≥180 µg/mL (+S9) Precipitation at ≥120 µg/mL (+/-S9) Negative result (+/-S9)	[redacted] (2005) M-266066-01-1
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	Chinese hamster V79 cells (HPRT) Concentrations limited by solubility 4, 8, 16, 32, 64, 128, 256 µg/mL (+/-S9)	Cytotoxicity at 256 µg/mL (-S9) No cytotoxicity (+S9) Precipitation at ≥128 µg/mL Negative result (+/-S9)	[redacted] (2006) M-268775-01-1

b) Genotoxicity/mutagenicity tests in mammalian somatic or germ cells *in vivo*

One study of genotoxicity/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 information 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); JMAFF (2000) 2000 PCEs scored (current guideline states 4000)	Fluopyram (94.7% purity) Batch 08528/0002	NMRI mice (5♂/group) 250, 500, 1000 mg/kg bw (2 x ip injection)	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)	[redacted] (2005) M-263710-02-1

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 *in vitro* 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 risk Assessment (RAC-31), on 4th December 2014. The CLH opinion adopted at RAC-31 concluded that no classification for germ cell mutagenicity was required.

11. Carcinogenicity

a) Animal studies on long-term toxicity and carcinogenicity

A combined chronic toxicity/carcinogenicity study in the rat and a carcinogenicity 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

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
Combined chronic toxicity/carcinogenicity study (dietary) OECD 453 (1981); EEC B33 (1987); EPA OPPTS 870.4300 (1998); JMAFF (2000) No deviations Rat (Wistar) 60/sex/group (24 months) 10/sex/group (12 months)	Fluopyram (94.5% purity) Batch 08528/0002 0, 30, 150, 750/375 (from Week 85) ppm (♂) 0, 30, 150, 1500 ppm (♀)	General toxicity NOAEL: ♂/♀ 30/30 ppm (1.20/1.68 mg/kg bw/d) LOAEL: ♂/♀ 150/150 ppm (6.0/8.6 mg/kg bw/d) Target organs: liver (♂/♀), kidney (♂), thyroid (♂,♀), eye (♀) ↑mortality (♂) 750 ppm Eye (retina) lesions (♀) 1500 ppm Liver toxicity (♂,♀) ↑liver weight, gross changes, histopathology Nephropathy (♂) Thyroid follicular hypertrophy (♂,♀) Carcinogenicity NOAEL: ♂/♀ 750/150 ppm (29/8.6 mg/kg bw/d) LOAEL: ♂/♀ -/1500 ppm (-/89 mg/kg bw/d) Increased incidences of hepatocyte adenoma and carcinoma in high dose females only	[redacted] (2008) M-298339-01-1
Carcinogenicity study (dietary) OECD 451 (1981); EEC B.32 (1987); EPA	Fluopyram (94.5-94.7% purity) Batch 08528/0002	General toxicity NOAEL: ♂/♀ 30/30 ppm (4.2/5.3 mg/kg bw/d) LOAEL: ♂/♀ 150/150 ppm (20.9/26.8 mg/kg bw/d)	[redacted] (2007) M-295688-01-1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
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 (♂,♀), kidney (♀) Nephropathy (♀) 750 ppm Thyroid follicular cell hyperplasia (♂,♀) Centrilobular to panlobular hepatocyte hypertrophy, single cell necrosis (♂,♀) Thyroid gland follicular cell Carcinogenicity NOAEL: ♂/♀ 150/750 ppm (20.9/129 mg/kg bw/d) LOAEL: ♂/♀ 750/- ppm (105/- mg/kg bw/d) The incidence 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 carcinogenicity

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 follicular tumours seen in high dose males in the mouse study. Data are described in detail in a Position Paper (Wason, 2013; M-465168-01-2) and Expert Summary Report (Geter *et al*, 2013; M-454439-02-1); the key points are summarised in Table 16, below.

Table 16: Summary table of other studies relevant to long-term toxicity and carcinogenicity

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline mechanistic study Rat (Wistar) ♀ 15/group	Fluopyram (94.7% purity) Batch 08528/0002 0, 3000 ppm (0, 195 mg/kg bw/d) 7 days	↑liver weight (40-43%) Minimal/slight centrilobular to panlobular hepatocyte hypertrophy ↑BrdU (4x) labelling of perilobular and centrilobular hepatocytes ↑liver P450 content ↑EROD activity (slight) ↑BROD activity (moderate) ↑PROD activity (moderate) ↑UDPGT activity (marked)	[redacted] (2008) M-299274-01
Non-guideline mechanistic study Rat (Wistar) ♀ 15/group	Phenobarbital 80 mg/kg bw/d 7 days	↑liver weight (19-22%) Slight centrilobular to panlobular hepatocyte hypertrophy ↑BrdU (2x) labelling of perilobular and centrilobular hepatocytes ↑liver P450 content ↑BROD activity (moderate) ↑PROD activity (moderate) ↑UDPGT activity (moderate)	[redacted] (2008) M-299491-01

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline mechanistic study Rat (Wistar) ♀ 15/group	Fluopyram (94.7% purity) Batch 08528/0002 0, 30, 75, 150, 600, 1500 ppm 3 and 7 days	30 ppm: no effects 75 ppm: ↑*cyp3a3* expression (marginal) 150 ppm: ↑hepatocyte proliferation, ↑*cyp2B1* expression, ↑*cyp3a3* expression, ↑*cyp1a1* expression 600 ppm: ↑liver weight, ↑hepatocyte proliferation, hepatocyte hypertrophy, ↑BROD/PROD activity, ↑UDPGT activity, ↑*cyp2b1* expression, ↑*cyp3a3* expression, ↑cyp1a1 expression, ↑*udpgtr2/gstm4/ephx1* expression 1500 ppm: ↑liver weight, ↑hepatocyte proliferation (marked), hepatocyte hypertrophy, ↑EROD/BROD/PROD activity, ↑UDPGT activity, ↑*cyp2b1* expression, ↑*cyp3a3* expression, ↑*cyp1a1* expression, ↑*udpgtr2/gstm4/ephx1* expression.	[redacted] (2011) M-408029-01-1
Non-guideline mechanistic study Rat (Wistar) ♀ 15/group	Fluopyram (94.7% purity) Batch 08528/0002 0, 30, 75, 150, 600, 1500 ppm 28 days (with 1-month recovery)	30 ppm: ↑*cyp3a3* expression 75 ppm: enlarged liver, ↑hepatocyte proliferation 150 ppm: ↑liver weight (slight), enlarged liver, ↑hepatocyte proliferation, ↑*cyp2b1* expression, ↑*cyp1a1* expression, ↑*udpgtr2/gstm4* expression, ↑EROD activity, ↑UDPGT activity 600 ppm: ↑liver weight, enlarged liver, hepatocyte hypertrophy, ↑hepatocyte proliferation, ↑*cyp2b1* expression, ↑*cyp1a1* expression, ↑*udpgtr2/gstm4/gsta2/ephx1* expression, ↑EROD/BROD/PROD activity, ↑UDPGT activity. 1500 ppm: ↑liver weight, enlarged liver, hepatocyte hypertrophy, ↑hepatocyte proliferation (marked), ↑*cyp2b1* expression, ↑*cyp1a1* expression, ↑*udpgtr2/gstm4/gsta2/ephx1* expression, ↑EROD/BROD/PROD activity, ↑UDPGT activity 1-month recovery 1500 ppm, hepatic changes showed signs of reversibility	[redacted] (2012) M-427431-01-1
Non-guideline mechanistic study Cultured primary human hepatocytes	Fluopyram (98.7% purity) Batch EDFL03235 0, 3, 10, 30, 100, 300 µM 96 hours	Slight cytotoxicity at 100 µM; more marked cytotoxicity at 300 µM No increase in replicative DNA synthesis ↑PROD activity (1-100 µM) ↑BROD activity (3-300 µM) ↑BQ activity (3-30 µM)	[redacted] (2013) M-450156-01-1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline mechanistic study Cultured primary rat hepatocytes	Fluopyram (98.7% purity) Batch EDFL03235 1, 3, 10, 30, 100, 300 µM 96 hours	Cytotoxicity at 300 µM ↑replicative DNA synthesis (1-100 µM) ↑PROD activity (1-300 µM) ↑BROD activity (1-100 µM) ↑BQ activity (1-100 µM)	[redacted] (2013) M-450157-01-1
Non-guideline mechanistic study Porcine thyroid microsomes	Fluopyram (94.7% purity) Batch 08528/0002 0, 3, 30, 300 µM	No inhibition of TPO activity by fluopyram at any concentration	[redacted] (2008) M-299276-01-1
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 15/group	Fluopyram (94.7% purity) Batch 08528/0002 2000 ppm 3 and 14 days	3 days - 14 days ↑liver weights (~11%/~22%) diffuse centrilobular/panlobular hepatocyte hypertrophy ↑mitoses Single cell necrosis ↓T4 (-30%/-27%) ↑TSH (+18%/+7%) ↑liver P450 content (+116%/+71%) ↑EROD activity (+116%/+165%) ↑BROD activity (+2890%/+2163%) ↑PROD activity (+8717%/+9061%)	[redacted] (2008) M-299522-01-1
Non-guideline mechanistic study Mouse (C57BL/6J) ♀ 15/group	Phenobarbital (99.6 % purity) Batch 06100228 80 mg/kg bw/day 3 and 14 days	3 days - 14 days ↑liver weights (~60%/~60%) hepatocyte hypertrophy ↓T4 (-10%/-19%) ↑T3 (+10%/-2%) ↑liver P450 content (+146%/+36%) ↑EROD activity (+297%/+375%) ↑BROD activity (+4930%/+2844%) ↑PROD activity (+1381%/+1345%) ↑UDPGT (no changes)	[redacted] (2008) M-299521-01-1
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 5/group	Fluopyram (94.7% purity) Batch 08528/0002 2000 ppm 3 days	↑clearance of ^{125}I-thyroxine from the blood	[redacted] (2008) M-308369-01-1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 10/group	Fluopyram (94.7% purity) Batch 08528/0002 2000 ppm 3 days	↑*cyp1b1* transcription ↑*cyp2b9* transcription ↑*cyp3a11* transcription ↑sulfotransferase transcription ↑UDPGT transcription	[redacted] (2008) M-308073-01-1
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 8/group	Fluopyram (94.7% purity) Batch 08528/0002 2000 ppm 4 days	↑clearance of ^{125}I-thyroxine from the blood	[redacted] (2008) M-328662-01-1
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 15/group	Fluopyram (94.7% purity) Batch 08528/0002 100, 300 mg/kg bw/d 3 days	100 mg/kg bw/d: ↓T4 (-26%) ↑*tsh* transcription (+21%) 300 mg/kg bw/d: ↓T4 (-34%) ↑*tsh* transcription (+46%)	[redacted] (2011) M-408352-01-1
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 15/group	Fluopyram (94.7% purity) Batch 08528/0002 100, 300 mg/kg bw/d 3 days	100 mg/kg bw/d ↓T4 (-26%) 300 mg/kg bw/d ↓T4 (-41%)	[redacted] (2012) M- 426994-01-1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 15/group	Fluopyram (94.7% purity) Batch 08528/0002 0, 30, 75, 150, 300, 750 ppm 28 days	30 ppm: ↓T4 (-28%) 75 ppm: ↓T4 (-31%) ↑liver weight (+6%) 150 ppm: ↓T4 (-25%) ↑liver weight (+11%) ↑liver UDPGT activity 300 ppm: ↓T4 (-37%) ↑liver weight (+27%) ↑liver UDPGT activity ↑pituitary *tsh* transcription 750 ppm: ↓T4 (-38%) ↑liver weight (+36%) ↑liver UDPGT activity ↑pituitary *tsh* transcription	[redacted] (2012) M-428031-02-1
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 15/group	Fluopyram (94.7% purity) Batch 08528/0002 750 ppm 28 days	Enlarged liver ↑thyroid BrdU labelling	[redacted] (2012) M-428303-01-1
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 15/group	Fluopyram (94.7% purity) Batch 08528/0002 0, 30, 75, 150, 600, 750, 1500 ppm 28 days	Enlarged liver ↑thyroid BrdU labelling at 150 ppm (1.21x), 600 ppm (1.40x), 750 ppm (1.61x), 1500 ppm (2.33x). reversible after 28 days (1500 ppm)	[redacted] (2013) M-449821-03-1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline mechanistic study Mouse (C57BL/6J) ♂ 15/group Mouse CAR/PXR KO	Fluopyram (94.7% purity) Batch 08528/0002 0, 750, 1500 ppm 28 days	WT mice / 750 ppm: Enlarged livers ↑liver weight (+39-42%) Hepatocyte hypertrophy, single cell necrosis ↑thyroid BrdU labelling (1.8x) ↑liver P450 content (3.6x) ↑PROD (70x), ↑BQ (5.5x), ↑T4-GT (1.8x), ↑bilirubin-GT (1.8x) ↑pituitary tsh transcription (1.6x) WT mice / 1500 ppm: Enlarged livers ↑liver weight (+39-42%) Hepatocyte hypertrophy, single cell necrosis ↑thyroid BrdU labelling (2.6x) ↑liver P450 content (3.7x) ↑PROD (150x), ↑BQ (7.9x), ↑T4-GT (1.9x), ↑bilirubin-GT (2.0x) ↑pituitary *tsh* transcription (1.7x) CAR/PXR KO mice 750 ppm ↑liver weight ↑PROD (1.4x), ↓BQ (1.5x) CAR/PXR KO mice 1500 ppm ↑liver weight ↑PROD (1.4x), ↓BQ (1.7x), ↑T4-GT (1.3x), ↓pituitary *tsh* transcription (1.2x)	[redacted] (2013) M-449890-01-1
Non-guideline mechanistic study Mouse (C57BL/6J) 15/group	Fluopyram (94.7% purity) Batch 08528/0002 0,, 30, 150, 600 750 ppm 28 days	↑PROD activity up to 4715.8% (30-750 ppm) ↑BQ activity up to 622.6% (30-750 ppm)	[redacted] (2013) M-451628-01-1
Non-guideline mechanistic study Primary cultures of cryopreserved male human and Wistar rat hepatocytes	Fluopyram (94.5% purity) Batch 08528/0002 10, 30 and 100 µM 3 & 7 days	Rat hepatocytes (10 - 100 µM) ↑ CYP 3A, ↑ CYP 1A, ↑ UGT 2B1, ↑UGT-T4 Human hepatocytes (10 - 100 µM) ↑ CYP 1A2, ↑ CYP 2B6, ↑ CYP 3A4 ↑ UGT 1A1 No activation of UGT-T4	[redacted] (2020) M-759019-01-1

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 resulted in the induction of cytochrome P450 content; increased cytochrome P450 (principally PROD and BROD) and UDPGT isoenzyme activities and corresponding changes in gene expression; hepatocyte 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 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 in the rodent for liver tumours in 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 not in human cells in response to fluopyram 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 fluopyram is via activation of the CAR/PXR nuclear receptors. Furthermore, the lack of proliferation in primary human hepatocytes (compared to rat hepatocytes), an essential key step in the formation of liver tumours via the postulated MoA, provides convincing evidence that the liver tumours seen in the rat are non-relevant to humans. Other MoA have effectively been 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.

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 a number of mechanistic studies. Administration of fluopyram to male mice resulted in cytochrome P450 induction (increased BROD/PROD activities), reduced T4 and increased TSH levels, and a more rapid clearance of T4. The pivotal CAR/PXR wild-type (WT) and knock-out (KO) mice study showed significant liver enlargement, hepatocyte hypertrophy, and liver enzyme induction in WT but not in KO mice, this study provides 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. Phase I and II liver enzymes were induced in both species, but crucially UGT-T4 was induced in rat hepatocytes but not in human hepatocytes. This MoA (increased TSH secondary to 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 an inhibitor 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 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.

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 fertility

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for sexual function and fertility, parents) - target tissue/organ - critical effects at the LOAEL	Reference
2-generation reproductive toxicity study (oral) OECD 416 (2001) EPA OPPTS 870.3800 (1998) JMAFF (2000) No deviations Rat (Wistar) 30/sex/group	Fluopyram (94.7% purity) Batch No. 08528/0002 0, 40, 220, 1200 ppm 10 weeks pre-mating	Parental toxicity NOAEL ♂♀ (220/220 ppm): 14.5/17.2 mg/kg bw/d LOAEL ♂♀ (1200/1200 ppm): 82.8/96.0 mg/kg bw/d ↓body weight gain (♀) during pre-mating, gestation ↓haemoglobin, haematocrit (♀) Kidney toxicity and clinical chemistry changes (♂) ↑liver weights, centrilobular hypertrophy (♂♀) Offspring toxicity NOAEL (220 ppm): 14.5 mg/kg bw/d LOAEL (1200 ppm): 82.8 mg/kg bw/d ↓weight gain ↑ Delayed sexual maturation, secondary to body weight (♂) Reproductive toxicity NOAEL ♂♀ (1200/1200 ppm): 82.8/96.0 mg/kg bw/d LOAEL (>1200 ppm) No effects on reproduction	[redacted] [redacted] (2008) M-299334-01-1

b) Human data on adverse effects on sexual function and fertility

No human data are available

c) Other studies relevant for toxicity on sexual function and fertility

Studies of repeated dose toxicity with fluopyram (summarised in Section 9, above) do not indicate any effects of potential relevance to sexual function and fertility.

d) Summary of sexual function and fertility and conclusion on classification

There was no evidence of reproductive toxicity in the two-generation oral reproductive toxicity study in the rat. Studies of repeated dose toxicity do 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.

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 reproductive toxicity (effects on sexual function and fertility) was required.

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

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for parent and for developmental effects) - target tissue/organ - critical effects at the LOAEL	Reference
Pre-natal developmental toxicity study (oral) OECD 414 (2001); EPA OPPTS 870.3700 (1998); JMAFF (2000) No deviations Rat (Sprague-Dawley) 23♀/group	Fluopyram (94.6% purity) Batch No. 08528/0002 0, 30, 150, 450 mg/kg bw/d GD 6-20	Maternal toxicity NOAEL: 30 mg/kg bw/d LOAEL: 150 mg/kg bw/d ↓body weight gain ↓food consumption ↑liver weight Centrilobular hepatocyte hypertrophy Developmental toxicity: NOAEL: 150 mg/kg bw/d LOAEL: 450 mg/kg bw/d ↓foetal weight (-5%) ↑visceral variations (thymus remnant, convoluted/dilated ureter ↑skeletal variations (bipartite thoracic centrum)	[redacted] (2008) M-299438-01
Pre-natal developmental toxicity study (oral) OECD 414 (2001); EPA OPPTS 870.3700 (1998); JMAFF (2000) No deviations Rabbit (NZW) 23♀/group	Fluopyram (94.6% purity) Batch No. 08528/0002 0, 10, 25, 75 mg/kg bw/d GD 6-28	Maternal toxicity NOAEL: 25 mg/kg bw/d LOAEL: 75 mg/kg bw/d ↓body weight gain ↓food consumption Developmental toxicity: NOAEL: 25 mg/kg bw/d LOAEL: 75 mg/kg bw/d ↓foetal weight (-11%)	[redacted] (2006) M-299773-01

b) Human data on adverse effects on development toxicity

No human data are available.

c) Other studies relevant for developmental toxicity

No other relevant studies are available.

d) Summary of developmental toxicity and proposal for classification

Studies of developmental toxicity in the rat and rabbit do not indicate any specific developmental toxicity for fluopyram. In the rat study, slightly reduced foetal weight and slightly increased incidences of visceral variations (thymus remnant present, convoluted/dilated ureter) and skeletal variations (thoracic 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 rats (summarised above) assessed 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 fluopyram on or via lactation. Reduced offspring weight gain seen in the two-generation oral reproductive toxicity study in the rat was associated with (and likely to be secondary to) maternal toxicity. Fluopyram does not therefore require classification for reproductive toxicity (effects on or via lactation) according to the CLP criteria.