Document Title

Summary of the toxicological and metabolism studies for Aclonifen

Data Requirement(s)

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

Document MCA

Section 5: Toxicological and metabolism studies

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

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Author(s)

Battelle UK Ltd.

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[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'.

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CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

INTRODUCTION

Aclonifen was included in Annex I to Council Directive 91/414/EEC in 2008 (Directive 2008/116/EC, Entry into Force on 01 August 2009). This present dossier in support of approval renewal includes all the data submitted at the time of the Annex I inclusion, in summaries updated and re-evaluated as necessary to take account of current validity criteria and data requirements.

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

The absorption, distribution, metabolism and excretion (ADME) of aclonifen has been investigated in rats. Studies were performed using ^{14}C radiolabel in the nitrophenyl/aniline ring, or in the phenoxy ring, as listed below:

[aniline-UL-^{14}C]-aclonifen [phenoxy-UL-^{14}C]-aclonifen

* labelling position

Aclonifen – Summary of metabolism studies

Test	Reference	New study
The metabolic fate of aclonifen in the rat: Cleavage of the diphenyl ether bond. (Position paper, not summarised here, as now superseded by & 2017, above)	KCA 5.1.1/01 M-233223-01-1 , 2004	-
ADME using a single oral dose at a nominal dose rate of 30 mg/kg or 1000 mg/kg body weight (aniline label) ADME using a single oral dose at a nominal dose rate of 1000 mg/kg body weight (aniline label)	KCA 5.1.1/02 M-211055-01-1 &, 2002	-
Bile kinetics using a single oral dose at a nominal dose rates of 30 or 1000 mg/kg body weight (aniline label)	KCA 5.1.1/03 M-210558-01-1 , 2002	-
Blood and plasma kinetics using a single oral dose at the nominal dose rates of 30 or 1000 mg/kg body weight (aniline label)	KCA 5.1.1/04 M-210562-01-1 2002	-
ADME using 14 consecutive daily doses of radiolabelled aclonifen at a nominal dose rate of 30 mg/kg body weight (aniline label)	KCA 5.1.1/05 M-211131-01-1 2002	-
Distribution and excretion during and following seven consecutive daily doses at a nominal dose rate of 30 mg/kg body weight (aniline label)	KCA 5.1.1/06 M-219328-01-1	-
ADME using a single oral dose at a nominal dose rate of 2 mg/kg body weight (phenoxy label)	KCA 5.1.1/07 M-598008-01-1	New

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Test	Reference	New study
Plasma kinetics using a single oral dose at the nominal dose rate of 2 mg/kg body weight (phenoxy label)	2017	
Liver, fat and plasma analysis at plasma C_{max} (phenoxy label)		
Bile kinetics using a single oral dose at a nominal dose rate of 2 mg/kg body weight (phenoxy label)		
Comparative *in-vitro* metabolism study in hepatocytes from mice, rats and humans (aniline & phenoxy label)	KCA 5.1.1/08 M-577083-01-1 , 2017	New
Metabolic stability of aclonifen in cryopreserved hepatocytes from rabbit, dog and human (aniline & phenoxy label)	KCA 5.1.1/09 M-674506-01-1 , 2019	New
In vitro binding of aclonifen to plasma proteins (phenoxy label)	KCA 5.1.1/10 M-569675-01-1 , 2016	New

Following rapid absorption, aclonifen was rapidly and extensively eliminated in both urine and faeces following both single and repeated dosing, with a mean of 97% of the administered radioactivity being eliminated over a seven-day period following cessation of dosing. The majority of this elimination occurred in the first 24 hours post dose following a single oral low dose and in the first 48 hours post dose for a single oral high dose and repeated low dose. The blood and plasma kinetic data for the aniline label indicated a terminal phase half-life of *ca* 103 h for both sexes following a single oral dose of 30 mg/kg, while the elimination rate from plasma was faster, with terminal phase elimination half-lives of 13 and 24 hours for males and females respectively. The data from bile cannulated animals showed that a large proportion of the radioactivity found in the faeces, particularly at the lower doses, was absorbed and then eliminated in the bile.

The oral bioavailability of [^{14}C]-aclonifen at the 30 mg/kg dose level was 81% and 86% for males and females respectively. At the high dose level of 1000 mg/kg there was clear evidence that the quantity of material was too great to permit complete dissolution in the gut and hence the proportional level of bioavailability was reduced to *ca* 27 %. This conclusion was supported by the blood kinetic AUC data.

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Table 5.1-1: Recovery data from the GLP ADME studies in rats, in terms of % of radioactivity administered

Study & Dose	**Single 2 mg/kg**		**Single 30 mg/kg**		**Single 1000 mg/kg**		**14d Repeat 30 mg/kg**	
Radiolabel	**phenoxy label**		**aniline label**					
Duration	**72 h**		**168 h**					
Sample / Sex	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Urine	52.03	48.76	31.83	58.47	33.82	34.79	43.51	49.44
Cage wash	[A]	[A]	4.31	7.09	5.80	5.88	6.39	9.14
Faeces	50.51	45.87	60.76	33.64	56.61	58.79	47.01	37.02
Total excreted	102.54	94.63	96.90	99.20	96.23	99.46	96.91	95.60
Tissues	0.181 [B]	0.264 [B]	0.14 [C]	0.17 [C]	0.13 [C]	0.13 [C]	0.13 [C]	0.09
GIT	0.073	0.078	-	-	-	-	-	-
Total in body	0.254	0.342	0.14	0.17	0.13	0.13	0.13	0.09
Balance	102.80	94.98	97.03	99.36	96.36	99.58	97.04	95.69

[A] Collected with urine
[B] Excluding gastrointestinal tract
[C] Excluding intestinal content from small and large intestine
GIT = Gastrointestinal tract

Table 5.1-2: Recovery data from bile cannulated rats, in terms of % of radioactivity administered

Dose	**Single 2 mg/kg**	**Single 30 mg/kg**		**Single 1000 mg/kg**	
Radiolabel	**phenoxy label**	**aniline label**			
Duration	**48 h**	**48 h**			
Sample / Sex	**Males**	**Males**	**Females**	**Males**	**Females**
Urine	21.81	35.91	34.29	9.56	6.17
Cage wash	[A]	4.68	2.68	0.83	0.94
Bile	55.22	39.65	47.61	15.68	16.40
Faeces	17.05	13.54	9.18	39.83	14.01
Total excreted	94.09	93.78	93.76	65.89	37.52
Tissues	0.267 [B]	1.14 [C]	1.02 [C]	1.60 [C]	1.68 [C]
GIT	1.165	2.04 [D]	0.61 [D]	26.44 [D]	57.93 [D]
Total in body	1.432	3.18	1.63	28.03	59.61
Balance	95.53	96.96	95.39	93.92	97.14
Absorption [E]	80.5 [F]	81.4	85.6	27.7	25.2

[A] Collected with urine
[B] Excluding gastrointestinal tract
[C] Sum of % dose in intestine, stomach, cardiac blood, cardiac plasma & residual carcass
[D] Sum of % dose in intestine contents & stomach contents
[E] Sum of % administered dose in urine, cage wash, bile, tissues
[F] Absorption rate reported as 80.9% based on % dose recovered in M-598008-01-1
GIT = Gastrointestinal tract

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Table 5.1-3: Mean concentration of radioactivity in tissues of the rat following oral dose of [^{14}C]-aclonifen, as µg aclonifen equivalents/g tissue

Study & Dose	**Single 2 mg/kg**		**Single 30 mg/kg**		**Single 1000 mg/kg**		**14d Repeat 30 mg/kg**	
Radiolabel	**phenoxy**		**aniline**					
Duration	**72 h**		**168 h**					
Tissue / Sex	**Males Test 1**	**Females Test 2**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Blood Cells	0.0071	0.0079	-	-	-	-	-	-
Plasma	0.0021	0.0039	-	-	-	-	-	-
Heart	0.0016	0.0032	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Brain	0.0006	0.0015	BLQ	BLQ	BLQ	BLQ	0.006	BLQ
Kidney	0.0115	0.0221	BLQ	BLQ	0.295	BLQ	0.667	0.423
Muscle	0.0010	0.0014	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Liver	0.0346	0.0554	0.146	0.148	3.502	2.790	1.339	0.474
Lung	0.0025	0.0062	BLQ	BLQ	BLQ	BLQ	0.285	0.350
Thymus	-	-	0.002	BLQ	BLQ	BLQ	0.031	0.028
Fat	0.0047	0.0024	0.007	0.016	0.204	0.501	0.131	0.128
Adrenals	0.0039	0.0108	BLQ	0.005	BLQ	0.445	0.099	0.140
Pancreas	-	-	BLQ	BLQ	BLQ	BLQ	0.112	0.118
Large intestine	-	-	BLQ	BLQ	BLQ	BLQ	0.041	BLQ
Small intestine		-	BLQ	BLQ	BLQ	BLQ	0.112	0.047
Stomach	-	-	BLQ	BLQ	BLQ	BLQ	0.092	0.065
Spleen	0.0026	0.0056	BLQ	BLQ	BLQ	BLQ	0.055	0.199
Skin & fur	0.0023	0.0055	0.131	0.086	3.820	2.861	0.401	0.244
Bone & marrow	0.0017	0.0028	BLQ	BLQ	BLQ	BLQ	0.048	0.006
Thyroid	0.0097	0.0094	BLQ	BLQ	BLQ	BLQ	0.468	0.167
Testis	0.0007	-	BLQ	-	BLQ	-	0.030	-
Ovary	-	0.0049	-	0.006	-	BLQ	-	0.115
Uterus	-	0.0050	-	0.016	-	BLQ	-	0.050
Carcass	0.0016	0.0028	0.040	0.059	1.087	1.293	0.475	0.380

BLQ = below limit of quantification (twice background, considered as zero)

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Table 5.1-4: Mean concentration of radioactivity in selected rat tissues at plasma t_{max} following single oral dose of [^{14}C]-aclonifen, as µg aclonifen equivalents/g tissue

Study & Dose	**Single 2 mg/kg**	
Radiolabel	**phenoxy label**	
Duration	**2 h (at organ preparation for metabolism)**	
Tissue / Sex	**Males Test 3**	**Females Test 4**
Blood Cells	0.7147	0.3591
Plasma	2.9034	1.7765
Liver	12.1688	9.3291
Fat	0.4229	0.2694
Skin	0.4776	0.2886

The blood and plasma maximum concentrations increased with the dose level, but this increase was not proportional to the dose ratio, which increased *ca* 33-fold whilst blood C_{max} increased 7.5 and 5.7-fold for male blood and plasma respectively and the C_{max} for female blood and plasma increased 2.6 and 2.2-fold. The time to achieve maximal blood/plasma concentrations was similar between the sexes at both dose levels (*ca* 2.6 hours at 30 mg/kg and *ca* 13 hours at 1000 mg/kg). There was no significant sex difference in the terminal elimination half-life values observed for the blood and plasma at either dose level. There was however a much more rapid elimination from the plasma (*ca* 21 hours) compared to whole blood (*ca* 74 to 103 hours). As observed for the maximal concentrations of radioactivity, the AUC data demonstrated that the males received a higher systemic exposure to the aclonifen derived radioactivity than the females at both dose levels. At the 30 mg/kg dose level the 0-168 hour AUC values were approximately 3.4 times higher for the males compared to the females in both blood and plasma. At the higher dose level of 1000 mg/kg the difference was reduced to 1.8 times and 2.3 times for the blood and plasma respectively. The increases in the 0-168 hour AUC values between the low and high dose groups were not proportional to the increase in dose rate (33-fold), being approximately 10-fold for the blood and plasma for the males and 19 and 14-fold for the female blood and plasma results respectively. It is clear that the 1000 mg/kg dose rate surpassed the threshold for maximum proportional absorption.

Plasma protein binding for aclonifen was shown to be very high (99.8-99.9%) in mice, rats and humans, with negligible interspecies differences.

Table 5.1-5: Plasma kinetics in rats (phenoxy label):

	Dose normalized		**Not dose normalized**	
Dose, oral (mg/kg bw)	**Males** 2 mg/kg bw oral	**Females** 2 mg/kg bw oral	**Males** 2 mg/kg bw oral	**Females** 2 mg/kg bw oral
Actual dose (mg/kg bw)	1.90	1.96	1.90	1.96
t_{max} [h]	2	1	2	1
C_{max} [g/g]; [mg/kg]	1.647	0.864	3.13	1.70
t½ absorption [h]	1.14	0.29	1.15	0.28
t½ elimination [h]	13.4	23.6	16.8	24.9
$AUC_{0-\infty}$ [g/g•h]; [mg/kg•h]	8.18	6.82	15.4	13.5

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Table 5.1-6: Blood and plasma kinetics in rats (aniline label), not dose normalized:

	Whole Blood				Plasma			
Dose, oral (mg/kg bw)	Single 30 mg/kg		Single 1000 mg/kg		Single 30 mg/kg		Single 1000 mg/kg	
Sex	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
t_{max} [h]	2.90	2.55	12.72	28.18	2.46	2.54	6.39	4.70
C_{max} [mg/kg]	17.45	8.67	131.40	22.75	30.22	15.18	171.00	33.75
$t_{1/2\ e}$ [h]	102.65	102.93	71.75	75.67	12.73	23.75	24.79	23.28
$AUC_{0-\infty}$ [mg/kg•h]	264.26	77.95	2681.87	1513.77	404.58	120.46	3912.80	1693.65

Amounts of radioactivity in the tissues were low. At 168 h post dosing, the mean recovery of radioactivity in the tissues of the animals from all the GLP study dose groups ranged from 0.09% to 0.17% dose. After a single oral dose the majority of the tissues had radioactivity concentrations below the limit of quantification. The highest concentrations were present in the skin & fur (means of 0.11 and 3.3 µg equiv./g for the low and high dose groups respectively) and liver (means of 0.15 and 3.2 µg equiv./g for the low and high dose groups respectively). Following 14 daily repeated dose of [14C]-aclonifen most of the tissues showed measurable levels of radioactivity, the highest levels being in the liver (mean of 1.06 µg equiv./g) and kidney (mean of 0.55 µg equiv./g). Tissue content was approximately proportional to dose.

The biotransformation of aclonifen occurred *via* six principal enzymatic reactions as identified from the metabolites observed in the rat: hydroxylation, methylation, reduction of the nitro group, N-acetylation, cleavage of the diphenyl ether bond and phase II type conjugation to sulphate or glucuronic acid.

In studies with [aniline-UL-14C]- aclonifen the major metabolic reaction was hydroxylation of the phenyl ring to form aclonifen-4-OH, which was then conjugated with glucuronic acid or sulfuric acid, together accounting for 10-38% of administered dose in urine and 1-12% in faeces in single dose experiments, or 16 to 28% in urine and 2-3% in faeces in the repeat dose experiment. Another important reaction was reduction of the nitro group to an amide with subsequent acetylation, leading to formation of diamino-acetyl compounds, in total up to 8% of dose in urine and 7% in faeces in single dose experiments. In the repeat dose experiment the levels were similar at 6-12% in urine and 4-12% in faeces. Unchanged parent compound was not detected in urine but was in faeces at 2-5% of the administered dose in both the single and repeat low dose experiments at 30 mg/kg and at 25-27% in the faeces of the single high dose experiment (1000 mg/kg). Cleavage of the diphenyl ether link was only observed in urine, leading to the formation of sulfate and glucuronide conjugates of the single aniline ring. In total these accounted for 7-10% of the administered dose in urine.

In the study with [phenoxy-UL-14C]- aclonifen chromatographic analysis was undertaken of bile, liver extracts, plasma and fat extracts, in addition to urine and faecal extracts. Unchanged parent compound was found at <0.5% of the administered dose in the faeces of intact rats, while in bile-duct cannulated rats it was detected at 13% of the dose. As observed for the aniline label, the major metabolic reaction was hydroxylation of the phenyl ring to form aclonifen-4-OH, which was then conjugated with glucuronic acid or sulfuric acid, together accounting for 44% in bile of the administered dose, 18-27% in urine and 1-3% in faeces. Reduction of the nitro group with subsequent acetylation led to formation of diamino-acetyl compounds at 4% of the administered dose in bile, 8-13% in urine and 7-10% in faeces. A further 24-30% of the dose was characterised as acid labile conjugates of aclonifen-diamino-acetyl-4-OH in faeces. Intra-molecular condensation (cyclisation) of the diamino-acetyl compounds and similar minor metabolites was identified. Cleavage of the diphenyl ether link was only observed in urine and liver, or as a very minor pathway only in bile, leading to formation of single phenoxy ring metabolites, such as aclonifen-4-OH-phenylsulfate, aclonifen-OH-phenylsulfate, aclonifen-OH-methoxy-phenylsulfate, aclonifen-phenylsulfate and aclonifen-phenyl-glucuronic acid. In total these accounted for 7% of the administered dose in urine. In plasma, hydroxylation of the

phenyl ring forming aclonifen-4-OH, with subsequent conjugation with sulfuric acid to form aclonifen-4-sulfate, was the main metabolic pathway. Formation of both diamino-acetyl compounds conjugated with glutathione and glucuronic acid conjugates was also observed in plasma. Parent compound and aclonifen-4-sulfate were the main compounds detected in perirenal fat. In the liver, a series of diamino-acetyl compounds were the prominent metabolites, the most abundant of which was aclonifen-diamino-aconitic acid-4-sulfate, together with a single acetylated glutathione conjugate, aclonifen-amino-acetyl-GSH-4-sulfate. Hydroxylation of the phenyl ring to form aclonifen-4-OH and its conjugate aclonifen-4-sulfate were also observed in the liver.

Two *in vitro* metabolism studies using cryopreserved hepatocytes have been conducted. The first compared mice and rats with humans, then a second study using the same methods compared rabbits and dogs with humans. The pattern of chromatographic peaks representing the metabolites was consistent, interspecies differences being predominantly quantitative. There was extensive metabolism in all species, the rat showing the most rapid rate of metabolism, the human being the slowest. No human-specific aclonifen metabolites exceeding 5% of total radioactivity were detected. The results are summarized in the table below, showing the inter-species comparison for those metabolites exceeding 5%.

Table 5.1-07: Summary metabolic profile for aclonifen (*in vitro*, hepatocytes) for inter-species comparison

Metabolite[a]	Relative retention time, approx.	Mouse		Rat		Rabbit		Dog		Human	
		Phen	Anil	Phen	Anil	Phen	Anil	Phen	Anil	Phen	Anil
M5	0.61			✓✓	✓✓						
M9 (M6)	0.64					✓✓	✓✓	✓	✓	✓	✓
M10 (M7)	0.66	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓
M13 (M36)	0.76			✓✓	✓✓	*	*			✓✓	✓
M14 (M22)	0.83	✓✓	✓✓	✓	✓	*	*	*	*	✓	✓
M17 (M9)	0.85	*	*	✓✓	✓✓	*	*		*	✓✓	✓✓
M18 (M30)	0.86	*	*		✓✓		*	*	*	✓✓	✓✓
M19	0.89	*	*	*	✓					*	*

a: Metabolite codes from the comparative study with human, rabbit and dog are in (parentheses). Equivalence with the mouse/rat/human comparative study are inferred from the relative retention times.
✓✓: Metabolites observed at ≥10%
✓: Metabolites observed at ≥5<10%
*: Metabolites observed at <5%

The metabolic pathway for aclonifen in the rat can be proposed as shown in the figure below:

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Figure 5.1-01: Proposed metabolic pathway for aclonifen in the rat

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CA 5.1.1 Absorption, distribution, metabolism and excretion by oral exposure

Data Point:	KCA 5.1.1/01
Report Author:	███
Report Year:	2004
Report Title:	The Metabolic Fate of Aclonifen in the Rat: Cleavage of the Diphenyl Ether Bond
Report No:	C042750
Document No:	M-233223-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Now is no longer acceptable

Assessment and conclusion by applicant:

This position paper was submitted in the previous renewal of aclonifen in place of conducting a metabolism study labelled on the phenyl ring. For this renewal a new metabolism study has been conducted with phenyl ring label therefore the position paper is now fully superseded by the ADME study of ███ ███ (2017), M-598008-01-1, summarized in KCA 5.1.1/07.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.1.1/02
Report Author:	;
Report Year:	2002
Report Title:	Absorption, distribution, metabolism and elimination of total radioactivity after an oral administration of [14C]-Aclonifen to male and female rats at a low dose (30 mg/kg) and a high dose (1000 mg/kg).
Report No:	C021364
Document No:	M-211055-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC part B; JMAF: 59 NohSan 4200; USEPA (=EPA): OPPTS 870,7485
Deviations from current test guideline:	Current Guideline: OECD 417, 2010 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

Wistar rats received a single dose of [aniline-UL-^{14}C]-aclonifen in PEG 200 at nominal dose levels of 30 or 1000 mg/kg bw. Urine was sampled for analysis at 12, 24, 48, 72, 96, 120, 144 and 168 h post dose, faeces at the same intervals from 24 h. Tissues were sampled for analysis after 168 h (7 days): liver, kidney, heart, brain, lungs, spleen, thyroids, uterus, thymus, bone and marrow, testes, ovaries, skeletal muscle, skin and fur, fat, large intestine, small intestine, stomach, pancreas, adrenals and the residual carcass.

Recovery of radioactivity 7 days post dose was satisfactory, with means between 96.4% and 99.6%. Estimated mean values for absorption were 40% and 51% for the high and low doses, respectively. The major route of excretion was the faeces in both sexes for the high dose (approximately 58% of administered radioactivity over the 168 h post dose). The corresponding value for urinary elimination was approximately 34%. The major routes of excretion in the low dose group were the faeces for the males (61% dose) and the urine for the females (59% dose). The majority of the administered radioactivity was eliminated during the first 24 hours at 30 mg/kg bw and by 48 hours post dose at 1000 mg/kg bw.

At seven days post dose the tissue levels of radioactivity were low to very low, between 0.13% and 0.17% of administered radioactivity. The highest mean specific tissue residues at 30 or 1000 mg/kg bw were in the liver, the skin and fur, and in fat. Apart from these tissues, only the adrenals, thymus, ovary, uterus and the carcass contained small, but measurable levels of radioactivity. All the remaining tissues showed concentrations that were BLQ.

The biotransformation of aclonifen appeared to occur in the rat *via* five main phase I enzymatic reactions: hydroxylation, methylation, reduction of the nitro group, N-acetylation, cleavage of the diphenyl ether link and phase II type conjugations. Up to 12 radioactive fractions were observed in the urine from the male rats and up to 11 fractions from the females. In the 30 mg/kg bw group only one fraction in the male urine (M11U) and two fractions in the female urine (M11U and M5U+M6U) represented more than 5% of the administered dose. M11U was the major fraction in urine and accounted for 9.8 % in males and 36.3% for females. It was identified by LC/MS/MS as being a sulphate of hydroxylated aclonifen (RPA 407074). M5U plus M6U (co-eluted metabolites) was the other major fraction and ranged from 5.9% to 10.3%. M5U was identified as a sulphate of the 2-chloro-3 hydroxy-6-nitroaniline (RPA 508285), and M6U as aclonifen after reduction of the nitro group, N-acetylation, hydroxylation and subsequent conjugation to sulphate. In urine at 1000 mg/kg bw, only two fractions in the urine from both sexes represented more than 5% of the

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administered dose. As for the low dose group these major fractions were M11U (12.9% in males; 9.6% in females) and M5U + M6U (7.8% in males; 7.6% in females). Enzymatic hydrolysis confirmed the urinary metabolites M1U, M8U and M9U as glucuronide conjugates. M1U seemed to correspond to glucurono-conjugated RPA 508285. Comparison with reference substances confirmed the presence of RPA 407074 (M12U), sulpho-conjugated RPA 508285 (M5U) and sulphated and hydroxylated aclonifen in the para position (M11U or sulpho-conjugated RPA 407074).

Up to 7 different radioactive fractions were observed in the faeces from the male rats and up to 9 fractions from the females. In males at 30 mg/kg bw, the major metabolites were M8F (12.1%), M3F (7.3%) and aclonifen (5.1%). Other metabolites were present at no more than 3.9% (M4F). In females, the main metabolite was M7F, at 2.1%. M3F was identified as a derivative of aclonifen with reduction of the nitro group, N-acetylation and hydroxylation. M8F was identified as hydroxylated aclonifen (RPA 407074). In the faeces from the 1000 mg/kg bw group, aclonifen was the major peak and represented 26.9% and 25.3% of the administered dose in males and females respectively. The second major fraction was M8F (2.6% in males and 5.7% in females).

It was concluded that aclonifen was rapidly and extensively eliminated following single oral dosing, with a mean of between 96% and 99% of the administered radioactivity being eliminated over a seven day postdosing period, the majority being eliminated in the first 24 h post dose at 30 mg/kg bw, and in the first 48 h at 1000 mg/kg bw. A minimum estimate of oral absorption was 40% and 51% for the high dose and low dose groups respectively.

At 168 hours post dosing, the mean recovery of radioactivity in the tissues from both dose groups ranged from 0.13% to 0.17% dose. Most tissues showed radioactivity concentrations below the limit of quantification, the highest being in the skin and fur (0.11 and 3.3 µg equiv/g for the low and high dose groups respectively) and liver (0.15 and 3.2 µg equiv/g for the low and high dose groups respectively).

The biotransformation of aclonifen occurred *via* five principal phase I enzymatic reactions, as identified from the metabolites observed in the rat: hydroxylation, methylation, reduction of the nitro group, N-acetylation, cleavage of the diphenyl ether link and phase II type conjugations to sulphate or glucoronic acid.

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I. MATERIALS AND METHODS

Study dates Start: 20 March 2001
In-life: 22 March to 03 April 2001
Completion: 05 March 2002

A. MATERIALS

1. **Test Item (unlabelled)** Aclonifen

Batch No.:	BES1572
Purity:	99.6%
Appearance:	Yellow powder
Storage:	*ca* 4°C, in brown glass
Expiry date:	April 2008

Test Item (labelled):

Batch No.:	PJS 968
Radiopurity:	>99% by TLC
Specific activity:	881.3 MBq/mmol (23.82 mCi/mmol)

+ = position of uniformly labelled ring

2. Test Organism Wistar rat

Source:	Iffa-Credo, France
Number, sex:	8 males, 8 females
Age:	6-10 weeks
Weight:	189-210 g at administration
Acclimatization:	5 days
Feed:	Powdered diet (RM1(E)SQC, SDS) Tap water *ad libitum*
Housing:	Metabolism cages

B. STUDY DESIGN AND METHODS

1. Treatment

The rats were dosed with [^{14}C]-aclonifen as a solution or suspension in PEG 200 at nominal dose levels of 30 or 1000 mg/kg bw.

Dose group	Nominal dose level (mg/kg)	Dose route	No. of rats	Nominal radioactive dose (µCi/kg)
SOLD	30	Oral	4M, 4F	125
SOHD	1000	Oral	4M, 4F	125

SOLD = Single Oral Low Dose, SOHD = Single Oral High Dose

2. Sampling

Dose groups	Sampling times (hours post dose)		
	Urine	Cage wash[a]	Faeces
SOLD & SOHD	12, 24, 48, 72, 96, 120, 144, 168	12, 24, 48, 72, 96, 120, 144, 168	24, 48, 72, 96, 120, 144, 168

a = cage washes performed with distilled water at all time points plus an additional water/methanol (50:50 v/v) rinse at 168 hours

Tissues were either removed or sampled following exsanguination under ketamine anaesthesia 7 days post dose; these were the liver, kidney, heart, brain, lungs, spleen, thyroids, uterus, thymus, bone and marrow, testes, ovaries, skeletal muscle, skin and fur, fat, large intestine, small intestine, stomach, pancreas and adrenals. The residual carcass was also retained for analysis.

3. Radioassay

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The amounts of radioactivity in the various samples were determined by liquid scintillation counting. Samples were counted in an appropriate scintillation cocktail using a Packard Tri-Carb 2100 TR counter with on-line computing facilities in which quenching effects were determined using an external standard and spectral quench parameter (tSIE) method. Counting was carried out with 1% precision or for a maximum duration of 10 to 30 minutes. For each biological matrix the background radioactivity was determined using two aliquots of the appropriate blank samples taken from a control animal. The mean of the background radioactivity was subtracted from the measured radioactivity for each sample. The limit of quantification (LOQ) was determined to be equal to twice the background in dpm. Values under this limit were noted BLQ (below limit of quantification). BLQ values were considered to be zero for mean calculations.

4. Chromatography

The metabolite profile was investigated using radio-HPLC of either pooled urine or pooled faecal samples. The HPLC used a Lichrospher 100-5RP18ec reverse phase column and a gradient elution using 20 mM ammonium acetate buffer containing 0.05% formic acid and acetonitrile containing 0.05% formic acid as the mobile phase. The identification of the metabolites was performed by LC/MS and LC/MS/MS. Enzymatic hydrolysis of urine was performed using a sulphatase from *Helix pomatia* H-1 and a beta-glucuronidase from bovine liver.

II. RESULTS AND DISCUSSION

Recovery of radioactivity from rats at 7 days postdose at 30 or 1000 mg/kg bw was satisfactory, with means between 96.4% and 99.6%.

Table 5.1-7: Recovery of radioactivity following single oral administration of [^{14}C]-aclonifen at 30 or 1000 mg/kg bw (% of administered dose)

Sample	30 mg/kg				1000 mg/kg			
	Males		Females		Males		Females	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Urine	31.83	6.92	58.47	5.48	33.82	4.95	34.79	4.18
Cage wash	4.31	0.90	7.09	2.30	5.80	2.11	5.88	2.75
Faeces	60.76	4.81	33.64	5.24	56.61	3.22	58.79	1.20
Tissues	0.14	0.03	0.17	0.04	0.13	0.02	0.13	0.04
Total	**97.03**	**3.74**	**99.36**	**0.32**	**96.36**	**0.26**	**99.58**	**1.26**

SD = standard deviation

A minimum estimate of the degree of oral absorption was made from the sum of the radioactivity found in the urine, tissues and cage washes which yielded mean values for the degree of absorption of 40% and 51% for the SOHD and SOLD groups respectively.

The major route of excretion was found to be the faeces in both sexes for the high dose group accounting for between 57% and 59% of the administered radioactivity over the 168 hours post dose. The corresponding values of urinary elimination were between 34% and 35%.

The major route of excretion in the low dose group was in the faeces for the males (61% dose) and the urine for the females (59% dose).

The majority of the administered radioactivity was eliminated during the first 24 hours at 30 mg/kg and by 48 hours post dose at 1000 mg/kg.

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Table 5.1-8: Elimination of radioactivity following administration of [14C]-aclonifen at 30 or 1000 mg/kg b/w (% of radioactivity)

Sample	Collection period (h)	30 mg/kg				1000 mg/kg			
		Males		Females		Males		Females	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Urine	0-12	22.77	2.75	46.89	6.08	8.55	1.48	7.12	1.99
	0-24	30.56	4.61	56.88	5.82	18.80	4.39	15.18	4.13
	0-48	31.35	6.91	58.22	5.52	33.08	5.12	33.51	4.42
	0-72	31.70	6.93	58.33	5.50	33.56	5.01	34.52	4.34
	0-96	31.77	6.92	58.39	5.49	33.69	4.98	34.67	4.23
	0-120	31.80	6.92	58.43	5.49	33.75	4.97	34.74	4.20
	0-144	31.82	6.92	58.46	5.48	33.80	4.96	34.76	4.20
	0-168	31.83	6.92	58.47	5.48	33.82	4.95	34.79	4.18
Faeces	0-24	53.62	4.63	26.57	4.05	29.09	8.82	24.27	9.67
	0-48	60.16	4.87	33.31	5.21	54.99	3.27	56.48	2.56
	0-72	60.61	4.80	33.48	5.24	56.35	3.18	58.48	1.29
	0-96	60.70	4.81	33.55	5.23	56.48	3.19	58.65	1.30
	0-120	60.74	4.81	33.59	5.23	56.55	3.21	58.71	1.26
	0-144	60.76	4.81	33.62	5.24	56.59	3.21	58.75	1.23
	0-168	60.76	4.81	33.64	5.24	56.61	3.22	58.79	1.20
Cage Wash	0-12	2.91	0.93	5.40	2.13	1.50	0.69	1.32	0.70
	0-24	3.83	0.96	6.65	2.17	3.70	1.78	3.31	1.89
	0-48	4.14	0.93	6.89	2.25	5.41	1.99	5.38	2.39
	0-72	4.20	0.92	6.96	2.27	5.59	2.07	5.64	2.57
	0-96	4.24	0.91	7.02	2.29	5.69	2.12	5.75	2.67
	0-120	4.26	0.91	7.04	2.30	5.73	2.12	5.79	2.70
	0-144	4.27	0.91	7.05	2.30	5.74	2.13	5.81	2.72
	0-168	4.31	0.90	7.09	2.30	5.80	2.11	5.88	2.75
Total	0-168	96.90	3.71	99.19	0.29	96.23	0.28	99.45	1.27

SD = Standard Deviation

At seven days post dose the tissue levels of radioactivity were low to very low, between 0.13% and 0.17% of administered radioactivity.

Table 5.1-9: Mean concentrations of [14C]-aclonifen in the tissues of rats following a single oral dose of 30 or 1000 mg/kg bw (µg aclonifen equivalents/g tissue)

Tissue	1000 mg/kg		30 mg/kg	
	Males	Females	Males	Females
Heart	BLQ	BLQ	BLQ	BLQ
Brain	BLQ	BLQ	BLQ	BLQ
Kidney	0.295	BLQ	BLQ	BLQ

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Tissue	1000 mg/kg		30 mg/kg	
	Males	Females	Males	Females
Muscle	BLQ	BLQ	BLQ	BLQ
Liver	3.502	2.790	0.146	0.148
Lung	BLQ	BLQ	BLQ	BLQ
Thymus	BLQ	BLQ	0.002	BLQ
Fat	0.204	0.501	0.007	0.016
Adrenals	BLQ	0.445	BLQ	0.005
Pancreas	BLQ	BLQ	BLQ	BLQ
Large intestine	BLQ	BLQ	BLQ	BLQ
Small intestine	BLQ	BLQ	BLQ	BLQ
Stomach	BLQ	BLQ	BLQ	BLQ
Spleen	BLQ	BLQ	BLQ	BLQ
Skin & fur	3.820	2.861	0.131	0.086
Bone & marrow	BLQ	BLQ	BLQ	BLQ
Thyroid	BLQ	BLQ	BLQ	BLQ
Testis	BLQ	-	BLQ	-
Ovary	-	BLQ	-	0.006
Uterus	-	BLQ	-	0.016
Carcass (estimated)	1.087	1.293	0.040	0.059

BLQ = below limit of quantification (twice background, considered as zero)

The highest mean specific tissue residues following administration with [^{14}C]-aclonifen at 30 or 1000 mg/kg bw were in the liver, the skin and fur, and in fat. Apart from these tissues, only the adrenals, thymus, ovary, uterus and the carcass contained small, but measurable levels of radioactivity. All the remaining tissues showed concentrations that were BLQ.

The biotransformation of aclonifen appears to occur *via* five main phase I enzymatic reactions, as identified from the metabolites observed in the rat: hydroxylation, methylation, reduction of the nitro group, N-acetylation, cleavage of the diphenyl ether link and phase II type conjugations.

Table 5.1-10: Quantification of metabolites in urine from rats following a single oral dose of [^{14}C]-aclonifen at 30 or 1000 mg/kg bw (% of administered radioactivity)

Metabolite	30 mg/kg (%dose)		1000 mg/kg (%dose)	
	Males	Females	Males	Females
M1U	1.38	1.42	0.92	1.87
M2U	n.d.	0.32	0.20	1.01
M3U	n.d.	0.38	0.90	1.28
M4U	0.21	1.49	1.17	0.77
M5U	4.27	n.d.	0.52	n.d.
M6U	2.45	n.d.	1.07	n.d.

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Metabolite	30 mg/kg (%dose)		1000 mg/kg (%dose)	
	Males	Females	Males	Females
M5U+M6U	3.55	5.87	7.82	7.64
M7U	1.12	1.29	0.85	1.28
M8U	n.d.	1.53	1.24	0.91
M9U	n.d.	n.d.	0.24	3.66
M10U	n.d.	0.21	n.d.	1.06
M11U	9.84	36.33	12.87	9.59
M12U	0.18	0.21	n.d.	n.d.
Aclonifen	n.d.	n.d.	n.d.	n.d.
Total	**23.00**	**49.05**	**27.80**	**29.07**

n.d. = not detected.

Up to 12 radioactive fractions were observed in the urine from the male rats and up to 11 fractions from the female rats. In the 30 mg/kg bw low dose group only one fraction in the male urine (M11U) and two fractions in the female urine (M11U and M5U+M6U) represented more than 5% of the administered dose. M11U was the major fraction in urine and accounted for 9.8 % in males and 36.3% for females. It was identified by LC/MS/MS as being a sulphate of hydroxylated aclonifen (RPA 407074).

M5U plus M6U (co-eluted metabolites) was the other major fraction and ranged from 5.9% to 10.3%. M5U was identified as a sulphate of the 2-chloro-3 hydroxy-6-nitroaniline (RPA 508285), and M6U as aclonifen after reduction of the nitro group, N-acetylation, hydroxylation and subsequent conjugation to sulphate.

A minor metabolite, M7U (nominal mass 354) accounted for 1.1% in males and 1.3% in females. LC/MS/MS analysis was unsuccessful in proposing a structure for this peak, although some structural information was deduced from the daughter-ion spectra: the presence of a fragment ion at m/z 108 (in negative ion mode) seemed to indicate a hydroxyl on the phenyl group, allowing subsequent conjugation, and the substituted phenyl group contained an even number of nitrogen atoms.

M7Ubis, a minor fraction of M7U, could be a positional isomer from M6Ubis. Other minor metabolites were also identified: M1U (1.4%) was identified as a glucuronide conjugate of the 2-chloro-3-hydroxy-6-aniline (RPA 508285), and M8U (1.5% in females only) as the glucuronide conjugate of hydroxylated aclonifen (RPA 407074).

In urine from the 1000 mg/kg bw high dose group, only two fractions in the urine from both sexes represented more than 5% of the administered dose. As for the low dose group these major fractions were M11U (12.9% in males; 9.6% in females) and M5U + M6U (7.8% in males; 7.6% in females). The other metabolites for which a structure has been proposed from the high dose group were: M1U (0.9% for males and 1.9% for females), M8U (1.2% for males and 0.9% for females), and M9U which could be a positional isomer of M8U (0.2% for males and 3.7% for females). Aclonifen was not detected in urine from the high dose group.

Enzymatic hydrolysis confirmed identification of the urinary metabolites M1U, M8U and M9U as glucuronide conjugates. M1U seemed to correspond to glucurono-conjugated RPA 508285. Comparison with reference substances (RPA 508285 and RPA 407074) confirmed the presence in urine of RPA 407074 (M12U), sulpho-conjugated RPA 508285 (M5U) and sulphated and hydroxylated aclonifen in the para position (M11U or sulpho-conjugated RPA 407074).

Up to 7 different radioactive fractions were observed in the faeces from the male rats and up to 9 fractions from the female rats.

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Table 5.1-11: Quantification of metabolites in faeces from rats following a single oral dose of [14C]-aclonifen at 30 or 1000 mg/kg bw (% of administered dose)

Metabolite	30 mg/kg (%dose)		1000 mg/kg (%dose)	
	Males	**Females**	**Males**	**Females**
M1F	n.d.	1.68	n.d.	n.d.
M2F	n.d.	1.25	n.d.	n.d.
M3F	7.29	n.d.	0.74	2.17
M4F	3.85	1.58	0.68	0.33
M5F	2.32	1.73	n.d.	n.d.
M6F	3.20	1.36	n.d.	n.d.
M7F	2.07	2.12	2.01	1.64
M8F	12.05	1.21	2.59	5.74
Aclonifen	5.13	1.52	26.92	25.29
Total	**35.91**	**12.45**	**32.94**	**35.17**

n.d. = not detected.

In males of the 30 mg/kg bw group, the major metabolites were M8F (12.1%), M3F (7.3%) and aclonifen (5.1%). Other metabolites were present at no more than 3.9% (M4F). In females, the main metabolite was M7F, at 2.1%. M3F was identified as a derivative of aclonifen with reduction of the nitro group, N-acetylation and hydroxylation. M8F was identified as hydroxylated aclonifen (RPA 407074).

In the faeces from the 1000 mg/kg bw group, aclonifen was the major peak and represented 26.9% and 25.3% of the administered dose in males and females respectively. The second major fraction was M8F (2.6% in males and 5.7% in females).

III. CONCLUSION

Aclonifen was rapidly and extensively eliminated following single oral dosing, with a mean of between 96% and 99% of the administered radioactivity being eliminated over a seven day postdosing period. The majority of this elimination occurred in the first 24 h post dose at 30 mg/kg bw, and in the first 48 h post dose at 1000 mg/kg bw.

A minimum estimate of the degree of oral absorption was made from the sum of the radioactivity found in the urine, tissues and cage washes, which yielded mean values of 40% and 51% for the high dose and low dose groups respectively.

At 168 hours post dosing, the mean recovery of radioactivity in the tissues from both dose groups ranged from 0.13% to 0.17% dose. After a single oral dose the majority of the tissues showed radioactivity concentrations below the limit of quantification. The highest mean concentrations were in the skin and fur (0.11 and 3.3 µg equiv/g for the low and high dose groups respectively) and liver (0.15 and 3.2 µg equiv/g for the low and high dose groups respectively).

The biotransformation of aclonifen occurred *via* five principal phase I enzymatic reactions, as identified from the metabolites observed in the rat: hydroxylation, methylation, reduction of the nitro group, N-acetylation, cleavage of the diphenyl ether link and phase II type conjugations to sulphate or glucuronic acid.

A proposed metabolic pathway for the rat based upon the results of this experiment is presented in Figure 5.1.1-1 below.

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Figure 5.1-3: Proposed metabolic pathway for aclonifen in the rat

M5U
M1U
RPA 508285
M8U or M9U
RPA 407074
M12U & M8F
M11U
ACLONIFEN
RPA 407291
M3F
M6U
M6Ubis/M7Ubis
{ } = postulated intermediates

Assessment and conclusion by applicant:
A good, acceptable, compliant study that produced valid results and conclusions.

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Assessment and conclusion by RMS:

Data Point:	KCA 5.1.1/03
Report Author:	[redacted]
Report Year:	2002
Report Title:	Rat bile excretion study Aclonifen
Report No:	C021085
Document No:	M-210558-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC Part B; JMAF: 59 NohSan No 4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	Current Guideline: OECD 417; 2010 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

Bile cannulated Wistar rats received a single dose of [14C]-aclonifen in PEG 200 at nominal dose levels of 30 or 1000 mg/kg bw. Urine and bile were sampled for analysis for the periods 0-6, 6-24 and 24-48 h post dose, faeces at 24 and 48 h. The intestinal tract, its contents, the stomach and its contents, and the residual carcass, were taken as samples for analysis after 2 days. The amounts of radioactivity in the samples were determined by liquid scintillation counting.

Recovery of radioactivity from rats at 2 days postdose at 30 or 1000 mg/kg bw was between 93.9% and 97.1%. The elimination of radiolabel *via* the bile at 30 mg/kg bw was slightly greater than that *via* the urine, which was greater than that *via* the faeces in both sexes. The difference between bile and urine was more marked for the female rats. The radioactivity in bile was higher in females than in males, while values in faeces were higher in males than in females. The mean proportions of the administered dose eliminated for the males and the females from the low dose group were 39.7% and 47.6% in bile, 35.9% and 34.3% in urine, 13.5% and 9.2% in faeces. In terms of total elimination (sum of urine, faeces, cage wash and bile), the elimination of [14C]-aclonifen and/or its metabolites appeared similar in males and in females (93.8% for both). At 1000 mg/kg bw the elimination of radiolabel *via* the faeces was greater than that *via* the bile, which was greater than that *via* the urine in male rats. In females, the elimination was approximately equal *via* faeces and bile and these two elimination routes were greater than that *via* urine. The mean proportions of the administered dose eliminated for the males and the females from the high dose group were 15.7% and 16.4% in bile, 39.8% and 14.0% in faeces, 9.6% and 6.2% in urine. Therefore, a significant difference in rate of elimination was observed between sexes: 65.9% of the administered dose was eliminated in male rats (bile + faeces + urine + cage wash), while 37.5% of was eliminated in females. At this dosage, the elimination of the radiolabel occurred principally *via* the faeces in male rats and via faeces and bile in female rats.

The biliary excretion study showed the highest levels of radioactivity were in the intestinal contents and residual carcass (for both sexes) in the low dose group. For the high dose group, highest levels were found in the stomach and intestinal contents. The results demonstrated that at the low dose,

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aclonifen was almost completely eliminated at 48 hours post-dose (no tissues presented significant levels of radioactivity).

It was demonstrated that biliary elimination was occurring and that the degree of oral absorption at the 30 mg/kg dose level was 81% and 86% for the males and females respectively (mean 83.5%). At the higher dose level of 1000 mg/kg there was clear evidence that the quantity of material was too great to permit complete dissolution in the gut and hence the level of bioavailability was reduced (*ca* 27 %).

I. MATERIALS AND METHODS

Study dates	Start:	12 April 2001
	In-life:	25 April 2001 to 08 January 2002
	Completion:	26 February 2002

A. MATERIALS

1.	Test Item (unlabelled):	Aclonifen
	Batch No.:	BES1572
	Purity:	99.6%
	Appearance:	Yellow powder
	Storage:	*ca* 4ºC, in brown glass
	Expiry date:	April 2008
	Test Item (labelled):	
	Batch No.:	PJS 968
	Radiopurity:	99.3% by TLC
	Specific activity:	881.3 MBq/mmol (23.82 mCi/mmol)

* = position of uniformly labelled ring.

2.	**Test Organism**	Wistar rat
	Source:	[redacted]
	Number, sex:	8 males, 8 females
	Age:	6-10 weeks
	Weight:	270-379 g at administration
	Acclimatization:	At least 5 days
	Feed:	Certified rodent diet A04C (UAR, France)
		Tap water *ad libitum*
	Housing:	Metabolism cages

B. STUDY DESIGN AND METHODS

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1. Treatment

Bile duct cannulated rats were dosed with [^{14}C]-aclonifen as a solution or suspension in PEG 200 at nominal dose levels of 30 or 1000 mg/kg bw.

Dose group	Nominal dose level (mg/kg)	Dose route	No. of rats	Nominal radioactive dose (µCi/kg)
SOLD	30	Oral	4M, 4F	125
SOHD	1000	Oral	4M, 4F	125

SOLD = Single Oral Low Dose, SOHD = Single Oral High Dose

2. Sampling

Dose groups	Sampling times (hours post dose)			
	Urine	Cage wash[a]	Bile	Faeces
SOLD and SOHD	0-6, 6-24, 24-48	24, 48	0-6, 6-24, 24-48	24, 48

a = cage washes performed with distilled water at 24 h intervals plus an additional acetonitrile rinse at 48 h post dose

The rats were exsanguinated under anaesthesia and the intestinal tract, intestinal tract contents, stomach and the stomach contents taken as samples. The residual carcass was also retained for analysis and plasma was prepared from cardiac blood samples by centrifugation.

3. Radioassay

The amounts of radioactivity in the samples were determined by liquid scintillation counting. Samples were counted for 10 minutes or for 2 sigma % in an appropriate scintillation cocktail using a Packard 1900 TR counter with on-line computing facilities, in which quenching effects were determined using an external standard and spectral quench parameter (tSIE) method. Efficiency correlation curves were prepared for each scintillation cocktail and were regularly checked by the use of [^{14}C]-n-hexadecane standards. The scintillation counter was recalibrated when a deviation of greater than 2% was observed when counting quality control standards. The limit of detection was taken to be twice the background values for blank samples in appropriate scintillation cocktails.

II. RESULTS AND DISCUSSION

Recovery of radioactivity from rats at 2 days postdose at 30 or 1000 mg/kg bw was satisfactory, with means between 93.9% and 97.1%.

Table 5.1-19: Recovery of radioactivity following single oral administration of [^{14}C]-aclonifen at 30 or 1000 mg/kg bw (% of administered dose)

Sample	30 mg/kg				1000 mg/kg			
	Males		Females		Males		Females	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Urine	35.91	13.03	34.29	10.96	9.56	6.28	6.17	2.46
Faeces	13.54	3.87	9.18	1.63	39.83	4.81	14.01	11.05
Bile	39.65	10.27	47.61	8.20	15.68	3.27	16.40	3.21
Cage wash	4.68	2.89	2.68	0.87	0.83	0.51	0.94	0.36
Tissues	3.18	2.15	1.63	0.75	28.03	12.42	59.61	18.84

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Sample	30 mg/kg				1000 mg/kg			
	Males		Females		Males		Females	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total	**96.96**	**1.13**	**95.39**	**1.50**	**93.92**	**2.75**	**97.14**	**5.29**

SD = standard deviation

The elimination of radiolabel *via* the bile at 30 mg/kg bw was slightly greater than that *via* the urine, which was greater than that *via* the faeces in both sexes. The difference between bile and urine was more marked for the female rats. The radioactivity in bile was higher in females than in males, while values in faeces were higher in males than in females. The mean proportions of the administered dose eliminated for the males and the females from the low dose group were 39.7% and 47.6% in bile, 35.9% and 34.3% in urine, 13.5% and 9.2% in faeces. In terms of total elimination (sum of urine, faeces, cage wash and bile), the elimination of [^{14}C]-aclonifen and/or its metabolites appeared similar in males and in females (93.8% for both).

In contrast, at 1000 mg/kg bw the elimination of radiolabel *via* the faeces was greater than that *via* the bile, which was greater than that *via* the urine in male rats. In females, the elimination was approximately equal *via* faeces and bile and these two elimination routes were greater than that *via* urine. The mean proportions of the administered dose eliminated for the males and the females from the high dose group were 15.7% and 16.4% in bile, 39.8% and 14.0% in faeces, 9.6% and 6.2% in urine. Therefore, a significant difference in rate of elimination was observed between sexes: 65.9% of the administered dose was eliminated in male rats (bile + faeces + urine + cage wash), while 37.5% of was eliminated in females. At this dosage, the elimination of the radiolabel occurred principally *via* the faeces in male rats and via faeces and bile in female rats.

The biliary excretion study showed the highest levels of radioactivity were in the intestinal contents and residual carcass (for both sexes) in the low dose group. For the high dose group, highest levels were found in the stomach and intestinal contents. These results demonstrated that at the low dose, aclonifen was almost completely eliminated at 48 hours post-dose (no tissues presented significant levels of radioactivity).

The absorption level of aclonifen can be evaluated from the recoveries obtained in urine (plus cage wash), bile and tissues (excluding stomach and intestinal contents), as shown below.

Table 5.1-20: Radioactivity in tissues following single oral administration of [^{14}C]-aclonifen at 30 or 1000 mg/kg bw (% of administered dose)

Tissue	30 mg/kg				1000 mg/kg			
	Males		Females		Males		Females	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cardiac Blood	0.018	0.005	0.015	0.006	0.040	0.021	0.012	0.007
Content Intestine	1.955	1.690	0.596	0.218	3.144	1.928	18.116	10.639
Residual Carcass	1.021	0.363	0.950	0.553	1.306	0.529	1.330	0.185
Cardiac Plasma	0.005	0.002	0.004	0.002	0.018	0.009	0.003	0.002
Intestine	0.074	0.058	0.044	0.024	0.088	0.074	0.143	0.071
Stomach	0.026	0.026	0.009	0.006	0.143	0.106	0.195	0.120
Content Stomach	0.081	0.147	0.011	0.014	23.295	13.815	39.815	16.458
Total	**3.180**	**2.151**	**1.629**	**0.749**	**28.033**	**12.416**	**59.614**	**18.838**

SD = standard deviation

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Table 5.1-21: Absorption of radioactivity following single oral administration of [^{14}C]-aclonifen at 30 or 1000 mg/kg bw (% of administered dose)

	% dose	
	30 mg/kg bw	1000 mg/kg bw
Males	81.4	27.7
Females	85.6	25.2
Mean	83.5	26.5

III. CONCLUSION

The bile excretion experiment demonstrated that biliary elimination was occurring and that the degree of oral absorption at the 30 mg/kg dose level was 81% and 86% for the males and females respectively (mean 83.5%). At the higher dose level of 1000 mg/kg there was clear evidence that the quantity of material was too great to permit complete dissolution in the gut and hence the level of bioavailability was reduced (*ca* 27 %).

Assessment and conclusion by applicant:

A good, acceptable, compliant study that produced valid results and conclusions.

Assessment and conclusion by RMS:

Data Point:	KCA 5.1.1/04
Report Author:	[redacted]
Report Year:	2002
Report Title:	Rat blood/plasma kinetics study Aclonifen
Report No:	C021087
Document No:	M-210562-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC Part B; JMAF: 59 NohSan No 4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	Current Guideline: OECD 417, 2010 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

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Wistar rats were dosed with [^{14}C]-aclonifen as a solution in PEG 200 at nominal dose levels of 30 and 1000 mg/kg b/w. Blood samples were taken at 0, 0.5, 1, 2, 3, 4, 6, 8, 24 h post dose and at 24 hour intervals up to 168 hours post dose. Whole blood and plasma were assayed for radioactivity by liquid scintillation counting.

it was indicated that the mean maximum concentration achieved in blood and plasma was always greater for males than females at both high and low dose levels, but the increase was not dose-proportional. The time to achieve maximal blood/plasma concentrations was similar between the sexes at both dose levels. There was no significant sex difference in the terminal elimination half-life values observed for the blood and plasma at either dose level, but there was a much more rapid elimination from the plasma compared to whole blood.

The AUC data demonstrated that the males received a higher systemic exposure to aclonifen than the females at both dose levels in both blood and plasma. At 30 mg/kg bw it was approximately 3.4 times higher for the males than for the females, while at 1000 mg/kg bw the difference was reduced to approximately 2 times. The increases in the 0-168 hour AUC values between the low and high dose groups were not proportional to the dose rate, and it was clear that the 1000 mg/kg bw dose surpassed the threshold for proportional absorption.

I. MATERIALS AND METHODS

Study dates	Start:	25 September 2001
	In-life:	18 October 2001 to 11 January 2002
	Completion:	19 February 2002

A. MATERIALS

1. Test Item (unlabelled) Aclonifen

Batch No.:	BES1572
Purity:	99.6%
Appearance:	Yellow powder
Storage:	*ca* 4ºC, in brown glass
Expiry date:	April 2008
Test Item (labelled):	
Batch No.:	PJS968
Radiopurity:	99.3% by TLC
Specific activity:	881.3 MBq/mmol (23.82 mCi/mmol)

+ = position of uniformly labelled ring.

2. Test Organism Wistar rat

Source:	[redacted]
Number, sex:	8 males, 8 females
Age:	6-10 weeks

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Weight:	206-247 g at administration
Acclimatization:	At least 5 days
Feed:	Certified rodent diet A04C (UAR, France)
	Tap water *ad libitum*
Housing:	Metabolism cages

B. STUDY DESIGN AND METHODS

1. Treatment

Wistar rats were dosed with [^{14}C]-aclonifen as a solution in PEG 200 at nominal dose levels of 30 and 1000 mg/kg b/w:

Dose Group	Nominal Dose level (mg/kg)	Dose route	N° of Rats	Nominal Radioactive dose (µCi/kg)
SOLD	30	Oral	4M, 4F	125
SOHD	1000	Oral	4M, 4F	125

SOLD = Single Oral Low Dose. SOHD = Single Oral High Dose.

2. Sampling

Dose Groups	Sampling Times (hours post dose)
	Blood (& Plasma)
SOLD and SOHD	0, 0.5, 1, 2, 3, 4, 6, 8, 24 and at 24 hour intervals up to 168 hours post dose

Plasma samples were prepared from the whole blood samples by centrifugation of the heparinised capillary collection tubes.

3. Radioassay

The amounts of radioactivity in the samples were determined by liquid scintillation counting. Samples were counted for 10 minutes or for 2 sigma % in an appropriate scintillation cocktail using a Packard 1900 TR counter with on-line computing facilities, in which quenching effects were determined using an external standard and spectral quench parameter (tSIE) method. Efficiency correlation curves were prepared for each scintillation cocktail and were regularly checked by the use of [^{14}C]-n-hexadecane standards. The scintillation counter was recalibrated when a deviation of greater than 2% was observed when counting quality control standards. The limit of detection was taken to be twice the background values for blank samples in appropriate scintillation cocktails.

II. RESULTS AND DISCUSSION

The actual achieved mean dose rates were 29.8 and 976.0 mg/kg for the low and high dose groups respectively.

The mean concentrations of radioactivity in rat blood and plasma following single oral doses of [^{14}C]-aclonifen, and the resulting pharmacokinetic parameters, are shown in the following tables.

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Table 5.1-22: Concentration of radioactivity in whole blood following single oral administration of [^{14}C]-aclonifen at 30 or 1000 mg/kg bw (µg [^{14}C]-aclonifen equivalents/g blood)

Time (h post dose)	SOLD (30 mg/kg)				SOHD (1000 mg/kg)			
	Males		Females		Males		Females	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	n.d.		n.d.		n.d.		n.d.	
0.5	12.877	1.326	7.084	2.502	11.291	3.921	5.906	2.442
1	13.292	3.116	6.357	2.263	17.336	5.813	8.020	3.255
2	14.344	4.023	4.940	0.706	28.124	14.161	10.269	3.058
3	13.118	2.758	5.166	1.612	53.377	5.812	9.419	2.591
4	14.830	3.981	5.679	1.957	62.708	11.380	12.993	4.905
6	12.905	2.996	3.549	1.109	78.308	17.026	15.086	6.810
8	12.850	2.507	2.885	0.891	100.260	12.733	14.378	5.395
24	2.217	2.300	0.330	0.074	52.956	15.861	17.644	7.938
48	0.164	0.031	0.125	0.011	5.180	2.024	20.800	7.013
72	0.097	0.007	0.095	0.014	3.479	1.400	8.162	2.560
96	0.092	0.013	0.093	0.010	2.333	0.795	3.694	0.827
120	0.067	0.011	0.068	0.003	2.106	0.760	3.280	0.634
144	0.069	0.010	0.067	0.003	1.874	0.735	2.812	0.658
168	0.051	0.009	0.052	0.003	0.852	0.987	1.935	0.339

S.D. = standard deviation; n.d. = not detected

Table 5.1-23: Concentration of radioactivity in plasma following single oral administration of [^{14}C]-Aclonifen at 30 or 1000 mg/kg bw (µg [^{14}C]-aclonifen equivalents/g plasma)

Time (h post dose)	SOLD (30 mg/kg)				SOHD (1000 mg/kg)			
	Males		Females		Males		Females	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	n.d.	n.a.	n.d.	n.a.	n.d.	n.a.	n.d.	n.a.
0.5	21.419	1.929	12.709	4.326	18.290	5.297	10.690	4.639
1	23.180	5.414	11.556	3.965	28.183	10.203	13.304	5.250
2	23.373	5.761	8.770	1.272	53.546	15.342	18.289	8.072
3	21.977	4.799	9.625	3.240	105.290	10.467	22.595	7.541
4	24.468	6.178	10.242	3.738	147.750	5.451	27.636	10.752
6	21.606	5.888	6.384	1.480	169.720	3.522	24.961	9.796
8	20.399	5.454	5.028	1.567	156.340	17.186	22.327	9.389
24	3.072	3.080	0.420	0.081	80.359	14.166	25.011	11.557
48	0.173	0.067	0.116	0.019	4.288	1.981	26.520	10.271
72	0.069	0.011	0.068	0.006	1.894	1.353	7.491	3.616
96	0.016	0.032	0.035	0.041	n.d.		1.193	1.426
120	n.d.		n.d.		n.d.		n.d.	
144	n.d.		n.d.		n.d.		n.d.	
168	n.d.		n.d.		n.d.		n.d.	

S.D. = standard deviation; n.d. = not detected

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Table 5.1-24: Pharmacokinetic parameters following single oral administration of [14C]-aclonifen at 30 or 1000 mg/kg bw (mean ± standard deviation)

WHOLE BLOOD		C_{max} (µg equiv./g)	T_{max} (h)	$t_{0.5}$ (h)	$AUC_{(0-168h)}$ (µg.h/g)
30 mg/kg bw	Males	17.45 ± 4.44	2.90 ± 1.45	102.65 ± 16.30	264.26 ± 27.81
	Females	8.67 ± 1.92	2.55 ± 2.14	102.93 ± 9.66	77.95 ± 9.96
1000 mg/kg bw	Males	131.40 ± 38.71	12.72 ± 2.90	71.75 ± 11.91	2681.87 ± 560.58
	Females	22.75 ± 6.70	28.18 ± 18.67	75.67 ± 3.98	1513.77 ± 384.06
PLASMA					
30 mg/kg bw	Males	30.22 ± 7.12	2.46 ± 1.65	12.73 ± 7.81	404.58 ± 40.95
	Females	15.18 ± 3.44	2.54 ± 2.10	23.75 ± 6.65	120.46 ± 14.33
1000 mg/kg bw	Males	171.00 ± 4.62	6.39 ± 0.50	24.79 ± 14.04	3912.80 ± 526.72
	Females	33.75 ± 5.93	4.70 ± 1.39	23.28 ± 4.38	1693.65 ± 461.75

C_{max} = maximal concentration, T_{max} = time of maximal concentration, $t_{0.5}$ = terminal elimination half-life, AUC = area under curve.

The results from the low dose (30 mg/kg bw) group indicated that the mean C_{max} value for male rats was approximately twice that in females. The absorption of radioactivity was very rapid, with mean t_{max} values 2.5-3 h, followed by relatively rapid elimination over the first 24 hours post dose and then a slower terminal elimination. The area under the curve AUC (0 - 168h) indicated a higher systemic exposure for males than for females (approximately 3-3.5 times higher).
The results from the high dose (1000 mg/kg bw) group also indicated higher mean blood concentration for males than for females (5-6 times higher), but the increase in absolute levels was not proportional with dose. Absorption of the radiolabel appeared relatively slow, with mean t_{max} values of 13 h and 28 h for males and females respectively, followed by a decline slightly faster than observed for the low dose group. Systemic exposure as indicated by the AUC values indicated that male exposure was approximately 1.8 times that of females at this dose.

Comparison of the estimations of area under the curve AUC (0 - 168h) for the low dose group with the high dose group indicated that aclonifen was proportionately less well absorbed at the high dose level. The results obtained for the plasma reflected the results observed for whole blood.

III. CONCLUSION

Following single oral dosing of [14C]-aclonifen, it was indicated that the mean maximum concentration achieved in blood and plasma was always greater for males than females at both high and low dose levels, but the increase was not dose-proportional. The time to achieve maximal blood/plasma concentrations was similar between the sexes at both dose levels. There was no significant sex difference in the terminal elimination half-life values observed for the blood and plasma at either dose level, but there was a much more rapid elimination from the plasma compared to whole blood.

The AUC data demonstrated that the males received a higher systemic exposure to aclonifen than the females at both dose levels in both blood and plasma. At 30 mg/kg bw it was approximately 3.4 times higher for the males than for the females, while at 1000 mg/kg bw the difference was reduced to approximately 2 times. The increases in the 0-168 hour AUC values between the low and high dose groups were not proportional to the dose rate, and it was clear that the 1000 mg/kg bw dose surpassed the threshold for proportional absorption.

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Assessment and conclusion by applicant:
A good, compliant, acceptable study that produced valid results and conclusions.

Assessment and conclusion by RMS:

Data Point:	KCA 5.1.1/05
Report Author:	[redacted]; [redacted]
Report Year:	2002
Report Title:	Absorption, distribution, metabolism and elimination of total radioactivity after a repeated oral administration of [14C]-Aclonifen to male and female rats at a low dose (30 mg/kg.
Report No:	C021401
Document No:	M-211131-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC part B; JMAF: 59 NohSan 4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	Current Guideline: OECD 417, 2010 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

Wistar rats received 14 daily doses of [^{14}C]-aclonifen (labelled in the nitrophenyl ring) as a solution in PEG 200 at the nominal dose level of 30 mg/kg bw. From dose day 1 to 13 the urine and faeces were collected daily. For dosing day 14 the samples were collected at 12, 24, 48, 72, 96, 120, 144 and 168 h (urine) and at 24, 48, 72, 96, 120, 144 and 168 h (faeces). Tissues were sampled for analysis following exsanguination under anaesthesia 7 days after the 14th dose: the liver, kidney, heart, brain, lungs, spleen, thyroids, uterus, thymus, bone with marrow, testes, ovaries, skeletal muscle, skin and fur, fat, large intestine, small intestine, stomach, pancreas and adrenals, and the residual carcass. Radioactivity in the various samples was determined by liquid scintillation counting. The limit of detection was determined to be equal to twice background in dpm. Values under this limit were noted BLQ (below limit of quantification and considered as zero for mean calculations. The metabolite profile was investigated using radio-HPLC of either pooled urine or pooled faecal samples.

Recovery of radioactivity at 168 h after the 14th dose was quantitative, with means of 96-97%. Urine was the major route of excretion for females, accounting for (including cage wash) *ca* 59% of the dose, while in males it accounted for *ca* 50%. Excretion rate was high, with mean totals between 85% and 95% of the dose being eliminated over 24 h periods for the males and between 91% and 96% for the females. Following cessation of dosing, the majority of administered radioactivity was eliminated during the first 24 h after the 14th dose.

Tissue radioactivity levels in this repeated low dose group accounted for 0.13% dose in males and 0.09% in females and thus both sexes displayed similar and relatively low levels of radioactivity in the

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tissues 168 hours post-dosing. The highest mean tissue residues following repeated administration at 30 mg/kg body weight were in the liver. The second most significant radioactive residues were in the kidney. For the males, the carcass, thyroids, skin & fur and lungs had radioactivity concentrations greater than 0.2 µg equiv./g. The fat, pancreas and small intestine had concentrations between 0.1 µg equiv./g and 0.2 µg equiv./g, while the remaining tissues had concentrations <0.1 µg equiv./g. Muscle and heart were BLQ. For the females, the carcass, skin & fur and lungs had radioactivity concentrations greater than 0.2 µg equiv./g. The spleen, thyroid, adrenals, fat, pancreas and ovary showed concentrations between 0.1 µg equiv./g and 0.2 µg equiv./g, while the remaining tissues had concentrations <0.1 µg equiv./g. The brain, muscle, large intestine and heart were BLQ.

The biotransformation of aclonifen following repeated administration appeared to occur *via* five main enzymatic reactions, as identified from the metabolites observed in the rat: hydroxylation, methylation, reduction of the nitro group, N-acetylation and phase II type conjugations. In the urine from the repeat dose group, four metabolites were detected. Only two of them, MBU and MCU, represented more than 5% of the administered dose and the major part of the radioactivity; 12% (males) and 5.5% (females) for MBU and 15% (males) and 27% (females) for MCU. In faeces, at least nine metabolites of low abundance were detected. Aclonifen was present in both sexes and accounted for 3% in males and for 2.2% in females. The main metabolite was MDF (aka M3F in the single dose studies) and represented 12% of the total administered dose in the faeces from males and 4.7% in females.

It was concluded that aclonifen was rapidly and extensively eliminated following repeated dosing with a mean of between 96% and 97% of the administered radioactivity being eliminated over a seven-day period following cessation of dosing. The majority of this elimination occurred in the first 48 hours following the last of the fourteen oral low dose administrations. A minimum estimate of the degree of oral absorption gave mean values of 50% and 59% for the males and females respectively.

At 168 hours post dosing, the mean recovery of radioactivity in the tissues of the animals both sexes ranged from 0.09% (females) to 0.13% dose (males). The highest mean tissue residues following repeated administration with [^{14}C]-aclonifen at 30 mg/kg body weight were liver, the second most significant residues being in the kidney.

The biotransformation of aclonifen following repeated administration was qualitatively similar to that observed following single oral dosing and included five principal enzymatic reactions: hydroxylation, methylation, reduction of the nitro group, N-acetylation and phase II type conjugations.

I. MATERIALS AND METHODS

Study dates	Start:	20 March 2001
	In-life:	29 March to 26 April 2001
	Completion:	19 March 2002

A. MATERIALS

1. Test Item (unlabelled)	Aclonifen
Batch No.:	BES1572
Purity:	99.6%
Appearance:	Yellow powder
Storage:	*ca* 4ºC, in brown glass
Expiry date:	April 2008
Test Item (labelled):	
Batch No.:	PJS 968
Radiopurity:	>99% by TLC
Specific activity:	881.3 MBq/mmol (23.82 mCi/mmol)

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+ = position of uniformly labelled ring

2. Test Organism Wistar rat

Source:	
Number, sex:	4 males, 4 females
Age:	6-10 weeks
Weight:	174-201 g at administration
Acclimatization:	8 days
Feed:	Pelleted diet (RM1(E)SQC, SDS) during acclimatization, then powdered diet (RM1(E)SQC FG, SDS)
	Tap water *ad libitum*
Housing:	Metabolism cages

B. STUDY DESIGN AND METHODS

1. Treatment

Rats received 14 daily doses of [^{14}C]-aclonifen as a solution in PEG 200 at the nominal dose level of 30 mg/kg bw.

Dose Group	Nominal Dose level (mg/kg)	Dose route	N° of Rats	Nominal Radioactive dose (µCi/kg)
ROLD	30	Oral	4M, 4F	100

ROLD = Repeat Oral Low Dose

2. Sampling

From dose day 1 to dose day 13 the urine and faeces were collected daily. For dosing day 14 the samples were collected as shown below:

Dose Groups	Sampling Times in terms of hours post dose		
	Urine	Cage wash[a]	Faeces
ROLD	12, 24, 48, 72, 96, 120, 144, 168	12, 24, 48, 72, 96, 120, 144, 168	24, 48, 72, 96, 120, 144, 168

a = cage washes performed with distilled water at all time points plus an additional water/methanol (50:50 v/v) rinse at 168 hours

Tissues were either removed or sampled following exsanguination under anaesthesia 7 days after the 14th dose. These were the liver, kidney, heart, brain, lungs, spleen, thyroids, uterus, thymus, bone with

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marrow, testes, ovaries, skeletal muscle, skin and fur, fat, large intestine, small intestine, stomach, pancreas and adrenals. The residual carcass was also retained for analysis.

3. Radioassay

The amounts of radioactivity in the various samples were determined by liquid scintillation counting. Samples were counted in an appropriate scintillation cocktail using a counter with on-line computing facilities in which quenching effects were determined using an external standard and spectral quench parameter (tSIE) method. Counting was carried out with 1% precision or for a maximum duration of 10 to 30 minutes. For each biological matrix the background radioactivity was determined using two aliquots of the appropriate blank samples taken from a control animal. The mean of the background radioactivity was subtracted from the measured radioactivity for each sample. The limit of detection (LOD) was determined to be equal to twice background in dpm. Values under this limit were noted BLQ (below limit of quantification). BLQ values were considered as zero for mean calculations.

4. Chromatography

The metabolite profile was investigated using radio-HPLC of either pooled urine or pooled faecal samples. The HPLC used a reverse phase column and a gradient elution using 20 mM ammonium acetate buffer containing 0.05% formic acid and acetonitrile containing 0.05% formic acid as the mobile phase. Identification of the metabolites was performed by LC/MS and LC/MS/MS. Enzymatic hydrolysis of urine was performed using a sulphatase from *Helix pomatia* H-1.

II. RESULTS AND DISCUSSION

Recovery of radioactivity at 168 hours after the 14th dose administration was quantitative with means of 97.04 ± 1.36% and 95.69 ± 2.48% for the males and females respectively.

Table 5.1-25: Recovery of radioactivity following 14 daily oral administrations of [^{14}C]-aclonifen at 30 mg/kg bw (% of administered dose)

	14 days at 30 mg/kg			
	Males		Females	
Sample	**Mean**	**SD**	**Mean**	**SD**
Urine	43.51	1.80	49.44	4.83
Cage wash	47.01	1.83	37.02	2.59
Faeces	6.39	1.01	9.14	4.66
Tissues	0.13	0.08	0.09	0.02
Total	**97.04**	**1.36**	**95.69**	**2.48**

SD = standard deviation

Following 14 daily administrations of radiolabelled aclonifen at 30 mg/kg bw the urine was the major route of excretion for females, accounting for (including cage wash) *ca* 59% of the dose, while in males it accounted for *ca* 50% of the dose.

Table 5.1-26: Elimination of radioactivity during the first 13 repeat daily administrations of [^{14}C]-aclonifen at 30 mg/kg bw (% of administered dose)

Dose Day	Urine		Cage wash		Faeces		Total	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
1	42.13	58.14	2.26	3.50	40.79	29.21	85.18	90.85

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Dose Day	Urine		Cage wash		Faeces		Total	
	Males	Females	Males	Females	Males	Females	Males	Females
2	43.83	57.31	2.76	3.29	44.27	32.33	90.85	92.92
3	44.23	57.86	2.70	3.25	44.30	34.77	91.22	95.89
4	45.43	57.40	3.19	3.61	45.47	35.36	94.09	96.37
5	45.26	56.47	3.34	4.24	45.52	35.26	94.12	95.96
6	45.39	54.60	3.73	5.23	46.06	34.52	95.18	94.35
7	45.29	53.91	4.04	5.97	45.36	34.75	94.68	94.62
8	44.8	53.02	4.43	6.61	45.87	34.97	95.09	94.59
9	44.25	51.82	4.83	7.70	45.77	35.11	94.84	94.63
10	43.67	50.72	5.07	7.90	45.37	34.88	94.11	93.49
11	43.62	49.99	5.31	8.08	45.16	35.34	94.09	93.41
12	42.83	48.81	6.20	9.34	45.27	35.62	94.31	93.76
13	43.08	49.15	6.10	9.01	45.41	36.37	94.59	94.53

During the 13-day dosing period before the last dose, the excretion rate was high with mean totals of between 85% and 95% of the dose being eliminated over 24 h periods for the males and between 91% and 96% for the females. Following cessation of dosing, the majority of administered radioactivity was eliminated during the first 24 h after the 14th dose.

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Table 5.1-27: Elimination of radioactivity following the 14th repeat daily administrations of [^{14}C]-aclonifen at 30 mg/kg bw (% of administered dose)

Sample	Period (h)	30 mg/kg			
		Males		Females	
		Mean	SD	Mean	SD
Urine	0-12	41.86	1.67	48.62	4.79
	0-24	43.09	1.76	49.21	4.86
	0-48	43.42	1.79	49.34	4.85
	0-72	43.46	1.79	49.37	4.85
	0-96	43.49	1.80	49.38	4.85
	0-120	43.49	1.80	49.40	4.85
	0-144	43.50	1.80	49.42	4.84
	0-168	43.51	1.80	49.44	4.83
Faeces	0-24	46.12	1.86	36.50	2.70
	0-48	46.82	1.85	36.89	2.59
	0-72	46.91	1.83	36.94	2.59
	0-96	46.96	1.83	36.98	2.58
	0-120	46.98	1.83	36.99	2.59
	0-144	47.00	1.83	37.00	2.59
	0-168	47.01	1.83	37.02	2.59
Cage Wash	0-12	5.98	0.92	8.87	4.64
	0-24	6.24	1.00	9.00	4.65
	0-48	6.30	1.02	9.04	4.64
	0-72	6.32	1.01	9.06	4.64
	0-96	6.33	1.01	9.07	4.65
	0-120	6.34	1.01	9.08	4.65
	0-144	6.35	1.01	9.10	4.65
	0-168	6.39	1.01	9.14	4.66
Total	**0-168**	**97.02**	**1.36**	**95.68**	**2.48**

SD = standard deviation

Tissue radioactivity levels in this repeated low dose group accounted for 0.13% dose in males and 0.09% in females and thus both sexes displayed similar and relatively low levels of radioactivity in the tissues 168 hours post-dosing.

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Table 5.1-28: Mean concentration of [^{14}C]-aclonifen in the tissues of rats following 14 repeat daily oral doses at 30 mg/kg bw (µg aclonifen equivalents/g tissue)

Tissue	Repeat 30 mg/kg	
	Males	Females
Heart	BLQ	BLQ
Brain	0.006	BLQ
Kidney	0.667	0.423
Muscle	BLQ	BLQ
Liver	1.339	0.774
Lung	0.285	0.350
Thymus	0.031	0.028
Fat	0.131	0.128
Adrenals	0.099	0.140
Pancreas	0.112	0.118
Large intestine	0.041	BLQ
Small intestine	0.112	0.047
Stomach	0.092	0.065
Spleen	0.055	0.199
Skin & fur	0.401	0.244
Bone & marrow	0.048	0.006
Thyroid	0.468	0.167
Testis	0.030	-
Ovary	-	0.115
Uterus	-	0.050
Carcass	0.475	0.380

BLQ = below limit of quantification

The highest mean tissue residues following repeated administration at 30 mg/kg body weight were in the liver. The second most significant radioactive residues were in the kidney.

For the males, the carcass, thyroids, skin & fur and lungs were found to have radioactivity concentrations greater than 0.2 µg equiv./g. The fat, pancreas and small intestine had concentrations between 0.1 µg equiv./g and 0.2 µg equiv./g while the remaining tissues had concentrations <0.1 µg equiv./g. Muscle and heart were BLQ.

For the females, the carcass, skin & fur and lungs had radioactivity concentrations greater than 0.2 µg equiv./g. The spleen, thyroid, adrenals, fat, pancreas and ovary showed concentrations between 0.1 µg equiv./g and 0.2 µg equiv./g while the remaining tissues had concentrations <0.1 µg equiv./g. The brain, muscle, large intestine and heart were BLQ.

The biotransformation of aclonifen following repeated administration appeared to occur *via* five main enzymatic reactions, as identified from the metabolites observed in the rat: hydroxylation, methylation, reduction of the nitro group, N-acetylation and phase II type conjugations.

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Table 5.1-29: Quantification of metabolites in urine from rats following repeat oral doses of [^{14}C]-aclonifen at 30 mg/kg bw (% of administered dose)

Metabolite	Repeat 30 mg/kg	
	Males	Females
MAU	2.35	4.92
MBU	11.86	5.51
MCU	15.40	27.26
MDU	0.83	0.72
Total	**30.44**	**38.41**

Table 5.1-30: Quantification of metabolites in faeces from rats following repeat oral doses of [^{14}C]-aclonifen at 30 mg/kg bw (% of administered dose)

Metabolite	Repeat 30 mg/kg	
	Males	Females
M F	0.13	0.85
MBF	0.09	0.38
MCF	0.14	1.25
MDF	12.19	4.74
MEF	0.24	0.89
MFF	3.31	2.98
MGF	4.16	3.34
MHF	1.91	2.97
MIF	2.40	2.93
Aclonifen	2.94	2.24
Total	**27.51**	**22.57**

In the urine from the repeat dose group, four metabolites were detected. Only two of them, MBU and MCU, represented more than 5% of the administered dose and the major part of the radioactivity: 12% (males) and 5.5% (females) for MBU and 15% (males) and 27% (females) for MCU. Sulphatase hydrolysis confirmed the identification of MAU, MBU and MCU as sulphate conjugates. Comparison with reference substances (RPA 508285 and RPA 407074) indicated that MDU was RPA 407074, and MCU the sulpho-conjugate of RPA 407074. Hence, both metabolites were common to the single oral dose study with MAU being equivalent to M6U and MCU being equivalent to M11U (see CA 5.1.1/01). Additionally, MBUbis was found to be equivalent to M6Ubis from the single dose studies, with the following structure:

MBUbis

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In faeces, at least nine metabolites of low abundance were detected. Aclonifen was present in both sexes and accounted for 3% in males and for 2.2% in females. The main metabolite was MDF (aka M3F in the single dose studies) and represented 12% of the total administered dose in the faeces from males and 4.7% in females. It was identified as aclonifen with reduction of the nitro group, N-acetylation and hydroxylation:

MDF

MIF (aka M8F in the single dose studies) was identified as hydroxylated aclonifen, RPA 407074.

MIF

The metabolism of aclonifen was therefore not qualitatively different following repeated dosing, compared with that following a single oral dose.

III. CONCLUSION

Aclonifen was rapidly and extensively eliminated following repeated dosing with a mean of between 96% and 97% of the administered radioactivity being eliminated over a seven-day period following cessation of dosing. The majority of this elimination occurred in the first 48 hours following the last of the fourteen oral low dose administrations.

A minimum estimate of the degree of oral absorption yielded mean values of 50% and 59% for the males and females respectively.

At 168 hours post dosing, the mean recovery of radioactivity in the tissues of the animals both sexes ranged from 0.09% (females) to 0.13% dose (males). The highest mean tissue residues following repeated administration with [^{14}C]-aclonifen at 30 mg/kg body weight were liver, the second most significant residues being in the kidney.

The biotransformation of aclonifen following repeated administration was qualitatively similar to that observed following single oral dosing and included five principal enzymatic reactions: hydroxylation, methylation, reduction of the nitro group, N-acetylation and phase II type conjugations.

A proposed metabolic pathway for the rat based upon these results is presented in the figure below:

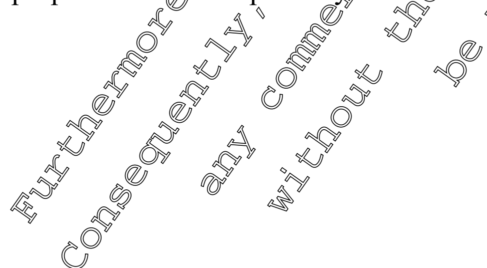

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Figure 5.1-6: **Proposed metabolic pathway for aclonifen in the rat following repeat oral low dose administration at 30 mg/kg bw**

RPA 407074
MIF
MCU
ACLONIFEN
RPA 407291
MDF
MBU
MBUbis
{ } = postulated intermediates

Assessment and conclusion by applicant:

A good, compliant, acceptable study that produced valid results and conclusions.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.1.1/06
Report Author:	[redacted]
Report Year:	1983
Report Title:	Investigations into the biokinetics of CME 127 in the rat
Report No:	C030405
Document No:	M-219328-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 417, 2010 Biliary excretion not assessed
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive summary

Rats were dosed orally with [^{14}C]-aclonifen (uniformly labelled in the aniline ring, ≥98% radiopurity) as a solution or suspension in PEG 400 at a nominal dose level of 30 mg/kg bw. Blood was sampled for kinetic analysis at 0.25, 0.5, 0.75,1, 2, 4, 6, 8, 24, 48, 72, 96, 120 and 168 h after a single dose. Urine and faeces were collected for analysis at 24 h intervals for up to 7 days after the last of 7 daily doses. Tissues were sampled for analysis at 1, 6, 24, 48 and 120 h after the last of 7 daily doses.

The radioactivity in the various samples was determined by liquid scintillation counting. One-dimensional thin-layer chromatography (TLC) was performed on urine and faecal extracts, using four different solvent systems. Mass spectrometry was used to aid identification of metabolites in the urine.

It was demonstrated that after both single and repeated dosing at a dose level of 30 mg/kg, aclonifen was rapidly absorbed and excreted, with the major route of excretion *via* the urine (62-66% of the administered dose) with the rest excreted in the faeces. The actual level of oral absorption was shown to be higher following a single oral dose, at *ca* 84% (see CA 5.1.1/01), as the result in the present study did not take into account any biliary excretion and the levels in tissues. After absorption the compound was extensively and rapidly metabolised by ring hydroxylation, methylation of the hydroxyl groups, reduction of the nitro group and acetylation of the amino group, prior to excretion. No indication of accumulation in the tissues was observed.

I. MATERIALS AND METHODS

Study dates Start: Not given
In-life: Not given
Completion: 26 January 1983

A. MATERIALS

1. Test Item (labelled) [^{14}C]-CME 127 (aclonifen)

Batch No.: Not given

Radiopurity: ≥98%

Specific activity: 2.80 MBq/mg (75.6 µCi/mg)

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+ = position of uniformly labelled ring

2. Test Organism Rat, Chbb:THOM (SPF)

Source:	
Number, sex:	13 males, 14 females
Age:	6-10 weeks
Weight:	174-209 g at administration
Acclimatization:	Not given
Feed:	FUKO 8013 diet for rats and mice, Tap water *ad libitum*
Housing:	Metabolism cages

B. STUDY DESIGN AND METHODS

1. Treatment

The rats were dosed orally with [^{14}C]-aclonifen as a solution or suspension in PEG 400 at a nominal dose level of 30 mg/kg bw.

Dose Group	Nominal dose level (mg/kg)	Dose type	N° of daily doses	Dose route	N° of rats	Nominal radioactive dose (µCi/kg)
Excretion	30	repeat	7	Oral	4M, 4F	25
Blood kinetic	30	single	1	Oral	4M, 5F	25
Tissue distribution	30	repeat	7	Oral	5M, 5F	25

2. Sampling

Dose Group	Sampling Times			
	Urine	Faeces	Blood	Tissues
Excretion	24 hour intervals up to 7 days following last dose	24 hour intervals up to 7 days following last dose	NA	NA
Blood kinetic	NA	NA	0.25, 0.5, 0.75,1, 2, 4, 6, 8, 24, 48, 72, 96, 120, 168 h	NA
Tissue distribution	NA	NA	NA	1, 6, 24, 48, 120 h after 7 doses.

NA = not applicable

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The daily urine samples were washed into volumetric flasks and 0.1 mL samples were then assayed for content of radioactivity. Faeces were freeze-dried, homogenized and extracted with 1:1 methanol and water using ultrasonic treatment. Radioactivity not recoverable by extraction was assayed by combustion. Blood was taken from the postorbital venous plexus and immediately centrifuged to extract the plasma.

Tissues that were either removed or sampled following exsanguination were: liver, kidney, heart, brain, lungs, spleen, thymus, gonads, skeletal muscle, fat, intestine, stomach and pancreas. The organs were freeze-dried and assayed for radioactivity by combustion.

3. Radioassay

The amounts of radioactivity in the various samples were determined by liquid scintillation counting, using either a Packard Tri-Carb 3380 or Packard Tri-Carb 460 CD counter. Quench correction was performed according to the external standard channels ratio method.

4. Chromatography

One-dimensional thin-layer chromatography (TLC) was performed on urine and faecal extracts, using four different solvent systems (di-isopropyl ether, acetone/water 9:1, chloroform/methanol 9:1, toluene/acetone 9:1) and the chromatograms analyzed either with the TLC scanner/multichannel analyzer or a linear analyzer. Mass spectrometry was used to aid identification of metabolites found in the urine.

5. Pharmacokinetic parameters

Certain of these parameters (elimination half-life, t½, AUC) were not provided in the report and have been calculated here using Topfit v2.0.

II. RESULTS AND DISCUSSION

The data demonstrated that elimination of radiolabel reached mean levels of *ca* 94% per day during the administration period. By day 7 approximately 99.9% of the administered dose had been eliminated by the males, with the majority of the remaining radioactivity (1.3% of the remaining 1.8% dose out of a total elimination of 101.7% dose) being eliminated during the first 24 hours post cessation of dosing. Similarly for the females, approximately 99.6% of the administered dose had been eliminated by day 7 with the majority of the remaining radioactivity (2% of the remaining 2.4% dose out of a total elimination of 102% dose) being eliminated during the first 24 hours post cessation of dosing. The minimum level of absorption (based on urinary excretion only) was of the order of 63% for males and 62% for females. The actual level of oral absorption was shown to be higher following a single oral dose, at *ca* 84% (see CA 5.1.1/01), as these figures did not take into account any biliary excretion and the levels in tissues.

Table 5.1-31: Mean proportions of administered dose in the urine and faeces of rats following 7 daily oral administrations of [^{14}C]-aclonifen at 30 mg/kg (% of dose administered)

Day	Males				Females			
	Urine		Faeces		Urine		Faeces	
	% of daily dose	% of cumulative dose	% of daily dose	% of cumulative dose	% of daily dose	% of cumulative dose	% of daily dose	% of cumulative dose
Treatment Phase								

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Day	Males				Females			
	Urine		Faeces		Urine		Faeces	
	% of daily dose	% of cumulative dose	% of daily dose	% of cumulative dose	% of daily dose	% of cumulative dose	% of daily dose	% of cumulative dose
1	50.9	50.9	22.1	22.1	60.8	60.8	15.0	15.0
2	64.1	57.4	41.3	31.5	65.7	63.2	36.8	25.9
3	65.7	60.1	46.8	36.6	59.1	64.9	49.3	33.5
4	59.9	60.0	37.8	36.9	64.4	62.5	43.5	36.0
5	69.1	61.9	38.4	37.2	61.4	62.3	44.8	37.7
6	65.6	62.5	37.1	37.2	59.1	61.8	38.0	37.8
7	65.5	63.0	34.8	36.9	62.0	61.8	37.7	37.8
Depuration Phase								
8	3.6	63.4	6.8	37.8	3.2	62.3	10.4	39.3
9	0.9	63.6	0.7	37.9	0.8	62.4	1.0	39.4
10	0.3	63.6	0.3	38.0	0.3	62.4	0.3	39.5
11	0.2	63.6	0.2	38.0	0.1	62.4	0.2	39.5
12	0.2	63.7	0.1	38.0	0.2	62.5	0.1	39.5
13	0.1	63.7	0.1	38.0	0.1	62.5	0.1	39.5
14	0.1	63.7	0.1	38.0	0.1	62.5	0.1	39.5

Table 5.1-32: Mean levels of radioactivity in the blood and plasma of rats following single oral administration of [^{14}C]-Aclonifen at 30 mg/kg (µg equiv./mL)

Time (h)	Blood		Plasma	
	Males	Females	Males	Females
0.25	3.29	2.37	6.17	4.42
0.5	9.86	3.77	19.61	6.82
0.75	13.51	3.53	23.50	6.25
1	14.92	3.24	25.17	5.67
2	14.93	2.44	24.18	4.18
4	15.71	2.38	24.59	3.79
6	20.22	3.90	30.60	6.11
8	15.52	5.85	23.50	8.59
24	2.33	0.72	3.52	0.97
48	0.18	0.08	0.08	0.11
72	0.09	0.08	0.02	0.05
96	0.07	0.03	0.01	0.03
120	0.08	0.01	<0.01	<0.01
168	0.02	<0.01	0.01	0.01

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Time (h)	Blood		Plasma	
	Males	Females	Males	Females
C_{max} (µg/mL)	20.2	5.85	30.6	8.59
T_{max} (h)	6	8	6	8
$t_{½}$ (h)	20.8	15.6	7.25	14.8
AUC_{0-168} (µg.hr/ml)	308	92.6	460	136

The blood kinetic data demonstrated that [^{14}C]-aclonifen was rapidly absorbed with maximal concentrations achieved between 6 and 8 hours post dose for both sexes. A clear sex difference was observed, with blood and plasma concentrations being higher in the males. The terminal elimination phase half-life values indicated rapid elimination, with values around 15 h for the females and between 21 and 7.25 for the blood and plasma for the males (the difference between the blood and plasma values for the males appears to be due to large inter-individual variations). Approximately 97% of the AUC value was achieved during the 0-48 time period, which is also indicative of rapid elimination.

Table 5.1-33: Concentration of radioactivity in tissues of rats following 7 daily oral administrations of [^{14}C]-aclonifen at 30 mg/kg (µg [14C]-aclonifen equivalents/g tissue)

Tissue	1 h		6 h		24 h		48 h		120 h	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver	67.53	37.29	35.84	33.88	19.30	12.43	1.33	1.25	0.56	0.57
Kidney	50.70	32.24	20.37	10.37	6.54	4.77	0.98	0.73	0.26	0.3
Heart	10.78	2.89	2.38	2.06	1.11	0.63	0.12	0.16	0.06	0.11
Lungs	14.80	5.85	3.91	3.14	1.21	0.98	0.73	0.4	0.28	0.28
Brain	1.72	0.85	0.97	0.58	0.11	0.23	0.02	0.01	0.02	0.02
Gonads	4.29	15.33	6.00	8.3	0.43	2.51	0.04	0.24	0.02	0.15
Spleen	7.03	4.94	3.67	2.25	0.94	0.98	0.16	0.25	0.10	0.21
Pancreas	9.74	8.13	3.30	2.95	2.95	1.76	0.36	0.18	0.14	0.11
Thymus	8.37	5.43	3.48	4.21	1.33	1.15	0.14	0.1	0.06	0.09
Muscle	3.58	1.9	2.00	0.72	0.36	0.33	0.03	0.03	0.03	0.04
Fat	7.29	18.21	3.85	3.55	1.42	2.01	0.17	0.12	0.05	0.19
Plasma	45.53	15.03	16.15	7.53	5.36	2.45	0.17	0.23	0.05	0.06
Stomach	614.25	1357.57	9.70	181.47	23.61	79.09	0.31	2.93	0.07	0.07
Intestine	98.07	146.78	111.93	83.11	29.49	26.7	2.89	8.66	0.23	0.49

Table 5.1-34: Percentage of administered dose in tissues of rats following 7 daily oral administrations of [^{14}C]-aclonifen at 30 mg/kg

Tissue	1 h		6 h		24 h		48 h		120 h	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver	1.65	0.64	0.66	0.75	0.52	0.27	0.04	0.03	0.02	0.02
Kidney	0.24	0.12	0.09	0.05	0.03	0.02	<0.01	<0.01	<0.01	<0.01

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Tissue	1 h		6 h		24 h		48 h		120 h	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Heart	0.02	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Lungs	0.04	0.01	0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Brain	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Gonads	0.03	0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Spleen	0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Pancreas	0.02	0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Thymus	0.01	0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Muscle	0.79	0.37	0.42	0.16	0.09	0.07	0.01	0.01	0.01	0.01
Fat	0.25	0.56	0.13	0.13	0.06	0.07	0.01	<0.01	<0.01	0.01
Stomach	2.63	4.69	0.04	5.48	0.10	0.37	<0.01	0.01	<0.01	<0.01
Intestine	3.97	5.62	3.89	3.40	1.22	1.07	0.10	0.24	0.01	0.02

The results from the tissue distribution study show that aclonifen and its metabolites were rapidly excreted, as the tissue radioactivity levels declined very rapidly. Two days after the last treatment, no residues exceeding 0.01% of the administered dose were found, with the exception of the liver (0.03%-0.04%) and intestine (0.10%-0.24%).

Chromatographic examination of urine and faecal extracts indicated only traces of aclonifen in the faeces (<5 %dose) and that it was not present in the urine.

The following compounds were identified in the urine by thin-layer chromatography and mass spectroscopy:

These were indicated in the report to be N-acetyl-[3-chloro-4-(4'-hydroxy)-phenoxy]-O-phenylenediamine, 2-chloro-6-nitro-3-(4'-hydroxy)-phenoxyaniline and 2-chloro-6-nitro-3-(4'-hydroxy-3'-methoxy)-phenoxyaniline, respectively, although the analyses were not presented in the report and these identities could not be verified.

III. CONCLUSION

This ADME study in rats demonstrated that after both single and repeated dosing of aclonifen (uniformly ^{14}C labelled in the aniline ring) at a dose level of 30 mg/kg, aclonifen was rapidly absorbed and excreted, with the major route of excretion *via* the urine (62-66% of the administered dose) with the rest excreted in the faeces. After absorption the compound was extensively and rapidly metabolised by ring hydroxylation, methylation of the hydroxyl groups, reduction of the nitro group and acetylation of the amino group, prior to excretion. No indication of accumulation in the tissues was observed.

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Assessment and conclusion by applicant:

Does not cover all the requirements of current guidance, but the results are considered valid and acceptable as supplementary data.

Assessment and conclusion by RMS:

Data Point:	KCA 5.1.1/07
Report Author:	[redacted]; [redacted]
Report Year:	2017
Report Title:	[Phenoxy-UL-14C]aclonifen - Absorption, distribution, excretion and metabolism in the rat
Report No:	EnSa-17-0284
Document No:	M-598008-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 of the European Parliament and of the Council amended by Commission Regulation (EU) No 283/2013 OECD Guideline for Testing Chemicals, 417 US EPA OCSPP 870.7485 Japanese MAFF Test Guideline 12 Nousan 8147
Deviations from current test guideline:	Current guideline: OECD 417, 2010 No deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

The objective of this study was to investigate the biokinetic behaviour (absorption, distribution and excretion of total radioactivity) of aclonifen and to identify and quantify the metabolites in urine, bile, faeces, plasma, liver and fat of male and female rats after a single oral low dose of aclonifen labelled with ^{14}C in the phenoxy moiety of the molecule.

Wistar rats (groups of 4/sex, plus one bile-cannulated group of 4 males) received [phenoxy-UL-^{14}C]aclonifen as a single oral gavage dose at 2 mg/kg bw, followed by sampling of urine, faeces, plasma over the next 72 h, and sampling of liver, peri-renal fat and plasma at termination. Analyses were conducted for the test compound and its metabolites in these samples. The parameter calculations used a two-compartment model.

Recovery of radioactivity from the urine, faeces, plasma, organs and tissues ranged from 95.0% to 105.2%. Aclonifen was rapidly absorbed and distributed. The maximum plasma concentration (C_{max}) was reached at approximately 2 h for males, 1 h for females, after administration. In general, males showed a two times higher plasma concentration compared to females. AUC-values from plasma of both genders were of the same magnitude.

Low amounts of radioactivity were detected in organs and tissues. Female rats showed higher organ concentrations compared to males, except for blood cells, thyroid and perirenal fat. Absorbed

radioactivity was quickly and efficiently eliminated within 72 h after administration, and for both genders, excretion was nearly completed after 48 hours. The urine and faecal excretion rate was nearly the same. In males, approximately 55% of the dose was excreted in bile. The absorption rate (based on radioactivity in urine, bile and body without GIT) amounted to 81% for males.

Parent compound was a prominent compound only in the faeces of bile-duct cannulated rats and in peri-renal fat (2 h after treatment). The main metabolite in urine was aclonifen-4-sulfate (25% of dose), with another prominent metabolite aclonifen-diamino- acetyl-4-sulfate (13% of dose in males, 6% in females). Other urinary metabolites amounted to not more than 3.8% of dose. A significant proportion of metabolites in the faeces of both sexes was characterized as residues based on aclonifen-diamino-acetyl-4-OH (24-30% of dose), and only 0.4% was parent. In bile, aclonifen-4-sulfate occurred at 41%, with aclonifen-glucA-4-sulphate at 5%, while other metabolites were not more than 2.7% of dose. Aclonifen-4-sulfate was again the main metabolite in plasma (85% of TRR in males, 66% in females), with aclonifen-4-OH (10% of TRR) and aclonifen-glucA-4-sulfate (8% of TRR for females), and no parent. In the liver, main metabolites were aclonifen-diamino-aconitic acid-4-sulfate (27% of TRR), aclonifen-amino-acetyl-GSH-4-sulfate (14% of TRR for males, 11% for females) and aclonifen-diamino-acetyl-4-sulfate (14% of TRR for males, 10% for females). Other prominent metabolites were between 4% and 8% of TRR and were aclonifen-diamino-acetyl-GSH-4- sulfate, aclonifen-diamino-acetyl-glycolic acid-GSH-4-sulfate, aclonifen-diamino- aconitic acid-4-sulfate-dehydrate, aclonifen-4-sulfate. Minor metabolites were between 0.2% and 2.2% of TRR. Parent was not found in the liver. Parent compound and aclonifen-4-sulfate were the main compounds in fat. Parent was 43% of TRR for males and 19% for females. Aclonifen-4-sulfate was at 40% of TRR for males and 61% for females. The minor metabolites amounted to between 0.9% and 3.0% of TRR.

The principal metabolic reactions of [phenoxy-UL-14C]Aclonifen in the rat were:

- Hydroxylation in position 4 of the phenyl ring leading to aclonifen-4-OH
- Conjugation of aclonifen-4-OH with glucuronic acid or sulphuric acid
- Reduction of the nitro group to an amine and the subsequent acetylation, formylation or addition of aconitic acid, leading to diamino-acetyl, diamino-formyl or diamino-aconitic acid compounds. Acetylation was also observed in the amino group of the parent compound.
- Intra-molecular condensation (cyclisation) of the diamino-acetyl compounds, the diamino-formyl compound and the diamino-aconitic acid compound
- Conjugation with glutathione and glycolic acid
- Cleavage of the phenyl ring moiety

All results were in good agreement with the results of ADME studies with the aniline label, and the toxicokinetic and metabolic behaviour of [phenoxy-UL-^{14}C]aclonifen is well understood in rats.

I. MATERIALS AND METHODS

Study dates Start: 28 January 2016
In-life: 15 February 2016 to 07 February 2017
Completion: 11 August 2017

A. MATERIALS

1. Test Item Aclonifen
Batch No.: KML 10134
Radiopurity: >99% by HPLC, TLC

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Specific activity: 6.79 MBq/mg

* = position of uniformly labelled ring.

Storage: Solution in acetonitrile, stored at approx. -18ºC

Dosing vehicle: 0.5% aqueous gum tragacanth

2. Test Organism Wistar rat, Unilever HsdCpb:WU

Source:

Number, sex: Groups of 4/sex, and one group of 6 males

Age: 6-9 weeks

Weight: Approximately 200 g at study initiation

Acclimatization: Approximately one week

Feed: Rat/mouse maintenance long-life diet (V1574-000 Ered I or V1534-000 R/M-H), Germany

Tap water *ad libitum*

Housing: Metabolism cages

B. STUDY DESIGN AND METHODS

The stock test material was concentrated to near dryness under nitrogen, then formulated in 0.5% aqueous gum tragacanth in an ultrasonic bath. The suspensions were stirred by magnetic stirrer overnight at approximately 4ºC, then at room temperature during administration. The radioactivity in each dosing suspension was determined by LSC.

Five tests were conducted, as follows:

Test ID	Administration		Animals		Duration	Description
	Dose	Route	No.	Sex		
1	2 mg/kg bw	p.o.	4	male	72 h	single dose
2	2 mg/kg bw	p.o.	4	female	72 h	single dose
3	2 mg/kg bw	p.o.	4	male	2 h	single dose, for organ analysis
4	2 mg/kg bw	p.o.	4	female	2 h	single dose, for organ analysis
5	2 mg/kg bw	p.o.	4	male	48 h	single dose, after bile-duct cannulation

Administration was by oral gavage, assuming an average body weight of 200 g at dosing. For tests 1-4 each animal received 2 mL of suspension, while for test 5 they received 1 mL.

Sampling was as follows:

Test	Test description	Times of sample collection (hours post-dose)

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No.		Urine	Faeces	Bile	Organs	Microplasma
1	4 male rats single oral dose (2 mg/kg bw)	4, 8, 12, 24, 48, 72	24, 48, 72	---	72	0.25, 0.5, 1, 2, 4, 7, 24, 48, 72
2	4 female rats single oral dose (2 mg/kg bw)	4, 8, 24, 48, 72	24, 48, 72	---	72	
3	4 male rats single oral dose (2 mg/kg bw) sacrifice 2 hours after plasma maximum	2	2	---	2 (liver, fat, plasma)	----
4	4 female rats single oral dose (2 mg/kg bw) sacrifice 2 hours after plasma maximum	2	2	---	2 (liver, fat, plasma)	----
5	6 bile-duct cannulated male rats [a] single oral dose (2 mg/kg bw)	4, 8, 24, 48	24, 48	4, 8, 24, 32, 48	48	----

[a] – Two animals not used for evaluation and metabolism investigation due to no bile sampling

The rats were sacrificed three days after dosing, or in case of bile-duct cannulation two days after dosing. For investigation of plasma, liver and perirenal fat the animals were sacrificed at plasma maximum approx. 2 h after dosing. The total radioactivity, including the test compound and the metabolites, was determined in plasma samples, urine, bile, faeces, organs and tissues at sacrifice. Metabolism was investigated in urine, bile and extracts of faeces and in addition in plasma, liver and fat at plasma maximum.

Analytical methods

Reversed phase HPLC with an acidic acetonitrile/water gradient was used to analyze parent compound and metabolites in urine, bile and plasma, and in liver, fat and faecal extracts. Peaks corresponding to a signal approximately 2.5x background noise were integrated. Electro-spray ionization mass spectroscopy and NMR were employed in compound identification and quantification.

The samples were processed as representative pools for each test. Urine pools were 0-4, 4-8, 8-12, 12-24 and 24-48 h in test 1, and 0-48 h for test 5; none were analyzed from tests 3 or 4. Bile was pooled as 0-48 h for test 5. Plasma was analyzed as 2 h pools for tests 3 and 4. Faeces were pooled as 0-24 and 24-48 h for test 1 and 0-48 h for tests 2 and 5; none were analyzed from tests 3 or 4. Liver and perirenal fat samples were analyzed as 2 h pools for tests 3 and 4.

II. RESULTS AND DISCUSSION

Actual dose levels were close to the target doses, as shown below:

Test ID	Nominal dose [mg/kg bw]	Actual dose [mg/kg bw]
1	2.00	1.90
2	2.00	1.96
3	2.00	1.99
4	2.00	2.03

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5	2.00	1.95

1. Recovery

The recovery from urine and faeces ranged from 95.1% to 101.1% of administered dose. Overall, from urine, faeces, plasma, organs and tissues, it ranged from 95.0% to 105.2%.

Table 5.1-12: Recovery of radioactivity from rats following a single oral dose of [^{14}C]-aclonifen at 2 mg/kg bw (% of administered dose), phenoxy label

	Test 1 male oral 2 mg/kg bw	**Test 2** female oral 2 mg/kg bw	**Test 3** male oral 2 mg/kg bw organ preparation	**Test 4** female oral 2 mg/kg bw organ preparation	**Test 5** male oral 2 mg/kg bw bile-duct cannulation
Urine	52.03	48.76	5.52	11.08	21.81
Bile	---	---	---	---	55.22
Faeces	50.51	45.87	0.02	0.02	17.05
Total excreted	**102.54**	**94.63**	**5.53**	**11.10**	**94.09**
Body excluding GIT	0.181	0.264	44.41	30.71	0.267
GIT	0.073	0.078	55.24	60.40	1.165
Total in body	**0.254**	**0.342**	**99.65**	**91.11**	**1.432**
Balance	**102.80**	**94.98**	**105.18**	**102.20**	**95.53**
Normalization factor	0.973	1.053	0.953	0.980	1.047
Total in body (% of recovered)	**0.247**	**0.360**	**94.79**	**89.06**	**1.508**
Absorption rate					**80.9%**

2. Absorption

The absorption of aclonifen started immediately after administration as shown by the results for plasma in tests 1 and 2. The highest plasma concentration was measured between 1 and 2 hours after treatment and therefore, analytical investigations in target organs (plasma, liver and perirenal fat) were performed at 2 hours after dosing (tests 3 and 4).

Female rats showed a two times lower absorption rate for plasma compared to male rats.

Test 5 with bile-duct cannulated males showed that about 55% of the recovered dose was detected in the bile. Absorption rates were calculated by summation of the recovered radioactivity in urine, bile and body without GIT and amounted to 80.9% for males.

3. Distribution and plasma kinetics

The distribution of the test substance to organs and tissues was measured from the concentration of total radioactivity in plasma.

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After a single oral administration of 2 mg/kg bw the maximum plasma concentration of the radioactivity (C_{max}) was measured approx. 2 h (t_{max}) after treatment for males and approx. 1 h (t_{max}) after treatment for females. The actual maximum concentrations amounted to 3.130 mg a.s./kg for males and 1.695 mg a.s./kg for females.

For all tests the plasma concentration declined to approx. 1% of the maximum concentration within 72 h post administration. This indicates no retention of parent compound related residues in the body of the animals.

Plasma concentrations from the test 1 and test 2 were calculated with a two-compartment model by TOPFIT based on dose normalized concentrations. The weighting of $1/y^2$ was used to achieve a good fit for the late time points. There was a very fast elimination phase after reaching the plasma maximum, followed by a slower elimination phase after approximately 15 h for males and 20 h for females.

The pharmacokinetic parameters are shown in the following table. Exponential analysis was done using the average values of dosage normalized plasma concentrations of four animals for the time range of 0 to 72 h post administration.

Table 5.1-13: Pharmacokinetic parameters (dose normalized) for rats following a single oral dose of [^{14}C]-aclonifen at 2 mg/kg bw, phenoxy label

	Test 1 Males	Test 2 Females
Actual dose (mg/kg bw)	1.90	1.96
Compartment model	two	two
Weighting of the TOPFIT calculation	$1/y^2$	$1/y^2$
t_{max} [h] measured	2	1
t_{max} [h] calculated	1.75	1.18
C_{max} [g/g] measured	1.647	0.864
C_{max} [g/g] calculated	1.700	0.897
t½ absorption [h]	1.14	0.29
t½ elimination [h]	13.4	23.6
$AUC_{0-\infty}$ [g/g•h]	8.18	6.82

4. Excretion

Generally, excretion was almost completed 48 h after administration. At this time, more than 98% of the recovered dose had been excreted via urine and faeces. The excretion rate for bile-duct cannulated male rates amounted to 98.5% of the recovered dose. In all tests the main portion of radioactivity was excreted at the latest after 24 hours.

For test 1 (male) and 2 (female) the renal and faecal excretion rates were nearly the same and amounted to approx. 50% of the dose. For bile-duct cannulated male rats (test 5) approx. 23% of the recovered dose was excreted via urine, approx. 58% via bile and approx. 18% via faeces.

The excretion rate in test 3 and 4, which were designed for organ preparation at plasma maximum, was below 11% of dose.

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Table 5.1-14: Cumulative excretion of radioactivity from rats following a single oral dose of [^{14}C]-aclonifen at 2 mg/kg bw (% of administered dose), phenoxy label

Time [h post admin.]	**Test 1** male oral 2 mg/kg bw	**Test 2** female oral 2 mg/kg bw	**Test 3** male oral 2 mg/kg bw organ preparation	**Test 4** female oral 2 mg/kg bw organ preparation	**Test 5** male oral 2 mg/kg bw bile-duct cannulation
Urine					
2	---	---	5.52	11.08	---
4	10.34	10.07	---	---	1.61
8	27.59	26.40	---	---	5.94
12	41.62	---	---	---	---
24	49.14	45.25	---	---	20.38
48	51.73	48.15	---	---	21.81
72	52.03	48.76	---	---	---
Bile					
4	---	---	---	---	17.03
8	---	---	---	---	31.96
24	---	---	---	---	51.96
32	---	---	---	---	53.85
48	---	---	---	---	55.22
Faeces					
2	---	---	0.02	0.02	---
24	45.61	37.82	---	---	11.61
48	50.01	45.36	---	---	17.05
72	50.51	45.87	---	---	---
Sum excreted:	**102.54**	**94.63**	**5.53**	**11.10**	**94.09**
Normalization factor	0.973	1.053	0.953	0.980	1.047
Sum excreted (% of recovered)	**99.75**	**99.64**	**5.21**	**10.94**	**98.49**

--- No sample collected

5. Residues in organs and tissues

Generally, there were very low residues in organs and tissues from all tests, except tests 3 and 4, which were designed for investigations of target organs at plasma maximum (approx. 2 h after dose). In general, female rats showed higher organ concentrations compared to males.

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Table 5.1-15: Equivalent concentrations in tissues of rats following a single oral dose of [^{14}C]-aclonifen at 2 mg/kg bw (mg a.i./kg, mean values), phenoxy label

Organs/ Tissues	**Test 1** male oral 2 mg/kg bw	**Test 2** female oral 2 mg/kg bw	**Test 3** male oral 2 mg/kg bw organ preparation	**Test 4** female oral 2 mg/kg bw organ preparation	**Test 5** male oral 2 mg/kg bw bile-duct cannulation
Blood Cells	0.0071	0.0079	0.7147	0.3591	---
Plasma	0.0021	0.0039	2.9034	1.7765	---
Carcass	0.0016	0.0028	0.3996	0.2928	0.0085
Heart	0.0016	0.0032	---	---	---
Brain	0.0006	0.0015	---	---	---
Kidneys	0.0115	0.0221	---	---	---
Liver	0.0346	0.0554	12.1688	9.3291	---
Testes	0.0007	---	---	---	---
Ovaries	---	0.0049	---	---	---
Uterus	---	0.0050	---	---	---
Adrenal	0.0039	0.0108	---	---	---
Thyroid	0.0097	0.0094	---	---	---
Spleen	0.0026	0.0056	---	---	---
Lung	0.0025	0.0062	---	---	---
Skin	0.0023	0.0055	0.4776	0.2886	0.0027
Bone femur	0.0017	0.0028	---	---	---
Peri-renal fat	0.0047	0.0024	0.4229	0.2694	---
Muscle (leg)	0.0010	0.0014	---	---	---

--- No sample collected

At sacrifice low portions of radioactivity between 0.181% and 0.267% of the administered dose (mean values, see Table 5.1.1/02-1) were found in the bodies excluding GIT of test 1, 2 and 5, except in bodies of test 3 and 4 (sacrifice at plasma maximum). Negligible amounts of radioactivity were detected in the GITs of test 1 (0.073% of dose) and test 2 (0.078%), excluding GITs from bile-duct cannulated rats (1.165%), showing that the elimination of the test compound related radioactivity was nearly completed at sacrifice.

The highest equivalent concentrations were detected in the liver in all tests. They ranged from 0.0346 mg/kg (test 1) to 12.17 mg/kg (test 3, sacrifice at plasma maximum). Concentrations in kidney amounted to 0.0115 mg/kg for males (test 1) and 0.0221 mg/kg for females (test 2). Concentrations in plasma and blood cells of test 1 and 2 at sacrifice were very low and amounted to ≤0.0079 mg/kg only. The concentrations in the other organs and tissues of test 1 and 2 ranged from 0.0007 (testes) to 0.0108 mg/kg (adrenal).

From the renal and faecal excretion and from the elimination kinetics of radioactivity in plasma, it was concluded that small amounts of residual radioactivity in organs and tissues are subject to further elimination.

6. Metabolism

For investigation of metabolism, urine, bile and faeces were sampled at different times in test 1, test 2 and test 5. Urine and bile samples were analyzed without sample preparation. Plasma at sacrifice (2 h after treatment) was analyzed after protein precipitation with acetonitrile.

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Faeces were conventionally extracted with a mixture of acetonitrile/water (8/2, v/v) followed by an exhaustive extraction with acetonitrile/water (1/1; v/v) and acetonitrile/water (1/1; v/v) plus formic acid with microwave assistance. Remaining residues in solids after the exhaustive extraction of test 1 (0 to 24 h) and test 2 (0 to 48 h) were further exhaustively extracted with 5N sodium hydroxide solution at 100°C. The radioactivity in these sodium hydroxide extracts were not further investigated, due to the high matrix content. The conventional extraction rate amounted to 46.5% for males 0 to 24 h, 43.1% for males 24 to 48 h, 38.6% for females 0 to 48 h and 80.9% for bile-duct cannulated males 0 - 48 h. After exhaustive extraction, between 1.20% and 4.10% of the dose were detected in the post extraction solids (PES) only. There were no losses during sample preparation.

Liver pool samples_(2 hours after treatment) were conventionally extracted with a mixture of acetonitrile/water (8/2, v/v). The TRRs were high and amounted to 12.169 mg/kg for males and 9.329 mg/kg for females. Remaining residues in solids (PES) amounted to 6.0% of the TRR for test 3 and 7.9% of the TRR for test 4 and were not further investigated. There were no losses during sample preparation.

Peri-renal fat pool samples_(2 hours after treatment) were conventionally extracted with a mixture of n-heptane and acetonitrile/water (8/2, v/v). Organic and aqueous phases were separated and the radioactivity was determined in the phases. The TRRs were moderate and amounted to 0.423 mg/kg for males and 0.269 mg/kg for females (both sacrifice 2 h after treatment). Remaining residues in solids (PES) amounted to 0.6% of the TRR for test 3 and 0.5% of the TRR for test 4. The main residues were detected in the aqueous phases and amounted to 95.3% of the TRR for males and 95.0% of TRR for females. The organic phases and the remaining solids were not further investigated. There were no losses during sample preparation.

Parent compound and metabolites were analyzed and quantified in urines, bile, plasma samples after protein precipitation and conventional extracts of faeces, liver and perirenal fat by radio-HPLC. For exhaustive extracts from faeces the profiles were analyzed using a neutral HPLC method. The identification of parent compound and metabolites were performed in isolated fractions from urine (test 2: female, 0 - 48 h), bile (test 5: male, 0 - 48 h), conventional extract of faeces (test 1: male, 0 - 24 h) and conventional extract of liver (test 3; male, 2 h) by structure elucidation or co-chromatography with reference compounds. All unknown metabolites were characterized based on their extraction and chromatographic behaviour and were not further investigated, due to their low radioactivity.

The distribution of parent compound and metabolites are shown in the following table.

Table 5.1-16: Balance of aclonifen and metabolites in rats following a single oral dose of [^{14}C]-aclonifen at 2 mg/kg bw (% of administered dose), phenoxy label

Compound	Found in	Test 1 (male, 2 mg/kg bw)	Test 2 (female, 2 mg/kg bw)	Test 5 (male, bile-duct can., 2 mg/kg bw)
		% of dose administered		
4-OH-phenylsulfate	Urine	1.92	2.82	---
OH-methoxy-phenylsulfate	Urine	0.17	0.47	---
phenylsulfate	Urine	3.83	2.41	3.63
phenyl-GlucA	Urine	1.15	1.01	---
diamino-acetyl-glycolic acid-GSH-4-sulfate	Urine	1.01	1.11	---
amino-acetyl-GSH-4-sulfate	Urine	2.40	0.87	---
OH	Urine	---	0.74	---
diamino-aconitic acid-4-sulfate	Urine, bile	0.48	0.58	1.06

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Compound	Found in	Test 1 (male, 2 mg/kg bw)	Test 2 (female, 2 mg/kg bw)	Test 5 (male, bile-duct can., 2 mg/kg bw)
		% of dose administered		
diamino-acetyl-4-sulfate	Urine, bile	12.73	6.08	2.67
4-OH-GlucA	Urine, bile	0.06	1.15	1.66
4-sulfate	Urine, bile, faeces	24.44	24.97	62.60
4-OH	Urine, bile, faeces	1.73	0.64	0.76
GlucA-4-sulfate	Bile	---	---	4.93
OH-phenylsulfate	Bile	---	---	0.69
diamino-acetyl-4-OH	Faeces	10.28	5.95	---
diamino-acetyl-4-OH-dehydrate and diamino-formyl-4-OH-dehydrate	Faeces	1.13	1.15	---
Parent compound	Faeces	0.43	---	12.65
Total identified		**61.78**	**49.96**	**90.65**
Total characterized in urine		**3.30**	**5.29**	---
Total characterized in bile		---	---	**2.15**
Total characterized in faeces as residues based on aclonifen-diamino-acetyl-4-OH		**23.64**	**29.98**	---
Exhaustive extract of faeces after treatment with NaOH		7.73	7.00	---
Solids of faeces (PES)		5.30	1.28	1.28
Urine not analyzed (48 - 72 h)		0.30	0.61	---
Faeces not analyzed (48 - 72 h)		0.50	0.51	---
Total		**102.54**	**94.63**	**94.09**

--- = not detected

Approximately 48% of the dose was identified in the urine of males in test 1 and approx. 43% in females in test 2. Parent compound was not detected in the urine. The main metabolite in urine was aclonifen-4-sulfate and amounted to 23.9% of the dose in males, 25.0% of the dose in females and 18.2% in the urine of bile-duct cannulated males. A prominent metabolite was aclonifen-diamino-acetyl-4-sulfate and was detected in males at 12.7% of the dose and in females at 6.1% of the dose. Other metabolites in urine amounted to ≤ 3.83% of the dose.

Approximately 53 % of the dose was identified in the bile of males (test 5). Parent compound was not found in bile. As for urine, the main metabolite in bile was aclonifen-4-sulfate and amounted to 41.3% of the dose. Another important metabolite was aclonifen-glucA-4-sulfate (4.9% of dose). The other five metabolites identified in bile amounted to not more than 2.7% of the dose.

Only small amounts of aclonifen (0.43% of dose) were found in the faeces of males (test 1). Aclonifen-diamino-acetyl-4-OH was the main compound in the conventional extracts and amounted to 10.3% of the dose for males (test 1) and 6.0% of the dose for females (test2). Some minor metabolites (≤1.2% of dose) were identified in the conventional extracts of faeces. Beside all these clearly identified metabolites in the conventional extract of faeces, a huge number of small metabolites were characterized after acidic cleavage with hydrochloric acid, as bound to matrix residues based on aclonifen-diamino-acetyl-4-OH.

In total, the characterized residues based on aclonifen-diamino-acetyl-4-OH in the conventional and exhaustive extracts amounted to 23.6% of the dose for males (test1) and 30.0% of the dose for females (test 2).

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In the conventional and exhaustive extracts of faeces from bile-duct cannulated males (test 5), 12.7% of the dose was quantified for parent compound and 3.1% of dose for aclonifen-4-sulfate.

Table 5.1-17: Aclonifen and metabolites in tissues of male rats following a single oral dose of [^{14}C]-aclonifen at 2 mg/kg bw, phenoxy label

Compound	Test 3 (male, oral, 2 mg/kg bw)					
	plasma		liver		peri-renal fat	
	% of TRR	mg/kg	% of TRR	mg/kg	% of TRR	mg/kg
4-OH-phenylsulfate	---	---	0.8	0.101	---	---
OH-phenylsulfate	---	---	1.6	0.190	---	---
diamino-acetyl-GSH-4-sulfate	1.8	0.052	6.6	0.798	---	---
diamino-acetyl-glycolic acid-GSH-4-	---	---	6.0	0.725	---	---
amino-acetyl-GSH-4-sulfate	---	---	13.6	1.656	---	---
diamino-aconitic acid-4-sulfate	---	---	27.0	3.286	---	---
diamino-acetyl-4-sulfate	---	---	14.3	1.742	1.6	0.007
diamino-aconitic acid-4-sulfate-dehydrate	---	---	7.3	0.883	2.3	0.010
diamino-acetyl-4-OH	---	---	2.2	0.269	---	---
GlucA-4-sulfate	1.4	0.042	---	---	---	---
4-OH-GlucA	0.6	0.017	---	---	---	---
4-sulfate	85.3	2.476	3.8	0.459	39.5	0.167
4-OH	10.2	0.297	0.3	0.039	2.5	0.011
parent compound	---	---	---	---	43.2	0.183
Total identified	**99.3**	**2.884**	**83.4**	**10.147**	**89.1**	**0.377**
unknown (L4)	---	---	0.9	0.114	---	---
unknown (L5, F1)	---	---	2.0	0.249	0.9	0.004
unknown (L6, F2)	---	---	2.9	0.354	5.3	0.022
unknown (L12)	---	---	2.3	0.283	---	---
unknown (L15)	---	---	1.0	0.119	---	---
unknown (L16)	---	---	0.4	0.043	---	---
unknown (L17)	---	---	0.4	0.051	---	---
unknown (L18)	---	---	0.5	0.057	---	---
unknown (P4)	0.7	0.019	---	---	---	---
unknown (L21)	---	---	0.2	0.025	---	---
Total characterized	**0.7**	**0.019**	**10.6**	**1.295**	**6.2**	**0.026**
Not analyzed fraction (heptane phase)	---	---	---	---	**4.1**	**0.017**
Total extracted	---	---	**94.0**	**11.442**	**99.4**	**0.421**
Solids (PES)	---	---	6.0	0.727	0.6	0.002
Total	**100.0**	**2.903**	**100.0**	**12.169**	**100.0**	**0.423**

--- = not detected

Table 5.1-18: Aclonifen and metabolites in tissues of female rats following a single oral dose of [^{14}C]-aclonifen at 2 mg/kg bw, phenoxy label

	Test 4 (female, oral, 2 mg/kg bw)		
	plasma	liver	peri-renal fat

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Compound	**% of TRR**	**mg/kg**	**% of TRR**	**mg/kg**	**% of TRR**	**mg/kg**
4-OH-phenylsulfate	---	---	0.9	0.085	---	---
OH-phenylsulfate	---	---	2.0	0.187	---	---
diamino-acetyl-GSH-4-sulfate	1.9	0.034	8.3	0.772	---	---
diamino-acetyl-glycolic acid-GSH-4-sulfate	---	---	4.0	0.377	---	---
amino-acetyl-GSH-4-sulfate	---	---	10.7	0.998	---	---
diamino-aconitic acid-4-sulfate	---	---	27.6	2.575	---	---
diamino-acetyl-4-sulfate	---	---	9.8	0.916	---	---
diamino-aconitic acid-4-sulfate-dehydrate	---	---	6.7	0.629	4.5	0.012
diamino-acetyl-4-OH	---	---	1.4	0.130	---	---
GlucA-4-sulfate	7.9	0.140	---	---	2.3	0.006
4-sulfate	65.9	1.170	6.0	0.559	60.9	0.164
4-OH	10.7	0.190	0.2	0.020	3.2	0.009
parent compound	---	---	---	---	19.4	0.052
Total identified	**86.4**	**1.534**	**77.7**	**7.249**	**90.3**	**0.243**
unknown (L3)	---	---	0.2	0.022	---	---
unknown (L4)	---	---	1.5	0.139	---	---
unknown (L5)	---	---	3.7	0.343	---	---
unknown (L6, F2)	---	---	3.7	0.349	1.6	0.004
unknown (L12)	---	---	2.2	0.202	---	---
unknown (L15)	---	---	1.2	0.108	---	---
unknown (L16)	---	---	0.4	0.038	---	---
unknown (L17)	---	---	0.5	0.045	---	---
unknown (L18)	---	---	1.0	0.094	---	---
unknown (P4)	4.5	0.081	---	---	---	---
unknown (F6, P5)	9.1	0.162	---	---	3.0	0.008
Total characterized	**13.6**	**0.243**	**14.4**	**1.340**	**4.7**	**0.013**
Not analyzed fraction (heptane phase)	---	---	---	---	**4.6**	**0.002**
Total extracted	---	---	**92.1**	**8.589**	**99.5**	**0.268**
Solids (PES)	---	---	7.9	0.740	0.5	0.001
Total	**100.0**	**1.777**	**100.0**	**9.329**	**100.0**	**0.269**

--- = not detected

About 99% and 86% of the TRR were identified in plasma at sacrifice (2 h after treatment) for male and females, respectively. Aclonifen was not found in plasma. Aclonifen-4-sulfate was the main metabolite in plasma at sacrifice (2 h after treatment) and amounted to 85.3% of the TRR for males in test 3 and 65.9% of TRR for females in test 4. Other prominent metabolites were aclonifen-4-OH (10.2% of TRR for males and 10.7% for females) and aclonifen-glucA-4-sulfate (7.9% of TRR for females). Minor metabolites identified in plasma were aclonifen- diamino-acetyl-GSH-4-sulfate and aclonifen-4-OH-glucA.

About 83% and 78% of the TRR were identified in liver at sacrifice (2 h after treatment) for male and females, respectively. Aclonifen was not detected in the liver. Main metabolites were aclonifen-diamino-aconitic acid-4-sulfate (approximately 27% of the TRR for males and females), aclonifen-amino-acetyl-GSH-4-sulfate (14% of the TRR for males and 11% of the TRR for females) and aclonifen-diamino-acetyl-4-sulfate (14% of the TRR for males and 10% of the TRR for females). Other prominent metabolites amounted to between 3.8 and 8.3% of TRR and were aclonifen-diamino-acetyl-GSH-4- sulfate, aclonifen-diamino-acetyl-glycolic acid-GSH-4-sulfate, aclonifen-diamino-

aconitic acid-4-sulfate-dehydrate, aclonifen-4-sulfate. Minor metabolites were between 0.2% and 2.2% of TRR.

Approximately 90% of the TRR was identified in peri-renal fat from males and females at sacrifice (2 h after treatment). Parent compound and aclonifen-4-sulfate were the main compounds in the fat. Parent amounted to 43.2% of TRR for males and 19.4% of TRR for females. Aclonifen-4-sulfate was detected as 39.5% of TRR for males and 60.9% of TRR for females. The minor metabolites amounted to between 0.9% and 3.0% of TRR.

7. Metabolic reactions

The most important metabolic reaction was the hydroxylation in position 4 of the phenyl ring leading to aclonifen-4-OH. Aclonifen-4-OH was conjugated with glucuronic acid or sulphuric acid leading to aclonifen-4-OH-glucA or aclonifen-4-sulfate, respectively.

Another important reaction was the reduction of the nitro group to an amine and the subsequent acetylation leading to diamino-acetyl compounds. For a minor metabolite, acetylation was also observed in the amino group of the parent compound.

Minor reactions were reduction of the nitro group to an amine and a subsequent formylation or an addition of aconitic acid.

Intra-molecular condensation (cyclisation) of the diamino-acetyl compounds, the diamino-aconitic acid compound and the diamino-formyl compound, was identified and observed during sample preparation.

Conjugation with glutathione and glycolic acid was observed for metabolites originating from the liver.

Cleavage of the phenyl ring moiety was only observed in urine and liver and led to metabolites like aclonifen-4-OH-phenylsulfate, aclonifen-OH-phenylsulfate, aclonifen-OH-methoxy-phenylsulfate, aclonifen-phenylsulfate and aclonifen-phenyl- glucA.

The principal metabolic reactions of [phenoxy-UL-14C]Aclonifen in the rat are listed as followed:

- Hydroxylation in position 4 of the phenyl ring leading to aclonifen-4-OH
- Conjugation of aclonifen-4-OH with glucuronic acid or sulphuric acid
- Reduction of the nitro group to an amine and the subsequent acetylation, formylation or addition of aconitic acid, leading to diamino-acetyl, diamino-formyl or diamino-aconitic acid compounds. Acetylation was also observed in the amino group of the parent compound.
- Intra-molecular condensation (cyclisation) of the diamino-acetyl compounds, the diamino-formyl compound and the diamino-aconitic acid compound
- Conjugation with glutathione and glycolic acid
- Cleavage of the phenyl ring moiety

Based on these results, the following metabolic pathway is proposed:

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Figure 5.1-4: Proposed metabolic pathway of [phenoxy-^{14}C]aclonifen in the rat (part A)

Aclonifen-4-OH-GlucA

Aclonifen-diamino-formyl-4-OH-dehydrate

Aclonifen-OH

Aclonifen-4-OH

Aclonifen

R1, R2 - H, $COCH_3$

Aclonifen-diamino-acetyl-4-OH

Aclonifen-4-sulfate

Aclonifen-GlucA-4-sulfate

Aclonifen-diamino-acetyl-4-OH-dehydrate

R1, R2 - H, $COCH_3$

Aclonifen-diamino-acetyl-4-sulfate

Aclonifen-amino-acetyl-GSH-4-sulfate

Aclonifen-diamino-aconitic acid-4-sulfate

R1, R2 - H, $COCH_3$

Aclonifen-diamino-acetyl-GSH-4-sulfate

Aclonifen-diamino-aconitic acid-4-sulfate-dehydrate

R1, R2 - H, $COCH_3$

Aclonifen-diamino-acetyl-glycolic acid-GSH-4-sulfate

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Figure 5.1-5: Proposed metabolic pathway of [phenoxy-^{14}C]aclonifen in the rat (part B, phenoxy label specific)

Cl
O
NH_2
NO_2
Aclonifen

HO
O
SO_3H
Aclonifen-OH-phenylsulfate

HO
O
SO_3H
Aclonifen-4-OH-phenylsulfate

O
SO_3H
Aclonifen-phenylsulfate

O
GlucA
Aclonifen-phenyl-GlucA

HO
O
SO_3H
H_3C-O
Aclonifen-OH-methoxy-phenylsulfate

III. CONCLUSION

Aclonifen was rapidly absorbed and distributed. The maximum plasma concentration (C_{max}) was reached at approximately 2 h for males, 1 h for females, after administration. In general, males showed a two times higher plasma concentration compared to females. AUC-values from plasma of both genders were of the same magnitude.

Low amounts of radioactivity were detected in organs and tissues. Female rats showed higher organ concentrations compared to males, except for blood cells, thyroid and perirenal fat. Absorbed radioactivity was quickly and efficiently eliminated within 72 h after administration, and for both genders, excretion was nearly completed after 48 hours. The urine and faecal excretion rate was nearly the same. In males, approximately 55% of the dose was excreted in bile. The absorption rate (based on radioactivity in urine, bile and body without GIT) amounted to 81% for males.

Parent compound was a prominent compound only in the faeces of bile-duct cannulated rats and in peri-renal fat (2 h after treatment). The main metabolite in the tests was aclonifen-4-sulfate. Other prominent metabolites were aclonifen-diamino- acetyl-4-sulfate, aclonifen-diamino-acetyl-4-OH, aclonifen-phenylsulfate and aclonifen-4-OH-phenylsulfate. A significant proportion in the faeces of both sexes was characterized as residues based on aclonifen-diamino-acetyl-4-OH.

The main metabolite in plasma and in peri-renal fat (approximately 2 h after treatment) was aclonifen-4-sulfate. Aclonifen-4-OH was also prominent in plasma. Aclonifen-diamino-aconitic acid-4-sulfate was the main compound in liver. Other prominent compounds were aclonifen-diamino-acetyl-GSH-4-sulfate, aclonifen-diamino-acetyl-glycolic acid-GSH-4-sulfate, aclonifen-amino-acetyl-GSH- 4-sulfate, aclonifen-diamino-acetyl-4-sulfate, aclonifen-aconitic acid-4-sulfate- dehydrate and aclonifen-4-sulfate. All other metabolites were detected only in low amounts.

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The most important metabolic reaction was the hydroxylation in position 4 of the phenyl ring, leading to aclonifen-4-OH. Aclonifen-4-OH was conjugated with glucuronic acid or sulphuric acid, leading to aclonifen-4-OH-glucA or aclonifen-4- sulfate, respectively. Another important reaction was reduction of the nitro group to an amine and the subsequent acetylation leading to diamino-acetyl compounds. For a minor metabolite, acetylation was also observed in the amino group of the parent compound. Minor reactions were the reduction of the nitro group to an amine and subsequent formylation or addition of aconitic acid. Intra-molecular condensation (cyclization) of the diamino-acetyl compounds, the diamino-aconitic acid compound and the diamino-formyl compound was identified and observed during sample preparation. Conjugation with glutathione and glycolic acid was observed for metabolites originating from the liver. Cleavage of the phenyl ring moiety was only observed in urine, bile and liver and led to metabolites such as aclonifen-4-OH-phenylsulfate, aclonifen-OH- phenylsulfate, aclonifen-OH-methoxy-phenylsulfate, aclonifen-phenylsulfate and aclonifen-phenyl-glucA.

All results were in good agreement with the results of ADME studies with the aniline label, and the toxicokinetic and metabolic behaviour of [phenoxy-UL-^{14}C]aclonifen is well understood in rats.

Assessment and conclusion by applicant:
A good, acceptable, compliant study that produced valid results and conclusions that are in agreement with the preceding study with the complementary label.

Assessment and conclusion by RMS:

Data Point:	KCA 5.1.1/08
Report Author:	
Report Year:	2017
Report Title:	[Phenoxy-UL-14C]aclonifen and [aniline-UL-14C]aclonifen: metabolic stability and profiling in hepatocytes from mice, rats and humans for inter-species comparison
Report No:	EnSa-16-0721
Document No:	M-577083-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 amended by the Commission Regulation (EU) No. 283/2013 (Europe) US EPA OCSPP Not Applicable
Deviations from current test guideline:	Not applicable (non-guideline study)
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 comparative metabolism of ^{14}C-aclonifen was studied using cryopreserved hepatocytes from mice, rats and humans. Experiments were conducted by incubating two analogues of ^{14}C-aclonifen

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([phenoxy-UL-^{14}C]aclonifen and [aniline-UL- ^{14}C]aclonifen) each at two concentrations (1 µM and 10 µM) with hepatocytes in suspension (0.7 x 10^6 cells/mL) at 37°C for 0, 30 and 120 min. Samples were analyzed following protein precipitation by reversed phase HPLC with radiochemical detection (HPLC-RAD).

Test item concentrations were chosen to assess possible concentration-dependent differences in its metabolism and to create enough sample material for identification of potential metabolites by chromatographic or spectroscopic methods. Test durations of 30 and 120 minutes were considered reasonable, because the metabolic activity is not affected by cell adaptation or/and survival when used in suspension. Likewise, positive results were obtained from the enzymatic reaction of testosterone to hydroxytestosterone at longer incubation times (3 h).

The potential cytotoxicity of unlabelled Aclonifen (1 µM and 10 µM) was assessed in the hepatocyte batches from the three species using the Alamar Blue® assay. After 120 minutes incubation, aclonifen was found to be non-cytotoxic.

The metabolic activity of the microsomes was demonstrated by determining 6β-hydroxytestosterone formed from testosterone by testosterone 6β-hydroxylase. This biochemical reaction is well-known for a CYP3A microsomal enzyme.

Measurement of the recovery of radioactivity after hepatocyte incubations demonstrated that no radioactivity was irreversibly bound to the hepatocytes after incubation with ^{14}C-aclonifen. However, non-specific binding (NSB) of ^{14}C-aclonifen and/or its metabolites occurred in the incubation tubes and in the HPLC-injection vials. Owing to this NSB effect, the calculation of relative abundance of ^{14}C-aclonifen and metabolites may be affected and the results of metabolite profiling should be considered as approximate.

^{14}C-aclonifen was extensively metabolized by the hepatocytes from all species used in the study. A total of 20 ^{14}C-labelled metabolites were detected (M-1 to M-20) based on their HPLC retention time. Six main metabolites, accounting for ≥10% of the relative percentage, were detected: M-5 and M-13 (rat), M-14 (mouse and human), M-17 and M-18 (rat and human) and M-10 (common to all three species tested).

The transformation rate of ^{14}C-aclonifen and relative formation of the different metabolites varied among the species, the incubation time and the ^{14}C-aclonifen concentration. The rat showed the highest metabolism rate.

No human-specific ^{14}C-aclonifen metabolites were detected.

Most of the metabolites were formed regardless of the ^{14}C-aclonifen analogue used for incubation, which means that the main metabolic pathways in mouse, rat and human hepatocytes do not involve remarkable hydrolysis of the phenoxyaniline moiety.

I. MATERIALS AND METHODS

Study dates: Start: 07 September 2016
Completion: 11 January 2017

A. MATERIALS

1a. Test Item (labelled) [Phenoxy-UL-^{14}C]aclonifen

Batch No.:	KML 10079
Radiopurity:	≥99%
Specific activity:	6.79 MBq/mg

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* = position of uniformly labelled ring

1b.	**Test Item (labelled)**	[Aniline-UL-^{14}C]aclonifen
	Batch No.:	KML 10212
	Radiopurity:	>99%
	Specific activity:	6.59 MBq/mg

+ = position of uniformly labelled ring

2a.	**Reference standard**	Aclonifen
	Batch No.:	AE F068300-PU-01
	Appearance:	Yellow crystalline powder
	Purity:	99.6%
	Expiry:	02 April 2018
2b.	**Reference standard**	6β-hydroxytestosterone
	Batch No.:	0455798-3 (Cayman Chemical Co.)
	Purity:	99.5%
	Expiry:	26 July 2017
2c.	**Reference standard**	Dexamethasone
	Batch No.:	SZBE094XV (Sigma Aldrich)
	Purity:	99.6%
	Expiry:	04 April 2019
3.	**Test system (cells)**	Cryopreserved hepatocytes from male CD-1 mice (pool from 36 individuals, Batch RSI), male Wistar rats (pool from 26, Batch ECP), humans (pool from 50 donors, both genders, Batch GOZ),

B. STUDY DESIGN AND METHODS

1. Cytotoxicity

The potential aclonifen-induced cytotoxicity was determined in the mouse, rat and human hepatocytes. Incubations were performed using two concentrations of unlabeled aclonifen (1 µM and 10 µM) for

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120 minutes (n=4). Cytotoxicity was evaluated by the AlamarBlue® assay using incubations with vehicle (DMSO) as negative controls (n=4). Two types of incubations were used as positive controls: 50 µM Tamoxifen and incubation without hepatocytes/aclonifen (n=4). Control samples containing incubation medium without cells and without AlamarBlue® (n=4) were used for absorbance background subtraction.

The incubations solutions were prepared in InVitroGRO KHB medium, to achieve final concentrations of 50 µM Tamoxifen, 20 or 2 µM aclonifen (unlabeled), or 10 µL DMSO in 4.99 mL medium.

After thawing, the hepatocyte viability was assessed using the Trypan Blue exclusion method. Suspensions were prepared at 1.4 x 10^6 viable cells/mL in medium. Aliquots of cell suspension were mixed with solutions containing the test item to a final cell density of 0.7 x 10^6 cells/mL, for incubation at 37ºC, with shaking. After 120 minutes incubation, 20 µL of Alamar Blue® were added directly to all samples, except for those used for absorbance background subtraction. Then the mixtures were protected from direct light and incubated at 37±1ºC in a Thermomixer device (500 rpm) for 3.25 to 3.37 additional hours. The actual time of incubation depended on visual inspection of the samples (change in color of control). At the end of the incubation time, the samples were centrifuged at 20±5ºC and the supernatants transferred to a 96-well plate for reading the absorbance of 570 nm and 600 nm in a plate reader. Analyses were performed the same day.

The radiochemical purity of [phenoxy-UL-14C]aclonifen and [aniline-UL-14C]aclonifen was assessed by HPLC-RAD prior to initiation of the experiments for linearity/LLOQ estimation and hepatocyte incubations. For this purpose, stock solutions of each were diluted with 1% formic acid in water. These solutions were stirred gently and kept at room temperature until analysis. Duplicate samples were analyzed by HPLC-RAD the same day of preparation. The acceptance criterion was set at ≥97.0%. Prior to analysis of test samples, the HPLC-RAD method was qualified for linearity of response and the Lower Limit of Quantitation (LLOQ). [Phenoxy-UL-14C]Aclonifen was used as standard for this purpose. Mean peak area values at each level were plotted versus the respective nominal injected dpm and linear regression analysis was carried out (acceptance criteria r ≥ 0.99). The criteria for accepting a value of injected radioactivity as the LLOQ were as follows: coefficient of variation (CV) of the mean peak area <20%; threshold for the remaining linearity levels <15%. The LLOQ value was set at 300 dpm for radioactivity detection (CV <20%), and thus compounds with peak areas below that for the LLOQ (area 1065.5) were not quantified.

2. Incubations with hepatocytes

Working solutions of each label of aclonifen were prepared fresh each day, each at 1 or 10 mM. Similarly, fresh solutions of 125 mM testosterone were prepared daily. Final concentrations in the cell suspensions were 1 or 10 µM for the aclonifen labels, 10 µM for unlabeled aclonifen, and 125 µM for testosterone. The cell density in the incubates was 0.7 x 10^6 hepatocytes/mL and the incubation volume was 250 µL.

Cryopreserved hepatocytes from each species were incubated separately (n=3) with [phenoxy-UL-^{14}C]aclonifen and [aniline-UL-^{14}C]aclonifen. Incubations were started by mixing 125 µL of hepatocyte suspension (1.4 x 10^6 viable cells/mL) with 125 µL of incubation solutions containing the test items or testosterone (for the positive metabolism controls) and were quenched after 30 or 120 min with 1 volume of acetonitrile at room temperature. Triplicate samples at T=0 were prepared by adding the same components as test samples but in different order (i.e. acetonitrile was added prior to addition of test item incubation solution). Two stability control samples were prepared each incubation day for each test item at the two concentrations. These samples were prepared by mixing 125 µL of culture medium with 125 µL of test item incubation solution and were also incubated for 30 or 120 minutes. As for test samples, 1 volume acetonitrile at room temperature was added at the end of the incubation period. Finally, one reference sample was prepared each day of incubation by mixing 125 µL of culture medium with 250 µL acetonitrile and 125 µL of unlabelled 20 µM aclonifen incubation solution. The samples were maintained at room temperature until analysis.

3. Recovery of radioactivity

After incubation of the hepatocyte samples, an aliquot of 50 µL of each incubate was analyzed by liquid scintillation counting (LSC). Following centrifugation an aliquot of 50 µL of the obtained supernatants was also analyzed by LSC. Aliquot weights were recorded. 10 mL of scintillation cocktail (Ultima Gold, Perkin Elmer) was added to the samples. After shaking, the samples were maintained at room temperature and protected from light for ≥24 h before LSC analysis. For LSC analysis, the samples were placed in the β-counter and analyzed for 10 minutes (maximum counting time if 2% of counting error was not achieved).

Recovery was determined as relative percentage of dpm/g recovered in supernatants as compared to whole incubates before centrifugation (initial dpm/g: 100% radioactivity).

4. Analysis for ^{14}C-aclonifen metabolism

The hepatocyte incubates were centrifuged at 16000 g for 15 minutes at 20±5°C. Then 100 µL of each supernatant was diluted with 400 µL of 1% formic acid in water. The samples were directly analyzed by HPLC-RAD without any further extraction procedure.

Instrument(s)	HPLC separation module, Alliance 2695 (Waters) UV-detector for HPLC, 2487 (Waters) Radioactivity flow-through detector (LB-509, Berthold) MassLynx Chromatography software V4.0
Column	Purospher STAR RP-18e, 250 x 4.6 mm, 5 µm (Merck)
Column temperature	40°C
Injector temperature	20°C
HPLC-run time	70 minutes
Injection volume	Variable (100 µL for standards, 350 µL for test samples)
Wash solvent	AcN : Milli-Q grade water = 80 : 20 (v/v)
Purge solvent	AcN : Milli-Q grade water = 80 : 20 (v/v)
Mobile phases	Eluent A: 1% Formic acid in Milli-Q grade water Eluent B: 1% Formic acid in AcN
Column flow rate	1.0 mL/min
UV-detection	254 nm
^{14}C-detection	500 µL admixture cell (Z-500, Berthold) Scintillation cocktail: Ultima Flo M (Perkin Elmer) Flow rate scintillation cocktail: 4.0

Gradient	Time (min.)	0	5	60	65	66	70
	% A	100	100	0	0	100	100
	% B	0	0	100	100	0	0
	Curve	1	6	6	6	6	6

The chromatograms were recorded electronically and quantitatively evaluated using the MassLynx® Chromatography software (V 4.0, Waters). The ^{14}C-trace of a chromatogram was divided into regions of interest, corresponding to the separated radioactive peaks. The area counts from all regions of interest were used for the percentage calculation of individual compounds.

The metabolic activity of the hepatocytes for a positive control sample was determined by measurement of 6β-hydroxytestosterone formed from testosterone by testosterone 6β-hydroxylase. This biochemical reaction is well-known for CYP3A enzyme.

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The hepatocyte suspensions of each species were incubated in triplicate with 125 µM testosterone at 37±1ºC following the incubation procedure. The reactions were started by the addition of testosterone incubation solution and were quenched after 3 h with 250 µL acetonitrile.

The in-study characterization of the 6β-hydroxytestosterone analytical method was performed together with test sample analysis. The analytical batch included a calibration curve, two blank samples and two blank samples spiked with internal standard (carry-over test), six quality controls interspersed in the sample analysis run and the incubation samples to be analyzed. The calibration curve, QCs and blanks were prepared in hepatocyte incubation medium (InVitroGRO™ KHB buffer). The method characterization included the evaluation of: linearity, accuracy and carry over. Dexamethasone was used as internal standard. 6β-hydroxytestosterone was determined by LC-MS/MS. For this purpose, calibration curve samples were prepared by spiking hepatocyte incubation medium with 6β-hydroxytestosterone standard and dexamethasone (internal standard). The calibration curve was obtained by plotting the response obtained for 6β-hydroxytestosterone in the sample versus the nominal concentration. The processing of the chromatograms, the calculation of correlation coefficients (r) and of the calibration curve slope and intercept values were performed using the AnalystTM quantification manager version 1.6.2 (AB Sciex). Linear regression with a $1/x^2$ weighting factor was used. Linearity was evaluated using eight concentrations (500-25000 pmol/mL). Accuracy of the method was determined by measuring the concentrations of the analyte in the QC samples and comparing them with the nominal concentrations. Assessment of carry-over considered that the peak area of the blank injected after the ULOQ should be less than 20% of the peak area of the LLOQ of the reference standard and less than 5% for the internal standard. The LLOQ was set at 1000 pmol/mL.

Instrument(s)	Acquity UPLC® System (Waters)					
Column	Acquity UPLC® BEH C18, 1.7 µm, 50 x 2.1 mm ID, (Waters)					
Column temperature	40°C					
Injector temperature	4°C					
HPLC-run time	5 minutes					
Injection volume	5 µL					
Weak wash solvent	MeOH : Milli-Q grade water = 25 : 75 (v/v) containing 0.1% Acetic acid					
Strong wash solvent	MeOH: AcN: Milli-Q grade water: 2 propanol (1:1:1:1 v/v/v/v) containing 1% Formic acid					
Mobile phases	Eluent A: MeOH : Milli-Q grade water : AcN = 64 : 30 : 6 (v/v) containing 0.1% Acetic acid Eluent B: MeOH : Milli-Q grade water = 25 : 75 (v/v) containing 0.1% Acetic acid					
Column flow rate	0.4 mL/min					
Gradient	Time (min)	0.00	3.00	4.00	4.10	5.00
	% A	45	100	100	45	45
	% B	55	0	0	55	55
	Curve	-	6	1	1	1

Analysis of cytotoxicity samples was performed by spectrophotometry by measuring the absorbance at 570 nm and at 600 nm. A correction factor was calculated for the contribution of the oxidized AlamarBlue® present in most of samples.

The relative percentages of unchanged ^{14}C-aclonifen and metabolites were calculated from the radiochromatographic profiles as:

$$\%Relative\ Pi = \frac{Area\ Pi}{\sum Area\ P} \times 100$$

Where area Pi was the mean area of the peak in the radiochemical chromatogram of a test sample, and ∑Area P was the sum of the total radioactive mean peak areas in the chromatogram.

II. RESULTS AND DISCUSSION

1. Cytotoxicity

The potential cytotoxicity of aclonifen was assessed in hepatocytes from the three species using two concentrations of unlabelled aclonifen (1 µM and 10 µM) for an incubation period of 120 minutes, corresponding to the longest incubation time for the subsequent metabolism evaluation.

The results demonstrated that aclonifen was non cytotoxic for the hepatocyte batches to be used in the study. The percent difference in Alamar Blue® reduction as compared to untreated cells (DMSO control) ranged from 94% to 131%. The positive control Tamoxifen 50 µM did not show cytotoxic effect, which was attributed to lack of metabolic activation due to the short incubation period used, a process necessary for Tamoxifen to induce cell toxicity.

Table 5.1-35: Cytotoxicity assay in mouse hepatocytes (AlamarBlue Assay)

Absorbance at 570 nm (AO_{570})						
	C_KHB (buffer)	**C_KHB +A**	**C_DMSO (Control)**	**Tamoxifen 50 µM**	**Aclonifen 1 µM**	**Aclonifen 10 µM**
Mean	0.026	0.736	0.937	1.186	0.909	1.007
Mean - buffer		0.710	0.911	1.160	0.883	0.981
Absorbance at 600 nm (AO_{600})						
Mean	0.026	1.064	0.634	0.830	0.634	0.654
Mean - buffer		1.038	0.608	0.804	0.608	0.628
Ratio 570/600	R_0 0.684		1.500	1.443	1.453	1.562
% Difference in reduction			100	123	94	111

A: Alamar Blue
KHB: Incubation buffer Control: untreated cells
C-KHB and C_KHB+A: samples without hepatocytes
R0: Correction factor for oxidized AlamarBlue
% Difference in reduction = (A570-(A600 x R0) for test well)/(A570-(A600 x R0) for untreated cells) x 100

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Table 5.1-36: Cytotoxicity assay in rat hepatocytes (AlamarBlue Assay)

Absorbance at 570 nm (AO_{570})						
	C-KHB (buffer)	C-KHB +A	C-DMSO (Control)	Tamoxifen 50 µM	Aclonifen 1 µM	Aclonifen 10 µM
Mean	0.025	0.742	0.802	0.804	0.774	0.916
Mean - buffer		0.717	0.777	0.779	0.749	0.891
Absorbance at 600 nm (AO_{600})						
Mean	0.026	1.073	0.678	0.716	0.646	0.693
Mean - buffer		1.047	0.652	0.691	0.621	0.667
Ratio 570/600	R_0 0.684		1.192	1.128	1.207	1.335
% Difference in reduction			100	93	98	131

A: Alamar Blue
KHB: Incubation buffer Control: untreated cells
C-KHB and C_KHB+A: samples without hepatocytes
R0: Correction factor for oxidized AlamarBlue
% Difference in reduction = (A570-(A600 x R0) for test well)/(A570-(A600 x R0) for untreated cells) x 100

Table 5.1-37: Cytotoxicity assay in human hepatocytes (AlamarBlue Assay)

Absorbance at 570 nm (AO_{570})						
	C-KHB (buffer)	C-KHB +A	C-DMSO (Control)	Tamoxifen 50 µM	Aclonifen 1 µM	Aclonifen 10 µM
Mean	0.025	0.769	0.834	0.885	0.958	0.913
Mean - buffer		0.744	0.809	0.860	0.934	0.888
Absorbance at 600 nm (AO_{600})						
Mean	0.025	1.118	0.552	0.742	0.595	0.638
Mean - buffer		1.094	0.527	0.717	0.570	0.613
Ratio 570/600	R_0 0.684		1.535	1.200	1.638	1.449
% Difference in reduction			100	83	121	105

A: Alamar Blue
KHB: Incubation buffer Control: untreated cells
C-KHB and C_KHB+A: samples without hepatocytes
R0: Correction factor for oxidized AlamarBlue
% Difference in reduction = (A570-(A600 x R0) for test well)/(A570-(A600 x R0) for untreated cells) x 100

Formation of 6β-hydroxytestosterone from testosterone demonstrated sufficient metabolic capability of the hepatocyte batches used in the study. Testosterone 6β-hydroxylase activity was as shown in the table below, for mice, rats and humans.

Table 5.1-38: Testosterone-6β-hydroxylase activity in hepatocytes (positive control)

	Mean (pmol/min/10^6 cells)	Coefficient of Variation (%)
Mouse hepatocytes (CD-1 males)	33.9	7.6%
Rat hepatocytes (Wistar males)	32.6	25.1%

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Human hepatocytes (mixed gender)	144.8	21.1%

2. Recovery of radioactivity after incubation

The recovery of radioactivity after hepatocyte incubations and sample preparation (protein precipitation with acetonitrile and centrifugation) is shown in the tables below.

Table 5.1-39: Mouse hepatocytes: Recovery of radioactivity

Sample	Incubation time (min)	Recovery (%)	Non-specific binding (% relative to T_0)
[Phenoxy-UL-^{14}C]aclonifen incubates 10 µM	0	105.7	0
	30	97.2	-3.5
	120	95.1	-5.8
[Phenoxy-UL-^{14}C]aclonifen incubates 1 µM	0	106.6	0
	30	108.8	-2.6
	120	101.5	-6.9
Buffer control 10 µM	30	96.1	29.2
	120	106.6	64.7
Buffer control 1 µM	30	94.7	25.5
	120	111.4	65.3
[Aniline-UL-^{14}C]aclonifen incubates 10 µM	0	109.2	0
	30	110.5	0.7
	120	107.1	-0.3
[Aniline-UL-^{14}C]aclonifen incubates 1 µM	0	108.1	0
	30	122.9	-8.9
	120	107.3	-10.3
Buffer control 10 µM	30	102.7	30.6
	120	106.2	64.0
Buffer control 1 µM	30	92.1	21.2
	120	106.1	62.0

Table 5.1-40: Rat hepatocytes: Recovery of radioactivity

Sample	Incubation time (min)	Recovery (%)	Non-specific binding (% relative to T_0)
[Phenoxy-UL-^{14}C]aclonifen incubates 10 µM	0	102.5	0
	30	100.2	38.8
	120	99.5	36.8
[Phenoxy-UL-^{14}C]aclonifen incubates	0	89.8	0
	30	98.4	38.9

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Sample	Incubation time (min)	Recovery (%)	Non-specific binding (% relative to T_0)
1 µM	120	99.2	38.7
Buffer control 10 µM	30 120	102.6 126.2	51.6 77.4
Buffer control 1 µM	30 120	109.0 112.6	58.1 85.3
[Aniline-UL-^{14}C]aclonifen incubates 10 µM	0 30 120	96.9 100.7 97.1	0 41.6 40.6
[Aniline-UL-^{14}C]aclonifen incubates 1 µM	0 30 120	98.0 99.7 98.2	0 42.5 40.7
Buffer control 10 µM	30 120	107.4 110.0	57.5 75.4
Buffer control 1 µM	30 120	104.8 110.1	58.0 79.1

Table 5.1-41: Human hepatocytes: Recovery of radioactivity

Sample	Incubation time (min)	Recovery (%)	Non-specific binding (% relative to T_0)
[Phenoxy-UL-^{14}C]aclonifen incubates 10 µM	0 30 120	103.4 101.5 100.0	0 3.7 7.9
[Phenoxy-UL-^{14}C]aclonifen incubates 1 µM	0 30 120	100.5 106.0 103.9	0 8.1 2.2
Buffer control 10 µM	30 120	113.9 115.4	53.8 79.6
Buffer control 1 µM	30 120	122.4 108.0	58.9 79.0
[Aniline-UL-^{14}C]aclonifen incubates 10 µM	0 30 120	101.4 100.9 98.7	0 6.1 6.5
[Aniline-UL-^{14}C]aclonifen incubates 1 µM	0 30 120	101.2 102.3 98.2	0 14.3 12.8
Buffer control	30	112.4	55.5

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Sample	Incubation time (min)	Recovery (%)	Non-specific binding (% relative to T_0)
10 µM	120	129.6	81.9
Buffer control 1 µM	30	113.6	57.4
	120	114.4	81.3

Recovery ranged from 89.8% to 122.9%, demonstrating that no radioactivity was irreversibly bound to the hepatocytes after incubation with ^{14}C-Aclonifen for 30 or 120 minutes, regardless of the analogue used and of the aclonifen concentration (1 µM and 10 µM). However, a decrease in the radioactivity was found in the samples as a function of the incubation time as compared to T=0 samples, meaning that non-specific binding (NSB) of ^{14}C-aclonifen and/or its metabolites occurred in the incubation tubes. The extent of NSB varied among the species, being higher in the rat hepatocyte incubations (36.8 % to 42.5%) and intermediate in the human hepatocyte incubations (2.2 % to 14.3%). No NSB was detected in the mouse hepatocyte incubations. Furthermore, the NSB seemed independent of the ^{14}C-aclonifen analogue and its concentration, while NSB was higher in control buffer samples (without hepatocytes) than in the hepatocyte incubates.

The binding of test item and potentially its labelled metabolites to the injection vials and to the hepatocyte incubation tubes requires a careful interpretation of the metabolite profiles, particularly because the calculation of their relative abundances may be affected. Therefore, the results described in the following section should be considered as approximate.

5. Metabolic profile

^{14}C-aclonifen was extensively metabolized by the hepatocytes from all species used in the study. A total of 20 ^{14}C-labelled metabolites were detected based on their HPLC retention times (numbered M-1 to M-20). Six main metabolites (those accounting for ≥10% of the relative percentage) were detected: M-5 and M-13 (rat), M-14 (mouse and human), M-17 and M-18 (rat and human) and M-10 (common metabolite in all the species). No human-specific metabolites were detected.

The transformation rate of ^{14}C-aclonifen and the relative formation of the different metabolites varied among the species, the incubation time and the ^{14}C-aclonifen concentration. The rat showed the highest metabolism rate.

Overall, most of the metabolites were formed regardless of the ^{14}C-aclonifen analogue used for incubation, which meant that the main metabolic pathways in mouse, rat and human hepatocytes do not involve remarkable hydrolysis of the phenoxyaniline moiety.

Table 5.1-42: Metabolic profile of ^{14}C-aclonifen in mouse hepatocytes, as relative percentage chromatographic peak areas

^{14}C-aclonifen conc.:		10 µM				1 µM			
Incubation time:		30 min		120 min		30 min		120 min	
^{14}C-aclonifen analog:		Phen	Anil	Phen	Anil	Phen	Anil	Phen	Anil
Metabolite	Retention time (min)								
M-1	23.4	0	0	0	0	0	0	0	0
M-2	27.2	0	0	0	0	0	0	0	0
M-3	28.0	0	0	0	0	0	0	0	0

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^{14}C-aclonifen conc.:		**10 µM**				**1 µM**			
Incubation time:		**30 min**		**120 min**		**30 min**		**120 min**	
^{14}C-aclonifen analog:		**Phen**	**Anil**	**Phen**	**Anil**	**Phen**	**Anil**	**Phen**	**Anil**
Metabolite	**Retention time (min)**								
M-4	28.3	0.6	0	0	0	0	0	0	0
M-5	29.0	0	0	0	0	0	0	0	0
M-6	29.8	0	1.2	0	BLLOQ	0	0	0	0
M-7	30.2	0	0	0	0	0	0	0	0
M-8	30.6	0	0	0	0	0	0	0	0
M-9	31.0	0	0	0	0	0	0	0	0
M-10	31.6	**20.3**	**18.4**	**28.0**	**32.5**	**36.8**	**35.1**	**55.4**	**46.8**
M-11	32.0	0	0	0	0	0	0	0	0
M-12	35.0	0	0.8	2.5	1.2	0	0	BLLOQ	8.8
M-13	36.3	0	1.1	0	0	0	0	0	0
M-14	39.8	**17.3**	**19.7**	**15.6**	**17.0**	**28.9**	**29.5**	**11.6**	**17.1**
M-15	40.2	0	0	0	0	0	0	0	0
M-16	40.4	3.2	3.0	5.7	4.7	0	0	0	0
M-17	40.7	BLLOQ	0.9	1.6	1.0	0	0	0	0
M-18	41.2	2.5	2.1	2.4	0	0	0	0	0
M-19	42.6	1.8	3.0	0	0	0	0	0	0
M-20	43.5	1.6	1.4	3.6	2.7	0	0	0	0
Phenoxy-^{14}C-aclonifen	47.7	**52.6**	-	**40.6**	-	**34.3**	-	**33.0**	-
Aniline-^{14}C-aclonifen	47.7	-	**48.6**	-	**40.8**	-	**35.4**	-	**27.3**

Metabolites representing >10% of relative percentage shown in bold

Table 5.1-43: Metabolic profile of ^{14}C-aclonifen in rat hepatocytes, as relative percentage chromatographic peak areas

^{14}C-aclonifen conc.:		**10 µM**				**1 µM**			
Incubation time:		**30 min**		**120 min**		**30 min**		**120 min**	
^{14}C-aclonifen analog:		**Phen**	**Anil**	**Phen**	**Anil**	**Phen**	**Anil**	**Phen**	**Anil**
Metabolite	**Retention time (min)**								
M-1	23.4	0	0	4.9	3.7	0	0	0	0
M-2	27.2	3.5	0	5.2	3.6	0	0	8.7	0
M-3	28.0	0	0	BLLOQ	0	0	0	0	0
M-4	28.3	0	0	BLLOQ	0	0	0	0	0

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^{14}C-aclonifen conc.:		10 µM				1 µM			
Incubation time:		**30 min**		**120 min**		**30 min**		**120 min**	
^{14}C-aclonifen analog:		**Phen**	**Anil**	**Phen**	**Anil**	**Phen**	**Anil**	**Phen**	**Anil**
Metabolite	**Retention time (min)**								
M-5	29.0	0	0	6.8	5.3	0	0	**48.1**	**42.2**
M-6	29.8	0	0	0	2.6	0	0	0	0
M-7	30.2	0	0	0	1.3	0	0	0	0
M-8	30.6	0	0	4.4	2.0	0	0	0	0
M-9	31.0	0	0	8.0	8.5	0	0	0	0
M-10	31.6	5.0	5.4	**10.9**	**14.9**	0	0	0	0
M-11	32.0	0	0	BLLOQ	0	0	0	0	0
M-12	35.0	0	0	0	0	0	0	0	0
M-13	36.3	9.0	7.7	**12.5**	**10.5**	0	0	8.8	0
M-14	39.8	5.7	5.9	3.5	3.7	0	0	0	0
M-15	40.2	BLLOQ	0	0	0	0	0	0	0
M-16	40.4	1.8	1.9	0	3.7	0	0	0	0
M-17	40.7	**19.6**	**21.5**	**23.9**	**21.0**	**83.5**	**19.6**	**34.4**	**57.8**
M-18	41.2	0	0	0	0	0	**53.8**	0	0
M-19	42.6	3.8	5.3	3.3	6.6	0	0	0	0
M-20	43.5	0	0	0	0	0	0	0	0
Phenoxy-^{14}C-aclonifen	47.7	**51.5**	-	**16.3**	-	**16.5**	-	0	-
Aniline-^{14}C-aclonifen	47.7	-	**52.2**	-	**12.6**	-	**26.6**	-	0

Metabolites representing >10% of relative percentage shown in bold

Table 5.1-44: Metabolic profile of ^{14}C-aclonifen in human hepatocytes, as relative percentage chromatographic peak areas

^{14}C-aclonifen conc.:		10 µM				1 µM			
Incubation time:		**30 min**		**120 min**		**30 min**		**120 min**	
^{14}C-aclonifen analog:		**Phen**	**Anil**	**Phen**	**Anil**	**Phen**	**Anil**	**Phen**	**Anil**
Metabolite	**Retention time (min)**								
M-1	23.4	0	0	0	0	0	0	0	0
M-2	27.2	0	0	0	0	0	0	0	0
M-3	28.0	0	0	0	0	0	0	0	0
M-4	28.3	0	0	0	0	0	0	0	0
M-5	29.0	0	0	0	0	0	0	0	0

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^{14}C-aclonifen conc.:		10 µM				1 µM			
Incubation time:		**30 min**		**120 min**		**30 min**		**120 min**	
^{14}C-aclonifen analog:		**Phen**	**Anil**	**Phen**	**Anil**	**Phen**	**Anil**	**Phen**	**Anil**
Metabolite	**Retention time (min)**								
M-6	29.8	0	0	0	0	0	0	0	0
M-7	30.2	0	0	0	0	0	0	0	0
M-8	30.6	0	0	0	0	0	0	0	0
M-9	31.0	0	0	0	0	0	0	0	0
M-10	31.6	4.6	4.3	**15.0**	**12.4**	7.1	5.8	**45.3**	**15.8**
M-11	32.0	0	0	0	0	0	0	0	0
M-12	35.0	0	0	0	0	0	0	0	0
M-13	36.3	0	0	1.2	BLLOQ	0	0	0	BLLOQ
M-14	39.8	8.3	8.5	5.3	5.2	**17.5**	**17.2**	0	0
M-15	40.2	0	0	0	0	0	0	0	0
M-16	40.4	0	0	0	0	0	0	0	0
M-17	40.7	0	0	**22.4**	**16.6**	0	0	**38.6**	**84.2**
M-18	41.2	7.6	7.4	0	0	**23.4**	**28.1**	**16.1**	0
M-19	42.6	0	0	4.3	2.6	0	0	BLLOQ	0
M-20	43.5	0	0	0	0	0	0	0	0
Phenoxy-^{14}C-aclonifen	47.7	**79.5**	-	**51.8**	-	**52.0**	-	BLLOQ	-
Aniline-^{14}C-aclonifen	47.7	-	**79.8**	-	**63.2**	-	**48.9**	-	BLLOQ

Metabolites representing >10% of relative percentage shown in bold

Table 5.1-45: Summary metabolic profile of ^{14}C-aclonifen in mouse, rat and human hepatocytes (main metabolites, >10%)

Metabolite	Retention time (approx., min)	Mouse		Rat		Human	
		Phenoxy	Aniline	Phenoxy	Aniline	Phenoxy	Aniline
M-5	29.0			✓	✓		
M-10	31.6	✓	✓	✓	✓	✓	✓
M-13	36.3			✓	✓		
M-14	39.8	✓	✓			✓	✓
M-17	40.7			✓	✓	✓	✓
M-18	41.2				✓	✓	✓

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In mice, ^{14}C-aclonifen was metabolized to 11 detectable metabolites, the relative percentage of unchanged parent being approximately 50% at 10 µM and 35% at 1µM after 30 min. After 120 min, these percentages declined to approximately 40% and 30%, respectively.

In rats, metabolism was the most extensive, with 18 detectable metabolites. The unchanged proportion was approximately 50% and 20% at 10 and 1 µM after 30 min, declining to approximately 15% and 0% after 120 min.

In humans, metabolism was the least extensive, with 6 detectable metabolites. The unchanged remainder was approximately 80% and 50% at 10 and 1 µM after 30 min, declining to approximately 55% and 0% after 120 min.

III. CONCLUSION

Aclonifen (1 µM and 10 µM) was found to be non-cytotoxic after 120 minutes incubation with all the hepatocytes used in the study, and no radioactivity was irreversibly bound to the hepatocytes after incubation. Non-specific binding of ^{14}C-aclonifen and/or its metabolites to the incubation tubes and the injection vials occurred. Owing to this binding effect, the results of the metabolite profiling should be considered as approximate.

^{14}C-aclonifen was extensively metabolized by the hepatocytes from all species used in the study, with a total of 20 labelled metabolites detected (M-1 to M-20, based on their HPLC retention time).

Six main metabolites, accounting for ≥10% of the relative percentage, were detected: M-5 and M-13 (rat), M-14 (mouse and human), M-17 and M-18 (rat and human) and M-10, (a metabolite common to all three species tested).

No human-specific ^{14}C-aclonifen metabolites were detected.

The transformation rate of ^{14}C-aclonifen and relative formation of the different metabolites varied among the species, the incubation time and the ^{14}C-aclonifen concentration. The rat showed the highest metabolism rate.

Very similar profiles were obtained using either [phenoxy-UL-^{14}C]aclonifen or [aniline-UL-^{14}C]aclonifen, which means that the main metabolic pathways in mouse, rat and human hepatocytes do not involve remarkable hydrolysis of the phenoxyaniline moiety.

Assessment and conclusion by applicant: A good, acceptable study that fulfils current understanding of requirements and produced valid results and conclusions.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.1.1/09
Report Author:	[redacted]
Report Year:	2019
Report Title:	The metabolic stability of [aniline-UL-14C]-aclonifen and [phenoxy-UL-14C]-aclonifen in cryopreserved hepatocytes from rabbit, dog and human
Report No:	180383
Document No:	M-674506-01-1
Guideline(s) followed in study:	US EPA OCSPP not applicable
Deviations from current test guideline:	Not applicable (non-guideline study)
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

[Aniline-UL-^{14}C]-aclonifen and [phenoxy-UL-^{14}C]-aclonifen (1 µM and 10 µM) were incubated with cryopreserved hepatocytes from New Zealand White rabbit, Beagle dog and human for 0, 60 and 120 min. Deactivated hepatocytes were incubated in parallel. The supernatants were analysed by TopCount (1 µM) and HPLC with online radio-detection (10 µM) to determine depletion of parent compound and quantify the major metabolites formed across species.

Radioactive recoveries were acceptable overall, with 66.5 – 115% of the total amount of radioactivity recovered. Metabolism of both radiolabels was rapid and extensive in all three species, with complete depletion in rabbit and dog within 120 min of incubation. In human hepatocytes, metabolism was 65.6% to 100% complete after 120 min.

In total, 100 metabolites were measured in dog, rabbit and human hepatocytes. All detected metabolites were characterized based on their chromatographic behaviour, but a metabolic pathway was not derived. Metabolites M6 and M7 were the main constituents observed in all three species at both concentrations and radiolabelled forms, with any species differences being of a quantitative rather than qualitative nature. No metabolite identification was carried out in the present study.

Compared to rabbit and dog, no metabolite with at least 5% of the total amount of radioactivity was found to be unique to human hepatocytes.

I. MATERIALS AND METHODS

Study dates Start: 27 May 2019
Completion: 29 November 2019

A. MATERIALS

1a. Test Item (labelled) [Phenoxy-UL-^{14}C]aclonifen
Batch No.: KML 10574
Radiopurity: 97.7%
Specific activity: 4.52 MBq/mg, 32.33 mCi/mmol

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* = position of uniformly labelled ring

1b. Test Item (labelled) [Aniline-UL-^{14}C]aclonifen
Batch No.: KML 10709
Radiopurity: >99%
Specific activity: 6.59 MBq/mg, 47.14 mCi/mmol

* = position of uniformly labelled ring

2. Reference standard Aclonifen
Batch No.: AE F068300-PU-01
Appearance: Yellow crystalline powder
Purity: 99.6%
Expiry: 27 February 2028

3. Test system (cells) Cryopreserved hepatocytes from female NZW rabbits (pool of 4 individuals, Batch LFG), Beagle dogs (mixed gender pool of 6, Batch OGY), humans (mixed gender, age and race, pool of 20 donors, Batch WGR), from USA
Culture medium: DMEM supplemented with 1% Glutamax

B. STUDY DESIGN AND METHODS

The supplied [aniline-UL-^{14}C]-aclonifen and [phenoxy-UL-^{14}C]-aclonifen were dissolved in acetonitrile at a nominal concentration of 5.0 mM. The radioactivity content of the stock solutions was measured by Liquid Scintillation Counting (LSC) and working solutions were prepared at 100x the final test item concentration in the incubations using radiolabeled material only. Working solutions were prepared at 0.1 and 1 mM in acetonitrile. The radioactive content of the working solutions was confirmed by LSC.

The solubility of the radiolabels in culture medium was determined, and acetonitrile was selected as the termination solvent. Hepatocyte viability was determined using the Trypan blue exclusion method.

Incubations of aclonifen label were performed in triplicate in 12-well plates with 10^6 viable cells/mL at 37°C in humidified air with 5% CO_2, in supplemented DMEM with solvent content 1% v/v. The incubations were terminated after 0, 60 or 120 min by removing into ice-cold acetonitrile. Blank

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incubations were performed in parallel with deactivated hepatocytes. The metabolic competence of the hepatocyte preparations was assessed in parallel incubations with [^{14}C]-testosterone (10 µM), which were terminated with ice-cold methanol.

The radioactive content of the dosing solutions and samples from incubations performed with aclonifen radiolabel was assessed by Liquid Scintillation Counting (LSC). Quadruple samples (from the dosing solutions) or triplicate samples (from incubations) were combined with Zinsser Aquasafe scintillant and water. LSC analysis was then conducted for each sample (dosing solutions and incubation supernatants) for together with representative blank samples, with automatic quench correction by an external method. Samples were allowed to stabilise with regard to light and temperature prior to analysis. Representative blank sample values were subtracted from sample count rate to obtain a net d.p.m. per sample, and a limit of reliable determination of 30 d.p.m. above background was used. The level of radioactivity in the sample supernatants was expressed as a percentage of dosed radioactivity in order to determine recovery of the dose in this fraction of the post incubation samples. The amount of [aniline-UL-^{14}C]-aclonifen, [phenoxy-UL-^{14}C]-aclonifen, [^{14}C]-testosterone and respective metabolites present in each sample was measured based on relative peak areas.

HPLC conditions

Aclonifen labels

Column	Purospher Star RP-18 endcapped (250 mm x 4.6 mm; 5 µm)
Column Temperature	40°C
Auto-sampler Temperature	4°C
Mobile Phase	A: MilliQ water : formic acid (99:1 v/v) B: Acetonitrile : water (99:1 v/v)

Gradient

Time (min)	%A	%B
0	100	0
5	100	0
60	0	100
65	0	100
66	100	0
70	100	0

Flow Rate	1.0 mL/min
u.v. Detector Wavelength	254 nm
Scintillan:	Proflow G+
Scintillant Flow Rate	2.0 mL/min.

Testosterone label

Column	Nova-Pak C18 (150 mm x 3.9 mm; 4 µm)
Column Temperature	50ºC
Auto-sampler Temperature	4°C
Mobile Phase	A: MilliQ water B: Methanol C: Acetontrile

Gradient

Time (min)	%A	%B	%C
0	75	25	0
40	30	64	6
45	75	25	0

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	55	75	25	0

Flow Rate	1.0 mL/min
u.v. Detector Wavelength	240 nm
Scintillant	Proflow G+
Scintillant Flow Rate	2.0 mL/min

II. RESULTS AND DISCUSSION

Poor solubility of [aniline-UL-^{14}C]-aclonifen and [phenoxy-UL-^{14}C]-aclonifen in DMEM supplemented with 1% (v/v) Glutamax was indicated, with radioactive recoveries ranging between 72.0 - 75.0% post-equilibrium and 62.2 - 65.9% post-centrifugation. In the presence of deactivated hepatocytes, the radioactive recoveries obtained with 1 µM and 10 µM of the aniline label post-equilibrium were 86.5% and 84.3%, respectively, whereas the radioactive recoveries obtained with 1 µM and 10 µM with the phenoxy label post-equilibrium were 87.0% and 91.9%, respectively, demonstrating that the presence of protein assisted test item solubility. Experiments performed to evaluate potential adsorption of the radiolabels (10 µM) to cell culture plates and select the most suitable termination solvent also demonstrated loss of radioactivity in the absence of deactivated hepatocytes, with radioactive recovery of 80.3 – 81.9% with either radiolabel and methanol or acetonitrile as the termination solvent. This loss of radioactivity was mitigated by the presence of protein, i.e. deactivated hepatocytes, with a recovery of 94.6% and 100% post-incubation with the aniline label and the phenoxy label respectively.

The viability of hepatocyte preparations was determined to be 86% (rabbit), 89% and 74% (dog) and 82% (human) respectively, indicating their suitability for use in the study.

The radioactive recoveries obtained from incubations with 1 µM [aniline-UL-^{14}C]-aclonifen ranged from 71.2% recovered at 0 min to 109% at 120 min. With the exception of the deactivated hepatocytes, there was an overall trend for increasing radioactive recoveries with increasing incubation time. The recoveries obtained from incubations with 1 µM [phenoxy-UL-^{14}C]-aclonifen were higher compared to those of [aniline-UL-^{14}C]-aclonifen, with recoveries ranging from 90.3% - 113%. The radioactive recoveries obtained from incubations with 10 µM [aniline-UL-^{14}C]-aclonifen followed a similar pattern to those from the low-dose incubations, with the highest recoveries observed at the last time-point of 120 min. To assess any discrepancy in radioactive recoveries owing to test item binding to the cells, the pellets were solubilized and subjected to LSC. The measurements were negligible, suggesting the test items did not bind to hepatic protein.

Table 5.1-46: Recovery of radioactivity following hepatocyte induction with [^{14}C]-aclonifen

Species	Time-point (min)	1 µM		10 µM	
		[Aniline-UL-^{14}C]-aclonifen	[Phenoxy-UL-^{14}C]-aclonifen	[Aniline-UL-^{14}C]-aclonifen	[Phenoxy-UL-^{14}C]-aclonifen
Rabbit	0	80.7 ± 3.5	94.9 ± 5.0	69.4 ± 7.9	88.1 ± 3.1
	60	79.3 ± 4.7	97.3 ± 4.0	81.6 ± 6.9	95.9 ± 5.5
	120	91.0 ± 3.6	93.4 ± 5.4	87.9 ± 3.2	97.8 ± 4.6
Dog	0	80.6 ± 4.8	90.3 ± 3.2	89.9 ± 5.4	75.1 ± 11.1
	60	99.5 ± 4.2	98.8 ± 6.2	89.1 ± 5.3	100 ± 6.0
	120	105 ± 4.9	113 ± 5.7	103 ± 5.9	110 ± 4.2
Human	0	78.3 ± 3.0	90.3 ± 4.5	70.2 ± 4.6	80.9 ± 1.2
	60	80.7 ± 5.1	91.7 ± 4.6	66.5 ± 7.0	73.1 ± 3.4

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Species	Time-point (min)	1 µM		10 µM	
		[Aniline-UL-^{14}C]-aclonifen	[Phenoxy-UL-^{14}C]-aclonifen	[Aniline-UL-^{14}C]-aclonifen	[Phenoxy-UL-^{14}C]-aclonifen
	120	109 ± 2.7	110 ± 5.0	108 ± 3.9	103 ± 9.2
Deactivated	0	71.2 ± 7.3	90.5 ± 5.1	81.8 ± 5.3#	85.4 ± 4.4
	60	75.0 ± 8.5	99.1 ± 4.1	84.3 ± 9.1	95.1 ± 9.3
	120	78.8 ± 4.5	93.3 ± 9.1	115 ± 8.7	115 ± 7.0

Each value is the mean of triplicate aliquots from each of triplicate incubation wells, apart from # where one replicate from one incubation well was removed as an outlier.

[^{14}C]-aclonifen was rapidly and extensively metabolized by hepatocytes from rabbit, dog and human. It was fully depleted within 60 min of incubation in rabbits and dogs, but metabolism was slower in human cells and was incomplete at 10 µM by 120 min. Metabolism was absent in deactivated hepatocytes.

Table 5.1-47: Metabolism of [aniline-UL-^{14}C]-aclonifen in hepatocytes

Species	Time-point (min)	1 µM		10 µM	
		Mean % Parent Remaining ± SD	Mean % Total Metabolism ± SD	Mean % Parent Remaining ± SD	Mean % Total Metabolism ± SD
Rabbit	0	100 ± 0.0	0.00 ± 0.0	97.2 ± 4.8	2.80 ± 4.8
	60	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0
	120	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0
Dog	0	99.0 ± 1.8	1.03 ± 1.8	99.2 ± 1.3	0.767 ± 1.3
	60	0.00 ± 0.0	100 ± 0.0	1.70 ± 1.5	98.3 ± 1.5
	120	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0
Human	0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0
	60	5.50 ± 2.3	94.5 ± 2.3	28.4 ± 26.6	71.6 ± 26.6
	120	0.00 ± 0.0	100 ± 0.0	3.93 ± 0.8	96.1 ± 0.8
Deactivated	0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0
	60	100 ± 0.0	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0
	120	100 ± 0.0	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0

Table 5.1-48: Metabolism of [phenoxy-UL-^{14}C]-aclonifen in hepatocytes

Species	Time-point (min)	1 µM		10 µM	
		Mean % Parent Remaining ± SD	Mean % Total Metabolism ± SD	Mean % Parent Remaining ± SD	Mean % Total Metabolism ± SD
Rabbit	0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0
	60	0.00 ± 0.0	100 ± 0.0	3.10 ± 0.9	96.9 ± 0.9
	120	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0
Dog	0	100 ± 0.0	0.00 ± 0.0	97.4 ± 2.6	2.57 ± 2.6

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Species	Time-point (min)	1 µM		10 µM	
		Mean % Parent Remaining ± SD	Mean % Total Metabolism ± SD	Mean % Parent Remaining ± SD	Mean % Total Metabolism ± SD
	60	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0
	120	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0
Human	0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0
	60	3.73 ± 3.6	96.3 ± 3.6	60.8 ± 2.6	39.2 ± 2.6
	120	0.00 ± 0.0	100 ± 0.0	34.4 ± 3.5	65.6 ± 3.5
Deactivated	0	100 ± 0.0	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0
	60	100 ± 0.0	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0
	120	100 ± 0.0	0.00 ± 0.0	100 ± 0.0	0.00 ± 0.0

Metabolite profiling of rabbit hepatocytes incubated with [^{14}C]-Aclonifen revealed Metabolite M7 as the major metabolite, with 45-50% of the total radioactivity being associated with this peak by 120 min at both 1 and 10 µM. M6 was also a major constituent, approximately 20% by 120 min at 1 µM, 33% at 10 µM. Other metabolites were <10% (mostly ≤5%) and/or transient.

Table 5.1-49: Metabolic profile with 1 µM [^{14}C]-aclonifen in rabbit hepatocytes

Metabolite	Relative retention time (tentative)	Time-point (min)	[Aniline-UL-^{14}C]-aclonifen 1 µM		[Phenoxy-UL-^{14}C]-aclonifen 1 µM	
		0 min	60 min	120 min	60 min	120 min
M2	0.40				0.400 ± 0.7	0.967 ± 1.7
M4	0.54-0.55		2.20 ± 2.3	4.13 ± 0.7	3.30 ± 0.9	3.77 ± 1.3
M6	0.63-0.65		18.6 ± 12.0	22.4 ± 3.2	19.0 ± 1.3	16.0 ± 0.5
M7	0.65-0.68		38.0 ± 6.3	49.7 ± 4.4	51.6 ± 9.8	45.5 ± 5.4
M9	0.82		0.100 ± 0.2	0.533 ± 0.9		0.500 ± 0.9
M18	0.57		3.43 ± 0.8	1.03 ± 1.8	1.73 ± 0.5	0.733 ± 1.3
M19	0.74-0.75		0.267 ± 0.3			
M22	0.81	0.00 ± 0.0	0.133 ± 0.2		4.37 ± 4.2	7.73 ± 11.8
M30	0.86		1.90 ± 0.9	7.60 ± 6.9		
M36	0.78				0.400 ± 0.7	1.07 ± 1.8
M39	0.51		0.500 ± 0.4		0.300 ± 0.5	
M41	0.64		3.07 ± 5.3			
M42	0.42-0.43		0.900 ± 1.2		0.600 ± 0.6	0.433 ± 0.8
M52	0.79-0.80		0.300 ± 0.3			
M77	0.88		15.0 ± 13.6	0.500 ± 0.9		5.77 ± 10.0

Metabolites present in at least two replicates at one time-point in one species with an overall mean of ≥5% are shown. Relative retention times calculated based on mean parent retention time at 0 min.

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Table 5.1-50: Metabolic profile with 10 µM [^{14}C]-aclonifen in rabbit hepatocytes

Metabolite	Relative retention time (tentative)	Time-point (min)	[Aniline-UL-^{14}C]-aclonifen 10 µM		[Phenoxy-UL-^{14}C]-aclonifen 10 µM	
		0 min	60 min	120 min	60 min	120 min
M2	0.40				0.533 ± 0.6	
M4	0.54-0.55		1.80 ± 1.7	1.00 ± 0.4	3.00 ± 0.7	0.333 ± 0.6
M6	0.63-0.65		32.6 ± 1.2	32.4 ± 4.5	35.3 ± 2.7	32.9 ± 2.8
M7	0.65-0.68		47.0 ± 0.2	51.7 ± 3.6	43.0 ± 3.6	47.5 ± 3.3
M9	0.82		0.633 ± 0.6		2.20 ± 0.9	0.500 ± 0.4
M18	0.57				0.200 ± 0.3	
M19	0.74-0.75					
M22	0.81	0.00 ± 0.0			0.367 ± 0.6	7.53 ± 5.9
M30	0.86					
M36	0.78		0.200 ± 0.3		0.700 ± 0.6	2.50 ± 2.3
M39	0.51		0.233 ± 0.4		0.300 ± 0.5	1.07 ± 0.4
M41	0.64		5.73 ± 0.8	5.93 ± 1.1		
M42	0.42-0.43		0.433 ± 0.8			0.133 ± 0.2
M52	0.79-0.80			0.333 ± 0.6	0.300 ± 0.5	
M77	0.88					

Metabolites present in at least two replicates at one time-point in one species with an overall mean of ≥5% are shown. Relative retention times calculated based on mean parent retention time at 0 min.

In dog hepatocytes, the main component was again M7 (approximately 80% by 120 min), while M6 was present at only up to 5-8%. Otherwise, only M18 and M19 exceeded 5% (5-8% by 120 min).

Table 5.1-51: Metabolic profile with 1 µM [^{14}C]-aclonifen in dog hepatocytes

Metabolite	Relative retention time (tentative)	Time-point (min)	[Aniline-UL-^{14}C]-aclonifen 1 µM		[Phenoxy-UL-^{14}C]-aclonifen 1 µM	
		0 min	60 min	120 min	60 min	120 min
M2	0.40					0.967 ± 1.1
M4	0.54-0.55		0.433 ± 0.5			
M6	0.63-0.65	0.00 ± 0.0	8.67 ± 1.8	7.73 ± 0.4	7.57 ± 2.4	5.07 ± 0.6
M7	0.65-0.68		73.1 ± 10.2	82.0 ± 3.1	82.3 ± 10.0	83.5 ± 2.8
M9	0.82	1.03 ± 1.8[A]		0.400 ± 0.7		
M18	0.57			4.77 ± 1.3		
M19	0.74-0.75					
M22	0.81	0.00 ± 0.0	0.100 ± 0.2		0.833 ± 1.4	3.90 ± 3.4
M30	0.86		0.333 ± 0.3	0.167 ± 0.3	3.37 ± 5.8	1.27 ± 2.2
M36	0.78					

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Metabolite	Relative retention time (tentative)	Time-point (min)	[Aniline-UL-^{14}C]-aclonifen 1 µM		[Phenoxy-UL-^{14}C]-aclonifen 1 µM	
		0 min	60 min	120 min	60 min	120 min
M39	0.51					
M41	0.64					
M42	0.42-0.43		0.233 ± 0.4			
M52	0.79-0.80					
M77	0.88		8.37 ± 7.5	1.80 ± 3.1		

Metabolites present in at least two replicates at one time-point in one species with an overall mean of ≥5% are shown. Relative retention times calculated based on mean parent retention time at 0 min.
A: Aniline label only.

Table 5.1-52: Metabolic profile with 10 µM [^{14}C]-aclonifen in dog hepatocytes

Metabolite	Relative retention time (tentative)	Time-point (min)	[Aniline-UL-^{14}C]-aclonifen 10 µM		[Phenoxy-UL-^{14}C]-aclonifen 10 µM	
		0 min	60 min	120 min	60 min	120 min
M2	0.40					
M4	0.54-0.55	0.00 ± 0.0				
M6	0.63-0.65		2.77 ± 2.33	2.47 ± 2.0	1.03 ± 1.8	2.20 ± 2.2
M7	0.65-0.68	1.03 ± 1.8^A	83.6 ± 2.8	73.5 ± 20.5	85.7 ± 3.4	79.2 ± 10.8
M9	0.82		0.967 ± 0.6			
M18	0.57		3.40 ± 1.4	4.17 ± 4.2	6.03 ± 5.1	5.00 ± 6.0
M19	0.74-0.75		0.200 ± 0.3			8.43 ± 10.9
M22	0.81		0.300 ± 0.5			
M30	0.86					
M36	0.78	0.00 ± 0.0				
M39	0.51					
M41	0.64					
M42	0.42-0.43					
M52	0.79-0.80					
M77	0.88					

Metabolites present in at least two replicates at one time-point in one species with an overall mean of ≥5% are shown. Relative retention times calculated based on mean parent retention time at 0 min.
A: Phenoxy label only.

In human hepatocytes, the main component was again M7, with 50-60% by 120 min at 1 µM, but at 10 µM up to 74% of the aniline label and approximately 30% of the phenoxy label. This was followed by M9, with up to 14% by 120 min at 1 µM, but generally appeared transient). M6 was present at only 6-7%, while M4 was up to approximately 5% at 1 µM but did not exceed 1% at the higher dosage.

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Table 5.1-53: Metabolic profile with 1 µM [^{14}C]-aclonifen in human hepatocytes

Metabolite	Relative retention time (tentative)	Time-point (min)	[Aniline-UL-^{14}C]-aclonifen 1 µM		[Phenoxy-UL-^{14}C]-aclonifen 1 µM	
		0 min	60 min	120 min	60 min	120 min
M2	0.40				1.67 ± 0.7	0.733 ± 0.8
M4	0.54-0.55		1.00 ± 1.2		0.633 ± 1.1	5.23 ± 3.9
M6	0.63-0.65		5.63 ± 1.8	5.97 ± 1.9	5.90 ± 0.4	6.93 ± 1.6
M7	0.65-0.68		63.7 ± 2.9	51.7 ± 10.4	49.3 ± 3.7	58.9 ± 8.5
M9	0.82		0.667 ± 1.2	13.5 ± 2.7	9.77 ± 1.5	3.53 ± 3.9
M18	0.57					
M19	0.74-0.75			0.900 ± 0.8		0.633 ± 0.6
M22	0.81	0.00 ± 0.0	0.667 ± 1.2			
M30	0.86		1.40 ± 1.2	2.13 ± 1.9		
M36	0.78					4.63 ± 6.8
M39	0.51				0.233 ± 0.4	
M41	0.64		2.20 ± 1.9			
M42	0.42-0.43		0.667 ± 0.6	0.367 ± 0.4	0.767 ± 0.3	0.500 ± 0.5
M52	0.79-0.80			3.97 ± 6.9	9.63 ± 2.3	1.23 ± 2.1
M77	0.88		0.167 ± 0.3	0.967 ± 1.7		0.400 ± 0.7

Metabolites present in at least two replicates at one time-point in one species with an overall mean of ≥5% are shown. Relative retention times calculated based on mean parent retention time at 0 min.

Table 5.1-54: Metabolic profile with 10 µM [^{14}C]-aclonifen in human hepatocytes

Metabolite	Relative retention time (tentative)	Time-point (min)	[Aniline-UL-^{14}C]-aclonifen 10 µM		[Phenoxy-UL-^{14}C]-aclonifen 10 µM	
		0 min	60 min	120 min	60 min	120 min
M2	0.40					0.600 ± 0.6
M4	0.54-0.55		0.700 ± 1.2	1.03 ± 0.9		
M6	0.63-0.65		6.20 ± 3.8	6.67 ± 1.6	3.17 ± 0.2	4.50 ± 1.3
M7	0.65-0.68		44.8 ± 30.7	74.2 ± 1.1	17.8 ± 4.0	31.3 ± 2.1
M9	0.82		3.13 ± 3.2			
M18	0.57			0.800 ± 0.7		
M19	0.74-0.75	0.00 ± 0.0				
M22	0.81		0.300 ± 0.5		9.47 ± 1.8	11.8 ± 1.8
M30	0.86					
M36	0.78		5.03 ± 0.3	3.63 ± 1.7	8.27 ± 1.0	14.6 ± 2.3
M39	0.51					
M41	0.64					
M42	0.42-0.43					

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Metabolite	Relative retention time (tentative)	Time-point (min)	[Aniline-UL-^{14}C]-aclonifen 10 µM		[Phenoxy-UL-^{14}C]-aclonifen 10 µM	
		0 min	60 min	120 min	60 min	120 min
M52	0.79-0.80					
M77	0.88					

Metabolites present in at least two replicates at one time-point in one species with an overall mean of ≥5% are shown. Relative retention times calculated based on mean parent retention time at 0 min.

Generally, the two radiolabels behaved similarly across the three species.

While metabolite M9 was present at up to 10% in human hepatocytes, it appeared likely to be eventually transient and was present in small quantities (≤2%) in dog and rabbit hepatocytes. M36 was present in human cells at 5-15% and appeared also in rabbits (up to 2%), although not in dogs. M22 appeared in human cells at up to 12% at the higher concentration of 10 µM, but only transiently at approximately 1% at 1 µM; it was present at 4-7% in rabbits and dogs. The other detected human metabolites were only at generally low levels and appeared transient.

There were no human-specific metabolites in excess of 5% of total radioactivity.

Metabolic competence of the hepatocytes

All hepatocyte preparations displayed extensive depletion of [^{14}C]-testosterone, indicating their metabolic competence and suitability for use in this study.

Table 5.1-55: Testosterone metabolism in hepatocytes from rabbit, dog and human

Species	Assay	Time-point (min)	Mean % Testosterone Remaining ± SD	Mean % Testosterone Metabolism ± SD
Rabbit	1	0	94.8 ± 1.4	5.17 ± 1.4
		60	19.3 ± 0.2	80.7 ± 0.2
		120	2.73 ± 0.2	97.3 ± 0.2
Dog	1	0	98.4 ± 0.9	1.60 ± 0.9
		60	9.83 ± 2.2	90.2 ± 2.2
		120	2.20 ± 1.2	97.8 ± 1.2
	2	0	98.9 ± 0.1	1.07 ± 0.1
		60	19.7 ± 1.7	80.3 ± 1.7
		120	0.00 ± 0.0	100 ± 0.0
Human	1	0	97.1 ± 0.5	2.87 ± 0.5
		60	7.93 ± 7.6	92.1 ± 7.6
		120	6.33 ± 10.5	93.7 ± 10.5

III. CONCLUSION

Radioactive recoveries were acceptable overall, with 66.5 – 115% of the total amount of radioactivity recovered. Metabolism of both radiolabels was rapid and extensive in all three species, with complete depletion in rabbit and dog within 120 min of incubation. In human hepatocytes, metabolism was 65.6% to 100% complete after 120 min.

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In total, 100 metabolites were measured in dog, rabbit and human hepatocytes. Metabolites M6 and M7 were the main constituents observed in all three species at both concentrations and radiolabelled forms, with any species differences being of a quantitative rather than qualitative nature. No metabolite identification was carried out in the present study.

Compared to rabbit and dog, no metabolite with at least 5% of the total amount of radioactivity was found to be unique to human hepatocytes.

Assessment and conclusion by applicant:

A good, acceptable study that fulfils current understanding of requirements and produced valid results and conclusions.

Assessment and conclusion by RMS:

Data Point:	KCA 5.1.4/10
Report Author:	████
Report Year:	2016
Report Title:	[Phenoxy-UL-14C] Aclonifen: In vitro determination of binding to plasma proteins from mouse, rat and humans
Report No:	EnSa-16-0718
Document No:	M-569675-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 amended by the Commission Regulation (EU) No. 283/2013 (Europe) US EPA OCSPP not applicable
Deviations from current test guideline:	Not applicable (non-guideline study)
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

The binding of [phenoxy-UL-^{14}C] aclonifen to plasma proteins was determined for mouse, rat and human, using an ultrafiltration technique.

Pooled blank plasma for each species, from at least three different individuals, was spiked with [phenoxy-UL-^{14}C] aclonifen at two concentration levels (1 and 10 µM) and incubated at 37 ± 1°C for 15 min. The unbound [phenoxy-UL-^{14}C] aclonifen fraction was then obtained by ultrafiltration in Centrifree® devices. Sample analysis was performed by liquid scintillation counting of radioactivity.

The binding percentage of [phenoxy-UL-^{14}C] aclonifen 10 µM to plasma proteins was 99.82% in mice, 99.89% in rats, and 99.85% in humans, with evidently only negligible interspecies differences.

Owing to the high extent of binding to plasma proteins in all species, the radioactivity found in the ultrafiltrate fraction in samples corresponding to 1 µM [phenoxy-UL-^{14}C] aclonifen was detectable,

but was below the lower limit of quantitation. The binding at 1 µM [phenoxy-UL-^{14}C] aclonifen in all species is indicated to be very high (>99%), and values may be expected to be in the same range as those found at 10 µM.

I. MATERIALS AND METHODS

Study dates	Start:	05 August 2016
	Completion:	20 September 2016

A. MATERIALS

1. Test Item (labelled) [Phenoxy-UL-^{14}C]aclonifen

Batch No.:	KML 10079
Radiopurity:	≥99%
Specific activity:	6.79 MBq/mg

* = position of uniformly labelled ring

2. Test Item (unlabelled) Aclonifen

Batch No.:	AE F068300-PU-01
Purity:	99.6%
Appearance:	Yellow crystalline powder
Expiry:	02 April 2018

3. Test system (plasma) Pooled blank plasma from male CD-1 mice (batch MSE228790) and humans (batches BRH1101573, BRH1101574), from

Pooled blank plasma from Wistar rats (batch RM609XLJ5406), from Envigo RMS (UK) Ltd. Obtained from whole blood from at least 3 individuals; lithium heparin used as anticoagulant.

B. STUDY DESIGN AND METHODS

A stock solution of [phenoxy-UL-^{14}C] aclonifen at 18.5 MBq/ml (0.5 mCi/mL) was prepared in acetonitrile and stored in aliquots at -80 ± 10°C. Appropriately diluted working solutions of this were freshly prepared for each day of experimentation.

The radiochemical purity of [phenoxy-UL-^{14}C] aclonifen was assessed prior to initiation of the experiments for plasma protein binding. For this purpose, a working solution of [phenoxy-UL-^{14}C] aclonifen at 0.0167 MBq/ml (0.45 µCi/mL) was prepared. Duplicate 500 µL aliquots of this sample were analyzed the same day of preparation by HPLC-RAD.

To minimize binding to the filter membranes, solutions of 15 µM unlabeled aclonifen were filtered through the Centrifree® devices to be used, by centrifugation at room temperature. This filtered solution was discarded, and the sample reservoirs with the ultrafilters were left to dry at room

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temperature for 3 days. Similarly, to prevent non-specific binding to the ultrafiltrate recipients, the inner side of the recipients was rinsed with 10% (v/v) Triton X-100 solution and left to dry for 1 hour before use.

Plasma was centrifuged at room temperature after thawing, then aliquots of 995 µL were spiked with 5 µL of working solutions of [phenoxy-UL-^{14}C] aclonifen at 1 or 10 µM concentrations. The samples were incubated at 37 ± 1°C for 15 min in a Thermomixer device with agitation. After incubation, 100 µL aliquots were taken from each incubate to measure the initial radioactivity by LSC.

Incubated samples were transferred into the sample reservoir of the Centrifree® devices and filtration was accomplished by centrifugation at 1500 g for 10-15 minutes at 20 ± 5ºC. Aliquots of each ultrafiltrate (50 or 100 µL) were then used to measure the radioactivity of unbound [phenoxy-UL-^{14}C] aclonifen by LSC. Radioactivity was also measured in the plasma remaining in the reservoir of the Centrifree® device to assess total recovery.

Blank ultrafiltered plasma was obtained by filtration of whole blank plasma in Centrifree devices by centrifugation at 20 ± 5°C for 30 minutes at 1500 g. The blank ultrafiltered plasma was used to determine the background radioactivity in ultrafiltrate samples during LSC analysis.

Sample aliquots were placed in weighed 20 mL LSC vials. Afterwards, 10 mL of Ultima Gold scintillation cocktail were added to each vial. After shaking, the samples were maintained at room temperature and protected from light for 20 hours. For LSC analysis, the samples were placed in the β-counter and analyzed for 10 minutes (maximum counting time if 2% of counting error was not achieved). Three replicated aliquots of whole blank plasma and of blank ultrafiltered plasma were used to determine the background radioactivity. Blank samples were prepared for each species. Two-fold the mean background radioactivity of the three replicates was considered the lower limit of quantitation (LLOQ). For calculations, the mean background radioactivity in blank samples was subtracted from the radioactivity in test samples.

The percentage of [phenoxy-UL-^{14}C] aclonifen bound to plasma proteins was calculated as follows:

$$Bp(\%) = 100 - (fu\ x\ 100)$$

$$fu = \frac{Cu}{UBm\ x\ Ci}$$

$$UBm = \frac{\text{Amount of analyte in ultrafiltrate + Amount of analyte remaining in reservoir}}{\text{Initial amount of analyte}}$$

where:

Bp(%) = percentage bound
fu = fraction unbound (free fraction)
UBm = fraction unbound to the Centrifree® device
Cu = concentration in ultrafiltrate (dpm/mg)
Ci = initial plasma concentration before ultrafiltration (after 15 minutes of incubation at 37±1°C, dpm/mg).

II. RESULTS AND DISCUSSION

The radiochemical purity of [phenoxy-UL-^{14}C] aclonifen stock solution, prior to the protein binding assay, was 99.21%.

The binding percentage of [phenoxy-UL-^{14}C] aclonifen to plasma proteins for each species (mean values of three replicates) is shown in the following table.

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Table 5.1-56: Percentage binding of [phenoxy-UL-^{14}C] aclonifen to mouse, rat and human plasma proteins

[Phenoxy-UL-^{14}C] aclonifen concentration	Mouse (%)		Rat (%)		Human (%)	
	Mean	SD	Mean	SD	Mean	SD
10 µM	99.82	0.01	99.89	0.01	99.85	0.02
UBm (recovery range)	0.69 - 0.77		0.75 - 0.87		0.80 - 0.90	
1 µM	>99[a]	-	>99[a]	-	>99[a]	

a dpm in the ultrafiltrate were below the LLOQ
SD Standard Deviation

Owing to the very high extent of binding to plasma proteins, the radioactivity found in ultrafiltrate samples corresponding to the incubates with 1 µM [phenoxy-UL-^{14}C] aclonifen was detectable in all species, but was below the lower limit of quantitation. (The LLOQ for the 1 µM incubations corresponded to values of <0.01 for the fraction unbound). Calculation of the protein binding percentage using the dpm values without background subtraction gave values higher than 99%. Therefore, the results are expressed as >99% of binding and are expected to be in the same range as the binding percentages found at the 10 µM concentration.

Variations among the three species were negligible.

The fraction unbound to the ultrafilter membranes (UBm, *ie* the recovery) was in the range of 0.69 to 0.90 in the incubations with 10 µM [phenoxy-UL-^{14}C] aclonifen. As the radioactivity in ultrafiltrates from incubations with 1 µM concentrations were below the LLOQ, recovery from these could not be determined.

III. CONCLUSION

Plasma protein binding for [phenoxy-UL-^{14}C] aclonifen at a concentration of 10 µM was very high (99.8-99.9%) in mice, rats and humans, with negligible interspecies differences. Owing to this high extent of binding, the radioactivity in samples from the 1 µM concentrations, although detectable, was below the lower limit of quantitation, but may be expected to be in the same range as was found at 10 µM.

Assessment and conclusion by applicant:
A good, acceptable study that produced valid supplementary results and conclusions.

Assessment and conclusion by RMS:

CA 5.1.2 Absorption, distribution, metabolism and excretion by other routes

No studies by other routes.

CA 5.2 Acute toxicity

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The acute toxicity package of aclonifen comprises several studies already submitted and evaluated during the Annex I inclusion. However, as some of those studies were not run under GLP, the acute oral and dermal toxicity studies in the rat and the eye and skin irritation studies in the rabbit have been carried out following a request from non-European Authorities. Toxicological data generated in the new studies are in line with the previous one. Aclonifen was on very low acute oral (rat and mouse), dermal, and inhalation toxicity. Aclonifen is not irritating to the skin and eye. In skin sensitisation studies in the guinea pig, aclonifen was not a sensitiser in the Buehler test, but was a strong sensitiser in the Magnusson and Kligman assay.

Due to new data requirements a phototoxicity study is required if the molar extinction coefficient is higher than 1000 L x mol $^{-1}$ x cm $^{-1}$. This is the case for aclonifen so a phototoxicity study has been conducted and results showed no phototoxic potential.

Aclonifen – Summary of acute toxicity studies

Type of test	Species	Result	Reference	GLP	New study
Oral route	Rat (♀♂)	LD_{50} >5000 mg/kg	KCA 5.2.1/01 M-174876-01-1 1981	No	-
	Mouse (♀♂)	LD_{50} >5000 mg/kg	KCA 5.2.1/01 M-174876-01-1 ., 198	No	-
	Rat (♀)	LD_{50} >2000 mg/kg	KCA 5.2.1/02 M-465474-01-1 , 2013	Yes	New
Dermal route	Rat (♀♂)	LD_{50} >5000 mg/kg	KCA 5.2.2/01 M-174876-01-1 1981	No	-
	Rat (♀♂)	LD_{50} >2000 mg/kg	KCA 5.2.2/02 M-465477-01-1 , 2013	Yes	New
Inhalation (dust aerosol)	Rat (♀♂)	LC_{50} > 5.06 mg/L	KCA 5.2.3/01 M-174311-01-1 S.M. 1990	Yes	-
Skin irritation	Rabbit (♀♂)	Slight transient irritation (not classified)	KCA 5.2.1/01 M-174876-01-1 1981	No	-
	Rabbit (♂)	Not irritating	KCA 5.2.4/02 M-465334-01-1 , 2013	Yes	New
Eye irritation	Rabbit (♀♂)	Not irritant	KCA 5.2.5/01 M-174876-01-1 1981	No	-
	Rabbit (♂)	Not irritating	KCA 5.2.5/02 M-465328-01-1 , 2013	Yes	-
Sensitisation (Buehler 9 induction)	Guinea-pig (♀)	Not sensitising	KCA 5.2.6/01 M-174308-01-1 1990	Yes	-

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Sensitisation (Magnusson and Kligman)	Guinea-pig (♀♂)	Sensitiser Category 1A	KCA 5.2.6/02 M-212028-01-1 2002	Yes	-
Phototoxicity *in vitro*	BALB/c 3T3 cells	Not phototoxic	KCA 5.2.7/01 M-634103-01-1 2017	Yes	New

Classification

According to the classification criteria laid down in Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures, aclonifen is classified as a Category 1A for skin sensitisation and assigned the pictogram GHS05, the signal word « Danger » and the risk phrase H317: « May cause an allergic skin reaction ».

CA 5.2.1 Oral

In addition to the acute dermal toxicity study already available in the Monograph and Baseline Dossier a new acute oral toxicity study was conducted in 2013 in order to support a registration in Korea.

Data Point:	KCA 5.2.1/01
Report Author:	;
Report Year:	1981
Report Title:	KUB 3359 - Studies for acute toxicity in mice with oral, and in rats with oral, intraperitoneal and dermal treatment, as well as for primary skin and eye irritation in rabbits
Report No:	R007415
Document No:	M-174876-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 423, 2001 Dose volume for rats in acute oral study exceeded recommended levels. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In an acute oral toxicity limit test, groups of fasted Wistar-AF/HAN-EMD (SPF) rats and NMRI-EMD (SPF) mice, 5/sex/dose were given a single oral dose (gavage) of aclonifen (lot T5/81 – purity 96.2%) 5000 mg/kg bw in 0.5% aqueous carboxymethyl cellulose mucilage (CMC) vehicle and were observed for 14 days.

There were no mortalities. Clinical signs on the day of dosing or within three days after dosing included reduced activity, ataxia and piloerection. Yellow stained urine was seen in both species.

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In conclusion aclonifen was found to be of a low order of acute oral toxicity following exposure in both rats and mice.

In rats Oral LD_{50} > 5000 mg/kg bw in both sexes
In mice Oral LD_{50} > 5000 mg/kg bw in both sexes

There were some limitations to the study as it does not strictly follow OECD test guidelines, is not to GLP.

Table 5.2.1-1 Aclonifen – acute oral toxicity study in rats and mice – summary of findings

Dose (mg/kg bw)	Males	Females	Combined
		Rats	
5000	5/5/0	5/5/0	10/10/0
		Mice	
5000	5/5/0	5/5/0	10/10/0

Number of animals dosed/clinical signs/mortality

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description: Aclonifen (KUB 3359)

Lot/Batch: Lot T5/81

Purity: 96.2%

Appearance: Not mentioned

Stability of test compound: Not mentioned

2. Vehicle and /or positive control Vehicle: 0.5% aqueous carboxymethyl cellulose mucilage (CMC)

3. Test animals

Species and strain: Rat – Wistar-AF/HAN-EMD (SPF). Mouse – NMRI-EM (SPF)

Source: Not mentioned

Number of animals: 5 per sex and species

Sex: Male and female

Age: Not mentioned

Weight at treatment: Rat – 163g (range 153-186g). Mouse 24g (range 19-28g)

Acclimation period: Not mentioned

Diet: Altromin Standard, Diet TPF(R) N 1324, Lot 1442 (6 July, 1981)

Water: Not mentioned, ad libitum access

Cage type: Makrolon cages type III

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Housing: Grouping per sex; [redacted]

Environmental conditions: Temperature: 23-28°C
Humidity: 43-56 %
Air changes: Not mentioned
Photoperiod: Not mentioned

B. STUDY DESIGN AND METHODS

1. In life dates

26 August to 15 September 1981

2. Animal assignment and treatment

Aclonifen was tested for acute oral toxicity by dosing 5 male and 5 female rats and mice with a single oral dose of 5000 mg/kg bw in 0.5% aqueous CMC (20 g to 100 mL in rats, 20 g to 100 mL in mice). The volume of administration was 2.5 mL / 100 g bw in rats and 2.0 mL / 100 g bw in mice.

For clinical examinations, the behaviour and general condition all rats were monitored in the 5 to 6 hours after treatment and then checked daily during the follow-up period after dosing. All animals were weighed before treatment and on days 1, 3, 5, 7, 10, 12 and 14 after treatment.

Pathologico-anatomical examinations were carried out on all animals which died during the study. Animals that survived to the end of the study were not killed.

II. RESULTS AND DISCUSSION

1. Mortality

No deaths occurred.

2. Clinical observations

In both species clinical signs commenced 1 to 2 hours after dosing, and consisted of reduced spontaneous activity, ataxia, piloerection and yellow urine. Clinical signs completely disappeared 24 hours after treatment in rats, and 4 days after treatment in mice. Yellow staining of the urine was noted in both species and persisted up to 6 days in rats and 4 days in mice. The clinical signs are presented in the table below.

Table 5.1.1- 1: Aclonifen – acute oral toxicity study in rats and mice – clinical signs

Time after dosing		0 – 1 hour	2-6 hr	6-24 hr	Day 2 -3	Day 4-5	Day 6-14
Rats							
Reduced activity		0	10	0	0	0	0

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Ataxia		0	1	0	0	0	0
Piloerection		0	0	0	0	0	0
Yellow urine		0	10	10	10	10	0
Mice							
Reduced activity		10	10	0	0	0	0
Ataxia		0	5	0	0	0	0
Piloerection		0	0	5	2	0	0
Yellow urine		0	10	10	0	0	0

Number of animals with clinical signs (out of 10 animals 5 m / 5 f)

3. Body weight

Rats displayed a slight inhibition of body weight development up to the third day of the trial. There was no effect on body weight in mice.

4. Necropsy

Gross necropsy did not reveal any treatment-related changes.

III. CONCLUSION

Under these test conditions the oral LD50 of aclonifen in rats and mice was >5000 mg/kg/bw. Aclonifen is concluded to be of a low order of acute oral toxicity in both species.

Assessment and conclusion by applicant:

This study was not conducted to GLP, and it does not follow OECD test guidelines.

This study was deemed to be acceptable in the DAR (2006), however a new study has since been conducted (see document M-465474-01-1 below) which addresses the deficiencies in the current study.

Under these test conditions the oral LD50 of aclonifen in rats and mice was >5000 mg/kg/bw. Aclonifen is concluded to be of a low order of acute oral toxicity in both species.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.2.1/02
Report Author:	████
Report Year:	2013
Report Title:	Aclonifen technical (AE F068300) - Acute oral toxicity study in rats
Report No:	13/275-001P
Document No:	M-465474-01-1
Guideline(s) followed in study:	OECD 423 (2001); EEC Directive 440/2008, B.1.tris (2008); US-EPA 712-C-98-190, OPPTS 870.1100 (1998)
Deviations from current test guideline:	Current guideline: OECD 423, 2001 No deviations
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In an acute oral toxicity test, following the acute toxic class method, 3 fasted female Wistar rats, were given a single oral dose (gavage) of aclonifen (batch PEA1000235, purity 99.5%) 2000 mg/kg bw in 0.5% aqueous methylcellulose 400 vehicle and were observed for 14 days. The test item did not cause mortality in the first group; therefore a confirmatory group of 3 female rats was treated at the same dose level. The test item did not cause mortality in the confirmatory group

Yellow stained urine was seen in all animals. There were no clinical signs of toxicity, no treatment-related effects on body weight, and no gross pathological findings at necropsy.

In this well-conducted study aclonifen was found to be of a low order of acute oral toxicity in female rats.

In female rats Oral LD_{50} > 2000 mg/kg bw

The study follows current OECD test guidelines, was conducted to GLP with a high purity batch of the test item.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen technical (AE F068300)
Lot/Batch:	PEA1000235
Purity:	99.5% w/w
Appearance:	Yellow powder
Stability of test compound:	Not mentioned

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2. Vehicle and /or positive control Vehicle: 0.5% of methylcellulose 400

3. Test animals

Species and strain:	Rat – RccHan:WIST
Source:	
Number of animals:	6 animals, 3 per group
Sex:	Female
Age:	8 weeks
Weight at treatment:	152 – 170 g
Acclimation period:	At least 5 days
Diet:	Ssniff® SM R/M "Autoclavable complete diet for rats and mice breeding and maintenance" *ad libitum*. Batch 247 9652.
Water:	Water quality analysed every three months, microbial content checked monthly
Cage type:	Makrolon cages type III
Housing:	3 per cage
Environmental conditions:	Temperature: 22 ± 3 °C Humidity: 30 - 70 % Air changes: 15 – 20 per hour Photoperiod: 12 hours daily

B. STUDY DESIGN AND METHODS

1. In life dates

04 to 19 September 2013

2. Animal assignment and treatment

Based on preliminary toxicological information, 2000 mg/kg bw was selected to be the starting dose. Following an overnight fast, 3 female rats (Group 1) were administered a single oral dose of 2000 mg/kg bw aclonifen in 0.5% aqueous CMC (200 mg/mL, dose volume of 10 mL/kg bw). The test item did not cause mortality in this group; therefore a confirmatory group (Group 2) was treated at the same dose level. The test item did not cause mortality in the confirmatory group, so no further testing was required

Animals were monitored for 6 hours after treatment and then checked daily during the 14-day follow-up period after dosing. Body weight was recorded on the day before treatment, on the day of dosing, and weekly thereafter.

For clinical examinations, the behaviour and general condition of all rats were monitored in the 6 hours after treatment and then checked daily.

For pathologico-anatomical examinations, all animals which died during or were sacrificed at the end of the trial were subjected to a necropsy and evaluated. Surviving animals were sacrificed by

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exsanguination under pentobarbital anaesthesia. After examination of the external appearance, the cranial, thoracic and the abdominal cavities were opened and the organs and the tissues were observed. Macroscopic abnormalities were recorded.

II. RESULTS AND DISCUSSION

1. Mortality

No deaths occurred.

2. Clinical observations

Yellow staining of the urine was noted in all animals which was coloured by the test item.

3. Body weight

There were no treatment-related effects on body weight or body weight gain.

4. Necropsy

Gross necropsy did not reveal any treatment-related changes.

III. CONCLUSION

Under these test conditions the oral LD50 of aclonifen in female rats was >2000 mg/kg/bw. Aclonifen is concluded to be of a low order of acute oral toxicity in the rat.

The study result triggers the following classification/labelling:

- Regulation (EC) No 1272/2008 (CLP): none

- GHS (rev. 4) 2011: CAT. 5 or "Unclassified"

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Assessment and conclusion by applicant:

An acute oral toxicity study was conducted in 1981, but had deficiencies of not being conducted to GLP, not strictly according to OECD test guidelines. Nevertheless the study was considered to be acceptable during the evaluation of the 2006 DAR (see M-174876-01-1, above).

This new study was conducted at the request of the Korean authorities and is a well-conducted study, with a high purity of test substance, conducted to GLP and in accordance with current OECD test guidelines.

Under these test conditions the oral LD50 of aclonifen in female rats was >2000 mg/kg/bw. Aclonifen is concluded to be of a low order of acute oral toxicity in the rat.

Assessment and conclusion by RMS:

CA 5.2.2 Dermal

In addition to the acute dermal toxicity study already available in the Monograph and Baseline Dossier a new acute dermal toxicity study was conducted in 2013 in order to support a registration in Korea.

Data Point:	KCA 5.2.2/01
Report Author:	[redacted]; [redacted]
Report Year:	1981
Report Title:	KUB 3359 - Studies for acute toxicity in mice with oral, and in rats with oral, intraperitoneal and dermal treatment, as well as for primary skin and eye irritation in rabbits
Report No:	R007415
Document No:	M-174876-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 423, 2001 Dose volume for rats in acute oral study exceeded recommended levels. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In an acute dermal toxicity test, groups of Wistar-AF/HAN-EMD (SPF) rats 5/sex/dose were given a single dermal dose of aclonifen (lot T5/81 – purity 96.2%) 5000 mg/kg bw, moistened with water, and applied to shaven unbroken skin in an area of 6 x 6 cm. The skin was covered in tin foil kept in place by a rubber sleeve. The test item was removed by washing 24 hours after dose administration and the animals were observed for 14 days.

There were no mortalities and no clinical signs of toxicity. Yellow stained urine was seen in both sexes, and a compound-related local yellow discolouration of the skin could be observed.

In conclusion aclonifen was found to be of a low order of acute dermal toxicity following exposure in rats.

In rats Dermal LD_{50} > 5000 mg/kg bw in both sexes

There were some limitations to the study as it does not strictly follow OECD test guidelines, is not to GLP, does not specify the purity of the test substance.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen (KUB 3359)
Lot/Batch:	Lot T5/81
Purity:	96.2%
Appearance:	Not mentioned
Stability of test compound:	The test item was administered as a single dose without using vehicle.

2. Vehicle and /or positive control Moistened with water

3. Test animals

Species and strain:	Rat – Wistar-AF/HAN-EMD (SPF)
Source:	Not mentioned
Number of animals:	5 per sex
Sex:	Male and female
Age:	Not mentioned
Weight at treatment:	180 (range 165-197) g
Acclimation period:	Not mentioned
Diet:	Altromin Standard, Diet TPF(R) N 1324, Lot 1442 (6 July, 1981)
Water:	Not mentioned, ad libitum access
Cage type:	Makrolon cages type III
Housing:	Individually,
Environmental conditions:	Temperature: 23-28°C Humidity: 43-56 % Air changes: Not mentioned Photoperiod: Not mentioned

B. STUDY DESIGN AND METHODS

1. **In life dates**

26 August to 15 September 1981

2. **Animal assignment and treatment**

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Aclonifen (KUB 3359) was tested for acute dermal toxicity by dosing 5 male and 5 female Wistar-AF/HAN-EMD rats with a single dermal dose of 5000 mg/kg (moistened).

The backs and abdomens of the rats designed for dermal treated were shaven with an electric hair clipper approximately one hour before treatment. The test material was moistened and applied to the shaven, unscarified skin (area of 6 x 6 cm). The time of exposure was 24 hours. During this time the treated part of skin was covered by tin foil which was kept in place and sealed by a rubber sleeve (modified method of ██████████, 1969). Then, the rubber sleeve and the tin foil were removed and the remaining material, if there was any left, washed off carefully with water.

For clinical examinations, the behaviour and general condition of all rats were monitored in the 5 to 6 hours after treatment and then checked daily during the 14-day follow-up period after dosing. All animals were weighed before treatment and on days 1, 3, 5, 7, 10, 12 and 14 after treatment. The local changes in the area of application were assessed according to the scale of ████ (█████ *1959*).

For pathologico-anatomical examinations, all animals which died during or were sacrificed at the end of the trial were subjected to a necropsy and evaluated.

II. RESULTS AND DISCUSSION

1. Mortality

No deaths occurred.

2. Clinical observations

General motility was reduced during the day of treatment, but this was considered to be due to the bandage with the rubber sleeve. The only clinical signs of reaction to the treatment were yellow urine and yellow skin which persisted up to days 7 and 14, respectively.

3. Body weight

The animals displayed an inhibition of body weight development on the first day of treatment, but this was considered to be bound to the fixation of the rubber sleeve. Body weight development was normal after removal of the rubber sleeve.

4. Necropsy

Gross necropsy did not reveal any treatment-related changes.

III. CONCLUSION

Under these test conditions the dermal LD50 of aclonifen in male and female rats was > 5000 mg/kg/bw. Aclonifen is concluded to be of a low order of acute dermal toxicity in the rat.

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Assessment and conclusion by applicant:

This study was not conducted to GLP, and it does not follow OECD test guidelines.

This study was deemed to be acceptable in the DAR (2006), however a new study has since been conducted (see document M-465477-01-1 below) which addresses the deficiencies in the current study.

Under these test conditions the dermal LD50 of aclonifen in male and female rats was > 5000 mg/kg/bw. Aclonifen is concluded to be of a low order of acute dermal toxicity in the rat.

Assessment and conclusion by RMS:

Data Point:	KCA 5.2.2/02
Report Author:	[redacted]
Report Year:	2013
Report Title:	Aclonifen technical (AE F068300) - Acute dermal toxicity study in rats
Report No:	13/275-002P
Document No:	M-465477-01-1
Guideline(s) followed in study:	OECD 402 (1987); US-EPA OPPTS 870.1200 (1998); EC 440/2008 (2008)
Deviations from current test guideline:	Current guideline: OECD 402 (2017) Animals were not administered the dose in a sequential manner and were housed individually throughout the study. These deviations are less desirable for animal welfare reasons but do not affect the validity of the test.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In an acute dermal toxicity test, groups of RccHan:WIST (SPF) rats 5/sex were given a single dermal dose of aclonifen (batch PEA1000235 – purity 99.5%) 2000 mg/kg bw, moistened with water, and applied to shaven unbroken skin in an area of 5 x 5 cm. The skin was covered in gauze kept in place by adhesive hypoallergenic plaster and wrapped in semi occlusive plaster. The test item was removed by washing 24 hours after dose administration and the animals were observed for 14 days.

There were no mortalities and no clinical signs of toxicity. Yellow stained discolouration of the skin could be observed on all animals up to 3 days after dosing.

In conclusion, in this well conducted study, aclonifen was found to be of a low order of acute dermal toxicity following exposure in both rats.

In rats Dermal LD_{50} > 2000 mg/kg bw in both sexes

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The study largely follows current OECD test guidelines, was conducted to GLP with a high purity batch of the test item.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen technical (AE F068300)
Lot/Batch:	AE F068300-01-14; origin PEA1000235
Purity:	99.5 %
Appearance:	Yellow powder
Stability of test compound:	The test item was administered as a single dose without using vehicle.

2. Vehicle and /or positive control Not applicable

3. Test animals

Species and strain:	RccHan:WIST rats
Source:	[redacted]
Number of animals:	5 animals per sex
Sex:	Male - Female, nulliparous and non-pregnant
Age:	Young healthy adult rats
Weight at treatment:	Mean: 216-246 g
Acclimation period:	7 days
Diet:	ssniff® SM R/M autoclavable complete diet for rats and mice: breeding and maintenance ([redacted] Germany); *ad libitum*
Water:	tap water, *ad libitum*
Cage type:	Type II polypropylene/polycarbonate; Lignocel Bedding for Laboratory Animals
Housing:	Individual caging
Environmental conditions:	Temperature: 22±3 °C Humidity: 30-70 % Air changes: 15-20 exchanges per hour Photoperiod: 12 hours daily, from 6.00 am to 6.00 pm

B. STUDY DESIGN AND METHODS

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1. In life dates

28 August to 18 September 2013

2. Animal assignment and treatment

Justification of the dose:

The dose-level was selected based on the previous non-GLP study, in which the dermal LD50 of the test item was higher than 5000 mg/kg (M-174876-01-1, █████, █████; 1981). Therefore, a limit test was carried out by administering 2000 mg/kg to one group of ten animals (five males and five females).

Procedures:
The backs of the animals were shorn (approximately 10% area of the total body surface) approximately 24 hours prior to the treatment. Only those animals without injury or irritation on the skin were used in the test. On test day 0, the test item was applied as supplied at a single dose of 2000 mg/kg bw, and applied uniformly over the skin by use of a gauze pad (*ca.* 5 cm x 5 cm) on the skin throughout a 24- hour exposure period. The test item was moistened with water to ensure good contact with the skin. Sterile gauze pads were placed on the skin of rats at the site of application. These gauze pads were kept in contact with the skin by a patch with adhesive hypoallergenic plaster. The entire trunk of the animal was then wrapped with semi occlusive plastic wrap for 24 hours. At the end of the exposure period, residual test item was removed, using body temperature water.

Animals were weighed on Day 0 (beginning of the experiment) and on days 7 and 14. Clinical observations were performed on the day of treatment, at 1 and 5 hours after the application of the test item, and once each day for 14 days thereafter. Observations included the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, and somatomotor activity and behaviour pattern. Particular attention was directed to the observation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

All animals were subjected to gross macroscopic examination after a deep anaesthesia (RELEASE 300 mg/ml inj. A.U.V) and exsanguination. After examination of the external appearance, the cranial, thoracic and abdominal cavities were opened and the appearance of the tissues and organs were observed.

II. RESULTS AND DISCUSSION

1. Mortality

Aclonifen technical (AE F068300) did not caused mortality at a dose level of 2000 mg/kg bw.

The dermal LD50:
- for males was > 2000 mg/kg bw
- for females was > 2000 mg/kg bw

2. General observation/clinical signs

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There were no clinical signs noted in any animals throughout the study.

No local dermal signs were observed after treatment with the test item during the 14 day observation period. However, yellow staining was observed on the skin in all animals after dosing from Day 1 up to Day 3.

3. Body weight

Body weight and body weight gain of Aclonifen technical (AE F068300) treated animals showed no indication of a treatment-related effect.

4. Gross pathology / Organ weights / Histopathology

There was no evidence of any observations at a dose level of 2000 mg/kg bw at necropsy.

III. CONCLUSION

The median lethal dose of Aclonifen technical (AE F068300) after a single dermal administration was found to be greater than 2000 mg/kg bw in male and female RccHan:WIST rats.

The study result triggers the following classification/labelling:

- Regulation (EC) No 1272/2008 (CLP): none

<u>**Assessment and conclusion by applicant:**</u>

An acute dermal toxicity study was conducted in 1981, but had deficiencies of not being conducted to GLP, not strictly according to OECD test guidelines, and no information on the purity of the batch tested. Nevertheless the study was considered to be acceptable during the evaluation of the 2006 DAR (see M-174876-01-1, above).

This new study was conducted at the request of the Korean authorities and is a well-conducted study, with a high purity of test substance, conducted to GLP and in accordance with current OECD test guidelines.

<u>**Assessment and conclusion by RMS:**</u>

CA 5.2.3 Inhalation

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Data Point:	KCA 5.2.3/01
Report Author:	[redacted]
Report Year:	1990
Report Title:	Aclonifen (CME 127): Acute inhalation toxicity study four-hour exposure (nose only) in the rat
Report No:	R007148
Document No:	M-174311-01-1
Guideline(s) followed in study:	EU (=EEC): 84/449/EEC/B2; OECD: 403
Deviations from current test guideline:	Current Guideline: OECD 403 (2009) The MMAD should be 1-4 µm with GSD of 1.5-3. In this study the MMAD was 5.2 µm with GSD of 0.43. However 37.8% of particles were < 4 µm (within the respirable range). As the test is at a very high dose the % of the dose that was respirable is high enough to adequately investigate the acute inhalation toxicity. Overall study is acceptable.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In an acute inhalation toxicity study, groups of Sprague Dawley rats (5/sex), were exposed by the inhalation route (nose only) to aclonifen (91.3 % purity) in air for 4 hours to nose only at a concentration of 5.06 mg/L. Animals were observed for the following 14 days.

Inhalation LC50 males = > 5.06 mg/L
females = > 5.06 mg/L
combined = > 5.06 mg/L

Aclonifen was found to be of a low order of acute toxicity following exposure of rats *via* the inhalation route.
Clinical signs during exposure were wet fur and decreased respiratory rate. After exposure hunched posture, pilo-erection and yellow staining around the head and shoulders were observed, and a few animals also had red/brown staining around the snout. All signs regressed by the following day with the exception of one animal where signs persisted until day three.

From post-exposure days 3 to 14, all animals appeared normal. All animals showed normal bodyweight development. One female lost weight slightly in the second week but this was not considered abnormal for rats of this strain and age.

The only gross necropsy abnormality observed was the occurrence in one female of a slight general redness to the lungs with multiple dark foci approximately 1 mm in diameter on all lobes. The isolated incidence of this findings suggests it may not be related to treatment.

On the basis of this study, aclonifen does not warrant classification as being harmful or toxic by the inhalation route.

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The study was conducted to GLP and largely follows OECD test guidelines. A deficiency in the study is that the MMAD slightly exceeded test guideline requirements, however 37.8% of the particles were within the respirable range.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description: Aclonifen (CME127)

Lot/Batch: DA 618

Purity: 91.3%

Appearance: Yellow powder

Stability of test compound: not determined

2. Vehicle and /or positive control Not applicable

3. Test animals

Species and strain: Rat - Sprague-Dawley

Source: [redacted]

Number of animals: 5 animals per sex

Sex: Male - Female

Age: eight to ten weeks old

Weight at treatment: males: 219 - 241 g; females: 196 - 218 g

Acclimation period: at least five days

Diet: Rat and Mouse Expanded Diet No. 1, [redacted]); *ad libitum*

Water: *ad libitum*

Cage type: Polypropylene cages

Housing: Groups of 5 rats per cage / sex

Environmental conditions: Temperature: 19-24°C

Humidity: 46-64 %

Air changes: approximately 15 changes per hour

Photoperiod: 12 hours light and 12 hours darkness

B. STUDY DESIGN AND METHODS

1. In life dates

Date of exposure: 28 August 1990; Date completed: 11 September 1990.

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2. Animal assignment and treatment

Five male and five female rats were exposed to aclonifen for four hours, using a nose-only exposure system, to a dust atmosphere containing aclonifen.

The animals were observed for clinical signs at hourly intervals during the exposure period, one hour after termination of the exposure and once daily for a period of 14 days. Body weight was recorded on the day of exposure, on days 7 and 14 after exposure.

At the end of the 14 day observation period, animals were sacrificed by sodium phenobarbitone, and necropsied and examined for gross pathological changes. The respiratory tract was subjected to a detailed macroscopic examination for signs of irritancy or local toxicity.

3. Generation of the test atmosphere / chamber description

The dust atmosphere was generated by a Wright Dust feed mechanism.

The test atmosphere was sampled at least every 15 minutes using the gravimetric method to estimate the chamber concentration of aclonifen. A mean atmosphere concentration of 5.06 mg/l aclonifen (CME 127) with a range between 4.44 and 5.63 mg/L.

The oxygen levels, relative humidity and temperature inside the chamber was measured every 30 minutes throughout the exposure period.

The particle size distribution was determined twice using a Cascade Impactor.

The main exposure parameters were as follows:

Table 5.2.3- 1: Aclonifen - Acute inhalation toxicity study in rats – Exposure parameters

Parameters	Value
Flow rate (L/min)	14
Actual concentration (mg/L)	5.06 ± 0.38
Particle size (µm) MAAD[1] / GSD[2]	5.2 / 0.43
Particles < 10 µm (% w/w)	78.0
Particles < 7 µm (% w/w)	63.7
Particles < 4 µm (% w/w)	37.8

[1] Mass median aerodynamic diameter

[2] Geometric standard deviation

Temperature in the chamber was 23 ± 1° C, relative humidity was 42 ± 6%, and oxygen exceeded 20 % which are in accordance with the 2009 test guideline requirements.

II. RESULTS AND DISCUSSION

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1. Mortality

During the 14-day observation period there were no deaths and only minor signs of reaction to treatment were observed.

Table 5.2.3- 2: Aclonifen - Acute inhalation toxicity study in rats – Mortality

Males			Females		
Dose (mg/L)	**Mortality**	**Time of death**	**Dose** (mg/L)	**Mortality**	**Time of death**
5.06	0/5	-	5.06	0/5	-

2. Clinical observations

During the exposure period, wet fur and decreased respiratory rate were noted. On removal from the exposure chamber additional signs of hunched posture, pilo-erection and yellow staining around the head and shoulders were seen. These signs were still evident one hour after exposure, with the addition of a few signs of red/brown staining of the snout, but all signs had regressed by the following day in all but one animal which appeared normal on day three. All animals appeared normal for the remainder of the study.

3. Body weight

There was no treatment-related effect on body weight. All animals showed normal bodyweight development during the study. One female (animal number 7) appeared to lose weight slightly during the second week but the reduction was only by 3g (1%), this was not considered abnormal for rats of this strain and age.

4. Necropsy

At necropsy only one animal, a female (animal number 10), showed any gross abnormalities. The abnormalities observed were a slight general redness to the lungs and multiple dark foci on all lobes; however the isolated incidence of these findings suggests that they may not be related to treatment.

III. CONCLUSION

In this well conducted GLP study, acute inhalation LC50 of aclonifen in the Sprague-Dawley rats was greater than 5.06 mg/L (4-hour nose only exposure). A deficiency in the study is that the MMAD slightly exceeded the levels recommended in OECD 403 (2009).

The study result triggers the following classification/labelling:
- EU directive 1999/45/EC: none
- Regulation (EC) No 1272/2008 (CLP): none

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Assessment and conclusion by applicant:

The OECD guideline 403 (2009) supplemented by OECD guidance document No. 39 (2009) stipulates a desirable MMAD of ≤4 µm with a GSD of 1-3). In this study the particle size MMAD was 5.2 µm with GSD of 0.43. 37.8% of particles were below 4 µm (i.e. in the respirable range) so although the MMAD is larger than is stipulated, because a limit dose of 5.06 mg/L test atmosphere was achieved, the fact that only 37.8% was respirable still means that a fairly high dose was still respirable. Therefore despite this deficiency the test is considered to be sufficient to investigate the acute toxicity of aclonifen by the inhalation route. This study was deemed to be acceptable in the DAR (2006).

LC50 of aclonifen in male and female Sprague-Dawley rats was > 5.06 mg/L (4-hour nose only exposure).

Assessment and conclusion by RMS:

CA 5.2.4 Skin irritation

In addition to the skin irritation toxicity study already available in the Monograph and Baseline Dossier a new study was conducted in 2013 in order to support a registration in Korea.

Data Point:	KCA 5.2.4/01
Report Author:	[redacted]; [redacted]
Report Year:	1981
Report Title:	KUB 3359 - Studies for acute toxicity in mice with oral, and in rats with oral, intraperitoneal and dermal treatment, as well as for primary skin and eye irritation in rabbits
Report No:	R007415
Document No:	M-174876-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 423, 2001 Dose volume for rats in acute oral study exceeded recommended levels. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

In a primary dermal irritation study, 6 New Zealand rabbits, 3 males and 3 females, were exposed *via* the dermal route to 0.5 g of aclonifen (purity not stated) per animal. The test material was applied as a powder moistened with water for 4 hours to 8 cm^2 of the body surface area of each test animals. Animals then were observed for 14 days. Irritation was scored using the Draize scheme.

Slight erythema was noted on days 2 and 3 after treatment. This had resolved by day 7. No other effect was seen. In this study, aclonifen was not a dermal irritant. On the basis of this study, aclonifen does not warrant classification as being irritating to the skin.

There were some limitations to the study as it does not strictly follow OECD test guidelines, is not to GLP.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen (KUB 3359)
Lot/Batch:	Lot T5/81
Purity:	96.2%
Appearance:	Not mentioned
Stability of test compound:	Not mentioned

2. Vehicle and /or positive control	Not applicable

3. Test animals

Species and strain:	Rabbit – White New Zealand
Source:	Not mentioned
Number of animals:	3 per sex
Sex:	Male and female
Age:	Not mentioned
Weight at treatment:	3.46 (range 3.23-3.78) kg
Acclimation period:	Not mentioned
Diet:	Altromin Brand-Purina (R) sole feed for rabbits, manufactured by [redacted] *ad libitum*
Water:	*ad libitum* access
Cage type:	type KK 017 on metal grids (350 x 600 x 390 mm)
Housing:	Individually; [redacted].
Environmental conditions:	Temperature: 18-21°C

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Humidity: 49-84 %

Air changes: Not mentioned

Photoperiod: Not mentioned

B. STUDY DESIGN AND METHODS

1. In life dates

2 to 16 September 1981

2. Animal assignment and treatment

Aclonifen (500 mg) was moistened and applied for 24 hours to one intact and one abraded shaved area (4 cm^2 patch) on the back of each of 6 rabbits. During exposure the areas of application were wrapped with an occlusive dressing consisting of polyethylene foil which was kept in place by a leather sleeve. At the end of exposure the dressing was removed and the remaining test material was wiped off.

The rabbits were observed daily for clinical signs and mortality. They were weighed before the beginning of the experiment and after 4, 7, 10 and 14 days of dosing. Skin responses were evaluated 1, 2, 3, 7, 10 and 14 days after the end of the exposure period according to the technique of Draize. Gross. Pathologico-anatomical examinations were to be performed only in those cases where animals died in the course of the study. The surviving animals were not killed and subjected to a necropsy.

II. RESULTS AND DISCUSSION

1. Mortality

No deaths occurred.

2. Clinical observations

A single application of 500 mg of aclonifen to the dorsal skin of rabbits produced only slight irritation after a 24 hour period. A yellow discolouration of the skin was observed as well as slight erythema and on day 3 scale formation was observed. This effect was reversible and disappeared after 3 days. The mean value of scores for erythema and oedema were less than 2.3, i.e. below the trigger value for classification. A deficiency in this study is that only mean scores for each time point are given but no data on the scores for individual animals. However given that the mean scores are low, the individual values are not likely to affect the overall classification of the test substance.

Table 5.4.1- 1: Aclonifen - Primary dermal irritation in rabbit – mean findings

	Intact skin	**Scarified skin**
Local finding	Group mean scores	Group mean scores
Erythema*		
1 day	0.0	0.0
2 day	0.3	0.5
3 days	0.5	0.7
7 days	0.0	0.0

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Mean scores (days 1,2,3)	0.3	0.4
Criteria for classification ≥ 2.3		
Edema*		
1 day	0.0	0.0
2 day	0.0	0.0
3 days	0.0	0.0
7 days	0.0	0.0
Mean scores (days 1,2,3)	**0**	**0**
Criteria for classification ≥ 2.3		
* = Score for erythema and oedema: 0 = no irritation; 1 = questionable; 2 = slight; 3 = pronounced; 4 = severe		

3. Body weight

The body weight development was not different from that of untreated rabbits of the same age.

4. Necropsy

All animals survived the 14-day observation period. Animals were not killed for necropsy.

III. CONCLUSION

Under the conditions of this test, aclonifen was considered as a negligible irritant to rabbit skin.

The study result triggers the following classification/labelling:

- Regulation (EC) No 1272/2008 (CLP): none

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Assessment and conclusion by applicant:

This study was not conducted to GLP, and it does not follow OECD test guidelines.

This study was deemed to be acceptable in the DAR (2006), however a new study has since been conducted (see document M-465334-01-1 below) which addresses the deficiencies in the current study.

Under the conditions of this test, aclonifen was not a skin irritant in the rabbit.

Assessment and conclusion by RMS:

Data Point:	KCA 5.2.4/02
Report Author:	[redacted]
Report Year:	2013
Report Title:	Aclonifen technical (AE F068300) - Acute skin irritation study in rabbits
Report No:	13/275-006N
Document No:	M-465334-01-1
Guideline(s) followed in study:	OECD 404 (2002); US-EPA OPPTS 870.2500 (1998); EC No 440/2008, B.4 (2008)
Deviations from current test guideline:	Current guideline: OECD 404, 2015 An in vitro skin irritation study should have been conducted in accordance with the Integrated Approaches to Testing and Assessment for skin irritation/corrosion (Current guideline: OECD 2014). The test item was applied to an area of 10 x 10 cm, which is larger than the area required in the test guideline (6 cm2). These deviations do not affect the reliability of the study.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

In a primary skin irritation study, 3 young adult male New Zealand White Rabbits, were exposed *via* the dermal route to 0.5g of Aclonifen technical (AE F068300, purity 99.5%). The test item was moistened with water and applied on the skin of rabbits for 4 hours to an area of 10 x 10 cm. Animals were observed for 3 days. Irritation was scored according to the Draize scheme.

In this well-conducted study, aclonifen technical was not irritating to the skin according to the Draize classification system.

The study follows current OECD test guidelines, was conducted to GLP with a high purity batch of the test item.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description: Aclonifen technical (AE F068300)

Lot/Batch: AE F068300-01-14; PEA1000235

Purity:	99.5 %
Appearance:	Yellow powder
Stability of test compound:	The test item was administrated in its original form

2. Vehicle and /or positive control Vehicle: not applicable

3. Test animals

Species and strain:	New Zealand White Rabbit
Source:	
Number of animals:	3 animals
Sex:	Male
Age:	13 weeks
Weight at treatment:	3220 - 3259 g
Acclimation period:	Not specified
Diet:	Uni diet for rabbits ( ); *ad libitum*
Water:	tap water, *ad libitum*
Cage type:	AAALAC open wire structure, metallic cages for rabbit
Housing:	Individual caging; cages are placed together to allow social interaction with rabbit(s) in adjoining cages
Environmental conditions:	Temperature: 20±3 °C Humidity: 41-86 % Air changes: 15-20 exchanges per hour Photoperiod: 12 hours daily, from 6.00 am to 6.00 pm

B. STUDY DESIGN AND METHODS

1. In life dates

14 August to 6 September 2013

2. Animal assignment and treatment

The following considerations were taken before starting the study:

- According to OECD Guideline 404, a test item does not need to be tested if the pH-value is less than 2 or greater than 11.5, owing to its predictable corrosive properties. The pH of the test item was measured before the study initiation date and was found to be 5.0.
- No sign of corrosivity/irritation to rabbit skin was observed in the previous *in vivo* non-GLP toxicity study after a single skin application of 500 mg aclonifen (M-174876-01-1, ; ; 1981).

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Approximately 24 hours prior to dosing the hair was clipped from the back and flanks of young adult New Zealand White rabbit with an electric clipper.

On the day of treatment, 0.5 g of Aclonifen technical (AE F068300) was placed on a surgical gauze pad (*ca*. 10 cm x 10 cm) and sufficient water was added to dampen the material to ensure good contact with the skin. This gauze pad was applied to the clipped area of skin and was kept in contact with the skin by a patch with a surrounding adhesive hypoallergenic plaster. The entire trunk of the animals was then wrapped with plastic wrap held in place with an elastic stocking. After 4 hours the dressing was removed and the skin was washed with tap water.

Initially, a single animal was treated. As no significant irritant effect was observed after the 1-hour exposure, the test was completed using the 2 remaining animals with an exposure period of 4 hours.

The body weights were recorded on the day of application and at termination of observation. The clinical signs were recorded daily.

The skin reaction was assessed for erythema and oedema at approximately 1, 24, 48 and 72 hours after the end of exposure (removal of the dressing, gauze patch and test item).

At the end of the observation period, animals were sacrificed (intramuscular injections of CP-Ketamin 10% and CP-Xylazin 2% followed by intravenous injection of pentobarbital sodium), without gross necropsy.

II. RESULTS AND DISCUSSION

1. Mortality

No mortality occurred.

2. General observation/clinical signs

At 1, 24, 48 and 72 hours after patch removal, there were no observed clinical signs noted on the skin of the treated animals. The test item stained the application site yellow between 1 and 24 hours after patch removal. However this did not obscure the assessment of any skin irritation.

Table 5.4.1- 2: Aclonifen – Individual and mean skin irritation scores according to the Draize scheme

Animals Identification	Observations after patch removal	1h	24h	48h	72h	Mean scores (12, 24, 72 hr)
Animal 1 (00104)	Erythema (redness) and eschar formation	0	0	0	0	0.00
	Oedema formation	0	0	0	0	0.00
Animal 2 (00101)	Erythema (redness) and eschar formation	0	0	0	0	0.00
	Oedema formation	0	0	0	0	0.00
Animal 3	Erythema (redness) and eschar formation	0	0	0	0	0.00

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(00115)	Oedema formation	0	0	0	0	0.00

As no clinical signs and no skin irritation effects were observed at 72 hours after patch removal, the study was terminated after the 72 hours observation.

No clinical signs of systemic toxicity were observed in the animals during the study.

3. Body weight

The body weights of all rabbits were considered to be within the normal range of variability.

4. Gross pathology / Organ weights / Histopathology

Animals were not subjected to necropsy.

III. CONCLUSION

Aclonifen technical (AE F068300) is not irritating to the skin according to the Draize classification system.

The study result triggers the following classification/labelling:
- Regulation (EC) No 1272/2008 (CLP): none

Assessment and conclusion by applicant:

A skin irritation study was conducted in 1981, but had deficiencies of not being conducted to GLP, not strictly according to OECD test guidelines. Nevertheless the study was considered to be acceptable during the evaluation of the 2006 DAR (see M-174876-01-1, above).

This new study was conducted at the request of the Korean authorities and is a well-conducted study, with a high purity of test substance, conducted to GLP and is largely in accordance with current OECD test guideline (2015). The study deviates from the current test guidelines because an *in vitro* skin irritation study should have been conducted as a replacement for *in vivo* testing in accordance with the Integrated Approaches to Testing and Assessment for skin irritation/corrosion (OECD 2014). The test item was applied to an area of 10 x 10 cm, which is larger than the area required in the test guideline (6 cm^2). These deviations do not affect the reliability of the study.

Under these test conditions aclonifen was not a skin irritant in the rabbit.

Assessment and conclusion by RMS:

CA 5.2.5 Eye irritation

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In addition to the eye irritation toxicity study already available in the Monograph and Baseline Dossier a new study was conducted in 2013 in order to support a registration in Korea.

Data Point:	KCA 5.2.5/01
Report Author:	████; ████
Report Year:	1981
Report Title:	KUB 3359 - Studies for acute toxicity in mice with oral, and in rats with oral, intraperitoneal and dermal treatment, as well as for primary skin and eye irritation in rabbits
Report No:	R007415
Document No:	M-174876-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 423, 2001 Dose volume for rats in acute oral study exceeded recommended levels. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In a primary eye irritation study, 0.1g of aclonifen (96.2 % purity) was instilled into the conjunctival sac of the right eye of 6 New Zealand White rabbits (3 males, 3 females). Animals then observed for the following 7 days. A further 3 rabbits (2 males, 1 female) had the same treatment except that their eyes were rinsed after 30 seconds. Irritation was scored using the Draize scheme. Aclonifen caused no observed signs of irritation.

In this study, aclonifen was not irritating to the eyes according to the Draize classification system.

There were some limitations to the study as it does not strictly follow OECD test guidelines, is not to GLP.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description: Aclonifen (KUB 3359)

Lot/Batch: Lot T5/81

Purity: 96.2%

Appearance: Not mentioned

Stability of test compound: Not mentioned

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2. Vehicle and /or positive control Not applicable

3. Test animals

Species and strain:	Rabbit – White New Zealand
Source:	Not mentioned
Number of animals:	5M + 4F
Sex:	Male and female
Age:	Not mentioned
Weight at treatment:	3.82 (range 3.49-4.15) kg
Acclimation period:	Not mentioned
Diet:	Altromin Brand-Purina (R) sole feed for rabbits, manufactured by
Water	*ad libitum* access
Cage type:	type KK 017 on metal grids (350 x 600 x 390 mm)
Housing:	Individually, Rabbit
Environmental conditions:	Temperature: 18-21°C Humidity: 49-84 % Air changes: Not mentioned Photoperiod: Not mentioned

B. STUDY DESIGN AND METHODS

1. In life dates

2 to 16 September 1981

2. Animal assignment and treatment

The eyes of the animals were examined 24 hours before the beginning of trial by instillation of 0.15 % fluorescein solution (and subsequent examination with a hand lamp according to the method of Eisenhut (Basle). Only those animals were accepted to the trial that did not display any changes.

0.1 grams of aclonifen was instilled into the left eye of 9 rabbits. The right eye served as a control. In 6 of these rabbits (3 males and 3 females) the eyes were not rinsed, in the remaining 3 (2 males and 1 female) the eyes were rinsed with lukewarm water 30 seconds after instillation of the test material and for a period of 60 seconds.

All rabbits were weighed before the beginning of the trial and on the 4-th and 7th day of observation. Behaviour and general condition as well as the occurrence of local changes were recorded daily for a period of 7 days. Eye irritation was scored according to the Draize scale. Pathologico-anatomical examinations were to be performed only in those cases where animals died in the course of the study. The surviving animals were not killed and subjected to a necropsy.

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II. RESULTS AND DISCUSSION

1. Mortality

No deaths occurred.

2. Clinical observations

After the single instillation of 0.1 g of aclonifen, no signs of irritation were observed. Aclonifen produced a yellow colouration of the hair around the eyes which persisted throughout the 7 day observation period. There were no clinical signs of reaction to treatment throughout the entire observation period.

Table 5.2.5- 1: Aclonifen – Eye irritation in rabbit - (without flushing the eyes) mean score of 6 rabbits

Findings		Day 1	Day	Day 3	Day 7	Mean score
Cornea	Opacity	0	0	0	0	0
	Area involved	0	0	0	0	0
Iris	Intensity	0	0	0	0	0
Conjunctivae	Redness	0	0	0	0	0
	Chemosis	0	0	0	0	0
	Discharge	0	0	0	0	0

Table 5.2.5- 2: Aclonifen – Eye irritation in rabbit (with eyes flushed) mean score of 3 rabbits

Findings		Day 1	Day	Day 3	Day 7	Mean score
Cornea	Opacity	0	0	0	0	0
	Area involved	0	0	0	0	0
Iris	Intensity	0	0	0	0	0
Conjunctivae	Redness	0	0	0	0	0
	Chemosis	0	0	0	0	0
	Discharge	0	0	0	0	0

3. Body weight

The body weight development was not different from that of untreated rabbits of the same age.

4. Necropsy

All animals survived the 14- day observation period, therefore animals were not killed for necropsy.

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III. CONCLUSION

Aclonifen was not irritant to the eye and does not require labelling as an eye irritant according to EU criteria.

Assessment and conclusion by applicant:

This study was not conducted to GLP, and it does not follow OECD test guidelines.

This study was deemed to be acceptable in the DAR (2006), however a new study has since been conducted (see document M-465328-01-1 below) which addresses the deficiencies in the current study.

Under the conditions of this test, aclonifen was not an eye irritant in the rabbit.

Assessment and conclusion by RMS:

Data Point:	KCA 5.2.5/02
Report Author:	███
Report Year:	2013
Report Title:	Aclonifen technical (AE F068300) - Acute eye irritation study in rabbits
Report No:	13/275-005N
Document No:	M-465328-01-1
Guideline(s) followed in study:	OECD 405, US-EPA 712-C-98-195, OPPTS 870.2400; Commission Regulation (E)C No 440/2008, B5
Deviations from current test guideline:	Current guideline: OECD 405, 2017 No deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

In a primary eye irritation study, 0.1g of aclonifen (99.5 % purity) was instilled into the conjunctival sac of the right eye of 3 young adult male New Zealand White rabbits. Animals then observed for the following 3 days. Irritation was scored using the Draize scheme. Aclonifen caused conjunctiva redness and transient discharge. The effects were fully reversible within 72 hours.

In this well-conducted study, aclonifen was not irritating to the eyes according to the Draize classification system.

The study follows current OECD test guidelines, was conducted to GLP with a high purity batch of the test item.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen technical (AE F068300)
Lot/Batch:	AE F068300-01-14; origin PEA1000235
Purity:	99.5 %
Appearance:	Yellow powder
Stability of test compound:	The test item was administrated in its original form

2. Vehicle and /or positive control Not applicable

3. Test animals

Species and strain:	New Zealand White Rabbit
Source:	
Number of animals:	3 animals
Sex:	Male
Age:	14 weeks
Weight at treatment:	3444 – 3604 g
Acclimation period:	at least 27 days
Diet:	UNI diet for rabbits ( ); *ad libitum*
Water:	tap water, *ad libitum*
Cage type:	AAALAC open wire structure, metallic cages for rabbit
Housing:	Individual caging; cages are placed together to allow social interaction with rabbit(s) in adjoining cages
Environmental conditions:	Temperature: 20±3 °C Humidity: 41-86 % Air changes: 15-20 exchanges per hour Photoperiod: 12 hours daily, from 6.00 am to 6.00 pm

B. STUDY DESIGN AND METHODS

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1. In life dates

14 August to 17 September 2013

2. Animal assignment and treatment

The following considerations were taken before starting the study:

- the pH of the test item was found to be 5.0. In accordance with OECD Guideline 405, the test item does not need to be tested if the pH values is of high acidity (pH ≤2) or alkalinity (pH ≥11.5).

- No sign of corrosivity/irritation to rabbit eye was observed in the previously *in vivo* non-GLP toxicity study after a single instillation of 0.1 g of aclonifen (M-174876-01-1, ██████; ████ ██; 1981).

Sixty minutes prior to test substance application a systemic opiate analgesic (buprenorphine) was administered by subcutaneous injection. Five minutes prior to the test substance application a topical ocular anaesthetic (oxybuprocaine) was applied to each eye. Eight to nine hours after the test substance application a systemic buprenorphine and a nonsteroidal anti-inflammatory drug (Meloxicam) were administered as subcutaneous injection. Buprenorphine was administered again after a further 12 hour period.

Initially only one rabbit was treated with test item. As the local effect in the first rabbit showed scores of above zero but not severe, then a second rabbit was treated after the 48 hour observation of the first rabbit. As the result in the second rabbit is not severe, a third rabbit was treated 48 hours after the treatment of the second rabbit. A pre-emptive pain management was performed under direct Veterinary supervision prior and after test substance application as per the regulatory guideline.

A single dose of 0.1 g of test item Aclonifen technical (AE F068300) was administered to the animals (*n*=3). The test substance was placed in the conjunctival sac of the left eye of each animal. The untreated contralateral eye was served as the control. The treated eye was rinsed with physiological saline solution at the first observation time point in the all animals at one hour after the application of test item. Individual body weight was recorded on the day of treatment and before euthanasia.

For clinical observations, the eyes were examined at 1, 24, 48 and 72 hours after application. Fluorescein staining (2%) was performed 24 hours before administration of the test item and 24, 48 and 72 hours after treatment to examine corneal damage. The duration of the observation period was sufficient to identify reversibility or irreversibility of changes. The eye irritation scores were evaluated according to the scoring system by ████ (1977) and OECD 405.

Any clinical signs of toxicity or signs of ill- health during the study were recorded. At the end of the observation period, animals were sacrificed (intramuscular injections of CP-Ketamin 10% and CP-Xylazin 2% followed by intravenous injection of pentobarbital sodium), without gross necropsy.

II. RESULTS AND DISCUSSION

1. Mortality

There was no mortality observed during the study.

2. General observation/clinical signs

Initial Pain Reaction (IPR/PR) was not observed in the animals.

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At one hour after treatment, a clear discharge (grades 1-3) and a slight chemosis were noted in all animals, persisting on day 1 for one of them. Test item staining was observed around the eye area, only at this time point. A slight to moderate redness of the conjunctiva (grade 1 or 2) was observed in all animals from one hour after treatment until day 1 for two animals, and persisting in one of them until day 2. At day 3 after treatment, no clinical signs, and no conjunctival effects were observed.

The mean scores calculated for each animal following grading at 24, 48 and 72 hours after installation of the test material were 0.00, 0.00, and 0.33 for chemosis; 1.00, 0.33, and 0.67 for redness of the conjunctiva; and all values were calculated at 0.00 for corneal opacity and iritis.

Table 5.2.5- 3: Aclonifen – Eye irritation scores according to the Draize scheme

Animals identification	Time after treatment:	1h	24h	48h	72h	Mean scores 24-72 hours	Response	Reversible (day)
Animal 1 (00102)	Corneal opacity	0	0	0	0	0.00	--	n.a.
	Iritis	0	0	0	0	0.00	--	n.a.
	Redness conjunctivae	2	2	1	0	1.00	--	3
	Chemosis conjunctivae	1	0	0	0	0.00	--	n.a.
	Discharge	3	0	0	0	0.00	--	n.a.
Animal 2 (00113)	Corneal opacity	0	0	0	0	0.00	--	n.a.
	Iritis	0	0	0	0	0.00	--	n.a.
	Redness conjunctivae	2	1	0	0	0.33	--	2
	Chemosis conjunctivae	1	0	0	0	0.00	--	n.a.
	Discharge	1	0	0	0	0.00	--	n.a.
Animal 3 (00116)	Corneal opacity	0	0	0	0	0.00	--	n.a.
	Iritis	0	0	0	0	0.00	--	n.a.
	Redness conjunctivae	2	1	0	0	0.67	--	3
	Chemosis conjunctivae	1	1	0	0	0.33	--	2
	Discharge	3	2	0	0	0.67	n.a.	2

n.a. = not applicable;
Responses for mean scores: (Corneal opacity: CO; Iritis: IR; Conjunctival Redness: R; Conjunctival Oedema: OE)

Negative: --

CO <1	IR <1	R <2	OE <2	Regulation (EC) No 1272/2008 and GHS

Mild irritant: (+)

CO ≥1 - <3	IR ≥1 - <2	R ≥2	OE ≥2	GHS category 2B (effects reversible within 7 days)

Irritant: +

CO ≥1 - <3	IR ≥1 - <2	R ≥2	OE ≥2	GHS category 2B (effects reversible within 7 days)
CO ≥1 - <3	IR ≥1 - <2	R ≥2	OE ≥2	Regulation (EC) No 1272/2008 (GHS) category 2

Irreversible effect/serious damage: ++

CO ≥3	IR ≥2	R -	OE -	Regulation (EC) No 1272/2008 and GHS category 1

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The general state and behaviour of animals were normal throughout the study period.

3. Body weight

The body weight and body weight changes were considered to be normal with no indication of any treatment related effect.

4. Gross pathology / Organ weights / Histopathology

As there were no clinical signs observed, therefore animals were euthanised after the 72 hours observation and were not subjected to necropsy.

III. CONCLUSION

The test item Aclonifen technical (AE F068300), applied to the rabbits' eye mucosa, caused conjunctival redness and transient discharge. The effects were fully reversible within 72 hours.

The study result triggers the following classification/labelling:
- Regulation (EC) No 1272/2008 (CLP): none

<u>**Assessment and conclusion by applicant:**</u>

An eye irritation study was conducted in 1981, but had deficiencies of not being conducted to GLP, not strictly according to OECD test guidelines. Nevertheless the study was considered to be acceptable during the evaluation of the 2006 DAR (see M-174876-01, above*)*.

This new study was conducted at the request of the Korean authorities and is a well-conducted study, with a high purity of test substance, conducted to GLP and in accordance with current OECD test guideline (2015).

Under these test conditions aclonifen was not an eye irritant in the rabbit.

<u>**Assessment and conclusion by RMS:**</u>

CA 5.2.6 Skin sensitisation

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Data Point:	KCA 5.2.6/01
Report Author:	[redacted]
Report Year:	1990
Report Title:	Aclonifen (CME 127): Modified nine-induction Buehler delayed contact hypersensitivity study in the Guinea pig
Report No:	R007147
Document No:	M-174308-01-1
Guideline(s) followed in study:	EU (=EEC): 84/449/EEC,B6; OECD: 406
Deviations from current test guideline:	Current Guideline: OECD 406, 1992 Minor deviations, positive control tests were conducted 18 months before the study, the standard Buehler in the guideline stipulates 3 topical inductions, whereas the current test used 9 inductions. Overall no impact on study integrity
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In a skin sensitisation study by the Buehler method, aclonifen (91.3% purity) at a test concentration of 75 % w/v aclonifen in arachis oil was applied to female Albino Dunkin-Hartley guinea pigs (20 treated and 10 controls). The treatment regime involved induction by dermal application to give nine exposures over a period of 3 weeks, and a challenge by topical administration on day 28.

There was no dermal response either at the 24- or 48-hour observation points following the challenge application. Appropriate historical control data using 2,4-dinitrochlorobenzene demonstrated a positive response.

In this modified Buehler study, aclonifen was not a skin sensitiser in the guinea pig.

This study was conducted to GLP and is largely in compliance with current OECD test guidelines. The study used 9 topical inductions which exceeds the 3 topical inductions. The main deficiency is that the positive control study was conducted 18 months prior to the current study (it should be conducted within 6 months of the study).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen (CME 127)
Lot/Batch:	DA 618
Purity:	91.3 %
Appearance:	Yellow powder
Stability of test compound:	Not determined

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2. Vehicle and /or positive control Vehicle: 75% (w/w) in arachis oil B.P.

Positive control: 2,4-Dinitrochlorobenzene

3. Test animals

Species and strain:	Guinea pigs – albino Dunkin-Hartley guinea pigs
Source:	[redacted]
Number of animals:	34
Sex:	Female
Age:	8 to 12 weeks old
Weight at treatment:	Mean: 342 ± 17 g
Acclimation period:	Minimum period of 5 days
Diet:	Guinea Pig FD1 Diet, [redacted] *ad libitum*
Water	tap water, *ad libitum*
Cage type:	Polypropylene cages
Housing:	Groups of three animals
Environmental conditions:	Temperature: 19-23°C Humidity: 41-67 % Air changes: 15 changes per hour Photoperiod: 12 hours of continuous artificial light; 12 hours darkness

B. STUDY DESIGN AND METHODS

1. In life dates

16 August to 3 October 1990

2. Animal assignment and treatment

The concentrations of test material for the topical induction and topical challenge stages of the main study were determined in preliminary tests. In each test 2 females guinea pigs were treated with 0.5 ml of different concentrations of the test material in arachis oil. To select the concentration for topical induction 75%, 50%, 25% and 10% w/w aclonifen in arachis oil was applied. The highest concentration of test material which did not produce excessive irritation 24 or 48 hours after 6-hour occlusive dermal exposure was selected for the topical induction stage. To select the concentration for topical challenge 75% and 50% w/w aclonifen in arachis oil was applied. The highest concentration of test material which did not produce dermal irritation 24 or 48 hours after a 6-hour occlusive dermal exposure was selected for the topical challenge stage.

On the basis of the preliminary tests 75% w/w aclonifen in arachis oil was chosen for topical induction and challenge dose.

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For the main study 20 test animals + 10 controls were used. In the treated animals, the hair was removed from the left flank with clippers. 0.5 mL of the test material was applied on absorbent lint (approx. size 15 mm x 35 mm) which was held in place with surgical adhesive tape and covered with aluminium foil. The patch and foil were secured with elastic adhesive bandage wound round the torso of each animal. This occlusive dressing was kept in place for 6 hours. The application was repeated on the same area of skin over a period of three weeks, to give a total of nine 6-hour exposures (on days 0, 2, 4, 7, 9, 11, 14, 16 and 18). In the control animals the same procedure was followed as for the treated animals except that only the vehicle (arachis oil) was applied to the skin.

On day 28 a challenge dose of 0.5 mL of the test material, 75% w/w aclonifen in arachis oil, was applied to the clipped skin of the right flanks of the test and control animals for a 6 hour exposure period. The dose was applied on absorbent lint (approx. size 15 mm x 30 mm), held in place with surgical adhesive tape, and covered with aluminium foil which was then secured with a strip of adhesive bandage wrapped around the torso.

II. RESULTS AND DISCUSSION

1. Mortality

No deaths occurred.

2. Clinical observations

Yellow-staining caused by aclonifen was noted during the induction period but it did not prevent evaluation of the skin response, where aclonifen treatment produced only isolated incidents of scattered mild, moderate and diffuse redness; desquamation and oedema were also noted in a few cases.

There was no adverse reaction to aclonifen noted at either the 24 or 48-hour observation time points following the challenge application.

The validity of the study was confirmed in a positive control test using 2,4-Dinitrochlorobenzene which was conducted in an earlier study (30 March to 29 April 1989) which produced positive reactions in 15/19 animals.

Table 5.2.6- 1: Aclonifen – Buehler test – Number of animals with signs of allergic skin reaction

Test substance	Scored after	
	24 h	48 h
Negative control	0/10	0/10
Aclonifen	0/20	0/20
Positive control (0.5% DNCB)	15/19	15/19

3. Body weight

Bodyweight gains of guinea pigs in the test group, between day 0 and day 30, were comparable to those observed in the control group animals over the same period.

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III. CONCLUSION

It was concluded that aclonifen was not a skin sensitiser to guinea pig according to the method of Buehler.

The study result triggers the following classification/labelling:
- Regulation (EC) No 1272/2008 (CLP): none

Assessment and conclusion by applicant:

This modified Buehler sensitisation study was conducted to GLP and is largely in compliance with current OECD test guidelines. The study used 9 topical inductions which exceeds the 3 topical inductions. The main deficiency is that the positive control study was conducted 18 months prior to the current study (it should be conducted within 6 months of the study).

Under these test conditions aclonifen was not a skin sensitiser in the guinea pig by the modified Buehler method.

Assessment and conclusion by RMS:

Data Point:	KCA 5.2.6/02
Report Author:	[redacted]
Report Year:	2002
Report Title:	Skin sensitization test in guinea pigs Aclonifen
Report No:	C021874
Document No:	M-212028-01-1
Guideline(s) followed in study:	EU (=EEC): 96/54/EEC B6 1996; OECD: 406 1992; USEPA (=EPA): OPPTS 870.2600 1998
Deviations from current test guideline:	Current Guideline: OECD 406 1992 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

In a skin sensitisation study by the Magnusson and Kligman method, aclonifen (99.2% purity) in Freund's Complete Adjuvant (FCA) Emulsion was tested in young Hartley Guinea pigs (10/sex). The treatment regime involved induction of sensitisation by intradermal injection on day 1, induction of sensitisation by topical administration on day 8 and challenge by topical administration on day 22.

There was a dermal response in 19/20 animals in response to the challenge dose. There was no response in the control group of 10 animals. Appropriate historical control data using mercaptobenzothiazole demonstrated a positive response. On the basis of this study aclonifen should be classified as a strong sensitiser Category 1A according to Regulation (EC) 1272/2008.

This was a well-conducted GLP and guideline compliant study.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen
Lot/Batch:	OP9750062
Purity:	99.2 %
Appearance:	Yellow powder
Stability of test compound:	Not determined

2. Vehicle and /or positive control

Vehicle for intradermal injection: corn oil; Vehicle for topical application: ethanol/purified water (80/20 w/w); Positive control: mercaptobenzothiazole.

3. Test animals

Species and strain:	Guinea pigs – Hartley Crl.(HA) BR
Source:	[redacted]
Sex:	Male + female
Number of animals:	3M + 3F for preliminary tests; 15M + 15F for the main study
Age:	1-2 months old
Weight at treatment:	Mean males: 360 ± 25 g and mean females: 386 ± 23 g
Acclimation period:	Minimum period of 5 days
Diet:	106 pelleted diet, UAR, France; *ad libitum*
Water	filtered by a FG Millipore membrane; *ad libitum*
Cage type:	Polycarbonate cages with stainless steel lid (48 cm x 27 cm x 20 cm)
Housing:	Individually
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 30-70 % Air changes: 12 changes per hour

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Photoperiod: 12 hours of continuous artificial light; 12 hours Darkness

B. STUDY DESIGN AND METHODS

1. In life dates

11 October to 26 November 2001

2. Animal assignment and treatment

Preliminary test: The concentrations of the test material for the intradermal and cutaneous induction and challenge dose were determined by a preliminary test using 3 male and 3 female guinea pigs.

Intradermal tested concentrations were 1% and 0.1% w/w aclonifen in corn oil.

The dose of 1% caused irritation and was selected as the dose for the main study

Cutaneous tested concentrations were 50% and 25% w/w aclonifen in a vehicle of 80/20 (w/w) ethanol/water and covered under occlusive dressing for 48 hours.

Challenge phase tested concentrations were the same as for the cutaneous induction phase except that the exposure time was 24 hours.

The dose of 50% did not cause any irritation and was selected as the dose for the cutaneous induction and challenge dose for the main study.

For the main study, thirty guinea pigs were allocated to two groups: a control group of five males and five females and a treated group of ten males and ten females. Animals were clipped the day before each treatment, and for the cutaneous induction the animals were also shaved.

Intradermal induction: On Day 1, three pairs of intradermal injections of 0.1 mL were performed in the interscapular region of all animals:
- Freund's complete adjuvant (FCA) diluted at 50% (v/v) with 0.9% NaCl (both groups),
- Aclonifen, at the concentration of 1% (w/w, treated group) or corn oil (control group),
- Aclonifen at the concentration of 1% (w/w) in a mixture FCA/0.9% NaCl (50/50, v/v) (treated group) or corn oil at the concentration of 50% (w/v) in a mixture FCA/0.9% NaCl (50/50, v/v) (control group).

Cutaneous induction: On Day 8, the aclonifen at the concentration of 50% (w/w) in a 80/20 (w/w) mixture of ethanol/water (treated group) or a 80/20 (w/w) mixture of ethanol/water (control group) was applied topically to the same test site, approximately 8 cm^2, which was then covered by an occlusive dressing for 48 hours. As the test item was shown to be irritant during the preliminary test, a topical application with sodium lauryl sulfate was not necessary on day 7.

Challenge dose: On Day 22, all animals of the treated and control groups were challenged by a cutaneous application of aclonifen at the concentration of 50% (w/w) in a 80/20 (w/w) mixture of ethanol/water to the right flank. The left flank served as control and received the ethanol/water (80/20; w/w) only. The test item and vehicle were maintained under an occlusive dressing for 24 hours.

Skin reactions were evaluated approximately 24 and 48 hours after removal of the dressing.

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The animals were observed at least once a day during the study in order to check for clinical signs and mortality. The animals were weighed individually on the day of allocation into the groups, on the first day of the study (day 1) and on the last day of the study (day 25).

At the end of the study, animals were sacrificed without examination of internal organs. Skin samples were taken from the challenge application sites of all the animals and preserved in 10% formalin. No histological examination was performed.

The sensitivity of the assay was assessed by a positive control test using mercaptobenzothiazole conducted in October 2001.

II. RESULTS AND DISCUSSION

1. Mortality

No deaths occurred during the study.

2. Clinical observations

After the challenge application, no cutaneous reactions were observed in the animals of the control group.

In the treated group, at the 24-hour reading, a discrete or moderate erythema was noted in 11/20 and 8/20 animals, respectively. Discrete or moderate erythema persisted at the 48-hour reading in 9/20 and 10/20 animals, respectively.

Dryness of the skin was observed in almost all animals of the treated group at the 24 and 48-hour readings and an oedema was recorded in 1/20 animals of the treated group at the 48-hour reading.

A yellow coloration of the skin was noted in all animals of both groups at the 24 and 48-hour readings.

The cutaneous reactions observed in the animals of the treated group were attributed to delayed contact hypersensitivity.

Table 5.2.6- 2: Aclonifen – Number of animals exhibiting skin reactions (24 and 48 hours after removal of the dressing).

	Skin reactions after 24 hours		**Skin reactions after 48 hours**	
Groups	**Vehicle (LF)**	**Test (RF)**	**Vehicle (LF)**	**Test (RF)**
Control	0/10	0/10	0/10	0/10
Aclonifen 50%	0/20	**18/20**	0/20	**19/20**

LF = left flank (vehicle: ethanol/water)

RF = (Aclonifen at the concentration of 50% w/w)

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Hypoactivity and dyspnea was noted in one animal of the treated group from day 14 up to day 25. Such spontaneous clinical signs were sometimes observed in this species, it was not attributed to treatment with the test item.

3. Body weight

The body weight gain of the treated animals was similar to that of controls.

III. CONCLUSION

Under these experimental conditions and according to the maximisation method of Magnusson and Kligman, the test item aclonifen induces delayed contact hypersensitivity in 19/20 (95%) guinea pigs. 95% of animals showed a sensitivity reaction based on an intradermal induction dose of 4% aclonifen indicates that aclonifen is a strong sensitiser according to the CLP classification criteria.

The study result triggers the following classification/labelling:
- Regulation (EC) No 1272/2008 (CLP): Skin sensitisation Category 1A, H317: May cause and allergic skin reaction.

<u>**Assessment and conclusion by applicant:**</u>

In this well-conducted GLP and guideline compliant Magnusson and Kligman sensitisation study aclonifen was a strong sensitiser in the guinea pig.

Under Regulation (EC) No 1272/2008 (CLP) the following classification is triggered: Skin sensitisation Category 1A, H317: May cause and allergic skin reaction.

Aclonifen already has a Harmonised classification as a Category 1A skin sensitiser.

<u>**Assessment and conclusion by RMS:**</u>

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 (ς) 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 aclonifen in aqueous solution have been determined and showed maximum values of molar extinction coefficient on the absorbance at 238, 310 and 390 nm (see table below), and values from 3462 to 8431 L x mol^{-1} x cm^{-1} between 292-420 nm (M-161634-01-1).

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Table 5.2.7- 1: Aclonifen UV/VIS spectra (study submitted under point MCA 2.4)

Solvent	Wavelength [nm]	Molar extinction coefficient [L x mol^{-1} x cm^{-1}]	Reference
Aqueous solution (neutral medium)	238	18987	; M-161634-01-1
	310	8431	
	390	6143	

Therefore, according to the current requirements, a phototoxicity study was conducted.

Data Point:	KCA 5.2.7/01
Report Author:	
Report Year:	2017
Report Title:	Aclonifen technical AE F068300: Cytotoxicity assay in vitro with BALB/c 3T3 Cells: Neutral red (NR) test during simultaneous irradiation with artificial sunlight
Report No:	1767100
Document No:	M-634103-01-1
Guideline(s) followed in study:	Commission Regulation (EC) No. 440/2008, B.41 (2008); Committee for Proprietary Medicinal Products (CPMP) Note for Guidance on Photosafety testing, EMEA, CPMP/SWP/398/01 (2002); OECD 432 (2002)
Deviations from current test guideline:	Current guideline: OECD 432, 2019 Cells were seeded at 2 x 10 4 cells/well (the guideline recommends seeding at 1 x 10 4 cells/well). Microscopic evaluation of the cells was not conducted. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In a phototoxicity study, aclonifen (99.5% purity) was tested in BALB/c 3T3 cells. The treatment regime involved exposing cells treated with 8 concentrations of aclonifen to artificial sunlight for 50 minutes, and the cytotoxic effect measured in comparison to the solvent control. A duplicate plate of cells that were kept in the dark determined the cytotoxic effect of aclonifen without sunlight exposure. The poor solubility of aclonifen in the solvent (DMSO) limited the maximum concentration tested to 15.63 µg/mL. Cytotoxic effects were seen in cells exposed to aclonifen at the maximum concentration, both with and without irradiation with artificial sunlight, with the cytotoxic effect being slightly greater in cells exposed to artificial sunlight. Since the cell viability after treatment with the non-irradiated test item did not decrease below 50% in both experiments, the IC_{50} value could not be determined, therefore the photo-irritation-factor (PIF) values could not be calculated. The mean photo effect (MPE) values were determined as 0.000 and -0.002, in the range finder and main experiment, indicating no phototoxic potential of the test item. On the basis of this study aclonifen is not photo toxic.

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The solvent controls met the acceptance criteria, and positive control experiment confirmed the validity of the test conditions.

The study was conducted to GLP and was largely in compliance with current OECD test guidelines. One deviation was that the cells were seeded into the test wells at a higher density than the guideline recommends. A second deviation was that the treated cells were not examined microscopically. These deviations are not thought to invalidate the study.

I. MATERIALS AND METHODS

1. Test Material

Description:	Aclonifen Technical AE F068300
Lot/Batch:	PEA1000563
Purity:	>99.5%
Appearance:	Yellow powder
Stability of test compound:	Stable for at least 2 hours in aqueous/CMC formulations

2. Control materials

Solvent control for positive control:	EBSS.
Solvent control for test item:	EBSS containing 1% (v/v) DMSO
Positive control:	Chlorpromazine (CPZ, Sigma) dissolved in EBSS

3. Test Cells

BALB/c 3T3 cell line, clone 31

Source: [redacted]

4. Cell culture

Culture medium:	Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% new born calf serum
Culture incubation conditions:	37 ± 1.5 °C in a 7.5 ± 0.5% carbon dioxide atmosphere

5. Test Concentrations used

Preliminary test (µg/mL):	0.12, 0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63
Main test (µg/mL):	2.49, 3.23, 4.21, 5.47, 7.11, 9.25, 12.02, 15.63
Positive control (µg/mL):	Without irradiation: 6.25, 12.5, 25, 37.5, 50, 75, 100, 200
	With irradiation: 0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0
Number of replicates:	6 replicates for preliminary and main test and positive control, 12 replicates for solvent controls

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6. Solar Simulator	[redacted] 500 solar simulator
Filter:	H1 (UVB filter)
Wavelength:	> 320 nm
Irradiation treatment:	One hour through the lid of a 96-well plate at 1.68 mW/cm^2 (approximately 5 J/cm^2) for 50 minutes at 26°C.

B. STUDY DESIGN AND METHODS

1. Experimental dates

20 to 29 April 2016

2. Test item preparation

2 x 10^4 cells per well were seeded in 96-well plates and incubated for 24.75 hours.

3. Treatment

After seeding and incubation the cultures were washed with EBSS
− 8 dilutions of the solved test item were tested on two 96-well plates (100 µL/well)
− both plates were pre-incubated for 1 hour in the dark
− after one hour one 96-well plate was irradiated through the lid for 50 min, the other plate was stored for 50 mins in the dark.
− after irradiation the test item was removed and both plates were washed twice with EBSS
− fresh culture medium was added and the cells were incubated.

4. Determination of cell viability

The medium was removed and 0.1 mL serum free medium containing 50 µg Neutral Red/mL was added to each well. The plates were incubated for another 3 hours to allow uptake of the dye into the lysosomes. The medium was removed, the cells washed, then 0.15 mL of a solution of 49% (v/v) deionised water, 50% (v/v) ethanol and 1% (v/v) acetic acid was added to each well to extract the dye. After 10 min at room temperature and a brief agitation, the plates were transferred to a microplate reader (Versamax®, Molecular Devices) equipped with a 540 nm filter to determine the absorbance of the extracted dye. This absorbance shows a linear relationship with the number of surviving cells. Absorbance values were determined using the SoftMax Pro Enterprise (version 4.7.1) software.

5. Data and reporting

Arithmetic means ± standard deviation were calculated for every test group.
The IC_{50} values, the Photo-Irritancy-Factor (PIF), as well as the Mean Phototoxic Effect (MPE), were calculated using the software Phototox (Version 2.0) (distributed by [redacted] [redacted], and recommended by the OECD guideline).
The IC_{50} values (effective dose where only 50% of the cells survived) were determined by curve-fitting software.

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6. Evaluation criteria

If PIF ≤ 2 or MPE ≤ 0.1: no phototoxic potential predicted.
If PIF > 2 and ≤ 5 or MPE >0.1 and ≤0.15 a probable phototoxic potential is predicted.
If PIF > 5 or MPE > 0.15 a phototoxic potential predicted

The assay meets the acceptance criteria:
- if after irradiation with a UVA dose of ~ 5 J/cm² the cell viability of the solvent control is > 80% of non-irradiated cells;
- if for the positive control (chlorpromazine) the factor (PIF) between the two IC_{50} values is > 6; and
- if the mean OD_{540} of solvent controls is > 0.4.

II. RESULTS AND DISCUSSION

According to the OECD guideline no test item precipitation should occur in the irradiated cultures. Due to the limited solubility of the test item in DMSO, the highest applied concentration was 15.63 µg of the test item in 1 mL EBSS including 1% (v/v) DMSO. This was used as the highest concentration in the range finding experiment and in the main test.

Cytotoxic effects were observed after exposure of cells to the highest tested concentration of Aclonifen technical in the presence and absence of irradiation with artificial sunlight in both experiments. In the range finding experiment the IC_{50} value of aclonifen under irradiation was 13.2 µg/mL. In the main experiment the IC_{50} value of aclonifen under irradiation was 14.04 µg/mL (see table of summary of results below). Since the cell viability after treatment with the non-irradiated test item did not decrease below 50% without irradiation in both experiments, the IC_{50} values of the test item -UV could not be determined. For this reason the PIF values could not be calculated. The MPE values were determined as 0.000 and -0.002, respectively, indicating no phototoxic potential of the test item.

The mean of solvent control values of the irradiated group versus the non-irradiated group met the acceptance criteria as the cell viability of the irradiated solvent control is > 80% of non-irradiated cells and the OD_{540} of solvent controls is > 0.4.

The positive control chlorpromazine induced phototoxicity in the expected range (IC_{50} value is > 6) after irradiation with artificial sunlight.

Table 5.2.7- 2: Aclonifen phototoxicity study - Summary of Results

	Compounds	IC_{50} (+UV) [µg/mL]	IC_{50} (-UV) [µg/mL]	PIF[1]	MPE[2]	% viability of solvent control of irradiated versus non irradiated plate
Range finder	Aclonifen	13.02	-	-	0.000	106.1
	Positive control	0.68	12.58	18.62	0.577	98.9

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Main experiment	Aclonifen	14.04	-	-	-0.002	99.7
	Positive control	0.86	13.58	15.82	0.399	90.7

[1] PIF = Photo-Irritancy-Factor [2] MPE = Mean Phototoxic Effect

Table 5.2.7- 3: Phototoxicity - range finder: Treatment of BALB/c 3T3 with Aclonifen technical

With artificial sunlight				Without artificial sunlight			
Conc. [µg/mL]	**O.D.540 nm Mean Value**	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**O.D.540 nm Mean Value**	**Standard Deviation**	**% of Solv. Control**
Solvent Control	0.9578*	0.0280	100.00	**Solvent Control**	0.9027*	0.0569	100.00
0.12	0.9320	0.0076	97.31	**0.12**	0.8946	0.0610	99.11
0.24	0.9389	0.0319	98.03	**0.24**	0.8891	0.0696	98.49
0.49	0.9399	0.0189	98.13	**0.49**	0.9036	0.0716	100.10
0.98	0.9435	0.0418	98.51	**0.98**	0.9125	0.0647	101.09
1.95	0.9525	0.0287	99.44	**1.95**	0.8666	0.0431	96.01
3.91	0.9278	0.0301	96.87	**3.91**	0.8835	0.0506	97.88
7.81	0.9196	0.0257	96.02	**7.81**	0.8457	0.0447	93.68
15.63	0.1936	0.0215	20.21	**15.63**	0.4795	0.0886	53.11
* mean O.D.540 nm out of 12 wells							

Table 5.2.7- 4: Phototoxicity – range finder: Treatment of BALB/c 3T3 with the Positive Control (chlorpromazine)

With artificial sunlight				Without artificial sunlight			
Conc. [µg/mL]	**O.D.540 nm Mean Value**	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**O.D.540 nm Mean Value**	**Standard Deviation**	**% of Solv. Control**
Solvent Control	0.8591*	0.0249	100.00	**Solvent Control**	0.8689*	0.0304	100.00
0.125	0.8172	0.0178	95.12	**6.25**	0.8578	0.0171	98.73
0.250	0.7567	0.0140	88.08	**12.5**	0.4553	0.0516	52.39
0.500	0.6176	0.0113	71.88	**25.0**	0.0661	0.0059	7.61
0.750	0.3784	0.0345	44.04	**37.5**	0.0554	0.0011	6.38

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1.000	0.1848	0.0201	21.51	**50.0**	0.0542	0.0009	6.23
1.500	0.0715	0.0037	8.32	**75.0**	0.0575	0.0081	6.62
2.000	0.0708	0.0040	8.24	**100**	0.0585	0.0158	6.73
4.000	0.0800	0.0059	9.31	**200**	0.0525	0.0015	6.04

* mean O.D.540 nm out of 12 wells

Table 5.2.7- 5: Phototoxicity – main experiment: Treatment of BALB/c 3T3 with Aclonifen technical

With artificial sunlight				Without artificial sunlight			
Conc. [µg/mL]	**O.D.540 nm Mean Value**	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**O.D.540 nm Mean Value**	**Standard Deviation**	**% of Solv. Control**
Solvent Control	0.8422*	0.0204	100.00	**Solvent Control**	0.8446*	0.0406	100.00
2.49	0.8438	0.0167	100.19	**2.49**	0.8092	0.0139	95.81
3.23	0.8219	0.0091	97.59	**3.23**	0.7744	0.0164	91.69
4.21	0.7964	0.0194	94.56	**4.21**	0.7744	0.0154	91.69
5.47	0.7827	0.0155	92.94	**5.47**	0.7700	0.0267	91.17
7.11	0.7952	0.0125	94.42	**7.11**	0.7802	0.0228	92.38
9.25	0.7789	0.0390	92.48	**9.25**	0.7629	0.0211	90.32
12.02	0.6215	0.0207	73.80	**12.02**	0.6932	0.0243	82.08
15.63	0.3333	0.0229	39.57	**15.63**	0.5247	0.0408	62.12

* mean O.D.540 nm out of 12 wells

Table 5.2.7- 6: Phototoxicity – main experiment: Treatment of BALB/c 3T3 with the Positive Control (chlorpromazine)

With artificial sunlight					Without artificial sunlight			
Conc. [µg/mL]	**O.D.540 nm Mean Value**	**Standard Deviation**		**% of Solv. Control**	**Conc. [µg/mL]**	**O.D.540 nm Mean Value**	**Standard Deviation**	**% of Solv. Control**

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Solvent Control	0.8080*	0.0251		100.00	**Solvent Control**	0.8906*	0.0500	100.00
0.125	0.8303	0.0205		102.76	**6.25**	0.8153	0.0108	91.54
0.250	0.7821	0.0216		96.80	**12.5**	0.5251	0.0145	58.96
0.500	0.6581	0.0100		81.46	**25.0**	0.1276	0.0203	14.32
0.750	0.5117	0.0246		63.33	**37.5**	0.1195	0.0119	13.42
1.000	0.3196	0.0214		39.56	**50.0**	0.1235	0.0184	13.87
1.500	0.1691	0.0290		20.93	**75.0**	0.1144	0.0240	12.84
2.000	0.1875	0.0216		23.20	**100**	0.1230	0.0243	13.81
4.000	0.1542	0.0206		19.08	**200**	0.1275	0.0343	14.32

* mean O.D.$_{540\ nm}$ out of 12 wells

III. CONCLUSION

Under these experimental conditions aclonifen did not have phototoxic effects on BALB/c 3T3 cells.

Assessment and conclusion by applicant:

This phototoxicity study was conducted to GLP and is largely in compliance with current OECD test guidelines.

One deviation was that the cells were seeded into the test wells at a higher density than the guideline recommends. A second deviation was that the treated cells were not examined microscopically. These deviations are not thought to invalidate the study.

Cytotoxic effects were seen in cells exposed to aclonifen at the maximum concentration, both with and without irradiation with artificial sunlight, with the cytotoxic effect being slightly greater in cells exposed to artificial sunlight. However the mean phototoxic effect (MPE) was below the trigger value for phototoxicity.

Under these test conditions aclonifen was not phototoxic.

Assessment and conclusion by RMS:

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CA 5.3 Short-term toxicity

All the short-term studies reported here were already presented and evaluated during the EU process for Annex I listing. These studies were conducted between 1980 and 2002, and most bear some omissions with respect to the requirement of current OECD test guidelines, but overall are considered to be acceptable. The studies comprise a 28-day dietary mouse study, three 90-day dietary rat studies, a 26-week dietary dog study, and a 4-weeks dermal rat study are reported. In the dietary rat studies the same doses were applied (50 - 5000 ppm) and generally similar effects, more severe in males, have been reported (decreased body weight, changes in blood chemistry indicative of liver damage, increased liver and kidney weights, liver and kidney pathology). Thyroid hormones were measured in one of the 90-day rat studies (M-205288-01-1) and showed changes in T4 and TSH the correlated also with thyroid follicular hypertrophy in males. The lowest NOAEL obtained in these investigations was 3.6 mg/kg bw/day based on findings of follicular cell hypertrophy in the thyroid, histopathology and increased organ weights in liver and kidneys at higher doses. The NOAEL in the mouse dietary study was established at 121 mg/kg bw/day based on decreased body weight and toxicity in liver, kidneys and ovaries. From the dog study a NOAEL of 15 mg/kg bw/day (500 ppm) was derived based on decreased body weight, changes in clinical chemistry and hepatomegaly occurring at the highest dose of 5000 ppm. Dermal exposure for 4 weeks at the dose-levels of 250, 500 or 1000 mg/kg/day was well tolerated by Sprague-Dawley rats. The NOAEL in males was 500 mg/kg/day, while the NOAEL in females was 1000 mg/kg/day.

In the 28-day mouse study HPLC analysis of plasma samples obtained after 28 days of treatment detected a metabolite of aclonifen, but no unchanged parent material in the plasma. The findings indicate absorption of aclonifen and extensive metabolism, correlating with the findings in the metabolism studies in rats (see CA 5.1.1)

Aclonifen - Summary of short-term toxicity studies

Type of study Doses	NOAEL	Findings	Reference	GLP
Oral route				
4-week feeding mouse 0, 780, 3125, 12500, 50000 ppm 0, 121/143, 481/555, 2003/2336, 8906/12403 mg/kg bw/day in M/F	780 ppm males: 121 mg/kg bw/day females: 143 mg/kg bw/day	At 50000 ppm: ↓ bw gain, (m + f) ↓ food consumption (m + f), clinical signs of ill health (m + f). Liver: ↑ abs./rel. liver weight (m + f), hepatocellular hypertrophy (m + f). Kidney: ↑ rel. weight (m + f), necrosis of proximal convoluted tubules, cortical tubule dilation. Ovary: ↓ abs./rel. ovary weight, luteal deficiency. At 12500 ppm: Liver: ↑ abs./rel. liver weight (m + f), hepatocellular hypertrophy (m + f).	KCA 5.3.1/01 M-174234-01-1 ███ ██ 1988	Yes

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13-week feeding rat 0, 50, 500, 5000 ppm 0, 2.61/2.94, 26.41/29.43, 258/279 mg/kg bw/day in M/F	500 ppm males: 26 mg/kg bw/day females: 29 mg/kg bw/day	At 5000 ppm: ↓ bw gain, (m + f) ↓ food consumption (m + f), ↑ water consumption (m + f), ↑ ALT (m + f), ↓ total protein (m + f). In females ↑AP and ↑bilirubin and ↓ cholesterol. In males haematuria. Liver: ↑ abs./rel. liver weight (m + f). Kidney: ↑ abs./rel. kidney weight (m), and tubular nephropathy (m).	KCA 5.3.2/01 M-174843-01-2 █, 1982	No
13-week feeding rat 0, 50, 500, 5000 ppm 0, 3.56/4.15, 35.4/40.8, 341/390 mg/kg bw/day in M/F	50 ppm males: 3.6 mg/kg bw/day females: 4.2 mg/kg bw/day	At 5000 ppm: In females: ↓ bw gain and ↓ food consumption. In males: ↑ urea and ↑cholesterol and ↑albumin, ↑urinary volume and haematuria. Liver: ↑abs./rel. liver weight (m + f), hepatocellular hypertrophy (m + f). Kidney: ↑ abs./rel. kidney weight (m), and transitional cell hyperplasia of the pelvis, necrosis of the papilla (m). Thyroid: thyroid follicular hypertrophy (m + f). At 500 ppm: Thyroid: thyroid follicular hypertrophy (m + f).	KCA 5.3.2/02 M-174924-01-1 █ 1997	Yes
13-week feeding rat 0, 50, 500, 5000 ppm 0, 2.9/3.7, 29.4/36.3, 295/323 mg/kg bw/day	500 ppm males: 29 mg/kg bw/day females: 36 mg/kg bw/day	At 5000 ppm: ↓ bw gain, (m + f) ↓food consumption (m + f). In males: ↑ cholesterol, ↑creatinine, ↑blood urea, ↑albumin, ↓potassium, ↓globulin. In females: ↓ cholesterol. ↑ urinary volume (m + f), and haematururia (m). Liver: ↑ abs./rel. liver weight (m + f), hepatocellular hypertrophy (m + f), foci of hepatocellular necrosis (m). Kidney: ↑ abs./rel. kidney weight (m), corticotubular and papillary necrosis and medullary nephritis (m). Thyroid: follicular hypertrophy (m), ↑TSH and ↓T4 (m + f).	KCA 5.3.2/03 M-205288-01-1 █. 2001	Yes
26-week feeding dog 0, 100, 500, 5000 ppm 0, 3, 15, 142 mg/kg bw/day	500 ppm 15 mg/kg bw/day	At 5000 ppm: ↓ bw gain, (m + f) ↓ food consumption (f). Haematology changes (f): ↑ lymphocytes and ↑ monocytes, ↓ granulocytes. ↑ AP (m), ↑cholesterol (m + f). Liver: ↑ abs./rel. liver weight (m + f).	KCA 5.3.2/04 M-232142-01-2 █ █ 1982	Yes
Dermal route				

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30-day dermal rat 0, 250, 500, 1000 mg/kg bw/day in M & F	500 mg/kg bw/day	At 1000 mg/kg bw/day: ↓ bw gain, (m) ↓ food consumption (m), ↓ glucose (m).	KCA 5.3.3/01 M-212006-01-1 [redacted] 2002	Yes

CA 5.3.1 Oral 28-day study

The four-week study in the mouse was designed to select dose levels for the long-term oncogenicity study.

Data Point:	KCA 5.3.1/01
Report Author:	[redacted]
Report Year:	1988
Report Title:	CME 127 : Preliminary toxicity study by dietary administration to CD-1 mice for four weeks
Report No:	R007110
Document No:	M-174234-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 407, 2008 Significant deviations. There were no detailed clinical observations, no assessment of sensory and motor activity, no haematology or clinical chemistry. Plasma samples were analysed by HPLC to determine levels of aclonifen. Only five organs were examined histologically (a full histological examination is required in the guidelines). These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The test compound, aclonifen (CME 127, purity 81.3%), was administered continuously, via the diet, for 28 days at concentrations of 0, 780, 3125, 12500 or 50000 ppm (equivalent to 0, 121/143, 481/555, 2003/2335, 8906/12403 mg/kg bw/day in males/females respectively) to groups of CD-I mice, 12 animals of each sex per dose group.

A dosage-related absorption of Aclonifen and extensive metabolism was confirmed for all treated groups, by HPLC analysis of plasma samples obtained after 28 days of treatment, which detected a metabolite of aclonifen, but no unchanged parent material in the plasma.

At the top dose of 50000 ppm there was a severe reduction in body weight gain, clinical signs of ill health, and mortalities in both sexes indicating this dose exceeded the maximum tolerated dose. The main target organs were the liver and kidneys. There was a dose related increase in absolute and relative liver weight that was statistically significant at 12500 ppm, and accompanied by hepatocellular hypertrophy which was significantly increased in males at 3125 ppm and in females at 12500 ppm. In the kidneys necrosis of the proximal convoluted tubules was seen in male mice at 12500 ppm and in both sexes at 50000 ppm. In both sexes kidney cortical tubules were dilated and

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basophilic at 50000 ppm. In addition at 50000 ppm there was luteal deficiency in the ovaries and a reduction in absolute and relative ovary weight.

Overall, based on liver changes at 3125 ppm the NOAEL was 780 ppm, equivalent to 121 or 143 mg/kg/day in males and females, respectively.

The study was performed to GLP but has some significant deviations from the OECD guideline 407 (no clinical chemistry or haematology, and limited histopathology investigations). These deviations do not invalidate the study.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description: Aclonifen (CME127)

Lot/Batch: DA 504

Purity: 81.3 %

Appearance: Yellow powder

Stability of test compound: Acceptable stability of aclonifen in the diet was demonstrated over the treatment period. Concentrations averaged 110, 98, 95 and 101% of the intended concentration for the lowest to highest treatment levels respectively.

2. Vehicle and /or positive control Aclonifen (CME127) was incorporated into powdered rodent diet at the required concentrations.

3. Test animals

Species and strain: Mice – CD-1

Source: [redacted]

Number of animals: 12 m/f per dose group

Sex: Male - Female

Age: 21-28 days of age at start of acclimatisation period

Weight start of study: Males: 17-22 g; Females: 16-21 g (day after arrival)

Acclimation period: 13 days

Diet: Complete rodent diet, LAD-2 ([redacted]); *ad libitum*

Water: Tap water, *ad libitum*

Cage type: Type M2 cages (polypropylene, 33x15x13 cm)

Housing: Groups of 4 mice per cage / sex (except if the number was reduced by mortality)

Environmental conditions: Temperature: 21°C

Humidity: 55 %

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Air changes: approximately 15 changes per hour

Photoperiod: 12 hours light and 12 hours darkness

B. STUDY DESIGN AND METHODS

1. In life dates

From 1 to 30 September 1987.

2. Animal assignment and treatment

Aclonifen was administered orally via the diet to groups of CD-1 mice (12 animals/sex/dose group) for 4 weeks at doses of 0, 780, 3125, 12500, 50000 ppm (equivalent to 0, 121/143, 481/555, 2003/2335, 8906/12403 mg/kg bw/day in males/females respectively).

3. Diet preparation and analysis

Aclonifen was incorporated into powdered rodent diet. Batches were prepared weekly. Dose levels were 0, 780, 3125, 12500 or 50000 ppm. The effectiveness of the mixing procedure in producing homogeneous test diets and the chemical stability of the test material in diet were assessed analytically on trial mixes prepared before commencement of treatment. Samples (6 each) of the low (780 ppm) and high (50000 ppm) doses had mean concentrations of 705 and 47600 ppm respectively, 90% and 95% of target concentration respectively, with coefficient of variation of 2.6 and 2.2% in the low and high dose respectively. The same samples were assessed after 7 and 14 days storage at 21°C and demonstrated that aclonifen in the diet was stable for at least 14 days.

The concentrations of test material in mixes prepared for Weeks 1 and 4 of treatment were also determined for all the concentrations used and demonstrated the achieved concentration was between 93 and 113% of the target concentrations. The method of analysis to determine level of aclonifen in the rodent diet does not fulfil SANCO 3029, but can be regarded as fit for purpose.

Plasma samples from each animal were analysed by HPLC with chemical standards containing 4-nitro-aniline and aclonifen. The amount of aclonifen or metabolites in each sample was estimated quantitatively from the absorbance maximum compared with the absorbance maximum of the aclonifen standard. The method of analysis to determine level of aclonifen and metabolites in plasma does not fulfil SANCO 3029, but can be regarded as fit for purpose.

4. Statistics

Inter-group differences in bodyweight gain were assessed by Student's t-test using pooled within-group error variance. A least significant difference was calculated at the 99.9%, 99% and 95% confidence levels. Intergroup differences in mean absolute or bodyweight-relative organ weights were assessed using Dunnett's test at the 95% and 99% confidence levels. Fisher's Exact Probability test was applied as a two-tailed test, where considered appropriate, to the distribution of macroscopic and microscopic pathological entities.

5. Observations

Animals were observed twice daily for signs of toxicity. Food consumption (per cage) and individual body weight were recorded weekly. No haematological examinations, clinical biochemistry analyses or urine analysis were performed. Blood samples were collected on all surviving on day 28 and blood plasma analysed by HPLC using a chemical standards of aclonifen and 4-nitro-aniline.

All animals were necropsied at the end of the study after euthanasia by CO_2 inhalation. A gross pathology examination was conducted. Selected organs, i.e. adrenals, brain, heart, kidneys, liver, testes, ovaries and spleen were weighed. Fixing and staining was conducted on all gross lesions, all

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organs that were weighed, and the thyroids and pancreas. Kidneys, livers, ovaries, thyroids and pancreas of all animals were examined microscopically.

II. RESULTS AND DISCUSSION

1. General observation/clinical signs/ Mortality

One male and three female mice of the 50000 ppm group died or were sacrificed in extremis during the first week of treatment. In weeks 3 and 4 a further male at 50000 ppm died, and also a male and female in the control group. The cause of death could not be clarified. During blood sampling two males, one in control and one in 12500 ppm group also died.

A yellow staining of the coat and/or skin was apparent from week 1 of treatment in mice receiving 50000 ppm and from week 2 of treatment in mice receiving 12500 ppm and males receiving 780 or 3125 ppm. Similar staining was also noted in females receiving 3125 ppm during the period taken to complete the terminal sacrifice. In addition, yellow staining of the cage bedding was noted from the first week of treatment for mice receiving 3125 ppm or greater and bright yellow colouration was noted from week 2 of all treated groups.

Signs of ill health (including lethargy, body tremors, thin or hunched appearance, pilo-erection and pallor) were noted in some mice receiving 50000 ppm from week 2 of treatment, with evidence of recovery in the majority of animals being present by the final week.

2. Body weight / food consumption / Substance intake

Body weight loss or stasis and a markedly lower food intake were noted for mice receiving 50000 ppm during the first week of treatment compared to controls. Thereafter the bodyweight gains and food intake were higher than controls, although, the overall body weight gain and efficiency of food utilisation were markedly lower than controls. During the first three days of treatment only, the weight gains of other treated groups were marginally lower than controls. However, since the overall weight gain of these groups was similar to controls this difference is considered not to be of toxicological importance.

Table 5.3.1- 1: Aclonifen - 28-day toxicity study in mice – Bodyweight and food consumption

	Males					**Females**				
Diet concentration (ppm)	**0**	**780**	**3125**	**12500**	**50000**	**0**	**780**	**3125**	**12500**	**50000**
Bodyweight - necropsy (g)	35.3	32.9	34.2	33.7	**29.2** (↓17%)**	27.1	24.7	25.9	25.6	**21.6** (↓20%)**
Bodyweight gain Day 0-28 (g)	9.5	8.2	7.8	8.5	**3.5*** (↓63%)**	4.1	3.3	3.8	3.6	**0.2*** (↓95%)**
Bodyweight gain Day 0-28 as % of control	-	86	82	89	37	-	80	93	88	5
Food consumption (g/week)	137	134	138	143	136	132	128	127	127	147
Food conversion ratio (weeks 1-4)	14.1	16.4	17.6	16.8	43.1	31.8	38.4	33.1	35.5	263.7
statistically significant: ** $p < 0.01$; *** $p < 0.001$ **bold** : considered to be treatment-related										

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Table 5.3.1- 2: Aclonifen - 28-day toxicity study in mice – Achieved intake

	Males				**Females**			
Diet concentration (ppm)	**780**	**3125**	**12500**	**50000**	**780**	**3125**	**12500**	**50000**
Substance-intake (mg/kg /day)	121	481	2003	8906	143	555	2335	12403

4. HPLC plasma analysis

No aclonifen was found in the plasma of treated animals, but an unknown compound structurally similar to aclonifen was found in a dose-related concentration in the plasma of treated animals. This compound could be either a metabolite or a degradation product of aclonifen. The concentration of the presumed metabolite in plasma increased with dosage (see table below). The metabolite had a similar absorption spectrum to aclonifen but was of greater polarity. The apparent absence of aclonifen in any plasma sample indicates extensive metabolism or degradation following oral ingestion in the diet.

A difference in sex metabolism was noted at the top dose level, with males having the highest plasma levels of the unknown metabolite (which was statistically significantly higher $p < 0.001$ in males compared to females).

Table 5.3.1- 3: Aclonifen - 28-day toxicity study in mice – Plasma analysis by HPLC –Group mean metabolite concentration

	Males					**Females**				
Diet concentration (ppm)	**0**	**780**	**3125**	**12500**	**50000**	**0**	**780**	**3125**	**12500**	**50000**
Metabolite concentration (µg equivalent of Aclonifen/ml ± standard deviation)	0	10 ± 20	60 ± 0	70 ± 10	370 ± 90	0	0	50 ± 20	70 ± 10	130 ± 30

5. Gross pathology / Organ weights / Histopathology

Absolute and relative liver weight was increased in both sexes at 12500 and 50000 ppm and there was a decrease in absolute and relative ovary weights in females receiving 50000 ppm. There was a marked reduction in body weight in the 50000 ppm dose group therefore other changes in organ weights were considered not to be of toxicological significance.

Histopathological examination revealed treatment-related effects in the kidneys, liver and ovaries. Changes in the kidney comprised moderate to marked tubular dilation and, occasionally, proximal tubular necrosis, cystic tubules or basophilic epithelium, are indicative of a nephrotoxic potential of aclonifen at 12500 and 50000 ppm. There were no treatment-related findings in the thyroid or pancreas.

In the liver, treatment-related changes were noted for all treated male groups and for females which had received 3125 ppm and higher: these comprised, for animals receiving 50000 ppm panacinar hepatocytic enlargement (i.e. throughout the liver), and for the other affected groups, periacinar hepatocytic enlargement. These changes were considered to represent an adaptive response to the test item as there was no indication of liver damage. The ovaries of female mice receiving 50000 ppm had fewer luteal cells than their respective controls and a reduction in absolute and relative ovary weight. This was considered to suggest an interference with or failure to commence normal ovarian cycling.

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Table 5.3.1- 4: Aclonifen - 28-day toxicity study in mice – Organ weights and histopathology

	Males					Females				
Diet concentration (ppm)	**0**	**780**	**3125**	**12500**	**50000**	**0**	**780**	**3125**	**12500**	**50000**
Absolute and relative organ weights										
Numbers weighed	11	12	12	**12**	**10**	11	12	12	**12**	**9**
Liver weight (g)	2.07	1.78	2.06	**2.46*** **(↑19%)**	**2.67**** **(↑29%)**	1.52	1.35	1.51	**1.89**** **(↑24%)**	**1.65** **(↑9%)**
% bw	5.82	5.39	6.0	**7.30**** **(↑25%)**	**9.14**** **(↑57%)**	5.57	5.44	5.83	**7.35*** **(↑32%)**	**7.60*** **(↑36%)**
Kidney weight (g)	0.63	0.57	0.60	0.65	0.6	0.38	0.38	0.37	0.40	0.41
% bw	1.78	1.75	1.75	**1.93**	**2.05**** **(↑15%)**	1.42	1.51	1.43	**1.56**	**1.89**** **(↑33%)**
Ovarian weight (g)	/	/	/	/		0.011	0.012	0.011	0.011	**0.005**** **(↓55%)**
% bw	/	/	/	/		0.040	0.048	0.041	0.045	**0.022**** **(↓45%)**
Testes weight (g)	218	233	224	229	223	/	/	/	/	/
% bw	6210	7103	6598	6838	7642** (↑23%)	/	/	/	/	/
Histopathology										
Number examined	*12*	*12*	*12*	*12*	*11*	*12*	*12*	*12*	*12*	*12*
Liver - Periacinar hypertrophy	1	6	**9****	**9****	**0**	0	0	3	**11*****	**2**
Liver - Panacinar hypertrophy	0	0	0	**3**	**11*****	0	0	0	0	**10*****
Kidneys - Necrosis proximal Convoluted tubules	0	0	0	1	3	0	0	0	0	2
Kidneys - Cortical tubular dilatation	2	2	1	2	**6**	1	1	2	3	**8****
Kidneys - Basophilic cortical tubules	2	0	1	5	**7***	0	0	2	2	**8****
Ovaries - Luteal deficiency	-	-	-	-	-	2	1	0	0	**12*****

statistically significant: * p < 0.05, ** p < 0.01; *** p < 0.001; **bold** : considered to be treatment-related

III. CONCLUSION

The NOAEL in this 28-day study in the mouse was 780 ppm equivalent to 121 or 143 mg/kg/day in males and females respectively, based on liver changes at 3125 ppm.

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Assessment and conclusion by applicant:

This was a well-conducted GLP study, that is similar to OECD 407 (2008) but with significant omissions and additions to the protocol. It is acceptable as a range-finding study for the mouse carcinogenicity study.

The NOAEL was 780 ppm (121/143 mg/kg bw/day) based on increased absolute and relative liver weight, liver hypertrophy, and histopathological changes in the kidneys at 3125 ppm (481/555 mg/kg bw/day in males/females respectively).

Analysis of plasma samples collected on day 28 could not detect any levels of aclonifen, however a dose-related increase in a similar structure (assumed to be an aclonifen metabolite) was detected, indicating absorption of the test substance. The apparent absence of un-metabolised aclonifen in any plasma sample indicates extensive metabolism or degradation of aclonifen following oral ingestion in the diet, and correlates with the lack of un-metabolised aclonifen detected in the urine in the rat metabolism studies.

Assessment and conclusion by RMS:

CA 5.3.2 Oral 90-day study

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Data Point:	KCA 5.3.2/01
Report Author:	■
Report Year:	1982
Report Title:	KUB 3359 Subchronic toxicity study in rats with administration for 13 weeks
Report No:	C030416
Document No:	M-174843-01-2
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current guideline: OECD 408, 2018 T3, T4 and TSH, LDL and HDL and blood albumin not measured. It was not stated whether animals were fasted prior to blood sampling and necropsy. Organs not weighed: prostate weighed without seminal vesicles and coagulating gland. Epididymides and uterus not weighed. Histopathological examination not conducted on parathyroid, lungs, thymus, cervix, vagina, epididymis, seminal vesicles, coagulating glands, spinal cord and peripheral nerve and lymph nodes. Oestrus cycle not determined. Animals were 10 weeks old at start of study whereas guideline requires they should not be more than 9 weeks old. A higher number of animals per dose was used than stipulated in the guidelines. Animals were housed individually whereas for animal welfare reasons animals should be group housed. Photoperiod is not mentioned. Det1ailed weekly clinical observations and functional observations including motor activity were not conducted. No ophthalmological examination. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The test compound, aclonifen (KUB3359, purity 96.2%) was administered via the diet for 90 days to groups of albino rates, (12/sex/group), at concentrations of 0 or 50, 500 or 5000 ppm (equivalent to 0, 2.61/2.94, 26.41/29.43, 258/279 mg/kg bw/day in males/females respectively). Satellite groups of animals treated at 0 and 5000 ppm were sacrificed after a recovery period of 8 weeks

The only signs of toxicity were seen in the top dose of 5000 ppm, where the main target organ was the kidneys and urinary system, with effects being markedly more severe in males. Non-specific toxic effects were a marked reduction in food consumption and body weight gain in both sexes that was largely reversible in the recovery period. Urinalyses revealed substance-induced hematuria and haemoglobinuria in males, still present in the recovery period. Water intake was strongly increased in males, and slightly increased in females. During the recovery period the increase was reversible in females, and showed signs of recovery in males. In both sexes the most marked clinical chemistry finding was a statistically significant increase in alanine aminotransferase (ALT). Macroscopic examination revealed enlarged kidneys with granulated to roughly irregular surfaces mainly in males, and to a lesser extent in females. Absolute and relative kidney weight was also markedly increased in males and microscopic examination revealed all animals in both sexes had nephropathy in the kidney tubules which was more severe in males. An additional finding was a marked increase in relative liver weight in both sexes, and an increase in absolute liver weight in males.

No adverse findings were seen at 500 and 50 ppm.

The NOAEL in this study was 500 ppm (equating to 26.4 and 29.4 mg/kg/day in males and females, respectively) based on adverse findings in the kidneys and bladder.

The study was not performed to GLP and has some deviations from the OECD guideline 408. These deviations do not invalidate the study.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen (KUB 3359)
Lot/Batch:	T5/81
Purity:	96.2%
Appearance:	Not mentioned
Stability of test compound:	Fresh batches of aclonifen incorporated into the diet were prepared weekly. The substance is stable in the mixtures at room temperature for at least 8 days (no reference to any analysis in this study to demonstrate this, but analysis in other dietary studies have demonstrated aclonifen is stable in the diet for at least 8 days).

2. Vehicle and /or positive control Aclonifen was incorporated into pulverised rodent diet

3. Test animals

Species and strain:	Rat – Albino Chbbt:THOM (SPF)
Source:	[redacted]
Number of animals	12 per sex/dose group
Sex:	Male + Female
Age:	74 ± 2 days of age
Weight at treatment:	Mean males: 321g; Mean females: 207g (day of first treatment)
Acclimation period:	4 weeks
Diet:	FUKO 8014 fortified maintenance diet ([redacted]); *ad libitum*
Water:	Filtered water adjusted to pH 3, *ad libitum*
Cage type:	Makrolon cages, Type III
Housing:	Individually
Environmental conditions:	Temperature: 24 ± 2°C Humidity: 55 (50-65) % Air changes: yes, but number of changes/hour not mentioned

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Photoperiod: not mentioned

Study Design and methods

1. In life dates

20 July to 15 December 1981.

2. Animal assignment and treatment

Reasons for dose selection: The doses were selected based on a range finding study where the following concentrations were given: 0, 25, 250 and 2500 ppm in the diet for 4 weeks. No clinical, clinico-chemical or haematological changes occurred, except for a yellow discoloration of the urine, therefore the study was continued with doses of 0, 1000, 4.000 and 10 000 ppm for another 4 weeks. During the latter period the food consumption in the highest dose group dropped considerably, and blood was detected in the urine of the males of this group. Macroscopically and microscopically these animals showed inflammatory kidney changes. Based on these findings the dose levels of the present study, were set at 0, 50, 500 and 5000 ppm, a toxicological no-effect level was aimed at in the lowest dosage. In the highest dosage pronounced toxic reactions and in the medium dosage discrete substance-related effects were expected.

Procedures: In the main study aclonifen was administered orally via the diet to groups rats for 13 weeks (12 animals/sex/dose group) at dose levels of 0 or 50, 500 or 5000 ppm (equivalent to 2.61/2.94, 26.41/29.43, 258/279 mg/kg bw/day).

In addition two groups of 12 male and 12 female rats were administered 0 or 5000 ppm for 13 weeks and then maintained on control diet for a further 8 weeks recovery period.

In weeks 1, 4, 7 and 12 the substance concentrations were examined analytically (the results are not reported in the study report).

3. In-life observations

Animals were observed twice daily for signs of toxicity. Food consumption (per cage) and individual body weight were recorded weekly. Water consumption was determined in weeks 4, 7, 11, 15 and 19. A full haematology and clinician chemistry examination was performed on blood samples taken prior to treatment in week 1, and in week 7 and 13, and in the recovery animals prior to necropsy (week 21). Urinalyses was performed in study weeks 4, 8, 10, 12 of main study, and weeks 16 and 20 of the recovery period. Haematology, clinical chemistry and urine investigations were largely in accordance with OECD 408, 2018, except it was not stated if the animals were fasted prior to blood sampling, and blood albumin was not determined.

4. Investigations at necropsy

All animals were necropsied by i.p. injection of pentobarbital-Na and exsanguinated. It was not stated if animals were fasted prior to necropsy. A gross pathological examination was performed. Organs weighed were the lungs, thymus, thyroid, liver, kidneys, heart, spleen, prostate, gonads, adrenals, pituitary, brain and salivary glands. Representative tissue specimens from a wide range of organs were fixed and examined by light microscopy (largely in accordance with OECD 408, 2018 with exception that spinal cord and peripheral nerves and lymph nodes were not examined). In addition to H & E staining, the heart, lungs, liver, kidney and spleen were stained with Berlin blue to show levels of iron. The heart, liver, kidney and adrenals were stained with fatty red 7B to show lipids.

5. Statistics

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The statistical, evaluation was performed using the BARTLETT test, the simple variance analysis and the NEWMAN-KEULS test. The statistical tests were performed with a number of items > 4 for each biological parameter per time of examination and sex for investigating significant differences between the dose groups.

II. RESULTS AND DISCUSSION

1. General observation/clinical signs/ Mortality

Two animals from the control group died; one male most likely due to urinary retention and one female due to anaesthesia for blood sampling.

As early as 4 hours after the commencement of the treatment period a deep yellow coloration of the urine was observed in animals receiving 5000 ppm. In animals receiving 500 ppm this observation was made during the second week of the study. After 9 weeks of treatment the hair of the anogenital region and abdominal region of animals receiving 5000 ppm showed a deep yellow discolouration. Blood red urine was discharged by three males in the 5000 ppm dose during the recovery period.

2. Body weight / food consumption / Substance intake

Decreased body weight gain and food consumption were observed in both sexes during the treatment period in the top dose group. Water intake was markedly increased in males receiving 5000 ppm, but started to recover following withdrawal of treatment in the recovery group. In females there was a slight increase in water consumption at 5000 ppm which was reversible within the recovery period.

Table 5.3.2- 1: Aclonifen - 90-day toxicity study in rats – Bodyweight and food consumption

Sex	**Males**						**Females**					
Study duration	**13 Weeks**				**13 Weeks + 8 week recovery**		**13 Weeks**				**13 Weeks + 8 week recovery**	
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**5000**	**0**	**50**	**500**	**5000**	**0**	**5000**
Bodyweight - necropsy (g)	469.8	480.8	471.5	**395.5** (↓16%)**	495.0	**442.6** (↓10%)**	264.7	266.3	255.5	**232.1** (↓12%)**	276.8	261.9
Food consumption (g/week)	151.2	150.4	153.6	**128.7* (↓15%)**	133.1	133.5	103.9	98.7	97.1	**86* (↓18%)**	95.3	93.7
Water consumption (g/week)	217.5	210.3	224.5	**342.7 (↑58%)**	193.7	**259.9 (↑34%)**	153.3	151.2	152.4	**174.5 (↑38%)**	155.3	160.3

statistically significant * p < 0.05, ** p < 0.01, *** p < 0.001;
bold : considered to be treatment-related; - = no finding;
Measurements taken in week 13 (week 21 for recovery animals)

Table 5.3.2- 2: Aclonifen - 90-day toxicity study in rats – Achieved intake

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	Males			Females		
Diet concentration (ppm)	**50**	**500**	**5000**	**50**	**500**	**5000**
Substance-intake (mg/kg /day)	2.61	26.4	258	2.94	29.43	279

3. Haematology / Clinical chemistry / Urinalysis

Haematology and clinical chemistry analysis were conducted in prior to treatment and in weeks 1, 7, 13 and 21. Aclonifen administration had no effect on haematological parameters. Statistically significant changes in clinical chemistry parameters were a slight increase in alanine aminotransferase (ALT) values in males and females receiving 5000 ppm. Slight increases in alkaline phosphatase (AP) and bilirubin as well as a decrease in cholesterol levels were noted in females receiving 5000 ppm. There were no changes in aspartate aminotransferase in either sex. Slight decreases in glucose and total protein serum levels were seen in the top dose group. These findings were largely reversible in the recovery group.

Urinalysis revealed treatment-related haematuria and haemoglobinuria in males receiving 5000 ppm, an effect that was also present in the recovery period. The haematuria in males correlated with macroscopic changes in the kidneys.

Table 5.3.2- 3: Aclonifen - Oral 90-day toxicity study in rats - Clinical chemistry and urinalysis

Sex	**Males**						**Females**					
Study duration	**13 Weeks**				**13 Weeks + 8 week recovery**		**13 Weeks**				**13 Weeks + 8 week recovery**	
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**5000**	**0**	**50**	**500**	**5000**	**0**	**5000**
	Clinical chemistry											
Alanine aminotransferase (ALT) (u/L)	28.48	28.39	32.10	**33.17 (↑16%)**	25.04	30.39 (↑21%)	25.83	23.41	25.39	**33.58** (↑30%)**	26.51	25.77
Alkaline Phosphatase (AP) (u/L)	179.3	176.3	172.3	157.7*	175.18	204.33	114.0	110.7	115.5	**150.5** (↑32%)**	136.75	154.5
Bilirubin	3.36	2.84	2.46	3.43	3.22	2.62	2.506	2.124	2.851	**3.563** (↑42%)**	2.98	3.12
Cholesterol (mmol/L)	1.721	1.757	1.714	1.960	1.75	1.71	1.602	1.567	1.535	**1.256** (↓22%)**	1.61	1.668
Glucose (mmol/L)	5.946	6.438	6.082	6.133	6.53	6.382	6.192	6.646 *	5.689*	5.543**	6.64	6.970
Total protein	61.4	62.0	57.8*	**58.8** (↓4%)**	65.01	62.63* (↓4%)	67.7	65.6	63.0**	**65.2* (↓4%)**	67.95	66.00
	Urinalysis											
Haemoglobin	0	0	0	11/12	0	10/12	0	0	0	0	0	1/12
Erythrocytes	0	0	0	9/12	0	5/12	0	0	0	0	0	0

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statistically significant: * p < 0.05, ** p < 0.01, *** p < 0.001;
bold : considered to be treatment-related; - = no finding;
Clinical chemistry measurements taken in week 13 (week 21 for recovery animals)
Urinalyis measurements taken in week 12 (week 21 for recovery animals)

4. Gross pathology / Organ weights / Histopathology

Gross necropsy revealed enlarged kidneys with granulated to roughly irregular surfaces predominantly in males, although present in females receiving 5000 ppm. Changes in the urinary bladder were seen in top dose males but not in females. Five males had blood clots, and in the recovery group one had bloody urine, another with reddish sand-grain like formations, and another had a protein plug, and another had a solid brownish body 6mm x 3 mm in size.

Organ weight analysis revealed increased absolute and relative kidney weights in males, and increased relative liver weight in males and females receiving 5000 ppm. Absolute liver weight was only marginally increased in both sexes and did not reach statistical significance. In males of the 5000 ppm group significantly reduced absolute weights of heart, lung, spleen and in females receiving the same concentration a significant reduction in absolute heart and adrenal weights were noted but considered to be not relevant as they are a consequence of the reduced body weight.

Table 5.3.2- 4: Aclonifen - Oral 90-day toxicity study in rats – Organ weight

Sex	Males						Females					
Study duration	**13 Weeks**				**13 Weeks + 8 week recovery**		**13 Weeks**				**13 Weeks + 8 week recovery**	
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**5000**	**0**	**50**	**500**	**5000**	**0**	**5000**
Bodyweight - necropsy (g)	469.8	480.8	471.5	**395.5** (↓16%)**	495.0	**442.6** (↓10%)**	264.7	266.3	255.5	**232.1** (↓12%)**	276.8	261.9
Liver weight (g)	13.19	13.60	13.60	**14.18 (↑8%)**	13.12	11.47** (↓13%)	7.94	5.44	7.54	8.10 (↑2%)	8.32	7.64* (↓8%)
Liver weight % bw	2.79	2.83	2.88	**3.59** (↑29%)**	2.65	2.59	3.00	2.84	2.95	**3.49** (↑16%)**	2.95	2.92
Kidney weight (g)	2.69	2.79	2.81	**3.77** (↑40%)**	2.76	2.96	1.80	1.82	1.72	1.64*	1.81	1.78
Kidney weight % bw	0.57	0.58	0.60	**0.96* (↑68%)**	0.56	**0.67** (↑20%)**	0.68	0.69	0.67	0.71	0.66	0.68

statistically significant: * p < 0.05, ** p < 0.01, *** p < 0.001;
bold : considered to be treatment-related; - = no finding;

The only treatment-related changes noted during histopathological examination were confined to the kidneys of animals in the high dose group. Histologically examination of the kidneys revealed the occurrence of tubular nephropathy with haematuria, hydronephrosis and consecutive interstitial nephritis (chronically recurring pyelonephritis) which were a probable consequence of the formation

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of brownish deposits in the tubular epithelium. The brownish deposits appeared to enter the tubular lumen, forming aggregates which were also observed in the renal pelvis and urinary bladder. These changes were pronounced in males of both the treated and reverse phase groups. Female animals showed a clearly weaker and reversible nephropathy with moderate hydronephrosis, missing haematuria or interstitial nephritis.

Table 5.3.2- 5: Aclonifen - Oral 90-day toxicity study in rats – Histopathology findings

Sex	Males						Females					
Study duration	**13 Weeks**				**13 Weeks + 8 week recovery**		**13 Weeks**				**13 Weeks + 8 week recovery**	
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**5000**	**0**	**50**	**500**	**5000**	**0**	**5000**
Grade	Kidney tubular nephropathy											
Not detected	12	12	12	0	12	0	12	12	12	0	12	12
Minimal	0	0	0	0	0	0	0	0	0	0	0	0
Mild	0	0	0	0	0	6	0	0	0	6	0	0
Moderate	0	0	0	8	0	5	0	0	0	6	0	0
Severe/very severe	0	0	0	4	0	1	0	0	0	0	0	0

III. CONCLUSION

In this 90-day study in the rat the NOAEL in this study was 500 ppm (equating to 26.4 and 29.4 mg/kg/day in males and females, respectively.

Assessment and conclusion by applicant:

The study was not conducted to GLP and is similar to OECD 408 (2018) but with some omissions and additions.

The NOAEL in this 90-day study in the rat was 500 ppm (equating to 26.4 and 29.4 mg/kg/day in males and females, respectively) based on reduced food consumption, reduced body weight gain, and nephropathy in both sexes at 5000 ppm (258/279 mg/kg bw/day).

Assessment and conclusion by RMS:

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Data Point:	KCA 5.3.2/02
Report Author:	████
Report Year:	1997
Report Title:	Aclonifen - 90-day toxicity study in the rat by dietary administration
Report No:	R007437
Document No:	M-174924-01-1
Guideline(s) followed in study:	EU (=EEC): 92/69 Annex V B26; OECD: 408; USEPA (=EPA): 82-1
Deviations from current test guideline:	Current Guideline: OECD 408, 2018 Minor deviations. Seminal vesicles and coagulating glands not weighed. Histopathology not conducted on cervix and coagulating glands. T3, T4 and TSH, LDL and HDL not measured. Oestrus cycle of females not determined. Oestrus cycle of females not determined. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

Aclonifen (batch 95-335-02, purity 99.9%) was administered via the diet for 90 days to groups of Sprague-Dawley rats (10/sex/group) at concentrations of 0, 50, 500 and 5000 ppm (equivalent to 0, 3.56/4.15, 35.4/40.8, 341/390 mg/kg bw/day in males/females respectively).

At the top dose of 5000 ppm the main target organs were the kidney, thyroid and liver, with findings being more severe in males. In females at 5000 ppm compared to controls there was a 19% reduction in bodyweight at necropsy, and a reduction in food consumption throughout the study (22% reduction in week 13), but a similar effect was not seen in males. In males at 5000 ppm haematology findings were 10% reduction in red blood cells, a 7% reduction in haematocrit, a 6% reduction in haemoglobin, and mean corpuscular haemoglobin increased by 4%. In clinical chemistry, urea, cholesterol and albumin were increased in males at 5000 ppm. Urine samples presented an abnormal yellow fluorescent colour in both sexes at 500 and 5000 ppm. Crystals, different from those usually observed in urines were noted at 5000 ppm in 5/10 males and 1/10 female. Mean urinary volume was increased in males at 5000 ppm, occult blood with red blood cells were noted in 2/10 males at 5000 ppm and white blood cells in 2/10 males at 5000 ppm. At 5000 ppm in both sexes an increased absolute (37%/12% in M/F) and relative higher liver weight (39% in M & F) with hepatocellular hypertrophy in all males and all but one female. At 500 ppm a centrilobular hypertrophy in the liver of a single rat. Males also had an increase in absolute (14%) and relative (16%) kidney weight. with transitional cell hyperplasia of the pelvis in males and females, necrosis of the papilla in two males. Thyroid follicular hypertrophy was observed in a high proportion of rats of both sexes at 5000 ppm, and in 7 males and 3 females at 500 ppm.

The NOAEL was 50 ppm in both sexes (approximately 3.6 and 4.2 mg/kg/day) in males and females respectively based on follicular cell hypertrophy in the thyroid in both sexes.

The study was performed to GLP and OECD 408 (although there are some omissions in meeting the current 2018 guidelines).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen
Lot/Batch:	95-335-02
Purity:	99.9 %
Appearance:	Yellow powder
Stability of test compound:	The test substance formulations given to the animals were freshly prepared every three weeks and stored at approximately -18°C and defrosted one day before use. Acceptable stability of aclonifen in the diet was demonstrated for the lowest and highest concentrations from samples that were frozen at approximately -18°C for 4 weeks, then defrosted and kept one week at ambient temperature before analysis.

2. Vehicle and /or positive control

Test substance was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required dietary concentrations.

3. Test animals

Species and strain:	Rat – Sprague Dawley rats Ico: OFA. SD. (IOPS Caw)
Source:	[redacted]
Number of animals:	45 M + 45 F
Sex:	Male + Female
Age:	6 - 7 week old
Weight at treatment:	Males: 171-197 g; Females: 135-167g (first day of treatment)
Acclimation period:	8 days
Diet:	Rodent diet AO4C PI ([redacted] [redacted]); *ad libitum*
Water:	Filtered and softened water, *ad libitum*
Cage type:	Stainless steel, wire mesh cages
Housing:	Individually
Environmental conditions:	Temperature: 20-24°C Humidity: 40-70 % Air changes: 10-15 changes/hour Photoperiod: 12-hour light, 12-hour dark cycles

Study Design and methods

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1. In life dates

March 13 to June 21, 1996.

2. Animal assignment and treatment

Aclonifen was administered orally in the diet at concentrations of 0, 50, 500 and 5000 ppm to groups of rats (10/sex/group) for at least 90 days. Clinical signs were checked daily; body weight, food consumption and detailed physical examinations were recorded weekly. Ophthalmological examinations were performed on all animals during the acclimatisation phase and on all animals of the control and high dose groups during week 12. The week before necropsy a fasting blood sample was collected from the retro orbital venous plexus of each animal for haematology and clinical chemistry determinations. Prior to necropsy food and water were removed overnight and overnight urine samples were collected.

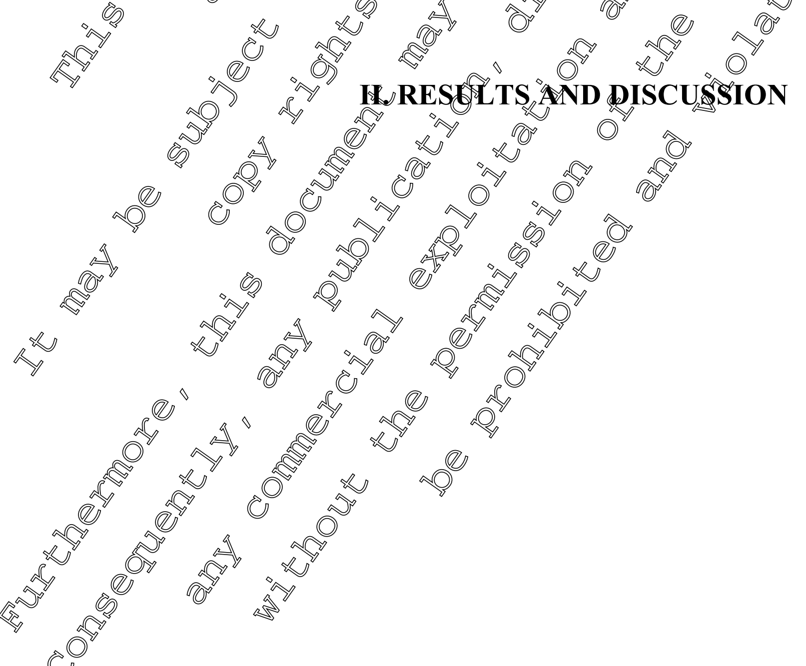
All animals were necropsied by exsanguination under deep anaesthesia, gross pathology examination was conducted, selected organs weighed and a range of tissues were taken, fixed and examined microscopically.

3. Statistics

Statistical analyses were performed using SAS programs.

Data were inter-compared for the exposed groups and the control group with PathTox computer system by use of DUNNETT'S test if BARTLETT'S test indicated homogeneous variances. If BARTLETT'S test indicated non-homogeneous variances, variables were analysed using SAS programs and a non-parametric statistical procedure was performed using the KRUSKAL-WALLIS non-parametric one-way analysis of variance by ranks. If the KRUSKAL-WALLIS test was significant, MANN-WHITNEY'S test was used to compare each group to the control.

The levels of signification for each statistical comparison were *0.05 and **0.01.



II. RESULTS AND DISCUSSION

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1. Analysis

Analytical method to determine aclonifen in the rodent diet fulfils nearly all SANCO 3029 criteria, with minor exception regarding precision data; nevertheless, method is fit for purpose. Homogeneity of the 50 and 5000 ppm preparations from the first formulation was checked from samples at the top middle and bottom. Acceptable homogeneity was achieved (84 to 104% of target dose for low dose samples, 90 to 93% of target dose for high dose samples). One concentration for the homogeneity of the 50 ppm diet was slightly below the target range (84% instead of 85% recovery).

Samples were then stored for 35 days (4 weeks at -15°C and one week at room temperature) then analysed to check for stability. Stability analysis showed aclonifen levels were at 83 to 91% of target concentrations after storage. One concentration for stability of the 50 ppm diet was slightly below the target range (83% instead of 85% recovery).

The concentration of all dietary preparations was checked. The results of concentration tests indicated that diet concentrations ranged from 86 to 102% of target doses.

Despite two results below target concentrations the homogeneity, concentration and stability of aclonifen in the diet were considered satisfactory.

2. General observation/clinical signs/ Mortality

There were no mortalities at any dose level during the study. The only treatment-related clinical signs observed were dark urine and soiled fur around the ano-genital area in animals treated at 500 and 5000 ppm.

No treatment-related ophthalmological abnormalities were observed.

3. Body weight / food consumption / Substance intake

Body weight gain and food consumption of the females treated at 5000 ppm were lower than those of controls throughout the study.

There was no effect on food consumption or weight gain in males at any dose level or in females at 50 or 500 ppm.

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Table 5.2.3- 3: Aclonifen - 90-day toxicity study in rats – Bodyweight and food consumption

	Males				Females			
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**50**	**500**	**5000**
Bodyweight - necropsy (g)	433	431	448	426	285	274	282	**230**** **(↓19%)**
Food consumption – week 13 (g/day)	22.6	24.6	23.8	22.2	18.5	17.2	18.7	**14.5*** **(↓22%)**
statistically significant: * p < 0.05, ** p < 0.01 **bold** : considered to be treatment-related								

Table 5.2.3- 4: Aclonifen - 90-day toxicity study in rats – Achieved intake

	Males			Females		
Diet concentration (ppm)	**50**	**500**	**5000**	**50**	**500**	**5000**

Substance-intake (mg/kg /day)	3.56	35.4	341	4.15	40.8	390

4. Haematology / Clinical chemistry / Urinalysis

In haematology a slight decrease in red blood cell count, decreased haemoglobin, decreased haematocrit and an increase in corpuscular haemoglobin was noted in males at 5000 ppm. The decrease in red blood cells of 4% at 500 ppm is not considered to be toxicologically adverse.

In clinical chemistry increased urea, cholesterol and albumin in males at 5000 ppm were considered to be treatment-related. Other minor changes are considered to be not toxicologically relevant or within normal ranges.

The urine samples collected at the end of the study presented an abnormal fluorescent yellow colour in both sexes at 500 and 5 000 ppm. Crystals, different from those usually observed in urine were noted at 5000 ppm in 5/10 males and 1/10 female. Mean urinary volume was increased in males at 5 000 ppm, occult blood with red blood cells were noted in 2/10 males at 5000 ppm and white blood cells in 2/10 males at 5 000 ppm.

Table 5.2.3- 5: Aclonifen - 90-day toxicity study in rats – Haematology, clinical chemistry and urinalysis

	Males				**Females**			
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**50**	**500**	**5000**
				Haematology				
Red Blood Count (10^{12}/L)	9.306	9.041	8.914* (↓4%)	**8.348** (↓10%)**	8.271	8.524	9.338	8.173
Mean Corpuscular Haemoglobin (pg)	16.86	17.22	17.25	**17.61* (↑4%)**	18.13	17.85	17.96	17.89
Haematocrit	0.471	0.464	0.457	**0.437* (↓7%)**	0.441	0.445	0.442	0.428
Haemoglobin g/100mL	15.68	15.56	15.36	**14.69** (↓6%)**	14.99	15.20	14.97	14.59
				Clinical biochemistry				
Urea (mmol/L)	4.494	4.589	4.780	**6.219** (↑38%)**	5.895	5.379	5.026	5.873
Cholesterol (mmol/L)	1.258	1.206	1.308	**2.363** (↑88%)**	1.530	1.325	1.451	1.665
Albumin (g/L)	37.4	37.0	38.4	**43.1**** (↑15%)	39.2	38.5	40.5	40.7
				Urinalysis				
Mean urinary volume (mL)	2.62	2.61	2.87	**5.41* (↑x2)**	2.53	1.08* (↓57%)	1.50	3.87
Occult blood	0	1	0	**2**	0	0	0	0
Red blood cells	0	0	0	**2**	0	0	0	0
White blood cells	0	0	0	**2**	0	0	1	**0**

statistically significant: * p < 0.05, ** p < 0.01;
bold : considered to be treatment-related

5. Gross pathology / Organ weights / Histopathology

At 5000 ppm all male and female rats showed yellowish-brown discoloration of the ventral fur, especially marked in the urogenital area. This change was associated with pale kidneys in a high proportion of rats of either sex, renal cortical yellowish deposits in three male rats. In addition, grossly enlarged livers were noted in male rats.

At 5000 ppm, liver weight was increased in both sexes and kidney weight was increased in males only.

Histopathology examinations revealed diffuse liver centrilobular hypertrophy in both sexes at 5000 ppm and centrilobular hypertrophy in one male at 500 ppm. The severity of the hypertrophy was minimal to moderate and the effect was more pronounced in males than in females.

Examination of the kidney revealed transitional cell hyperplasia of the pelvis in males and females, and necrosis of the papilla in two males at 5000 ppm.

In addition there was a dose-related higher incidence of thyroid follicular hypertrophy in both sexes at 500 and 5000 ppm, but there was no effect on thyroid weight.

Table 5.2.3- 6: Aclonifen - 90-day toxicity study in rats – Organ weight and histopathology

	Males				Females			
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**50**	**500**	**5000**
Bodyweight - necropsy (g)	433	431	448	426	285	274	282	**230**** **(↓19%)**
	Organ weights							
Liver weight (g)	10.59	11.01	11.56	**14.51**** **(↑37%)**	7.05	7.11	7.21	**7.89**** **(↑12%)**
Liver weight % bw	2.44	2.54	2.58	**3.40**** **(↑39%)**	2.48	2.57	2.59	**3.44**** **(39%)**
Kidney weight (g)	2.860	2.808	3.066	**3.261*** **(↑14%)**	2.002	1.787* (↓11%)	1.974	1.718** (↓14%)
Kidney weight % bw	0.660	0.654	0.686	**0.764**** **(↑16%)**	0.699	0.652	0.694	**0.748** (↑7%)
Thyroid weight (g)	0.020	0.020	0.023	0.024	0.018	0.027	0.018	0.019
Thyroid weight % bw	0.0047	0.0047	0.0050	0.0056	0.0064	0.0098	0.0062	0.0084
	Histopathology							
Liver – hepatocellular hypertrophy	0	0	1	**10**	0	0	0	**9**
Kidney – pelvis transitional cell hyperplasia, unilateral	0	0	0	**0**	0	1	2	**2**
Kidney – pelvis transitional cell hyperplasia, bilateral	0	0	1	**6**	1	1	0	**4**
Kidney – papillary necrosis (bilateral)	0	0	0	**2**	0	0	0	**0**
Thyroid – follicular cell hypertrophy	2	1	**7**	**9**	0	0	**3**	**7**

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statistically significant: * $p < 0.05$, ** $p < 0.01$;

bold : considered to be treatment-related. Statistical analysis not conducted on histopathology findings.

III. CONCLUSION

The No Observed Adverse Effect Level (NOAEL) in this 90-day study in the rat was 50 ppm in both sexes, equating to approximately 3.6 and 4.2 mg/kg/day in males and females, respectively.

Assessment and conclusion by applicant:

The NOAEL in this 90-day study in the rat was 500 ppm (equating to 3.6/4.2 mg/kg bw/day in males/females respectively) based on follicular cell hypertrophy in the thyroid in both sexes at 500 ppm (35/41 mg/kg bw/day in males/females).

The study was conducted to GLP and OECD 408, although it does not investigate all the parameters stipulated in the current OECD 409 (2018) (in particularT3, T4 and TSH, LDL and HDL) apart from these omissions overall the study is acceptable.

Assessment and conclusion by RMS:

Data Point:	KCA 5.3.2/03
Report Author:	███
Report Year:	2001
Report Title:	90-Day toxicity study in the rat by dietary administration Aclonifen
Report No:	C017982
Document No:	M-205288-01-1
Guideline(s) followed in study:	EU (=EEC): 92/69, V, B26 (1992); JMAF: 59 NohSan 4200 (1985); OECD: 408 (1998); USEPA (=EPA): OPPTS 810.3100 (1998)
Deviations from current test guideline:	Current Guideline: OECD 408, 2018 LDL and HDL not measured. Oestrus cycle of females not determined. Seminal vesicles and coagulating glands not weighed. Not stated if histopathology was conducted on coagulation glands. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

Aclonifen (batch 97013/03, purity 99.2%) was administered via the diet for 90 days to groups of Wistar rats (10/sex/group) at concentrations of 0, 50, 500 and 5 000 ppm (equivalent to 0, 2.9/3.7, 29.4/36.3, 295/323 mg/kg bw/day in males/females respectively).

At the top dose of 5000 ppm, body weight and body weight gain and food consumption were consistently reduced throughout the study. Clinical chemistry evaluation and urinary analysis revealed changes in several clinical chemistry in males, but only minor changes in females. Assessment of plasma thyroid hormones revealed an increase in TSH and a decrease in T4 levels in both sexes. Follicular hypertrophy of the thyroid gland was observed in 4/10 males.

In males absolute and relative kidney weight were increased, and at the macroscopic examination, abnormal shaped kidneys with yellow foci were observed. Treatment-related microscopic findings consisted of extreme changes in the kidney of all males including corticotubular nephrosis, acute to chronic medullary nephritis and necrosis of the papillary tip.

In both sexes absolute and relative liver weight were increased, there were macroscopic changes in liver shape and colour, and at microscopic examination centrilobular hepatocellular hypertrophy was present in all animals, and in males foci of hepatocellular necrosis.

At 500 ppm. the only treatment-related finding was dark yellow urine observed in all animals from study Day 2 onwards and slight changes in thyroid hormone levels. There were no treatment-related findings at 50 ppm.

The NOAEL was 500 ppm in both sexes (equating to approximately 29.4 and 36.3 mg/kg/day in males and females, respectively).

The study was performed to GLP and OECD 408 (although there are some minor omissions in meeting the current 2018 guidelines).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen
Lot/Batch:	97013/03
Purity:	99.2%
Appearance:	Yellow powder
Stability of test compound:	Stability was checked after 3, 5 and 7 weeks at ambient temperature, and after 2, 4 and 6 weeks at below -15°C followed by one week at ambient temperature. Aclonifen formulation was found to be stable over 7 weeks at ambient temperature.

2. Vehicle and /or positive control	Test substance was ground to a fine powder then incorporated into the diet by dry mixing to the required dietary concentrations.

3. Test animals

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Species and strain:	Wistar (AF) rats RJ: WI (IOPS AF)
Source:	
Number of animals	50 M + 50 F
Sex:	Male + Female
Age:	7 weeks old at start of treatment
Weight at treatment:	Males: 240-280 g; Females: 168-205 g (first day of treatment)
Acclimation period:	14 days
Diet:	Rodent powder diet "M 20 controle" (Pietrement, Provins, France); *ad libitum*, except before blood collections: fasting overnight.
Water:	Filtered and softened water, *ad libitum*
Cage type:	Stainless steel, wire mesh cages
Housing:	individually
Environmental conditions:	Temperature: 20-24°C Humidity: 40-70 % Air changes: 10-15 changes/hour Photoperiod: 12-hour light, 12-hour dark cycles

B. STUDY DESIGN AND METHODS

1. In life dates

September 27, 2000 to January 11, 2001

2. Animal assignment and treatment

Aclonifen (batch number 97013/03, 99.2% purity) was incorporated in rodent diet at concentrations of 50, 500 and 5000 ppm and prepared every three weeks.

Homogeneity, concentration and stability were checked on a pre-study mix at concentrations of 20 and 5000 ppm. Concentration was checked at all dose levels for each of the dietary preparations used on the study. Homogeneity was checked on the study mix at 50 and 5000 ppm, from the first formulation. Concentration was checked at all dose levels for each of the dietary preparations used in the study. When not in use the diet formulations were stored at approximately -15°C. Stability was checked after 3, 5 and 7 weeks at ambient temperature, and after 2, 4 and 6 weeks at below -15°C followed by one week at ambient temperature.

Groups of rats (10/sex/group) were administered aclonifen in the diet for a period of 90 days. Clinical signs were recorded daily, body weight and food consumption were measured weekly. A detailed physical examination was performed once during the acclimatisation phase and weekly throughout the study. In addition, a neurotoxicity assessment of the following reflexes were tested during the acclimatisation phase and in week 12: grasping, righting, corneal, pupillary, auditory startle and head shaking reflexes. Ophthalmologic examinations were performed on all animals during the acclimatisation phase and on all animals of the control and high dose groups during Week 12. The week before necropsy a blood sample was collected from each fasted animal for haematology and clinical chemistry determinations. In addition, a blood sample was collected during Weeks 2, 6 and 13

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from al1 fasted animals and frozen plasma samples prepared for T3, T4 and TSH analysis by radioimmunoassay by kit supplied by for TSH and for T3 and T4. At study termination, fasted urine samples were collected overnight from before necropsy from all animals. All animals were necropsied by exsanguination under deep anaesthesia, a gross pathology examination was conducted, selected organs were weighed, and a range of tissues were taken, fixed and examined microscopically.

4. Statistics

The treatment groups were compared using the following methods: Parametric ANOVA under the standard assumptions and robust linear regression methods (1986; 1986; 1967) which do not assume homogeneity of variance or normality. The homogeneity of variance assumption was examined via Levene's Test (1960). If Levene's test indicated lack of homogeneity of variance ($p<0.05$), robust linear regression methods were used to test all treatment effects. The robust regression methods use variance estimators that make no assumptions regarding homogeneity of variance or normality of the data. They were used to test for overall treatment group differences (via Wald chi-square tests), followed by individual t-tests for exposed vs. control group comparisons when the overall treatment effect was significant. If Levene's test did not reject the hypothesis of homogeneous variances, standard ANOVA techniques were applied for comparing the treatment groups. When a significant treatment effect was present each exposed group was compared to control via Dunnett's test.

II. RESULTS AND DISCUSSION

1. Analysis

Homogeneity tests conducted on the high and low dose indicated concentration range of 86 to 103% of the target concentrations. Tests on concentrations in the diet ranged from 86 to 100% of target concentration. Stability test concentrations after storage ranged from 86 to 113% of target concentrations and indicate that aclonifen is stable in the diet for over 7 weeks at ambient temperature.

All values were within the target range of 85-115% of the nominal concentrations. Analytical methods fulfilled SANCO 3029.

2. General observation/clinical signs/ Mortality

There were no mortalities during the study.

At 5000 ppm, treatment-related clinical signs consisted of dark yellow urine observed for all animals from study Day 2 onwards and soiled fur around the ano-genital area in the majority of animals. Neurotoxicity assessment revealed no treatment-related findings. No treatment-related ophthalmological abnormalities were observed.

3. Body weight / food consumption / Substance intake

Body weight and body weight gain were consistently reduced throughout treatment at 5000 ppm: At necropsy body weight was reduced by 14%/15% in males/females with overall body weight gain reduced by 25% in males and 41 % in females, compared with the controls. Food consumption was reduced throughout treatment in both sexes, the overall effect being a reduction of 13% in males and 21% in females.

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Table 5.2.3- 7: Aclonifen - 90-day toxicity study in rats – Bodyweight and food consumption

	Males				Females			
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**50**	**500**	**5000**
Bodyweight - necropsy (g)	498.1	491.6	490.5	**428.0*** (↓14%)**	264.1	267.2	2.58.8	**225.4*** (↓15%)**
Body weight gain: - weeks 1 – 13 (g)	263.9	254.1	253.1	**197.8** (↓25%)**	88.3	96.3	89.2	**52.2*** (↓41%)**
Food consumption - weeks 0 - 13 (g/day)	26.0	24.8	24.8	**22.5** (↓13%)**	18.0	18.8	17.8	**14.2*** (↓21%)**

statistically significant: * p < 0.05, ** p < 0.01, *** p < 0.001
bold : considered to be treatment-related

Table 5.2.3- 8: Aclonifen - 90-day toxicity study in rats – Achieved intake

	Males			Females		
Diet concentration (ppm)	**50**	**500**	**5000**	**50**	**500**	**5000**
Substance-intake (mg/kg /day)	2.9	29.4	294.5	3.7	36.3	322.5

4. Haematology / Clinical chemistry / Urinalysis

There were no relevant haematological findings.

Clinical chemistry evaluation revealed an increase in cholesterol, urea and creatinine concentration, and a decrease in potassium and globulin concentration in males. In addition, there was a slight increase in albumin concentration with a consequent increase in albumin/globulin ratio. A decrease in cholesterol concentration was observed in females, the slight increase in albumin in females was considered not clearly treatment related due to lack of a clear dose-response and the small magnitude of the change.

At the urinalysis examination an increase in urinary volume which was associated with lower pH and refractive index values was observed in males. At 5000 ppm red blood cells were found in 9/10 male urine samples and there was an increase in the number of white blood cells noted in 3/10 male urine samples. In females there was a slight increase in urinary volume at 5000 ppm but it did not achieve statistical significances. In both sexes at 5000 ppm dark yellow urine was observed in 8/10 males and 8/l0 females. At 500 ppm the only urinary finding was dark yellow urine in 2 males, and at 50 ppm dark yellow urine in 1 male.

Table 5.2.3- 9: Aclonifen - 90-day toxicity study in rats – Clinical chemistry and urinalysis

	Males				**Females**			
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**50**	**500**	**5000**
				Clinical biochemistry				
Urea (mmol/L)	4.81	5.23	4.90	**7.34** (↑53%)**	5.33	5.13	5.52	5.92
Creatinine (µmol/L)	30.9	30.8	30.7	**37.1* (↑20%)**	31.0	32.6	32.4	30.9

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Cholesterol (mmol/L)	1.908	2.171	2.212	**3.010*** (↑58%)**	2.187	1.842	1.916	**1.507*** (↓31%)**
Albumin (g/L)	40.8	40.7	42.1	**44.6*** (↑9%)**	42.1	43.4	41.5	45.1* (↑7%)
Globulin (g/L)	27.9	27.2	25.9	**21.3*** (↓24%)**	23.3	24.1	24.1	25.0
Albumin/globulin ratio	1.47	1.51	1.63	**2.10*** (↑43%)**	1.84	1.80	1.73	1.83
Potassium (mmol/L)	3.68	3.78	3.63	**3.39* (↓8%)**	3.32	3.24	3.38	3.66
	Urinalysis							
Mean urinary volume (mL)	4.09	4.51	3.57	**10.71*** (↑x2.5)**	2.44	2.71	1.95	**3.70 (↑52%)**
Refractive index (unit)	1.35	1.35	1.36	**1.34*** (↓1%)**	1.35	1.35	1.35	1.35
Urinary pH (unit)	6.30	6.40	6.05	**5.60****	5.50	5.55	5.50	5.85
Red blood cells	0	0	0	**9**	0	0	0	0
White blood cells	0	0	0	**3**	0	0	0	1
statistically significant: * p < 0.05, ** p < 0.01, *** p < 0.001; **bold** : considered to be treatment-related								

Assessment of the thyroid hormones T3, T4 and TSH revealed slight thyroid hormonal changes at 5000 ppm. These changes were clear decrease in T4 levels at Weeks 2, 6 and 13 in males and Weeks 2 and 6 in females, and an increase in TSH plasma levels in weeks 2, 6 and 13 in both sexes (though not statistically significant). Statistically significant decreases in T4 were also seen in males at 500 ppm in weeks 2 and 6.

Table 5.2.3- 10: Aclonifen - 90-day toxicity study in rats – Thyroid hormone analysis

	Males				**Females**			
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**50**	**500**	**5000**
	TSH (ng/mL)							
Week 2	7.208	7.260	12.377	**9.463 (↑31%)**	4.286	3.942	3.738	**5.821 (↑36%)**
Week 6	7.517	5.453	6.790	**9.462 (↑26%)**	2.989	3.569	3.755	**4.690 (↑57%)**
Week 13	9.828	7.304*	7.971*	**13.274 (↑35%)**	5.328	6.021	6.613	**6.859 (↑29%)**
	T4 (ng/mL)							
Week 2	30.942	30.920	25.015*** (↓19%)	**19.808*** (↓36%)**	21.940	23.127	20.692	**13.821** (↓37%)**
Week 6	34.469	31.951	28.319* (↓19%)	**20.743*** (↓40%)**	24.025	24.768	25.270	**17.841 (↓26%)**
Week 13	33.814	31.500	29.613	**22.018*** (↓35%)**	21.943	22.963	23.966	**14.482** (↓34%)**
	T3 (ng/mL)							
Week 2	0.325	0.388	0.453	0.453	0.393	0.395	0.462	0.365
Week 6	0.395	0.373	0.539	0.550	0.475	0.440	0.515	0.507
Week 13	0.445	0.359	0.433	0.297	0.375	0.334	0.360	0.383
statistically significant: * p < 0.05, ** p < 0.01, *** p < 0.001; **bold** : considered to be treatment-related								

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5. Gross pathology / Organ weights / Histopathology

At 5000 ppm, the absolute kidney weight was increased by 74% with relative kidney weight double that of control males, but there were no changes in female kidney weights. Absolute liver weight was increased by 25% in males and l8% in females with relative liver weight increased by 46% and 39% in males and females, respectively. The were no treatment-related changes in thyroid weight. An reduction in epididymis weight is likely to be secondary to reduced body weight as the relative epididymis weight was not affected.

At the macroscopic examination of the high dose animals, yellow foci were observed in the kidney of 6/10 males and the kidneys was abnormally shaped in 6/10 males. In addition, the livers of 2 males had rounded borders, whilst 4/10 females had dark coloured livers. Treatment-related microscopic findings consisted of extreme changes in the kidney of all males which included a corticotubular nephrosis, an acute to chronic medullary nephritis and a necrosis of the papillary tip. In the liver a centrilobular hepatocellular hypertrophy was noted in all animals of both species. In males foci of hepatocellular necrosis were seen in all dose groups and showed a dose-response. Follicular hypertrophy of the thyroid gland was also observed in 4/10 males in the top dose.

The spleen showed accumulation of haemosiderin in the controls and at 5000 ppm (intermediate doses were not fully investigated), but there was a slight increase in the severity of the accumulation of hemosiderin at 5000 ppm when compared to controls, and in males a higher incidence of cysts at the same dose. As these changes were isolated, it was considered of doubtful toxicological relevance.

Table 5.2.3- 11: Aclonifen - 90-day toxicity study in rats – Organ weights and gross pathology

	Males				**Females**			
Diet concentration (ppm)	**0**	**50**	**500**	**5000**	**0**	**50**	**500**	**5000**
Bodyweight - necropsy (g)	498.1	491.6	490.5	**428.0*** (↓14%)**	264.1	267.2	2.58.8	**225.4*** (↓15%)**
	Organ weights							
Liver weight (g)	11.60	11.58	11.72	**14.46*** (↑25%)**	6.19	6.16	6.27	**7.31*** (↑18%)**
Liver weight % bw	2.32	2.36	2.39	**3.38*** (↑46%)**	2.34	2.31	2.43	**3.25*** (↑39%)**
Kidney weight (g)	2.848	2.793	2.859	**4.947*** (↑74%)**	1.838	1.882	1.786	1.703
Kidney weight % bw	0.572	0.570	0.584	**1.154*** (↑x2)**	0.696	0.704	0.692	0.756
Thyroid weight (g)	0.023	0.023	0.027	0.024	0.018	0.018	0.018	0.018
Thyroid weight % bw	0.0047	0.0047	0.0054	0.0055	0.0069	0.068	0.0071	0.0080
Spleen weight (g)	1.073	1.081	0.998	1.095	0.771	0.769	0.758	0.619** (↓20%)
Spleen weight % bw	0.215	0.220	0.203	0.255* (↑19%)	0.292	0.287	0.293	0.275
Epididymis (g)	1.481	1.405	1.361* (↓8%)	1.320*** (↓11%)	-	-	-	-
Epididymis % bw	0.299	0.286	0.279	0.309	-	-	-	-
	Gross pathology							
Kidney – abnormal shape, and yellow foci	0	0	0	6	0	0	0	0
Liver - Dark	0	0	0	0	0	0	0	4
Liver - Rounded borders	0	0	1	2	0	0	0	0
	Histopathology							

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Liver – centrilobular hepatocellular hypertrophy	0	0	0	**10**	0	0	0	**10**
Liver – foci of hepatocellular necrosis, focal to multifocal	0	2	3	**5**	1	0	0	**0**
Kidney – corticotubular nephrosis	0	0	0	**10**	0	0	0	0
Kidney – medullary nephritis	0	0	0	**9**	0	0	0	0
Kidney – papillary necrosis (unilateral and/or bilateral)	0	0	0	**8**	0	0	0	0
Thyroid – follicular cell hypertrophy	0	0	0	**4**	0	0	0	0
Spleen – accumulation of hemosiderin, diffuse	9(10)	1(1)	1(3)	10(10)	10(10)	2(2)	3(3)	10(10)
slight	9	1	1	3	8	2	2	1
mild	0	0	0	7	2	0	1	9
Spleen – cyst (s), capsular	2(10)	1(1)	1(1)	4(10)	1(10)	2(2)	2(3)	0(10)

statistically significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$;

bold : considered to be treatment-related

When not all organs were examined the numbers examined are shown in brackets ( )

III. CONCLUSION

At 500 ppm, the only treatment-related finding was dark yellow urine observed in all animals from study Day 2 onwards. There were no treatment-related findings at 50 ppm.

The NOAEL of aclonifen in this 90-day study in the rat was 500 ppm in both sexes (equating to approximately 29.4 and 36.3 mg/kg/day in males and females, respectively).

Assessment and conclusion by applicant:

The NOAEL in this 90-day study in the rat was 500 ppm (equating to 29.2/36.3 mg/kg bw/day in males/females respectively) based on toxicity findings at 5000 ppm (295/323 mg/kg bw/day) which included reduced body weight gain and food consumption, with the main target organs being the liver in both sexes, and in males also the kidney and thyroid glands. Changes in circulating thyroid hormone level were seen in both sexes. There were also changes in urine and clinical chemistry parameters which were markedly more severe in males.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.3.2/04
Report Author:	███, W.
Report Year:	1982
Report Title:	KUB 3359 - Chronic Toxicity Study in Beagle Dogs with Oral Administration for 26 Weeks
Report No:	R007162
Document No:	M-232142-01-2
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 409, 1998. Animals were 11 months at start of study (guideline stipulates dogs should be no more than 9 months at study start). Detailed weekly clinical observations not conducted. It is not stated if animals were fasted prior to blood sampling for clinical chemistry tests. Thymus and uterus not weighed, Spinal cord, salivary glands, parathyroid and uterus not examined microscopically. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In a 26 week study, groups of 4 beagle dogs/sex/dose were administered aclonifen (purity 96.2%) in the diet at doses of 0, 100, 500, 5000 ppm (equivalent to 0, 3, 15, 142 mg/kg bw/day). The only signs of toxicity were seen in the top dose of 5000 ppm where there was a reduction in body weight gain in both sexes, absolute and relative liver weight were enlarged but without any accompanying histopathological findings. There were however minor changes in clinical chemistry (increased cholesterol and alkaline phosphatase in males) that suggest effects on the liver. The only other finding were some slight changes in white blood cell counts in females with increased granulocyte lymphocytes (neutrophils).

The NOAEL for this study was 500 ppm (15 mg/kg bw/day in both sexes), based on reduced body weight gain and changes in clinical chemistry parameters.

The study was performed to GLP, it was not performed to OECD test guidelines, but with some omissions and additions is broadly equivalent to OECD 409 (1998).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen (KUB 3359)
Lot/Batch:	T5/81
Purity:	96.2%
Appearance:	Not mentioned

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Stability of test compound:	The feed mixtures were analysed monthly to evaluate concentrations of the test substance in the feed. Stability in the diet was not reported.
2. Vehicle and /or positive control	Test substance was added to the pulverised feed at a fixed mixture ratio to provide the required dietary concentrations.

3. Test animals

Species and strain:	Dog – purebred beagle dogs, breeding strain Chbi:BEAGLE
Source:	
Number of animals	16 M + 16 F
Sex:	Male + Female
Age:	11 months
Weight at treatment:	Mean males: 13.0kg; Mean females: 11.5kg (start of treatments)
Acclimation period:	Not mentioned
Diet:	Pulverised ssniff HH sole diet for dogs (provided as an aqueous feed mush (ratio water : feed-substance mixture: 3:1). Availability: 10am – 7am. Ration of 400g/dog/day. The feed that was not consumed was weighed to determine daily consumption.
Water:	Drinking water, *ad libitum*
Cage type:	1.30 x 2.55 m inside room equipped with floor heating, and access to an outside kennel at all times
Housing:	individually
Environmental conditions:	Temperature: not mentioned Humidity: not mentioned Air changes: automatic supply of heated air Photoperiod: not mentioned

B. STUDY DESIGN AND METHODS

1. In life dates

August 5, 1981 to February 3, 1982.

2. Animal assignment and treatment

Aclonifen (Batch T5/81, purity 96.2%) was incorporated in pulverised canine diet at concentrations of 0, 100, 500 and 5000 ppm. Analyses of the dietary mix were performed monthly.

Groups of beagle dogs (4/sex/group) were administered aclonifen in the diet for a period of 26 weeks.

General health checks were made daily. Food consumption was measured daily. Body weights were recorded weekly. Ophthalmological examinations were performed on all animals at the beginning and end of the study. All animals in the high dose group and any showing unusual findings at the beginning of the study were also examined at 13 weeks.

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Blood samples were taken and subjected to haematological and clinical chemistry examinations before the start of the study, after 1, 6, 13, 19 and 26 weeks. Haematology tests included Heinz bodies, and methaemoglobin, and all parameters stipulated under OECD 409 (1998). Clinical chemistry tests conducted on serum samples measured sodium, potassium, calcium, chloride, glucose, total bilirubin, total cholesterol, urea nitrogen, creatinine, total protein, serum protein electrophoresis, albumin, globulin, albumin/globulin ratio, alpha-1-globulin, alpha-2-globulin, beta-globulin, gamma-globulin. In addition hepatic functional tests measured serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (AP). Bromosulfophthaleine liver function test was conducted in the control and high dose group at the end of the study. Gamma glutamyl transferase (GGT) and leucine arylamidase were measured at the end of the study.

Urinalysis was performed on all animals at the same time as the haematology and clinical chemistry investigations.

Animals were sacrificed by exsanguination under phenobarbital anaesthesia. After sacrifice, an autopsy with macroscopic evaluation was performed on all animals and organ weights were recorded of the heart, lungs, liver, kidneys, spleen, prostate, testes with epididymis, ovaries, adrenals, pituitary, thyroid, brain. Microscopic examination was conducted on a range of organs and tissues (as described in OECD 409, 1998, but note that salivary glands, spinal cord, parathyroids and uterus were not examined microscopically. Bone marrow liver, spleen, kidneys and lungs were stained with Berlin blue for determination of iron. Frozen samples of heart, liver, kidneys, adrenals were stained with fatty red 7B for determination of lipids.

3. Statistics

The statistical tests were performed for each biological parameter per time of examination, in connection with organ weights separately for males and females, for investigating significant differences between the dose groups. Data were processed using the Bartlett-test, ANOVA and the Newman-Keuls test.

II. RESULTS AND DISCUSSION

1. Analysis

The results for analyses of the feed mixtures in all groups were very close to the target concentrations (mean was 101% to 104.5%). Homogeneity, stability and method of analysis used is not described in the study report.

2. General observation/clinical signs/ Mortality

There were no intercurrent deaths.

No treatment-related clinical signs or behavioural changes were observed. In all dogs of the highest group the urine showed a clear yellow discoloration.

3. Body weight / food consumption / Substance intake

Body weight gain was reduced at the high dose group in both sexes, resulting in a slight loss of bodyweight over the 26 week study period.

Food consumption was not affected in males but was slightly reduced in females at 5000 ppm..

Table 5.2.3- 12: Aclonifen - 26-week toxicity study in dogs – Bodyweight and food consumption

	Males	Females

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Diet concentration (ppm)	**0**	**100**	**500**	**5000**	**0**	**100**	**500**	**5000**
Bodyweight - necropsy (kg)	**13.5**	**13.98**	**13.73**	**13.48**	12.50	12.95	12.53	**11.33 (↓9%)**
Bodyweight change – week 0 to 26 (kg)	0.73	1.16	0.96	**-0.04**	1.33	1.68	0.88	**-0.57**
Food consumption – week 26 (g/day)	391.0	400.0	381.3	399.8	340.8	371.5	371.5	**315.5 (↓7%)**
Statistical analysis not conducted								

Table 5.2.3- 13: Aclonifen - 26-week toxicity study in dogs – Achieved intake

	Males & Females		
Diet concentration (ppm)	**100**	**500**	**5000**
Substance-intake (mg/kg /day)	3	15	142

4. Haematology / Clinical chemistry / Urinalysis

At week 26, haematology revealed an increase in lymphoid cells in 3 females receiving 5000 ppm. In one of those females, the evaluation of the differential blood counts showed a clear 'shift to the left', coupled with a decrease in segmented granulocytes with simultaneous increase in lymphocytes, beginning in Weeks 13 to 26. There were no haematological findings in males.

Table 5.2.3- 14: Aclonifen – 26 week toxicity study in dogs – Haematology

	Males				**Females**			
Diet concentration (ppm)	**0**	**100**	**500**	**5000**	**0**	**100**	**500**	**5000**
Haematology week 13								
Leukocytes 1000/mm³ – week 13	10.4	11.45	10.82	11.72	10.9	12.65	11.35	9.95
Lymphocytes % – week 13	31.8	26.0	29.8	24.5	30.8	31.3	28.0	**37.5 (↑22%)**
Segmented granulocytes % – week 13	55.8	58.5	59.5	64.5	57.0	57.5	60.5	**40.0 (↓30%)**
Monocytes % – week 13	7.5	9.0	7.5	8.3	8.5	7.3	6.5	**10.8 (↑27%)**
Haematology week 19								
Leukocytes 1000/mm³ – week 19	10.7	11.12	10.65	9.67	12.2	14.05	11.67	10.62
Lymphocytes % – week 19	25.5	27.8	26.3	26.8	32.8	24.3	29.8	**43.0 (↑31%)**
Segmented granulocytes % – week 19	63.0	61.0	65.3	61.0	56.3	66.8	57.5	**43.0 (↓24%)**
Monocytes % – week 19	7.3	6.5	4.5	8.8	7.8	5.5	7.5	**9.8 (↑26%)**
Haematology week 26								
Leukocytes 1000/mm³ – week 26	8.95	10.35	10.17	11.55	11.45	13.62	10.47	10.17
Lymphocytes % – week 26	34.0	32.5	39.5	33.8	37.8	35.8	31.8	**43.8 (↑16%)**

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Segmented granulocytes % – week 26	62.8	62.8	55.5	58.3	57.0	58.0	62.0	**45.0 (↓21%)**
Monocytes % – week 26	0.8	1.3	1.3	0.3	1.3	1.5	0.8	0.8
Statistical analysis not conducted **bold** : considered to be treatment-related								

The clinico-chemical examinations showed slight to moderate increases in the alkaline phosphatase and the cholesterol level in one control animal and in a few animals of all treated groups. These increases were reversible in spite of continuous treatment until the end of the study. Besides these relatively mild transient changes, one female in the 500 ppm group and two males in the 5000 ppm group showed a strong increase in alkaline phosphatase during the entire treatment period. In one of these males from the 5000 ppm group, the cholesterol level was also markedly increased, starting in trial Week 1, and the bromosulfophthalein test performed at the end of the study showed a slight increase in retention. On the whole a certain dose-dependence is noticeable in connection with the increase in alkaline phosphatase and cholesterol levels regarding the frequency of their occurrence as well as their intensity.

Table 5.2.3- 15: Aclonifen – 26 week toxicity study in dogs – Clinical chemistry

	Males				Females			
Diet concentration (ppm)	**0**	**100**	**500**	**5000**	**0**	**100**	**500**	**5000**
Clinical chemistry week 6								
Alkaline phosphatase (U/L)	122	103	133	**354 (↑x3)**	103	121	143	103
Total cholesterol (mmol/L)	3.6	3.4	4.0	**5.4 (↑50%)**	3.6	3.6	4.1	**6.1 (↑69%)**
Clinical chemistry week 13								
Alkaline phosphatase (U/L)	131	118	139	**404 (↑x3)**	104	142	151	77
Total cholesterol (mmol/L)	3.5	3.1	3.3	**5.6 (↑60%)**	3.6	3.5	4.7	3.4
Clinical chemistry week 19								
Alkaline phosphatase (U/L)	123	109	**156**	**542 (↑x4)**	128	100	232	76
Total cholesterol (mmol/L)	2.8	2.6	3.3	**5.4 (↑92%)**	2.8	2.2	5.4	2.9
Clinical chemistry week 26								
Alkaline phosphatase (U/L)	106	91	105	**501 (↑x5)**	96	104	126	69
Total cholesterol (mmol/L)	2.1	2.3	2.4	**4.6 (↑x2)**	2.2	2.8	2.4	2.9
Statistical analysis not conducted **bold** : considered to be treatment-related								

In all animals of the 5000 ppm dose groups the urine showed a distinct yellow colouration. Apart from this finding, urinalysis showed no dose or time-dependent biologically relevant differences between treated or control groups.

5. Gross pathology / Organ weights / Histopathology

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Gross necropsy revealed no treatment related findings. Organ weight analysis revealed hepatomegaly in two male dogs and one female dog of the highest dosed group.

Table 5.2.3- 16: Aclonifen – 26 week toxicity study in dogs – Organ weights

	Males				Females			
Diet concentration (ppm)	**0**	**100**	**500**	**5000**	**0**	**100**	**500**	**5000**
Bodyweight - necropsy (kg)	**13.5**	**13.98**	**13.73**	**13.48**	12.50	12.95	12.53	**11.33 (↓9%)**
	Organ weights							
Liver weight (g)	396	418	438	**590 (↑49%)**	373	404	397	**461 (↑24%)**
Liver weight % bw	2.93	2.99	3.21	**4.38 (↑49%)**	2.97	3.12	3.22	**4.06 (↑37%)**

bold : considered to be treatment-related.
statistically significant: * $p < 0.05$, ** $p < 0.01$

Histopathological examination of tissues did not reveal any treatment-related changes in any tissue examined including the liver.

III. CONCLUSION

Based on the decreased body weight at 5000 ppm, the NOAEL in this 26-week study in the dog was 500 ppm (equivalent to approximately 15 mg/kg/day).

Assessment and conclusion by applicant:

The NOAEL in this 26-week study in the dog was 500 ppm (equating to 13 mg/kg bw/day in both sexes) based on decreased body weight gain at 5000 ppm (142 mg/kg bw/day in both sexes).

The study was conducted to GLP. It was not conduced to OECD guidelines but broadly follows OECD 409 (1998) except for a few omissions and the use of slightly older animals than recommended.

Assessment and conclusion by RMS:

CA 5.3.3 Other routes

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Data Point:	KCA 5.3.3/01
Report Author:	
Report Year:	2002
Report Title:	4-Week toxicity study by cutaneous route in rats Aclonifen
Report No:	C021862
Document No:	M-212006-01-1
Guideline(s) followed in study:	MAFF: 59 Nohsan 4200 1985; OECD: 410 (1981); USDA: 712-C-98-210 1998; USEPA (=EPA): OPPTS 870.3200 1998
Deviations from current test guideline:	Current Guideline: OECD 410, 1981 Minor deviation, not clear if porous gauze dressing was used. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In a 30-day percutaneous toxicity study, the aclonifen (batch OP9750062, purity 99.5%) was administered to groups of Sprague-Dawley rats (10 males and 10 females per dose) at concentrations of 0, 250, 500 and 1000 mg/kg bw per application. Duration and frequency of treatment was 6 hours per day, 5 days a week for a total of 30 days.

The dose was well-tolerated at the local level as no cutaneous reactions or histopathological findings at the application site were observed. Similarly the test substance was well-tolerated at the systemic level, the only change of possible toxicological significance being a reduction in body weight gain with reduced food consumption in males and lower blood glucose levels in males at the top dose of 1000 mg/kg bw/day. There were no adverse findings in females.

The NOAEL in male rats was considered to be 500 mg/kg/day while in female rats it was 1000 mg/kg/day.

This was a GLP and guideline compliant study.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen
Lot/Batch:	OP9750062
Purity:	99.5 %
Appearance:	Dark yellow powder
Stability of test compound:	The dosage forms were made at for up to 9 days of treatment and were stored at room temperature prior to use. Samples from

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	the low and high dose were sampled after 4- and 9-days storage at 4ºC.

2. Vehicle and /or positive control Vehicle: 0.5% aqueous methylcellulose solution.

3. Test animals

Species and strain:	Sprague-Dawley rats - Crl CD® (SD) IGS BR strain, *Caesarian Obtained, Barrier Sustained-Virus Antibody Free* (COBS-VAF®)
Source:	
Number of animals:	88 animals
Sex:	Males + Females
Age:	9 weeks
Weight at treatment:	Mean for males: 363 g (range: 328-390 g) – Mean for females: 238 g (range: 211-256 g)
Acclimation period:	11 days
Diet:	A04 C pelleted maintenance diet, batch No. 10227 ( ), *ad libitum*
Water:	Filtered tap water, *ad libitum*
Cage type:	Suspended wire-mesh cages (43.0 x 21.5 x 18.0 cm)
Housing:	Individual caging
Environmental conditions:	Temperature: 22±2 °C Humidity: 50±20 % Air changes: ~12 exchanges per hour Photoperiod: 12 hours daily

B. STUDY DESIGN AND METHODS

1. In life dates

17 May to 27 June 2001

2. Animal assignment and treatment

A total of 80 (40 males and 40 females) Sprague-Dawley rats were assigned to four groups of 10 males and 10 females each: one control group receiving the vehicle, three treated groups receiving the test substance suspended in the vehicle (0.5% methylcellulose) at dose-levels of 250, 500 or 1000 mg/kg/day under the constant dosage-volume of 2 mL/kg/day. The treatment period was 6 hours a day for 5 days a week for a period of 30 days.

The test substance was administered as a suspension in the vehicle. The test substance was ground to fine powder using a mortar and pestle, suspended in the vehicle in order to achieve the concentrations of 125, 250 and 500 mg/mL and then homogenised using a magnetic stirrer. The test compound was analysed for concentration, homogeneity and stability of the 125 and 500 mg/kg bw/day using gas liquid chromatography with electron capture detection. The analytical method fulfils nearly all SANCO 3029 criteria, with minor exception regarding calibration data due to reporting deficiencies;

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nevertheless, method is fit for purpose. Homogeneity was acceptable (coefficient of variation was 9% for the low dose and 8% for the high dose samples). Aclonifen was stable in the dosing suspension for at least 9 days (deviation from target concentration not more than 8%). Achieved concentration (from does prepared in weeks 1 and 4) were not more than 10% below target concentration.

Before the beginning of the treatment period, the dorsum of the animals was clipped free from hair, as close to the skin as possible, with an electric clipper. Before the first application, the treatment area was examined and any animals showing skin abnormality and/or irritation will be replaced from the spare animals ordered. During the treatment period, the animals were clipped whenever necessary, at least 4 hours before dosing and at least once a week.

The test substance was applied to the dorsum of the animals using a plastic dermal shield, held in place with a jacket. The animals were checked twice daily for mortality and clinical signs were observed once a day, including evaluation of possible cutaneous reactions at the application site.

All animals were subjected to a detailed clinical observation at the end of the treatment period for the evaluation of neurotoxic effects.

Body weight and food consumption were recorded before the beginning of the study and then once a week. Food consumption was calculated at weekly intervals. Ophthalmological examinations were performed on animals of the control and high-dose groups before the beginning of the study and on all animals at the end of the treatment period. Haematological and blood biochemical parameters were determined at the end of the treatment period after at least 14 hours fasting.

At the end of the treatment period animals were sacrificed by exsanguination following administration of carbon dioxide. A macroscopic *post-mortem* examination was performed on all animals, the adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes, thymus and uterus (with cervix) were weighed and a comprehensive list of tissues were preserved. A microscopic examination was carried out on tissues showing macroscopic abnormalities, on a range of organs and tissues from the animals of the control and high-dose groups, and on the kidneys, the lungs and the liver of all animals.

3. Statistics

Statistical analyses of body weight, food consumption, haematology, blood biochemistry and organ weight data were performed. Dunnett's test was used for data with normal distribution and homogenous variances between groups. Dunn's test was used for data with non-homogenous variances, or where distribution was not normal.

II. RESULTS AND DISCUSSION

1. Mortality

No mortality occurred during the study.

2. General observation/clinical signs

Local level

There were no cutaneous reactions at the application site.

Systemic level

No treatment-related clinical signs were observed in any group. At detailed clinical observation, there were no signs of neurotoxicity in any group. There were no ocular findings at the end of the treatment period.

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3. Body weight / Food consumption

When compared to the control values, a significantly lower body weight gain and a slight reduction in food consumption was noted in males treated at 1000 mg/kg/day throughout the study. There were no changes on body weight gain or on food consumption in females.

Table 5.3.3- 1 Aclonifen 30-day dermal - Bodyweight and food consumption

	Males				Females			
Dermal application (mg/kg bw/day)	**0**	**250**	**500**	**1000**	**0**	**250**	**500**	**1000**
Bodyweight - necropsy (g)	457	441	434	**417* (↓9%)**	273	269	274	275
Body weight change (day 1 *to* 29; g)	+88	+75	+69	**+60 (↓32%)**	+36	+32	+36	+37
Food consumption (mean weeks 1 - 4; g/animal/day)	31.4	31.2	30.5	**29.0 (↓8%)**	21.6	22.0	22.5	21.6
statistically significant: * p < 0.05, ** p < 0.01 ; **bold** : considered to be treatment-related								

4. Haematology/ Blood biochemistry

No variations in haematological parameters could be ascribed to treatment with the test substance in treated groups at 250 or 500 mg/kg/day.

When compared to the control values, lower total white blood cell count was noted in males and females treated at 1 000 mg/kg/day. This variation correlated with lower mean lymphocyte, monocyte and neutrophil counts (males only).

Table 5.3.3- 2 Aclonifen 30-day dermal – Haematology and clinical chemistry

	Males				Females			
Dermal application (mg/kg bw/day)	**0**	**250**	**500**	**1000**	**0**	**250**	**500**	**1000**
	Haematology							
Leukocytes (g/L)	12.94	12.95	12.62	10.01* (↓23%)	9.23	9.89	7.73	6.51* (↓30%)
Lymphocytes (g/L)	10.45	10.63	10.82	8.37 (↓20%)	8.00	8.58	6.62	5.48* (↓32%)
Monocytes (g/L)	0.40	0.34	0.28** (↓30%)	0.26** (↓35%)	0.29	0.22	0.21	0.17* (↓41%)
Neutrophils (g/L)	1.89	1.78	1.34* (↓29%)	1.22** (↓35%)	0.76	0.89	0.77	0.72 (↓5%)
	Clinical chemistry							
Glucose (mmol/L)	7.01	6.56	6.22	**5.86** (↓16%)**	6.24	6.19	6.37	6.12
statistically significant: * p < 0.05, ** p < 0.01 **bold** : considered to be treatment-related								

No variations in blood biochemical parameters could be ascribed to treatment with the test substance in treated groups at 250 or 500 mg/kg/day. When compared to the mean control values, slightly lower

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plasma glucose levels were noted among males treated at 1000 mg/kg/day (5.86 vs. 7.01 mmol/L, p<0.01). This variation in glucose levels was not observed in treated females.

Other differences were noted in blood biochemical parameters but were not considered to be the consequence of treatment with the test substance since they were slight (e.g. changes in sodium, chloride or calcium levels in males and/or females) or not dose-related (e.g. changes in albumin concentration in treated males).

5. Gross pathology / Organ weights / Histopathology

Organ weights:

No treatment-related changes were noted in organ weights.

Macroscopic *post-mortem* examination:

Local level

Yellowish coloration of the application sites was noted in all treated animals. This finding, which was without relevant histopathological abnormalities, was considered to be due to the colour of the test substance, and consequently of no toxicological importance.

Systemic level

No treatment-related necropsy findings were noted. All the necropsy findings encountered were commonly observed changes in the rat of this strain and age and considered to be of no toxicological importance.

Microscopic examination:

Treated skin

No treatment-related microscopic changes were noted. Minimal acanthosis was noted in some individuals from the control and treated groups. This was considered to be due to the mechanical irritation as a result of the preparation of the animals (clipping) before treatment. Consequently, it was considered to be of no toxicological importance.

Systemic level

No treatment-related microscopic changes were noted in any organ examined. The incidence, severity and morphological characteristics of the microscopic changes observed in all organs examined were similar in both control and treated animals and were recognised as commonly observed spontaneous changes in the rat of this strain and age. Consequently, they were considered to be of no toxicological importance.

III. CONCLUSION

The NOAEL was 500 mg/kg/day in males, based on reduced body weight, food consumption and lower glucose levels while in female rats was 1000 mg/kg/day, as the slight variation on white blood cell counts was not considered an adverse effect.

Assessment and conclusion by applicant:

This was a GLP and guideline compliant study.

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The NOAEL in male rats was 500 mg/kg/day based on reduced body weight gain and reduced food consumption at 1000 mg/kg bw/day. In female rats the NOAEL was 1000 mg/kg/day based on no adverse findings in females at the top dose in this study.

Assessment and conclusion by RMS:

CA 5.4 Genotoxicity testing

Aclonifen did not induce gene mutations in either procaryotes (Ames test) or mammalian eucaryotic cell cultures, nor any chromosome aberrations *in vitro* in cultured human lymphocytes or *in vivo* in mouse bone marrow cells, nor DNA damage in mammalian cells in the *in vitro* UDS assay (reported in section CA 5.8.2).

Taken together, the results demonstrate that aclonifen is not genotoxic and is unlikely to present a genotoxic hazard to humans.

Aclonifen – Summary of genotoxicity studies

Test system, Concentration range	Results	Remarks	Reference	New study
		***In vitro* studies**		
Ames test TA 98, 100, 1535, 1537, 1538. Plate incorporation 0, 50, 100, 150, 300, 600, 1200, 2,500 and 5,000 µg/plate	Negative +/- S9	Concentration range limited for some strains by cytotoxicity and/or precipitation	KCA 5.4.1/01 [redacted] 1982 M-174849-01-1	-
Ames test TA 98, 100, 1535, 1537, 102. Plate incorporation and pre-incubation 0, 16, 50, 158, 500, 1581 and 5000 µg/plate	Negative +/- S9	Weak cytotoxicity from 158 µg/plate in the plate incorporation assay No cytotoxicity up to the limit concentration of 5000 µg/plate in the pre-incubation assay	KCA 5.4.1/02 [redacted] 2006 M-174849-01-1	-
Clastogenicity assay in human cells *in vitro* Human lymphocytes 5, 10, 20, 30 µg/mL (-S9) 12.5, 25, 50, 100, 150 µg/mL (+S9)	Negative +/- S9	Reduced mitosis at ≥20 µg/mL (-S9), and at ≥100 µg/mL (+S9)	KCA 5.4.1/03 [redacted] 1992 M-174403-01-1	-

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Test system, Concentration range	Results	Remarks	Reference	New study
***In vitro* studies**				
Mutation assay in mammalian cells *in vitro* Chinese hamster V79 cells 0, 10, 50, 75, 150, 250, 500, 750, 1000 µg/mL	Negative +/- S9	Precipitation at ≥50 µg/mL Cytotoxicity without S9 at ≥25 µg/mL, ≥150 µg/mL with S9	KCA 5.4.1/04 [redacted] and [redacted] 1984 M-174850-01-1	-
Micronucleus test *in vitro* Human lymphocytes 8, 13.9, 24.4 µg/mL (4 h -S9) and 13.9, 24.4, 42.6 µg/mL (20 h -S9), Expt. I. 14.8, 22.2, 33.3 µg/mL (4 h +S9), Expt. II	Negative +/- S9	Precipitation at ≥24.4 µg/mL (-S9), ≥42.6 µg/mL (+S9), ≥75 µg/mL (-S9, Expt. II) No cytotoxicity in Expt. I up to levels with precipitation. Cytotoxicity in Expt. II at 33.3 µg/mL (-S9)	KCA 5.4.1/05 [redacted] 2019 M-664242-01-2	Yes
Mutation assay in mammalian cells *in vitro* Chinese hamster V79 cells 0, 1.6, 3.1, 6.3, 12.5, 25, 50 µg/mL Chinese hamster V79 cells 0, 1.6, 3.1, 6.3, 12.5, 25, 50 µg/mL	Negative +/- S9	Concentration range limited by precipitation (≥50 µg/mL) Cytotoxicity without S9 at ≥25 µg/mL, none with S9	KCA 5.4.1/06 [redacted] 2019 M-664619-01-1	Yes
Unscheduled DNA synthesis (autoradiographic) Primary rat hepatocytes 0.079, 0.25, 0.79, 2.5, 7.9 µg/mL	Negative	Cytotoxicity at ≥7.9 µg/mL	KCA 5.8.2 / 01 [redacted] 1991 M-174373-01-1	-
***In vivo* studies**				
Micronucleus study NMRI mice 1000, 3000, 10000 mg/kg	Negative	Only marginal/equivocal evidence of bone marrow cytotoxicity (for proof of tissue exposure)	KCA 5.4.2/01 [redacted] 1984 M-174855-01-1	-

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CA 5.4.1 *In vitro* studies

Data Point:	KCA 5.4.1/01
Report Author:	[redacted]
Report Year:	1982
Report Title:	In vitro assessment for mutagenic potential in bacteria with and without addition of a metabolizing system
Report No:	R007401
Document No:	M-174849-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: Not cited, but broadly similar to OECD 471 (1997) Minor deviation, no species to detect cross-linking mutagens. Overall study is acceptable.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

Aclonifen (purity >99%) was examined for mutagenic effects in five histidine-dependent autotrophs of *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1538 and TA 1537, using the plate incorporation method with and without rat liver S9 mix as a metabolizing system. Eight concentrations in DMSO, in the range 50 to 5000 µg/plate, were tested in parallel with suitable positive controls. This range was limited by inhibition of bacterial growth, or by solubility in the vehicle.

The positive controls showed the expected reversion properties, confirming validity and sensitivity of the test. Aclonifen did not show any genotoxic potential in the assay at up to 5000 µg/plate, the highest concentration tested.

I. MATERIALS AND METHODS

Study dates Experimental: 05 to 15 August 1980
Completion: 08 April 1982

A. MATERIALS

1. **Test Item** KUB 3359 (aclonifen)
   Batch No.: TH 1/80
   Purity: >99%
   Appearance: Yellow crystalline powder
   Expiry/retest: Not stated

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Structure:

Vehicle: DMSO

2 Positive controls

2-aminoanthracene (Sigma)
9-aminoacridine (Sigma)
Daunomycin (Serva)
1-ethyl-2-nitro-3-nitrosoguanidine (Ega Chemie)
Methyl methane sulphonate (Merck-Schuchardt)
N-methyl-N-nitro-N-nitrosoguanidine (Fluka)
2-nitrofluorene (Merck-Schuchardt)
4-nitro-1,2-phenylene diamine (Fluka)

3. Test system (strains)

Salmonella typhimurium
TA 100 (his G 46, uvrb, rfa + R-factor)
TA 1535 (his G 46, uvrb, rfa)
TA 98 (his D 3052, uvrb, rfa + R-factor)
TA 1538 (his D 3052, uvrb, rfa)
TA 1537 (his C 3076, uvrb, rfa)
Obtained from Ames, University of Berkeley, CA, USA

S-9 mix:

Liver homogenate S-9 fraction from Wistar AF/Han rats, induced with Aroclor 1254
$MgCl_2$/KCl (0.4M/1.65M)
Glucose-6-phosphate (1 M)
NADP (0.1 M)
Sodium phosphate buffer (0.2 M)

Medium: Merckoplate Minimal Glucose Agar (Merck)

B. STUDY DESIGN AND METHODS

Fresh suspensions of the tester strains were prepared for each test day. Aclonifen was dissolved in DMSO with aid of a sonicator, to prepare the necessary range of concentrations. Each solution and the S-9 mix were tested for bacterial contamination prior to use.

Eight plates were used for each concentration of test item or positive control (4 with and 4 without S-9), while 16 were used for negative control (8 with and 8 without S-9). A range of 8 concentrations of test item were used (50, 100, 150, 300, 600, 1200, 2500, 5000 µg/plate).

A preliminary experiment was conducted with concentrations of 50 to 10000 µg/plate, to determine the concentration range to be used in the main study, based on reduced spontaneous revertants, clearing of the background lawn, or degree of survival of the treated cultures.

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The main test was then conducted with concentrations from 50 to 5000 µg/plate, using the plate incorporation method:

Bacterial suspension:	0.1 mL
Sample (or solvent):	0.1 mL
Na-phosphate buffer (trials without S-9):	0.5 mL
S-9 mix (trials with activation):	0.5 mL
Soft agar:	2.0 mL
Incubation temperature:	37°C
Incubation period:	48 h

Revertants were then counted. Where this was rendered impossible because of test item precipitation, the colonies were stained with triphenyltetrazolium chloride to distinguish them and enable counting. Statistical evaluation was by the nonparametric Kruskal-Wallis test.

II. RESULTS AND DISCUSSION

The mutagenicity study results were as shown in the tables below:

Table 5.4.1-1: Mutagenesis assay results, Experiment I: Mean revertants ± SD

Substance	µg/plate	S-9	Base-pair substitution		Frame shift		
			TA 100	TA 1535	TA 98	TA 1537	TA 1538
DMSO		-	102 ± 20	13 ± 3	25 ± 4	7 ± 4	17 ± 8
Aclonifen	50	-	93 ± 10	-	21 ± 6	-	-
	100	-	92 ± 9	14 ± 5	25 ± 8	7 ± 3	17 ± 4
	150	-	91 ± 20	-	25 ± 6	-	-
	300	-	76 ± 12	9 ± 2	24 ± 8	5 ± 1	7 ± 5
	600	-	78 ± 12	10 ± 6	23 ± 5	4 ± 2	3 ± 2
	1200	-	77 ± 8	7 ± 1	25 ± 5	BG	7 ± 2
	2500	-	-	9 ± 2 P	-	2 ± 1 BG, P	2 ± 2 P
	5000	-	-	5 ± 5 P	-	BG, P	BG, P
DMSO		+	118 ± 17	14 ± 6	43 ± 12	11 ± 4	37 ± 8
Aclonifen	50	+	141 ± 21	-	39 ± 5	-	-
	100	+	136 ± 16	17 ± 7	36 ± 5	8 ± 3	28 ± 4
	150	+	129 ± 30	-	38 ± 6	-	-
	300	+	135 ± 10	14 ± 6	39 ± 10	7 ± 6	28 ± 3
	600	+	97 ± 10	9 ± 1	29 ± 8	5 ± 1	11 ± 2
	1200	+	102 ± 19	10 ± 8	34 ± 6	3 ± 1	9 ± 2
	2500	+	-	7 ± 1 P	-	BG, P	11 ± 3 P
	5000	+	-	5 ± 2 P	-	BG, P	11 ± 5 P
EtOH		-	-	-	-	9 ± 3	-
H_2O		-	-	-	29 ± 7	-	-

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Substance	µg/plate	S-9	Base-pair substitution		Frame shift		
			TA 100	TA 1535	TA 98	TA 1537	TA 1538
2-AA (DMSO)	1 1	- +	109 ± 13 1014 ± 107	17 ± 3 153 ± 8	26 ± 6 519 ± 31	11 ± 2 80 ± 7	26 ± 5 718 ± 64
9-AA (EtOH)	20 50	- -	- -	- -	- -	24 ± 3 232 ± 28	- -
Daun (H_2O)	10	-	-	-	835 ± 158	-	-
ENNG (DMSO)	2 10	- -	225 ± 11	- 90 ± 10	- -	- -	- -
MMS (DMSO)	500	-	838 ± 53	- -	- -	- -	- -
MNNG (DMSO)	4	-	-	1019 ± 162	- -	- -	- -
4-NP (DMSO)	10 20	- -	- -	- -	- -	- -	910 ± 71 1091 ± 40
2-NF (DMSO)	5	-	- -	- -	- -	- -	1056 ± 233

2-AA = 2-aminoanthracene; 9-AA = 9-aminoacridine; Daun = daunomycin; ENNG = 1-ethyl-2-nitro-3- nitrosoguanidine; MMS = methyl methane sulphonate; MNNG = N-methyl-N-nitro-N-nitrosoguanidine; 2-NF = 2-nitrofluorine; 4-NP = 4-nitro-1,2-phenylenediamine; DMSO = dimethylsulfoxide; EtOH = ethanol; BG = background toxicity; P = strong precipitation

Table 5.4.1-2: Mutagenesis assay results, Experiment II: Mean revertants ± SD

Substance	µg/plate	S-9	Base-pair substitution		Frame shift		
			TA 100	TA 1535	TA 98	TA 1537	TA 1538
DMSO		-	78 ± 6	11 ± 2	27 ± 6	7 ± 4	11 ± 2
Aclonifen	100	-	73 ± 12	9 ± 1	21 ± 7	7 ± 2	9 ± 5 BG
	150	-	72 ± 5	11 ± 5	22 ± 5	6 ± 5	4 ± 2
	300	-	67 ± 6	9 ± 1	19 ± 6	1 ± 1	1 ± 1 BG
	600	-	45 ± 6	8 ± 3	21 ± 4	3 ± 2	BG
	1200	-	49 ± 6	9 ± 3	14 ± 1	3 ± 1 BG	BG
	2500	-	56 ± 15 P	10 ± 2 P	17 ± 9 P	2 ± 2 BG, P	BG, P
DMSO		+	99 ± 15	12 ± 2	33 ± 7	9 ± 3	28 ± 7
Aclonifen	100	+	114 ± 16	10 ± 5	29 ± 4	11 ± 2	22 ± 2
	150	+	114 ± 8	12 ± 2	29 ± 5	7 ± 1	22 ± 4
	300	+	114 ± 13	10 ± 2	27 ± 9	8 ± 4	15 ± 2
	600	+	69 ± 3	9 ± 3	26 ± 4	4 ± 4	12 ± 0
	1200	+	74 ± 9	9 ± 4	17 ± 3	3 ± 1	13 ± 4 BG
	2500	+	71 ± 3 P	7 ± 2 P	15 ± 3 P	5 ± 3 BG, P	21 ± 11 BG, P

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Substance	µg/plate	S-9	Base-pair substitution		Frame shift		
			TA 100	TA 1535	TA 98	TA 1537	TA 1538
EtOH		-	-	-	-	7 ± 2	-
H_2O		-	-	-	26 ± 6	-	-
2-AA (DMSO)	1 1	- +	98 ± 11 748 ± 78	11 ± 2 79 ± 28	31 ± 8 336 ± 26	6 ± 2 66 ± 11	12 ± 4 387 ± 40
9-AA (EtOH)	20 50	- -	- -	- -	- -	12 ± 2 16 ± 5	- -
Daun (H_2O)	10	-	-	-	946 ± 107	-	-
ENNG (DMSO)	2 10	- -	253 ± 18 -	- 208 ± 36	- -	- -	- -
MMS (DMSO)	500	-	604 ± 39	- -	- -	- -	- -
MNNG (DMSO)	4	-	- -	1658 ± 236 -	- -	- -	- -
4-NP (DMSO)	10 20	- -	- -	- -	- -	- -	569 ± 185 808 ± 60
2-NF (DMSO)	5	-	-	-	-	-	591 ± 122

2-AA = 2-aminoanthracene; 9-AA = 9-aminoacridine; Daun = daunomycein; ENNG = 1-ethyl-2-nitro-3- nitrosoguanidine; MMS = methyl methane sulphonate; MNNG = N-methyl-N-nitro-N-nitrosoguanidine; 2-NF = 2-nitrofluorine; 4-NP = 4-nitro-1,2-phenylenediamine; DMSO = dimethylsulfoxide; EtOH = ethanol; BG = background toxicity; P = strong precipitation

The results for aclonifen, both in the presence and absence of S-9 metabolic activation, were all negative, with no statistically significant differences from control. Meanwhile, the positive and negative controls all yielded the expected ranges of mutant frequencies.

III. CONCLUSION

Aclonifen was negative under the conditions of this assay, both with and without metabolic activation.

Assessment and conclusion by applicant:

The study is considered to have produced valid supplementary results, showing that aclonifen was negative in the Ames assay, and is considered acceptable with the restriction that cross-linking mutagens were not specifically screened for.

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Assessment and conclusion by RMS:

Data Point:	KCA 5.4.1/01
Report Author:	
Report Year:	1982
Report Title:	In vitro assessment for mutagenic potential in bacteria with and without addition of a metabolizing system
Report No:	R007401
Document No:	M-174849-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: Not cited, but broadly similar to OECD 471 (1997) Minor deviation, no species to detect cross-linking mutagens. Overall study is acceptable.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

Aclonifen (purity 97.3 %) was examined for mutagenic effects in five histidine dependent autotrophs of *Salmonella typhimurium*: TA 98, TA 100, TA 1535, TA 102 and TA 1537, using the plate incorporation test with and without addition of liver postmitochondrial fraction as a metabolizing system (S-9). The following concentrations (in DMSO) were tested in the first trial (three plates for each concentration): 0, 16, 50, 158, 500, 1581 and 5000 µg/plate. Precipitation of the test substance was noted at the highest concentration. Aclonifen inhibited bacterial growth at concentrations above 16 µg/plate, so in the independent repeat test, using the preincubation method, concentrations from 1.6 to 500 µg/tube were employed. Suitable positive controls were tested in parallel.

Aclonifen techn. was non-mutagenic at concentrations up to 5000 µg per plate in either the presence or absence of hepatic S-9. The positive controls showed normal reversion properties, confirming sensitivity and validity of the test.

I. MATERIALS AND METHODS

Study dates Study start: 13 January 2006
Completion: 13 February 2006

A. MATERIALS

1 Test item Aclonifen techn.

Batch No.:	1219267-SP
Purity:	97.3%
Appearance:	Yellow powder

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Expiry: 12 January 2008

Structure:

Vehicle: DMSO

2 Positive controls

Sodium azide (Na-azide, Serva), for TA1535
Nitrofurantoin (NF, Sigma), for TA100
4-nitro-1,2-phenylene diamine (4-NPDA, Merck-Schuchardt), for TA1537 and 98
Mitomycin C (MMC, Fluka), for TA102 in plate incorporation
Cumene hydroperoxide (Cumene, Sigma), for TA102 in pre-incubation trials
2-aminoanthracene (2-AA, Aldrich), as control for the activating effect of the S-9 mix

3. Test system (strains)

Salmonella typhimurium

TA 100 (his G 46 + R-factor)
TA 1535 (his G 46)
TA 98 (his D 3052 + R-factor)
TA 1537 (his C 3076)
TA 102 (his G 428 + R-factor)

Obtained from [redacted]

S-9 mix:

Liver homogenate S-9 fraction from male Sprague-Dawley rats, induced with Aroclor 1254 (500 mg/kg bw)
$MgCl_2$
KCl
Glucose-6-phosphate
NADP
Sodium phosphate buffer
Protein content 23.4 mg/mL
Tested for sterility

B. STUDY DESIGN AND METHODS

The bacterial strains were cultured and tested for genotype, crystal-violet and UV sensitivity.

Experiment I was a plate incorporation test, with 3 plates for each strain and each dose, positive and negative control, with concentrations of 16, 5000 µg/plate:

Bacterial suspension:	0.1 mL
Sample (or solvent):	0.1 mL
Na-phosphate buffer (trials without S-9):	0.5 mL
S-9 mix (trials with activation):	0.5 mL

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Soft agar:	2.0 mL
Incubation temperature:	37°C
Incubation period:	48 h

Revertants were then counted with an automatic counter. Doses for Experiment II, the pre-incubation test, were based on results from Expt. I. Toxicity was assessed from reduced background lawn growth, dose-dependent marked reduction in mutants, and bacterial titer. The resulting concentrations chosen for the second test were then from 1.6 to 500 µg/tube.

Acceptance criteria:

a) Negative controls within expected range

b) Positive controls show sufficient effects

c) Titer determinations demonstrate sufficient bacterial density

Assessment criteria:

A reproducible and dose-related increase in mutant counts for at least one strain was considered a positive result. For TA 1535, TA 100 and TA 98 this increase had to be about twice that of negative controls, whereas for TA 1537, at least a threefold increase had to be reached. For TA 102 an increase of about 100 mutants was required. Otherwise, the result was evaluated as negative.

Data processing was by the Ames-Test III software (BioSys).

II. RESULTS AND DISCUSSION

The mean colony numbers are listed for each dose in the tables below. There was no indication of a bacteriotoxic effect of Aclonifen techn. at doses up to and including 16 µg per plate. The total bacterial counts consistently produced results comparable to the negative controls, or differed only insignificantly. No inhibition of growth was noted. Higher doses had a strong, strain-specific bacteriotoxic effect, such that they could only be used for assessment purposes up to and including 158 µg per plate. At 5000 µg per plate, the substance precipitated, although this did not interfere with scoring.

None of the five strains showed in the plate incorporation test a dose-related and biologically relevant increase in mutant counts over those of the negative controls, either with or without S9 mix, and this was confirmed by the preincubation trials. The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C, cumene hydroperoxide and 2-aminoanthracene increased mutant counts to well over those of the negative controls, and thus demonstrated sytem sensitivity and the activity of the S9 mix.

Table 5.4.1-3: Mutagenesis assay results, Experiment I, without S-9: Mean revertants

Group	Strain				
	TA 1535	TA 100	TA 1537	TA 98	TA 102
µg/plate	Plate incorporation				
0	17	137	8	26	245
16	17	135	6	25	254
50	12	116	7	17	237
158	15	75	4	14	178
500	13	78	3	10	139
1581	11	68	2	8	128

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Group	Strain				
	TA 1535	**TA 100**	**TA 1537**	**TA 98**	**TA 102**
5000	10	76	2	10	124
Na-azide	705				
NF		323			
4-NPDA			70	180	
MMC					519
µg/tube	Pre-incubation				
0	14	153	7	17	217
1.6	14	153	6	15	218
5	15	143	6	17	227
16	13	123	6	16	233
50	10	102	4	14	174
158	5	62	2	13	135
500	2	51	-	7	71
Na-azide	667				
NF		487			
4-NPDA			115	139	
Cumene					404

Table 5.4.1-4: Mutagenesis assay results, Experiment I, with S-9: Mean revertants

Group	Strain				
	TA 1535	**TA 100**	**TA 1537**	**TA 98**	**TA 102**
µg/plate	Plate incorporation				
0	10	184	9	53	291
16	8	175	7	43	308
50	7	188	6	33	287
158	5	145	6	27	260
500	6	83	6	10	217
1581	4	85	2	12	170
5000	3	86	2	8	139
2-AA	101	1456	356	1104	564
µg/tube	Pre-incubation				
0	12	170	9	30	287
1.6	13	172	7	27	281
5	10	195	7	26	277
16	10	203	7	27	290

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Group	Strain				
	TA 1535	TA 100	TA 1537	TA 98	TA 102
50	13	153	4	28	238
158	5	81	5	17	225
500	4	58	-	10	127
2-AA	131	1390	242	1069	562

III. CONCLUSION

Aclonifen techn. was non-mutagenic under the conditions of this assay, both with and without metabolic activation.

Assessment and conclusion by applicant:

The study is considered valid and acceptable, showing that aclonifen is non-mutagenic in the Ames test.

Assessment and conclusion by RMS:

Data Point:	KCA 5.4.1/03
Report Author:	███
Report Year:	1992
Report Title:	In vitro assessment of the clastogenic activity of Aclonifen in cultured human lymphocytes Final report
Report No:	R007188
Document No:	M-174403-01-1
Guideline(s) followed in study:	OECD: 473
Deviations from current test guideline:	Current Guideline: OECD 473, 2016 Minor deviation, no positive control for aneugenicity in absence of S9, however overall study integrity not affected
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

Aclonifen (purity 99.3%) was investigated for the potential to induce chromosomal aberration in human lymphocytes in the presence or absence of a hepatic S9 fraction from Aroclor pre-treated Sprague-Dawley CD rats. Test concentrations (in DMSO) ranged from 5-30 µg/mL without S9 mix

(24 h exposure) and from 12.5-100 µg/mL with S9 (3 h exposure). Cultures were prepared in triplicate.

Cell division was arrested by the addition of Colcemid, three hours before the cells were harvested; slides were then prepared for microscopic analysis. Mitotic indices were calculated for each culture, based on the number of metaphases observed per 1000 cells scored. Chromosome aberrations were scored by examination of 100 metaphases per culture (*ie* 300/concentration), and the frequencies of cells with one or more aberrations were calculated both including and excluding gap-type aberrations. A preliminary test was performed to investigate the toxicity of aclonifen to dividing lymphocytes. Subsequently the following aclonifen concentrations were tested in the main cytogenetic test:

- without S-9 mix: 5.0, 10.0, 20.0 and 30.0 µg/mL

- with S-9 mix: 12.5, 25.0, 50.0, 100.0 and 150.0 µg/mL.

Four aclonifen concentrations were tested in the absence of S-9 mix, and five in its presence, to ensure that an appropriate range of toxicity was covered. Slides prepared from cultures exposed to three concentrations only were selected for chromosomal analysis. The main test incorporated solvent (DMSO) and positive (cyclophosphamide and chlorambucil) control cultures.

In the absence of S9, aclonifen produced no evidence of toxicity (reduced mitotic activity) at 5 or 10 µg/mL, while reductions in mitotic activity of up to 57% were seen at 20 and 30 µg/mL. In the presence of S9, there was no evidence of toxicity of aclonifen at up to 50 µg/ml, while at 100 µg/mL a reduction in mitotic activity of approximately 65% was seen, and almost all cells were killed at 150 µg/mL.

No biologically or statistically significant increases in the frequency of aberrant cells over solvent control values were recorded at any tested concentration in either the presence or absence of S-9 mix ($p>0.05$). The positive controls induced significant increases in the frequency of chromosomal damage over the vehicle controls, demonstrating the sensitivity and validity of the test procedure, and the metabolic activity of the S-9.

It was concluded that aclonifen had no clastogenic activity in human lymphocytes under the conditions of this assay, in either the presence or absence of S-9 metabolic activation.

I. MATERIALS AND METHODS

Study dates	Study start:	20 February 1992
	Completion:	26 August 1992

A. MATERIALS

1. Test Item Aclonifen

Batch No.:	DA633
Purity:	99.3%
Appearance:	Yellow powder
Expiry:	23 April 1994 (retest)
Structure:	

Vehicle: DMSO

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2. Positive controls

Without activation:	Chlorambucil (Sigma Chemicals), dissolved in ethanol
With activation:	Cyclophosphamide (Endoxana, WB Pharmaceuticals), as aqueous solution

3. Test system (cells) Human lymphocytes from healthy non-smoking male not taking pharmaceuticals. Blood collected in heparinized vessels. Stimulated to divide with phytohaemagglutinin.

S-9 mix: Liver homogenate S-9 fraction, from male Sprague-Dawley rats induced with Aroclor 1254 (500 mg/kg bw)

S-9 supernatant:	1.5 mL
0.1 M KH_2PO_4 – Na_2HPO_4 buffer:	7.4 mL
0.4 M $MgCl_2.6H_2O$ / 1.65 M KCl:	0.2 mL
0.1 M NADP:	0.4 mL
0.1 M glucose-6-phosphate:	0.5 mL

Culture medium: RPMI 1640 with HEPES, sodium bicarbonate and L-glutamine, 10% fetal calf serum, 1% heparin and penicillin/streptomycin (5000 iu/mL / 5000 µg/mL)

B. STUDY DESIGN AND METHODS

Aclonifen was found to be soluble in DMSO at up to approximately 800 mg/mL. Concentrations were freshly prepared by serial dilution before addition to cultures.

After 48 h incubation, cell cultures were centrifuged and resuspended in culture medium. Freshly prepared S-9 was added to appropriate cultures and an aliquot of test material, solvent or positive control. Duplicate cultures were established for the preliminary cytotoxicity test, triplicate cultures for the main mutagenicity test. Incubation was at 37°C with shaking, for 3 h. Cultures without S-9 were then incubated for the remainder of the 24 h exposure period. Those with S-9 were centrifuged and washed twice with Hank's Balanced Salt solution and S-9 mix, resuspended in culture medium and incubated for a further 21 h. Three hours before harvesting, cell division was arrested with Colcemid (0.4 µg/mL). Harvest was by centrifugation and the cells were resuspended in hypotonic KCl solution (0.56%), recentrifuged and later fixed in methanol/glacial acetic acid (3:1 v/v). After 2 further changes of fixative, the cells were centrifuged and resuspended in fixative. Drops of the resulting cell suspension were placed on slides and air-dried. Two to four slides were prepared from each culture and stained with Giemsa.

Preliminary cytotoxicity test: Approximately 1000 lymphocytes per culture were examined and the mitotic index calculated, on the basis of which a concentration that should produce a depression of mitotic activity was selected as the highest level for the main test. Tested concentrations of aclonifen were:

6.4, 32, 160, 800, 4000 µg/mL, both with and without S-9 mix

Main test, chromosomal analysis and mitotic index: The concentrations selected were:

Without S-9: Aclonifen 5, 10, 20, 30 µg/mL, chlorambucil 2 µg/mL

With S-9: Aclonifen 12.5, 25, 50, 100, 150 µg/mL, cyclophosphamide 6 µg/mL

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Slides from cultures exposed to 3 concentrations only were selected for chromosome analysis. At least two randomly coded slides from each such culture were examined, 100 metaphases (with 46 centromeres) per culture, for:

Chromosome number

Specific aberrations: Gaps, breaks, fragments, exchanges, multiple aberrations (>8), endoreduplication, pulverization, polyploidy

Approximately 1000 cells scored to determine mitotic index

Statistical analysis used the one-tailed Fisher's Exact test to compare treated with corresponding solvent control.

II. RESULTS AND DISCUSSION

1. Preliminary cytotoxicity test

Precipitation of test material was observed at ≥800 µg/mL. Cultures with S-9 at ≥32 µg/mL were observed to be dark in colour after the 3 h exposure. Examination showed cells, but no metaphases, from cultures at 800 µg/mL, and from 3/4 cultures at 160 µg/mL.

In the presence or absence of S-9, no real toxicity was apparent at 6.4 µg/mL, but there was a reduction in mitotic index at 32 µg/mL. Cultures at 4000 µg/mL contained cells and metaphases, with reductions in mitotic index, but cell survival was considered consequent on rapid precipitation, reducing bioavailability.

Table 5.4.1-5: Toxicity test, mean results

Test group	Conc. µg/mL	S9 mix	Mitotic index (%)	Precipitation
Solvent control, DMSO	0	-	23.5	
Aclonifen	6.4	-	22.5	
	32	-	3.8	
	160	-	a	
	800	-	a	P
	4000	-	4.2	P
Solvent control, DMSO	0	+	17.3	
Aclonifen	6.4	+	17.8	
	32	+	14.6	
	160	+	b	
	800	+	a	P
	4000	+	10.3	P

a Few cells, no metaphases; not scored
b Few cells, no metaphases, in 3 of the 4 cultures; not scored

2. Main cytogenetic test

Cultures treated with aclonifen at ≥50 µg/mL in the presence of S-9 mix were observed to be dark in colour after the 3 h exposure period. In the absence of S-9, there was no reduction in mitotic activity at up to 10 µg/mL, but reductions occurred at ≥20 µg/mL. In the presence of S-9, there was no toxicity at up to 50 µg/mL, with reduced mitotic activity showing at ≥100 µg/mL.

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Table 5.4.1-6: Main cytogenetic test: Cytotoxicity, mean results

Test group	Conc. µg/mL	S9 mix	Mitotic index (%)	Precipitation
Solvent control, DMSO	0	-	13.8	None
Aclonifen	5	-	15.8	
	10	-	12.9	
	20	-	9.2	
	30	-	5.9	
Positive control (chloramb.)	2	-	11.1	
Solvent control, DMSO	0	+	12.3	None
Aclonifen	12.5	+	12.5	
	25	+	14.6	
	50	+	11.9	
	100	+	4.3	
	150	+	a	
Positive control (cyclophos.)	6	+	5.9	
a Few cells, no metaphases; not scored				

For each culture, 100 metaphases (with 46 chromosomes) were scored for aberrations, *ie* 300 per concentration. There were no meaningful increases in aberrant cell frequencies over concurrent solvent control, for any concentration of aclonifen, with or without S-9 metabolic activation. Meanwhile, the positive controls showed substantial and statistically significant increases in aberrant cell frequencies, demonstrating the sensitivity and validity of the system and efficacy of the S-9 mix.

Table 5.4.1-7: Main cytogenetic test: Aberrations, mean results

Test group	Conc. µg/mL	S9 mix	Mitotic index %	Cells with aberrations %	Cells with aberrations excluding gaps %
Solvent control, DMSO	0	-	13.8	2.7	0
Aclonifen	10	-	12.9	3.0	0.3
	20	-	9.2	3.7	0.7
	30	-	5.9	2.7	0
Positive control (chloramb.)	2	-	11.1	33.0 ***	17.7 ***
Solvent control, DMSO	0	+	12.3	2.7	0
Aclonifen	25	+	14.6	3.3	0.7
	50	+	11.9	2.7	0.7
	100	+	4.3	4.0	0.3
Positive control (cyclophos.)	6	+	5.9	26.3 ***	15.3 ***
Statistical significance of difference from solvent control: *** p≤0.001 (Fisher's Exact test)					

III. CONCLUSION

Under the conditions of the study, aclonifen showed no evidence of clastogenic activity in human lymphocytes, in either the presence or absence of S-9 metabolic activation.

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Assessment and conclusion by applicant:

The study is valid, reliable and acceptable, showing that aclonifen is not clastogenic in this mammalian (human) cell assay.

Assessment and conclusion by RMS:

Data Point:	KCA 5.4.1/04
Report Author:	████
Report Year:	1984
Report Title:	Mammalian cell (V79) mutagenic test in aclonifen
Report No:	R007402
Document No:	M-174850-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: Guideline not available, but broadly similar to OECD 476, 2016 Minor deviation, suboptimal number of cells/plate for express.ion period but overall study integrity not affected.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

Aclonifen (purity 95.5%) was teste for mutagenic potential by measuring its ability to induce mutations in V79 cells at the HGPRT locus. Dose levels were selected based on a preliminary cytotoxicity test.

In the absence of metabolic activation (S-9 mix), aclonifen did not show any toxic effect at concentrations of up to 15 µg/mL. At the next higher concentration, 25 µg/mL, the cloning efficiency was reduced by 27% compared with the solvent control, while at higher concentrations, virtually no cells survived. Based on these results, 25 µg/mL was selected as the highest concentration for the mutagenicity experiment without S-9. With S-9, toxicity only occurred at substantially higher concentrations, and concentrations of 10, 50, 75, 150, 250, 500, 750 and 1000 µg/mL were selected for the mutagenicity experiment. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a direct acting mutagen, and trans-7,8dihydroxybenzo(a)pyrene (BP-7,8-diol) and 7,12-dimethylbenz(a)anthracene (DMBA) were used as positive controls.

The mutagenicity experiment without S-9 clearly showed no effect, with very low mutant frequencies, indistinguishable from control, while the positive control exhibited strongly increased frequencies. No repeat experiment was required.

In the first experiment with S-9, aclonifen produced the strongest cytotoxicity at 250 µg/mL. Low mutation frequencies occurred at the other concentrations, indicative of a negative result. The repeat experiment confirmed this. Positive controls gave the expected high mutation frequencies.

It was concluded that aclonifen, when tested up to the highest feasible concentration, was negative in the V79 mammalian cell mutagenicity test, under the conditions of the study.

I. MATERIALS AND METHODS

Study dates Study start: 26 January 1984
Completion: 02 March 1984

A. MATERIALS

1. Test Item Aclonifen

Batch No.: Ho 06/81/1

Purity: 95.5%

Expiry: Not given

Structure:

Vehicle: DMSO

2. Positive controls:

Without activation: N-methyl-N-nitro-N-nitroso-guanidine (MNNG)

With activation: Trans-7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (BP), 7,12-dimethylbenz(a)anthracene (DMBA)

3. Test system (cells) V79 cell line (Chinese hamster lung fibroblasts). Master stock from [redacted], stored in liquid nitrogen. Screened for mycoplasm, karyotype stability and spontaneous mutant frequency.

S-9 mix: Liver homogenate S-9 fraction, from Sprague-Dawley rats induced with Aroclor 1254 (500 mg/kg bw)
3 volumes of S-9 fraction to 1 volume of cofactor solution

Culture medium: Dulbecco's MEM (minimal essential medium), with 10% calf serum and penicillin/streptomycin (DME-FCS). In S-9 mix experiments, exposure performed in Dulbecco's phosphate buffered saline with 20 mM HEPES

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Cofactor solution:	Glucose-6-phosphate	197 mM
	NADPH	28 mM
	NADH	26 mM
	NADPH in PBS-HEPES	11 mM

B. STUDY DESIGN AND METHODS

In the cytotoxicity experiment, 100 or 150 cells in DME-FCS were exposed after 4 h to a range of concentrations of aclonifen: 9 concentrations from 1-100 µg/mL plus zero control without S-9, and 9 concentrations from 10-1000 µg/mL plus control with S-9. In the absence of S-9, exposure was for 24 h, while with S-9 the medium was replaced with PBS-HEPES and S-9 mix and exposure was for 2 h. The cultures were then washed with PBS and incubated in DME-FCS for 8 days, then they were stained with Giemsa and the colonies counted. Incubations were in triplicate. The concentration that reduces the colonies by about 40-90% would be used as the highest concentration in the main mutagenicity test. The lower range of dosages should not show appreciable toxicity (relative cloning efficiency ≥0.8).

In the mutagenicity experiment, 1.5 x 10^6 cells were seeded in each dish. On the following day the cells were exposed to test compound as described above, but with larger volumes of media and test solution. After removal of the compound and washing with PBS, the cultures were maintained for 8 days in DME-FCS, with one subcultivation at day 5. The cells were then harvested by trypsinization and replated at 1 x 10^6 cells/dish in DME-FCS with 6-thioguanine for selection of mutants (6 replicates) or at 100/150 cells/dish in medium without 6-thioguanine for estimation of cloning efficiency (3 replicates). The plates were then fixed and stained after about 8 days (for cloning efficiency) or 12 days (6-thioguanine plates).

Assay acceptance criteria:

> Spontaneous mutant frequency in solvent control may be variable, but should not exceed 20 x 10^6.
>
> Positive controls should cause ≥10-fold increase in mutant frequency.

Assay evaluation:

> If solvent and positive controls give expected results, and the test compound does not increase mutant frequency more than 2-fold above solvent control under any condition, or if mutant frequency is always lower than 10 x 10^6 and if at least 10^6 cells/condition were evaluated, the compound is considered negative.
>
> If a dose-dependent increase in mutant frequency to at least 5-fold solvent control and at least 40 x 10^6 either with or without S-9, the compound is considered positive without additional experiments.
>
> In all other cases, the test is repeated with the suspected optimal concentration range. Then the result is considered positive if in both experiments (at similar concentrations) the mutant frequency is at least 2-fold above solvent control and at least 10 x 10^6. Otherwise, it is considered negative.

II. RESULTS AND DISCUSSION

1. Cytotoxicity

In the absence of metabolic activation (S-9 mix), aclonifen did not show any toxic effect at concentrations of up to 15 µg/mL. At the next higher concentration, 25 µg/mL, the cloning efficiency was reduced by 27% compared with the solvent control, while at higher concentrations, virtually no cells survived. Based on these results, 25 µg/mL was selected as the highest concentration for the mutagenicity experiment without S-9. With S-9, toxicity only occurred at substantially higher

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concentrations, and concentrations of 10, 50, 75, 150, 250, 500, 750 and 1000 µg/mL were selected for the mutagenicity experiment.

Table 5.4.1-8: Toxicity test, mean results

Test group	Conc. µg/mL	S9 mix	CE absolute[a] %	CE relative[b] %	Precipitation
Solvent control, DMSO	0	-	55	100	
Aclonifen	1	-	52	95	
	2.5	-	54	98	
	5	-	55	101	
	7.5	-	59	107	
	15	-	58	105	
	25	-	40	73	Precipitation
	50	-	1	2	Precipitation
	75	-	0	0	Precipitation
	100	-	0	0	Precipitation
Solvent control, DMSO	0	+	51	100	
Aclonifen	10	+	42	83	
	25	+	44	86	
	50	+	45	87	Precipitation
	75	+	38	75	Precipitation
	150	+	39	76	Precipitation
	250	+	0.4	1	Precipitation
	500	+	17	33	Precipitation
	750	+	29	56	Precipitation
	1000	+	25	49	Precipitation

a Number of cells grown to colonies divided by number of cells in the plates
b Absolute cloning efficiency of treatment group divided by absolute cloning efficiency of solvent control

2. Mutagenicity tests

Without S-9 mix, aclonifen showed very low mutant frequencies, ≤2.6 x 10^6, within the normally expected background range in solvent control, while the positive control strongly increased the mutant frequencies to over 2000 x 10^6. This clear result required no repetition of the experiment.

Table 5.4.1-9: Main mutagenicity tests, mean results, Experiment I

	Conc. µg/mL	P	S9 mix	No. of cells after expression period x 10^6	Cloning efficiency without thioguanine	No. of viable cells exposed to thioguanine x 10^6	Mutant colonies/ 10^6 cells
Solvent control (DMSO)	0		-	620	39%	2.3	0
Positive control (MNNG)			-	40	16%	0.8	2040
Aclonifen	1		-	470	30%	1.8	2.2
	2.5		-	740	50%	3.0	2.6
	5		-	610	48%	2.9	0
	7.5		-	690	51%	3.0	1.6
	15		-	660	45%	2.2	0.9
	25		-	730	14%	0.9	0
Solvent control (DMSO)	0		+	380	45%	2.7	2.2
	0		+	770	35%	2.1	1.9
Positive control (BP)			+	720	62%	3.7	56

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Aclonifen	10		+	1040	51%	3.0	1.0
	50		+	930	51%	3.1	0
	75	P	+	760	58%	3.5	4.3
	150	P	+	790	53%	3.1	4.1
	250	P	+	No surviving cells			
	500	P	+	40	44%	2.6	1.9
	750	P	+	730	43%	a	10.9
	1000	P	+	1090	55%	3.3	3.0

P = Precipitation visible
a Value obscured in the study report (printing error)

Table 5.4.1-10: Main mutagenicity tests, mean results, Experiment II

	Conc. µg/mL	P	S9 mix	No. of cells after expression period x 10^6	Cloning efficiency without thioguanine	No. of viable cells exposed to thioguanine x 10^6	Mutant colonies/ 10^6 cells
Solvent control (DMSO)	0		+	560	62%	3.7	0.3
	0		+	770	56%	3.3	0.3
	0		+	670	62%	3.7	3.7
Positive control (BP)	10		+	540	55%	3.3	18.5
	20		+	590	44%	2.6	367
Positive control (DMBA)			+	270	26%	1.6	666
Aclonifen	75	P	+	260	68%	4.4	0.2
	75	P	+	360	51%	3.0	2.0
	75	P	+	290	44%	2.6	6.1
	150	P	+	No surviving cells			
	150	P	+	No surviving cells			
	150	P	+	No surviving cells			
	750	P	+	680	47%	2.8	1.1
	750	P	+	850	53%	3.2	0.6
	750	P	+	1230	53%	3.2	0.6

P = Precipitation visible

In the first experiment with S-9, aclonifen produced the strongest cytotoxicity at 250 µg/mL, while at other concentrations, low mutant frequencies occurred (≤4.3 x 10^6), consistent with a negative result. The apparently increased frequency at 750 µg/mL was considered artefactual. The experiment was repeated including triplicate concentrations of 750 µg/mL and for control, yielding mutation frequencies ≤3.7 x 10^6. Positive controls substantially increased mutant frequencies, demonstrating sensitivity and validity of the assay.

III. CONCLUSION

Aclonifen, when tested up to the highest feasible concentrations (up to 1000 µg/mL) was completely negative in the V79 mammalian cell mutagenicity test, under the conditions of the study.

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Assessment and conclusion by applicant:

The study is considered valid and acceptable, showing that aclonifen is non-mutagenic in this mammalian cell assay.

Assessment and conclusion by RMS:

Data Point:	KCA 5.4.1/05
Report Author:	[redacted]
Report Year:	2019
Report Title:	Aclonifen technical AE F068300: Micronucleus test in human lymphocytes in vitro
Report No:	1939801
Document No:	M-664242-01-2
Guideline(s) followed in study:	OECD 87 (2016)
Deviations from current test guideline:	Current guideline: OECD 487, 2016 No deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

Aclonifen (99.9% purity), dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in two independent experiments. The following study design was used:

	Without S9 mix		With S9 mix
	Exp. I	Exp. II	Exp. I
Stimulation period	48 h	48 h	48 h
Exposure period	4 h	20 h	4 h
Recovery	16 h	-	16 h
Cytochalasin B exposure	20 h	20 h	20 h
Total culture period	88 h	88 h	88 h
Concentrations applied (µg/mL) Evaluated ones in **bold**	2.6, 4.5, **8.0, 13.9, 24.4**, 42.6, 74.6, 131, 229, 400, 1000	4.4, 6.6, 9.9, **14.8, 22.2, 33.3**, 50, 75, 150	4.5, 8.0, **13.9, 24.4, 42.6**, 74.6, 131, 229, 400, 1000

In each experimental group, two parallel cultures were analyzed, 1000 binucleated cells per culture being evaluated for cytogenetic damage. The highest applied concentration (1000 µg/mL of the test

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item) was chosen with regard to solubility properties. Dose selection for the cytogenetic experiment considered the toxicity data and the occurrence of test item precipitation.

In Experiment I in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest evaluated concentration of 24.4 µg/mL, which showed precipitation. In Experiment II in the absence of S9 mix after continuous treatment, cytotoxicity (47.6% cytostasis) was observed at the highest evaluated concentration of 33 µg/mL. The next higher tested concentration, however, showed clear cytotoxic effects (74.9% cytostasis) and was therefore too cytotoxic for cytogenetic evaluation.

In the absence and presence of S9 mix, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test item. Appropriate mutagens, used as positive controls, induced statistically significant increases in cells with micronuclei.

It was concluded that, under the experimental conditions, the test item did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes, and therefore, ACLONIFEN TECHNICAL AE F068300, specification 102000017430-02 is considered non-mutagenic, when tested up to cytotoxic or precipitating concentrations.

I. MATERIALS AND METHODS

Study dates Study start: 16 January 2019
Completion: 09 May 2019

A. MATERIALS

1. Test Item Aclonifen technical AE F068300, specification 102000017430-02

Batch No.: PTDF001324
Purity: 99.9%
Appearance: Yellow solid
Expiry: 13 November 2020 (retest)
Structure:

Vehicle: DMSO (99.93% purity)

2. Positive controls

Without activation: Mitomycin C (MMC), 98% purity, aqueous, 0.8 µg/mL
Demecolcin, ≥98% purity, aqueous, 75 ng/mL
With activation: Cyclophosphamide (CPA), 97-103% purity, in saline, 17.5 µg/mL

3. Test system (cells) Human lymphocytes from healthy non-smoking male not taking pharmaceuticals. Blood collected in heparinized vessels. Stimulated to divide with phytohaemagglutinin.

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S-9 mix:	Liver homogenate S-9 fraction, from rats induced with Phenobarbital/β-naphthoflavone. Each batch of S9 routinely tested for activity with the mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test.

$MgCl_2$	8 mM
KCl	33 mM
Glucose-6-phosphate	5 mM
NADP	4 mM
Sodium orthophosphate buffer	100 mM, pH 7.4
Protein concentration	30.4 mg/mL

Culture medium:	Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, 1:1) supplemented with 200 mM GlutaMAX™, 10% fetal calf serum, PHA (3 µg/mL), HEPES (10 mM), heparin (125 U/mL) and penicillin/streptomycin (100 U/mL / 100 µg/mL). Incubations all at 37°C, 5.5% CO_2 in humidified air.
Saline G:	Composition per litre (pH 7.2):

NaCl	8000 mg
KCl	400 mg
Glucose.H_2O	1100 mg
$Na_2HPO_4.2H_2O$	192 mg
KH_2PO_4	150 mg

B. STUDY DESIGN AND METHODS

1. Preliminary cytotoxicity test

With regard to the solubility of the test item, 1000 µg/mL was applied as top concentration for treatment of the cultures in the pre-test. Test item concentrations ranging from 2.6 to 1000 µg/mL (without S9 mix) and from 4.5 to 1000 µg/mL (with S9 mix) were chosen for the evaluation of cytotoxicity, as characterized by percentage reduction in the CBPI (cytokinesis-block proliferation index) in comparison with the controls (% cytostasis), assessed by counting 500 cells per culture. The experimental conditions in this preliminary test were identical to those described below for the mutagenicity assay.

The preliminary test was performed with 11 concentrations without S9 mix (2.6, 4.5, 8.0, 13.9, 24.4, 42.6, 74.6, 131, 229, 400, 1000 µg/mL) and 10 concentrations with S9 mix (4.5, 8.0, 13.9, 24.4, 42.6, 74.6, 131, 229, 400, 1000 µg/mL) of the test item, separated by no more than a factor of √10, plus a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hrs (with and without S9 mix). The preparation interval was 40 h after start of the exposure.

2. Cytogenetic experiment

Pulse exposure: About 48 hrs after seeding, 2 blood cultures were set up in parallel in 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 hrs the cells were spun down by gentle centrifugation. The supernatant was discarded and the cells were resuspended in and washed with "saline G". The washing procedure was repeated once. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and

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cultured for a 16-hour recovery period. After this, Cytochalasin B (4 µg/mL) was added and the cells were cultured for another approximately 20 h until preparation.

Continuous exposure (without S9 mix): About 48 hrs after seeding, 2 blood cultures were set up in parallel 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. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once, after which 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 another approximately 20 h until preparation.

Preparation of cells: The cultures were harvested by gentle centrifugation 40 hrs after beginning of treatment. The supernatant was discarded and the cells were re-suspended in saline G and spun down once again. Then the cells were then resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C. Ice-cold fixative (mixture of methanol and glacial acetic acid (19:1) was added to the hypotonic solution and the cells were resuspended. After removal of the solution by centrifugation the cells were resuspended in fixative and kept cold. Slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

Evaluation of cytotoxicity and cytogenetic damage: Micronuclei were counted in cells showing a clearly visible cytoplasm area. At least 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity was expressed as % cytostasis. A CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytostasis.

$$CBPI = \frac{(MONC \times 1) + (BINC \times 2) + (MUNC \times 3)}{n}$$

CBPI	Cytokinesis-block proliferation index
n	Total number of cells
MONC	Mononucleate cells
BINC	Binucleate cells
MUNC	Multinucleate cells

$$Cytostasis\ \% = 100 - 100\ [(CBPI_T - 1) / (CBPI_C - 1)]$$

T	Test item
C	Solvent control

3. Statistical analysis

Statistical analysis was by the Chi square test (α <0.05), 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, 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 would be judged as significant if $p < 0.05$.

4. Acceptability criteria

The micronucleus assay is considered acceptable if it meets the following criteria:

- The concurrent solvent control is within the laboratory historical solvent control data range.
- The concurrent positive controls produce a statistically significant increase in the micronucleus frequency, within the laboratory historical positive control data range.
- Cell proliferation criteria in the solvent control are considered acceptable.

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- All experimental conditions described above were tested, unless one exposure condition resulted in a clearly positive result.
- The quality of the slides allow evaluation of an adequate number of cells and concentrations.
- The criteria for the selection of top concentration are consistent with those described above.
-

5. Interpretation of results

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 exhibit a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase
- The results in all evaluated test item concentrations are within the range of the laboratory historical solvent control data

The test item is considered 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

II. RESULTS AND DISCUSSION

In Experiment I, precipitation of the test item in the culture medium was observed at 24.4 µg/mL and above in the absence of S9 mix and at 42.6 µg/mL and above in the presence of S9 mix, at the end of treatment. Precipitation also occurred in Experiment II, in the absence of S9 mix at 75.0 µg/mL and above at the end of treatment. No relevant influence on osmolarity or pH was observed.

In Experiment I in the absence or presence of S9 mix, no cytotoxicity was observed up to the highest evaluated concentration, which showed precipitation. In Experiment II in the absence of S9 mix after continuous treatment, cytotoxicity (47.6% cytostasis) was observed at the highest evaluated concentration. The next higher tested concentration, however, showed clear cytotoxic effects (74.9% cytostasis) and was therefore too cytotoxic for cytogenetic evaluation.

In the absence and presence of S9 mix, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test item. Appropriate mutagens, used as positive controls, induced statistically significant increases in cells with micronuclei.

Table 5.4.1-11: Summary results, micronucleus test in human lymphocytes *in vitro*

Exp	Prep. interval	Concentration µg/mL	Proliferation index CBPI	Cytostasis % of solvent control	Micronucleated cells % of 2000	95% Ctrl limit
Exposure period 4 h without S9 mix						
I	40 h	Solvent control[1]	1.82		0.10	0.01 – 1.20
		Positive control[2]	1.66	19.5	**5.25**[S]	2.66 – 22.74
		8.0	1.70	14.4	0.10	
		13.9	1.73	11.2	0.10	

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		24.4[P]	1.59	28.5	0.15	
Exposure period 20 h without S9 mix						
II	40 h	Solvent control[1]	1.90		0.15	0.00 – 1.14
		Positive control[3]	1.63	30.5	**2.55**[S]	1.15 – 6.44
		14.8	1.67	25.0	0.05	
		22.2	1.65	28.3	0.15	
		33.3	1.47	47.6	0.20	
Exposure period 4 h with S9 mix						
I	40 h	Solvent control[1]	1.70		0.20	0.00 – 1.24
		Positive control[4]	1.47	33.4	**6.00**[S]	1.01 – 7.34
		13.9	1.76	n.c.	0.15	
		24.4	1.60	13.6	0.35	
		42.6[P]	1.52	25.8	0.25	

[P] Precipitation occurred at the end of treatment
[S] Number of micronucleated cells statistically significantly higher than corresponding control
n.c. Not calculated as CBPI equal or higher than solvent control
[1] DMSO 0.5 % (v/v)
[2] MMC 0.8 µg/mL
[3] Demecolcine 75 ng/mL
[4] CPA 17.5 µg/mL

III. CONCLUSION

Aclonifen did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes, under the conditions of the study, when tested up to cytotoxic or precipitating concentrations. Therefore, Aclonifen Technical AE F068300, specification 102000017430-02, was considered to be non-mutagenic/clastogenic.

Assessment and conclusion by applicant:
The study is valid, reliable and acceptable, showing that aclonifen is not clastogenic in this mammalian (human) cell assay.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.4.1/06
Report Author:	[redacted]
Report Year:	2019
Report Title:	Aclonifen technical AE F068300 - Gene mutation assay in Chinese hamster V79 cells in vitro (V79-HPRT)
Report No:	1939802
Document No:	M-664619-01-1
Guideline(s) followed in study:	OECD 476 (2016); Commission Regulation (EC) No. 440/2008 B.17 (2008); US-EPA OPPTS Guideline 870.5300 (1998) Japanese Guidelines: Kanpoan No. 287-EPA, Eisei No. 127; Heisei 09/10/31 Kikyoku No. 2
Deviations from current test guideline:	Current guideline: OECD 476, 2016 No deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

This study was performed to investigate the potential of aclonifen technical (99.9% purity) to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster.

The treatment period was 4 hours with and without metabolic activation. The maximum test item concentration of the pre-experiment (1000 µg/mL) was based on the solubility properties of the test item. The highest concentration in the main experiment (100.0 µg/mL) was limited by precipitation observed in the pre-experiment.

No increase in mutant colony numbers was observed in the main experiment up to the maximum concentration scored for gene mutations, 50 µg/mL.

Appropriate positive controls induced distinct increases in mutant colonies, demonstrating the sensitivity of the test system and the activity of the S-9 metabolic activation system.

It was concluded that, under the experimental conditions, aclonifen technical was non-mutagenic in this HPRT assay.

I. MATERIALS AND METHODS

Study dates Study start: 31 January 2019
Completion: 26 February 2019

A. MATERIALS

1. Test Item Aclonifen technical

Batch No.:	PTDF001324
Purity:	99.9%
Appearance:	Yellow solid
Expiry:	13 November 2020

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Structure:

Vehicle: DMSO (99.3% purity)

2. Positive controls

Without activation: Ethyl methane sulfonate (EMS, 99%), in nutrient medium at 300 µg/mL, 2.4 mM

With activation: 7,12-dimethylbenz(a)anthracene (DMBA, ≥95%) in DMSO, final concentration in nutrient medium 0.5%

3. Test system (cells)

V79 cell line (Chinese hamster lung fibroblasts). Master stock from Laboratory for [redacted] [redacted] stored in liquid nitrogen.
Screened for mycoplasm, karyotype stability and spontaneous mutant frequency.

S-9 mix: Liver homogenate S-9 fraction, from rats induced with phenobarbital/β-naphthoflavone
$MgCl_2$ (8 mM)
KCl (33 mM)
Glucose-6-phosphate (5 mM)
NADP (4 mM)
Sodium orthophosphate buffer (100 mM, pH 7.4)
Protein content 30.4 mg/mL (0.74 mg/mL in the cultures)
Tested for activity

Culture medium: MEM (minimal essential medium), with Hank's salts, neomycin (5 µg/mL), 10% FBS, amphotericin B (1%)

PBS: Composition per litre:

NaCl	8000 mg
KCl	200 mg
KH_2PO_4	200 mg
Na_2HPO_4	150 mg

Saline G: Composition per litre:

NaCl	8000 mg
KCl	400 mg
Glucose.H_2O	1100 mg
Na_2HPO_4.$2H_2O$	192 mg
KH_2PO_4	150 mg

pH adjusted to 7.2

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B. STUDY DESIGN AND METHODS

A pre-test was performed to determine the toxicity of the test item, and the pH-value and osmolarity were measured. The general culturing and experimental conditions were the same as described below for the mutagenicity experiment.

In the pre-test approximately 1.5 million cells were seeded in 25 cm² flasks 24 hours prior to treatment. After approximately 24 hours the test item was added and the treatment proceeded for 4 h (duplicate cultures per concentration level). Immediately after treatment the test item was removed by rinsing with PBS. Subsequently, the cells were trypsinized and suspended in complete culture medium. After an appropriate dilution the cell density was determined with a cell counter. Toxicity of the test item is evident as a reduction of the cell density compared to a corresponding solvent control. The test was performed in the presence and absence of metabolic activation, with concentrations between 3.5 and 1000 µg/mL, as limited by solubility.

Relevant cytotoxicity, indicated by a relative cloning efficiency of <50% occurred at ≥55.6 µg/mL in the presence of metabolic activation. In the absence of metabolic activation no relevant cytotoxicity was noted. Precipitation occurred after 4 hours treatment at ≥27.8 µg/mL with and without metabolic activation. There was no relevant shift of pH and osmolarity of the medium, even at the maximum concentration of test item.

The doses thereby selected for the main gene mutation assay were as follows:

S9 mix	Concentrations (µg/mL)								
	Main experiment								
-	0.8	**1.6**	**3.1**	**6.3**	**12.5**	**25.0**	**50.0 P**	75.0 P	100.0 P
+	0.8	**1.6**	**3.1**	**6.3**	**12.5**	**25.0**	**50.0 P**	75.0 P	100.0 P

P = Precipitation, visible to naked eye at end of treatment
Concentrations in **bold** were selected for mutation rate analysis

Cultures at 0.8, 75 and 100 µg/mL were discontinued.

Two to four days after subcultivation stock cultures were trypsinized at 37 °C for approximately 5-10 min, then enzymatic digestion was stopped by adding complete culture medium with 10% FBS and a single cell suspension was prepared. The trypsin concentration for all subculturing steps was 0.2% in saline. Prior to the trypsin treatment the cells were rinsed with PBS. Approximately 0.7 to 1.2×10^7 cells were seeded in plastic flasks and grown for approx. 24 h prior to treatment.

The medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. 4 hours after treatment, this medium was replaced with complete medium following two washing steps with PBS. Immediately after the end of treatment the cells were trypsinized as described above and sub-cultivated. At least 2.0×10^6 cells per experimental point (concentration series plus controls) were subcultivated in flasks containing 30 mL medium. Two additional flasks were seeded per experimental point with approx. 500 cells each to determine the relative survival (RS) as a measure of test item induced cytotoxicity. The cultures were incubated at 37 ± 1.5°C in a humidified atmosphere with 1.5% ± 0.5 CO_2. The colonies used to determine the relative survival were fixed and stained approximately 6 to 8 days after treatment as described below.

Three or four days after the first sub-cultivation, at least 2.0×10^6 cells per experimental point were again sub-cultivated in flasks containing 30 mL medium. Following the expression time of approximately 7 days, 5 cell culture flasks were seeded with about 4 – 5×10^5 cells each in medium

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containing 6-TG (11 µg/mL). Two additional flasks were seeded with approximately 500 cells each in non-selective medium to determine viability. The cultures were incubated as before.

After 8 – 11 days the colonies were stained with 10% methylene blue in 0.01% KOH solution. Colonies with more than 50 cells were counted.

Acceptability criteria:

The gene mutation assay was considered acceptable if:

a) The mean values of the numbers of mutant colonies per 10^6 cells found in the solvent controls of both parallel cultures remained within the 95% confidence interval of the laboratory historical control data range.

b) Concurrent positive controls induced responses compatible with those generated in the historical positive control data base and produced a statistical significant increase compared with the concurrent solvent control.

c) Two experimental conditions (*i.e.* with and without metabolic activation) were tested unless one resulted in positive results.

d) An adequate number of cells and concentrations (at least four test item concentrations) were analyzable, even for the cultures treated at concentrations that caused 90% cytotoxicity during treatment.

e) The criteria for the selection of the top concentration were fulfilled.

Assessment criteria:

The test item is declared mutagenic if all the following criteria are met:

a) At least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

b) The increase is dose-related when evaluated with an appropriate trend test,

c) Any of the results are outside the distribution of the historical negative control data (*e.g.* Poisson-based 95% control limits).

A linear regression (least squares, calculated using a validated excel spreadsheet) was performed to assess a possible dose dependent increase of mutant frequencies. The number of mutant colonies (mean values) obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant if $p \leq 0.05$; however, both biological and statistical significance are considered together.

Calculations:

Pre-test

Cloning efficiency, absolute	mean cells/mL divided by the number of cells seeded x100
Cloning efficiency, relative	(cloning efficiency absolute divided by the mean cloning efficiency absolute of the corresponding control) x 100

Main test

Cloning efficiency I (survival)	cloning efficiency determined immediately after treatment to measure toxicity.

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Cloning efficiency II (viability)	cloning efficiency determined after the expression period to measure viability of the cells without selective agent.
Cloning efficiency I (survival, absolute)	mean number of colonies per flask divided by the number of cells seeded per test point
Cloning efficiency I (survival, relative)	cloning efficiency I absolute divided by the cloning efficiency I absolute of the corresponding control x 100
Relative density % of control	(cell density at 1st subcultivation divided by the cell density at 1st subcultivation of the corresponding control) x 100
Cloning efficiency II (viability, absolute)	mean number of colonies per flask divided by the number of cells seeded
Cloning efficiency II (viability, relative)	cloning efficiency II absolute divided by the cloning efficiency II absolute of the corresponding control x 100
Cells survived (after plating in TG containing medium)	number of cells seeded x cloning efficiency II absolute
Mutant colonies / 10^6 cells	mean number of mutant colonies per flask found after plating in TG medium x 10^6 divided by number of cells survived
Relative adjusted cloning efficiency I	relative cloning efficiency I x relative cell density at first subcultivation / 100

II. RESULTS AND DISCUSSION

Precipitation visible to the unaided eye was observed at 50.0 µg/mL at the end of treatment.

Relevant cytotoxic effects indicated by an adjusted cloning efficiency I below 50% occurred at ≥25 µg/mL in the absence of metabolic activation. The relative adjusted cloning efficiency I at 50 µg/mL was 12.2 and therefore met the required toxicity range of 10 – 20 %. In the presence of metabolic activation, no relevant cytotoxic effect was noted up to the highest concentration.

No increase in mutant colony numbers was observed in the main experiment up to the maximum concentration scored for gene mutations.

In the main experiment the mean mutant frequency of the solvent controls was 8.6 mutants per 10^6 cells in the absence of metabolic activation, and 7.6 mutants per 10^6 cells in the presence of metabolic activation. These values were well within the 95% confidence interval of the laboratory's historical solvent control data and therefore fulfilled the guideline requirements. Similarly, mean mutant frequencies of the groups treated with test item was in the range of 3.8 to 14.6 mutants per 10^6 cells, well within the 95% confidence interval of the laboratory's historical solvent control data. The positive controls EMS and DMBA showed distinct increase in induced mutant colonies, demonstrating sensitivity and validity of the assay.

Table 5.4.1-12: Toxicity test, mean results

Test group	Conc. µg/mL	S9 mix	CE absolute %	CE relative %	Precipitation
Solvent control, DMSO		-	67.8	100.0	
Aclonifen	3.5	-	64.6	95.3	
	6.9	-	63.8	94.1	

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Test group	Conc. µg/mL	S9 mix	CE absolute %	CE relative %	Precipitation
	13.9	-	63.2	93.2	
	27.8	-	54.4	80.3	precipitation
	55.6	-	50.9	75.1	precipitation
	111.1	-	48.6	71.7	precipitation
	333.3	-	40.8	60.2	precipitation
	1000.0	-	36.9	54.5	precipitation
Solvent control, DMSO		+	63.6	100.0	
Aclonifen	3.5	+	65.1	102.4	
	6.9	+	62.6	98.5	
	13.9	+	65.2	102.6	
	27.8	+	52.0	81.7	precipitation
	55.6	+	17.5	27.5	precipitation
	111.1	+	8.0	12.7	precipitation
	333.3	+	1.5	2.3	precipitation
	1000.0	+	2.3	3.7	precipitation

Table 5.4.1-6: Main mutagenicity tests, mean results

	Conc. µg/mL	P	S9 mix	Relative cloning efficiency I %	Relative cell density %	Rel. adjusted cloning efficiency I %	Mutant colonies/ 10^6 cells	95% confidence interval
Main experiment / 4 h treatment				Mean values of culture I and II				
Solvent control with DMSO			-	100.0	100.0	100.0	8.6	2.8 - 30.9
Positive control (EMS)	300.0		-	88.6	103.3	91.7	275.4	2.8 - 30.9
Aclonifen	0.8		-	89.3	105.0	93.6	#	#
	1.6		-	86.9	110.8	96.3	9.4	2.8 - 30.9
	3.1		-	88.7	115.9	103.3	11.6	2.8 - 30.9
	6.3		-	86.8	97.4	84.5	3.8	2.8 - 30.9
	12.5		-	88.7	99.7	89.0	9.5	2.8 - 30.9
	25.0		-	53.6	94.6	48.0	12.9	2.8 - 30.9
	50.0	P	-	12.1	100.2	12.2	14.6	2.8 - 30.9
	75.0	P	-	#	#	#	#	#
	100.0	P	-	#	#	#	#	#
Solvent control with DMSO	0.0		+	100.0	100.0	100.0	7.6	3.1 -30.7
Positive control (DMBA)	2.3		+	93.8	101.2	94.8	57.6	3.1 -30.7
Aclonifen	0.8		+	97.1	94.4	91.7	#	#
	1.6		+	101.1	86.7	87.7	9.2	3.1 -30.7
	3.1		+	93.5	103.1	96.4	7.0	3.1 -30.7
	6.3		+	94.3	92.2	87.3	4.9	3.1 -30.7
	12.5		+	92.5	92.9	85.8	9.6	3.1 -30.7
	25.0		+	91.6	104.4	95.9	7.5	3.1 -30.7
	50.0	P	+	83.4	69.3	56.4	10.7	3.1 -30.7
	75.0	P	+	#	#	#	#	#
	100.0	P	+	#	#	#	#	#

P = Precipitation visible at end of treatment
# = Culture not continued

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The linear regression analysis showed no significant dose dependent trend in the mutation frequency for any of the experimental groups.

III. CONCLUSION

Under the experimental conditions, aclonifen did not induce gene mutations at the HPRT locus in V79 cells. Therefore, aclonifen was considered non-mutagenic in this HPRT assay.

Assessment and conclusion by applicant:
The study is valid and acceptable, showing that aclonifen is non-mutagenic in the HPRT assay.

Assessment and conclusion by RMS:

CA 5.4.2 *In vivo* studies in somatic cells

Data Point:	KCA 5.4.2/01
Report Author:	[redacted]
Report Year:	1984
Report Title:	Cytogenetic Investigations in NMRI mice after a single oral administration of CME 127 (Aclonifen)
Report No:	R007404
Document No:	M-174855-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 474, 2016 Purity of test item not reported. Only 1000 PCE/animal evaluated, instead of the currently required 4000. No blood sampling and no change in the PCE/NCE ratio to confirm marrow exposure, although there were some other indications of slight impairment of erythropoiesis. Doses higher than current limit dose of 2000 mg/kg bw. Overall these deviations do not affect the integrity of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

Aclonifen (purity not stated) was examined for clastogenicity *in vivo* using the mouse micronucleus assay. Aclonifen, suspended in an aqueous 0.5% CMC solution was administered as a single oral dose to male and female NMRI mice at dose levels of 0 (control), 1000, 3000 or 10000 mg/kg bw (nominal). The positive control cyclophosphamide was administered orally at 40 mg/kg bw. The animals were sacrificed and bone marrow smears prepared at 16, 24 and 48 h after dosing from the control and 10000 mg/kg dose groups, while for the other groups only the 24 h interval was investigated. After staining, 1000 polychromatic erythrocytes were evaluated per animal for micronuclei and for normocytes with and without micronuclei.

The achieved dosages, as determined by analysis of the dose formulations, were 578, 1650 and 7260 mg/kg bw for the groups receiving aclonifen, *ie* 55-73% of the nominal levels. The expected range for analysis of a CMC suspension, quoted by the laboratory, was 65-85%. However, the highest dose group did receive the highest feasible dose concentration.

There were no clinical or gross pathological signs of toxicity. There was no increase in the number of polychromatic erythrocytes containing micronuclei at any dose level of aclonifen. There was no change in the ratio of polychromatic to normochromatic erythrocytes, although in some animals at 10000 mg/kg, microcytes, macrocytes and basophilic stippling were observed, giving some evidence of slight impairment of erythropoiesis.

It was concluded that under the conditions of the study, aclonifen did not have any clastogenic activity, and gave no indication of any inhibitory effect on mitosis.

I. MATERIALS AND METHODS

Study dates Study start: October 1983
Completion: 08 March 1984

A. MATERIALS

1. Test Item CME 127 (aclonifen)

Batch No.: 83/286

Purity: Not stated

Appearance: Yellow powder

Expiry: Not stated

Structure:

Vehicle: 0.5% aqueous carboxymethylcellulose (CMC)

2. Positive control Cyclophosphamide, in distilled water

3. Animals Male and female NMRI mice [redacted]

[redacted] mean bodyweight 31.3 g

Individually housed, with water and pelleted feed *ad libitum* ([redacted]

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B. STUDY DESIGN AND METHODS

Aclonifen was administered as a suspension in 0.5% aqueous CMC, orally by gavage to groups of NMRI mice (15/sex/group) as single doses of 0 (vehicle control), 1000, 3000 or 10000 mg/kg bw. The highest dose was the highest concentration feasible, at a dose volume of 20 mL/kg bw. The positive control, cyclophosphamide, was formulated as a solution in distilled water and administered orally by gavage as a single dose at 40 mg/kg bw using a dose volume of 10 mL/kg bw.

The dosing formulations were prepared immediately prior to dosing, and samples of the aclonifen formulations were analyzed for achieved concentration, following acetone extraction, by gas chromatography.

The mice were examined for any clinical signs of toxicity, after the administration. Five per sex were sacrificed at 16, 24 or 48 h after administration, according to the schedule shown below:

Test group	Sacrifice interval (h post-dose)	Dose administered	Number of mice (males/females)
1	16	Solvent control, CMC, 20 mL/kg bw	5/5
2	24		5/5
3	48		5/5
4	16	Aclonifen, 10000 mg/kg bw	5/5
5	24		5/5
6	48		5/5
7	24	Aclonifen, 3000 mg/kg bw	5/5
8	24	Aclonifen, 1000 mg/kg bw	5/5
9	24	Positive control, cyclophosphamide, 40 mg/kg bw	5/5

During examination at necropsy for any grossly visible changes, both femurs were removed from each animal for preparation of the marrow, which was flushed out of the diaphysis with fetal calf serum at room temperature. The resulting suspension was centrifuged, the supernatant was removed and the cells resuspended. Drops of this suspension were placed on slides and smears prepared and air dried. These were stained with eosin and methylene blue, rinsed and then stained with Giemsa, prior to clearing in xylene and embedding.

The smears were coded and examined microscopically, assessing 1000 polychromatic erythrocytes from each animal for the number exhibiting micronuclei. The number of normochromatic erythrocytes containing micronuclei was also recorded. The ratio of polychromatic to normochromatic erythrocytes was determined, as a measure of general effect on the bone marrow. The number of small micronuclei (<¼ of cell diameter) and large micronuclei (≥¼ of cell diameter) were also recorded.

Differences between treatments and control in terms of relative frequencies of cells with micronuclei were analyzed statistically using Fisher's Exact test and the Mann-Whitney U test. The relative frequencies were also analyzed sequentially to determine the significance of any apparent change through time (over the successive sacrifice intervals).

II. RESULTS AND DISCUSSION

Analysis of the dosing formulations gave the following results:

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Table 5.4.2-16: Micronucleus assay in mice, formulation analysis

Nominal concentration	Individual sample results (mg/mL)	Mean result (achieved dose, mg/kg bw) Coefficient of variation
10000 mg/20 mL	392 342 397 321	7260 10%
3000 mg/10 mL	179.8 156.2 148.2 175.8	1650 9%
1000 mg/10 mL	56.2 57.4 65.2 52.2	578 9%

Approximately 55-73% of the theoretical, nominal values could be determined analytically. The laboratory stated that a value between 65% and 85% would be usual for this analysis of a CMC suspension, and that regardless of the nominal values the high dose animals received the maximum dose that was feasible. The coefficients of variation were ≤10%, demonstrating homogeneity.

There were no clinical signs of toxicity in terms of appearance/behaviour of the animals, but the urine in all aclonifen groups showed a distinct yellow-green colour. There were no grossly visible pathological changes observed at necropsy.

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Table 5.4.2-17: Micronucleus assay in mice, summary results

Dose (mg/kg)	NCE/PCE ratio (%) 16, 24, 48h	Interval 16 h			Interval 24 h			Interval 48 h		
		NCE/ 1000 PCE	Micronucleated cells		NCE/ 1000 PCE	Micronucleated cells		NCE/ 1000 PCE	Micronucleated cells	
			/1000 PCE	/1000 NCE		/1000 PCE	/1000 NCE		/1000 PCE	/1000 NCE
Solvent control (0)	31, 37, 29	3102	1.9	1.29	3684	1.1	0.81	2892	2.0	2.07
Aclonifen (10000)	31, 30, 28	3071	2.4	2.61+	2990	2.0	0.33	2836	0.9+	0.71
Aclonifen (3000)	25	-	-	-	2471	1.5	2.43	-	-	-
Aclonifen (1000)	29	-	-	-	2944	0.7	-	-	-	-
Cyclophos. (40)	37	-	-	-	3696	21.9**	1.89	-	-	-

Statistical significance: Fisher's Exact and/or Mann-Whitney U: * p≤0.05, ** p≤0.01
Time trend: + p≤0.05

Aclonifen did not induce any increase in the incidence of micronuclei. Inhibition of erythropoiesis, as indicated by the NCE/PCE ratio, was not detected. However, in some animals at 10000 mg/kg bw, microcytes, macrocytes and basophilic stippling were occasionally observed, indicating some slight disturbance of erythropoiesis.

The number of normochromatic and polychromatic erythrocytes containing not only small micronuclei, but also large micronuclei, remained in the range of that of solvent control. Only 2 mice at 10000 mg/kg bw, and at 24 h only, each had a single cell out of the 1000 examined that included a large micronucleus together with small micronuclei, versus zero in solvent control and 4 mice in positive control.

The positive control induced a substantial, statistically significant increase in the incidence of micronuclei, demonstrating the validity and sensitivity of the test.

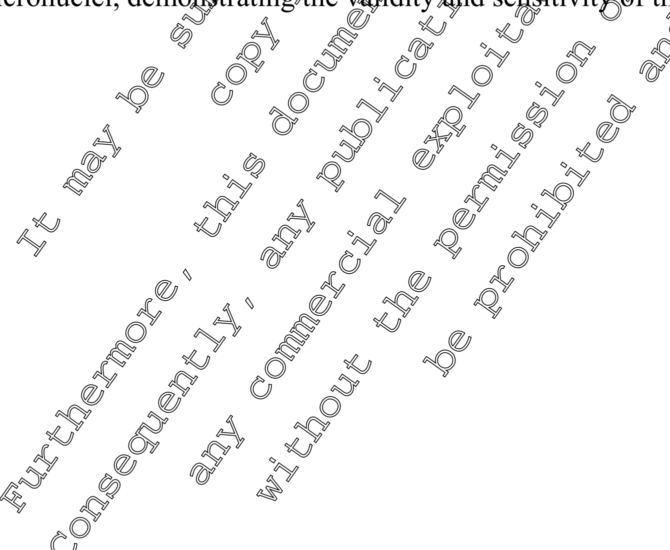

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Table 5.4.2-18: Micronucleus assay in mice, mean incidence of small/large micronuclei

Dose (mg/kg)	Polychromatic erythrocytes		
	With 1 MN ≤¼ cell diameter 16, 24, 48h	With 1 MN ≥¼ cell diameter 16, 24, 48h	With ≥2 MN 16, 24, 48h
Solvent control (0)	1.9, 1.1, 2.0	0, 0, 0	0, 0, 0
Aclonifen (10000)	2.4, 1.8, 0.9	0, 0.2, 0	0, 0, 0
Aclonifen (3000)	1.5	0	0
Aclonifen (1000)	0.7	0	0
Cyclophos. (40)	20.8	0.6	0.5

III. CONCLUSION

There were no biologically relevant, significant differences in the incidence of micronucleated erythrocytes between the solvent control and any dosage of aclonifen. Therefore, aclonifen had no clastogenic activity under the conditions of the study, and gave no indication of any inhibitory effect on mitosis.

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Assessment and conclusion by applicant:

There was no convincing evidence of exposure of the bone marrow, in terms of altered NCE/PCE ratio, but the ADME studies have shown the presence of aclonifen in blood cells and plasma and following repeat dosing it was measurable also in the bone marrow (ref. text under section CA 5.1).

The study is considered acceptable as supplementary information, to show that aclonifen does not have clastogenic activity.

Table showing evidence of bone marrow exposure to aclonifen

Evidence to check	Evidence found
Toxicity to the bone marrow observed in the *in vivo* mammalian erythrocyte micronucleus test	PCE not reduced. In some mice microcytes, macrocytes and basophil stippling may be slight evidence of toxicity. Suggests bone marrow exposure.
Systemic toxicity observed in the *in vivo* bone marrow micronucleus test	Yes. Aclonifen is yellow. Urine stained yellow indicating the test substance was absorbed into the blood. Bright yellow urine indicates metabolites are excreted in mouse urine therefore systemic exposure.
Systemic toxicity observed in toxicity studies in mice	Yes. In acute oral mouse study there were clinical signs of toxicity at 5000 mg/kg bw (; 1981, M-174876-01-1). In 28-day mouse study at 50000 ppm (8906/12403 mg/kg bw/day) there were severe reduction in body weight gain, clinical signs of ill health (including lethargy, tremors, hunched posture) and mortalities in both sexes (1988, M-174234-01-1). Systemic toxicity in acute and 28-day mouse studies demonstrates test substance is absorbed
Test substance (and/or metabolites) detected in the bone marrow in a toxicokinetic study in rats	Yes. Radioactivity detected in bone marrow in repeat-dose ADME study in rat at 168 hrs post dose (30 mg/kg bw/day after 14 days of repeat dosing 2002, M-211131-01-1). Detected in rat bone marrow.
Test substance (and/or metabolites) detected in blood/plasma toxicokinetic study	Yes. Radiolabelled aclonifen found in plasma and blood ADME study doses of 2, 30 and 1000 mg/kg bw (2002, M-210562-01-1, and 2017 M-598008-01-1). Detected in rat blood and plasma.
Test substance detected systemically in a specific blood/plasma analysis in mice.	Yes. Aclonifen metabolites detected in plasma in 28-day mouse study (1988, M-174234-01-1). Detected in mouse plasma.

Evidence compiled with reference to EFSA Journal 2017;15(12):5113 **Clarification of some aspects related to genotoxicity assessment section** 3.2 The adequacy to demonstrate target tissue exposure

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in *in vivo* studies, particularly in the mammalian erythrocyte Micronucleus Test

In conclusion there is sufficient evidence to demonstrate bone marrow exposure.

Assessment and conclusion by RMS:

CA 5.4.3 *In vivo* studies in germ cells

Studies in germ cells are considered unnecessary.

CA 5.5 Long-term toxicity and carcinogenicity

Two long-term toxicity and carcinogenicity studies have been performed in the rat and one in the mouse. All the studies are broadly in compliance with current guideline requirements and were performed according to GLP.

The two studies in the **rat** were run using the same strain and similar dose levels.

In the first study (1989, M-174241-01-1) aclonifen was orally administered, via the diet, at dietary levels of 0 (control), 40, 200 and 1600 ppm to groups of 70 male and 70 female Wistar (Chbb:THOM (SPF)) rats for 24 months. Reduced body weights and food consumption were observed in females at the top dose level. In both sexes at the top dose there were slight changes in clinical chemistry (increased serum protein, increased serum albumin, decreased triglycerides), a reduction in T4, and in males a reduction in T3. Necropsy revealed a slight increase in absolute and relative thyroid weight at 12 months.

At 24 months an equivocal increased incidence of thyroid C-cell tumours was seen in females at dose levels $\geq$ 200 ppm, however following further re-evaluation and investigation, these findings were considered to be within the normal background incidence for this strain of Wistar rat. Based on effects occurring at the 200 ppm dose level, the NOAEL was 200 ppm (equating to approximately 8 mg/kg/day).

The histological sections of the thyroids from the aclonifen rat carcinogenicity study were subsequently reviewed by an independent consultant pathologist, Professor (1990, M-175852-01-1), and the results from this and the original histopathology report (1988, M-232479-01-1) were analysed using appropriate statistics (Peto analysis) which takes account of mortality during the study (1990, M-174790-01-1). On the demand of the German Authorities, an additional review of the thyroid slides was carried out (1995, M-174790-01-1) and an additional statistical analysis was carried out of total tumour incidence (2001 M-205316-01-1).

The overall conclusion from all the reviews was that in the absence of any significant dose-related trend in both studies, the finding of statistical significance in tumour incidence was considered to be a chance phenomenon and not related to aclonifen administration.

However, due to no clear evidence of a no effect level a new study was performed.

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In a second study (2004b, M-234946-01-1) aclonifen was orally administered, via the diet, at dietary levels of 0 (control), 20, 40, 200 and 1600 ppm to groups of 70 male and 70 female Wistar WI-IOPS AF rats for 24 months. Treatment-related findings were only seen at the top dose of 1600 ppm. Reduced body weight and food consumption were observed in females and clinical chemistry analysis revealed in males an increase in both aspartate aminotransferase and alanine aminotransferase, and in both sexes an increase in total cholesterol. Females had a slight increase in absolute and relative liver weight, and in both sexes hepatocellular hypertrophy was evident. The incidence of thyroid C-cell tumours in treated groups was similar to that of control groups. In females four malignant astrocytoma were observed at 1600 ppm with one occurring also at 200 ppm and none in the control or 20, and 40 ppm dose groups. In males there were 2 astrocytomas at 1600 ppm, with one also occurring in the control, 40 and 200 ppm dose groups The incidence was not statistically different from control (but there was a dose-related increase in females which was statistically significant in a trend test). There is no evidence for a mechanistic explanation for the astrocytoma findings in females.

Based on effects occurring at 1600 ppm, the NOAEL was 200 ppm (equivalent to 7.6/11 mg/kg body weight/day in males/females respectively).

Aclonifen has a harmonised classification under (EC) No. 1272/2008 Carcinogenicity Category 2 H351, based on a low incidence of unusual brain tumours in female rats in the study by 2004b, (M-234946-01-1), due to the rarity of this tumour type and the absence of a mechanistic explanation, the finding in female rats was considered as limited evidence of carcinogenicity (RAC Opinion ECHA/RAC/ CLH-O-0000001543-79-03/A1 September 2010).

In **mice** aclonifen was administered via the diet at dietary levels of 0 (control), 70, 700 and 7000 ppm to groups of 52 male and 52 female CD-1 mice for 80 weeks, to determine carcinogenic potential.

Lower bodyweights gains were observed in male and female mice of the top dose group, as well as male mice of the intermediate dose level.

Treatment-related hepatomegaly occurred in both sexes at 7000 ppm, and an increase in aspartate aminotransferase and increased alanine aminotransferase was seen in males, but no adverse histopathological liver findings in either sex. An increase in T4 was seen at 700 and 7000 ppm but no adverse histopathological changes in the thyroid. In the bladder there was a higher incidence of transitional cell hyperplasia at 700 and 7000 ppm in both sexes as well as chronic inflammation at 7000 ppm. Three urinary bladder tumours (two in males and one in a female) were found at the top dose level suggesting an irritant effect. The NOAEL for CD-1 mice was 70 ppm (equivalent to intake of 7.1/8.3 mg/kg/day for males/females respectively).

A review of the urinary bladder histologic lesions (1994, M-174769-01-1), confirmed that the dose level of 700 ppm was a clear NOAEL for tumour development. Only one malignant bladder tumour was observed in the mouse study. Therefore any carcinomic response in aclonifen is weak and even questionable.

A special study (KCA 5.8.2/01 1995., M-174903-01-1) showed that aclonifen does not bind to liver and/or urinary bladder of CD-1 mice after oral administration, confirming that urinary tumours are triggered by a non-genotoxic mode of action.

Aclonifen Summary of long-term toxicity/carcinogenicity

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Type of study Doses	NOAEL	Findings	References
Rat, combined chronic/carcinogenicity study 12 and 24 months 0, 40, 200 and 1600 ppm (1.6/2.2, 8/11, 67/98 mg/kg bw/day in M/F respectively)	200 ppm males: 8 mg/kg bw/day females: 11 mg/kg bw/day	At 1600 ppm: Decreased food consumption and decreased body weight gain (F); increase in total protein and albumin and reduced triglyceride (M & F). Reduced T4 (M & F), reduced T3 (M only). Increased abs/rel thyroid weight (at 12 months M & F).	KCA 5.5/01 [redacted] 1989 M-174241-01-1; [redacted] 1989 M-174243-01-1 [redacted] 1988 M-232479-01-1; [redacted] & [redacted] 1986 M-232482-01-1; [redacted] 1989 M-232485-01-1.
Rat combined chronic/carcinogenicity study 12 and 24 months 0, 20, 40, 200 and 1600 ppm (0, 0.8/1.1, 1.6/2.1, 7.6/10.6, 61/86 mg/kg bw/day in M/F respectively)	200 ppm males: 7.6 mg/kg bw/day females: 11 mg/kg bw/day	At 1600 ppm: Decreased food consumption and body weight gain (F); liver hypertrophy (M & F) Increased aspartate aminotransferase and increased alanine aminotransferase (M only) Increased cholesterol (M & F) 4/60 malignant astrocytoma (F)	KCA 5.5/11 [redacted] 2004 M-234946-01-1
Mouse combined chronic/carcinogenicity study 9 and 18 months 0, 70, 700 or 7000 ppm (0, 7.1/8.3, 76/80, 892/984 mg/kg bw/day in M/F respectively)	70 ppm males: 7.1 mg/kg bw/day females: 8.3 mg/kg bw/day	At 7000 ppm: Decreased bodyweight gain (M & F) Increased absolute and relative liver weight (M & F) Decreased T4 (M & F) Increased aspartate aminotransferase and increased alanine aminotransferase (M only) In urinary bladder increased transitional cell hyperplasia and chronic inflammation (M & F). Urinary bladder tumours in two males and one female At 700 ppm: Decreased bodyweight gain (M only) Decreased T4 (M & F) In urinary bladder increased transitional cell hyperplasia (M & F).	KCA 5.5/12 [redacted] 1991 M-174334-01-1

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Data Point:	KCA 5.5/01
Report Author:	
Report Year:	1989
Report Title:	REPORT on the study of the chronic toxicity and oncogenic potential of ACLONIFEN (CME 127) in rats after 24-month administration in the diet Volume I
Report No:	R007113
Document No:	M-174241-01-1
Guideline(s) followed in study:	MAFF: (1985); OECD: 453; USEPA (=EPA): F,83-5
Deviations from current test guideline:	Current Guideline: OECD 453, 2018 Minor deviations, No differential leukocyte count. Uterus and epididymides not weighed. Histopathology not conducted on upper respiratory tract, Harderian gland, bone marrow. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

In a combined chronic and carcinogenicity study in Wistar rats (50 animals/sex/dose in main 24 month study, 10/sex/dose in the 12 month study, and 10/sex/dose in 24 month cohort used for haematology and clinical chemistry) were administered aclonifen (purity 95.5%) in the diet at doses of 0, 40, 200, 1600 ppm (equivalent to 0, 1.6/2.2, 8/11, 67/98 mg/kg bw/day in males/females respectively).

At the top dose of 1600 ppm food consumption and body weight gain was reduced in females. In both sexes there was an increase in serum albumin and increased serum protein, and decreased triglycerides. There was also a decrease in T4 which was more marked in males, and accompanied by males by a slight reduction in T3 and an increase in absolute and relative thyroid weight at 12 months though without any correlating histopathological findings in the thyroid at 12 months. An increase in C-cell tumours of the thyroid at 24 months was considered to be within the background incidence for this strain of rat (and following re-examination of the slides and additional statistical analysis – see M-175852-01-1, M-174790-01-1, M-174790-01-1 and M-205316-01-1). At the mid dose of 200 ppm the only finding was a slight reduction in serum albumin in males. There were no adverse findings at 40 ppm.

Overall tumour incidence was not affected by treatment.

The NOAEL was 40 ppm in both sexes (equating to approximately 1.6 and 2.2 mg/kg/day in males and females, respectively).

A slight increase in C-cell carcinoma of the thyroid in females at 200 and 1600 ppm was considered to be within the normal background incidence for this strain of rat. Therefore under these test conditions aclonifen displayed no oncogenic potential following 24 months administration in the Wistar rat.

The study was performed to GLP and OECD 435 (although there are some minor omissions in meeting the current 2018 these omissions to not affect the overall adequacy of the study).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	Aclonifen (CME 127)
Lot/Batch:	84/1
Purity:	95.5%
Appearance:	Not mentioned
Stability of test compound:	The mixture of test substance and food was freshly prepared at intervals of 7 - 21 days and stored at room temperature. Stability in the diet was demonstrated for a period of 4 weeks.

2. Vehicle and /or positive control Aclonifen was incorporated into pulverised rodent diet

3. Test animals

Species and strain:	Rat – Wistar ChbbTHOM (SPF)
Source:	
Number of animals:	50 per sex/dose group for main study (plus 2 satellite groups of 10 per sex/dose)
Sex:	Male + Female
Age:	30 days old at start of acclimatisation period
Weight at treatment:	Mean weight – main group: males: 182g (154-207g); females: 149g (130-173g). Satellite group: males 181g (162-201g); females 149g (134-167g)
Acclimation period:	12 days
Diet:	Ground Kliba rat/mouse/hamster GLP 343 maintenance diet of which was available *ad libitum*
Water:	Drinking water, *ad libitum*
Cage type:	Stainless steel wire cages, Type III, by and Co
Housing:	Individually
Environmental conditions:	Temperature: 20 - 24°C Humidity: 30 - 70 % Air changes: yes, but number of changes/hour not mentioned Photoperiod: 12 hours light/dark

B. STUDY DESIGN AND METHODS

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1. In life dates

18 January 1984 to 21 February 1986

2. Animal assignment and treatment

Aclonifen (Batch HO 06/84/1, purity 95.5 %) was orally administered, via the diet, at levels of 0, 40, 200 and 1600 ppm to groups of 70 male and 70 female Wistar (Chbb:THOM (SPF)) rats for up to 24 months.

In each dose group, the rats were divided into a main group (50 animals/sex) and two satellite groups, group I and group II (10 animals/sex/group). Satellite group I was terminated after 52 weeks. The rats of the main group and satellite group II were sacrificed after 24 months.

Table 5.5- 1: Aclonifen carcinogenicity study in rats – Test groups

Test group	Main group	Satellite I	Satellite II
Number of animals per dose/sex	50	10	10
Clinical signs	daily	daily	daily
Detailed clinical observations	weekly	weekly	weekly
Ophthalmoscopy	10 per sex/dos every 6 months	-	-
Haematology and clinical chemistry	-	-	3, 6, 12, 18 and 24 months
Urinalysis	-	-	3, 6, 12, 18 and 24 months
Necropsy	24 months	12 months	24 months
Organ weights	All dose groups	All dose groups	All dose groups
Histopathology	All dose groups	All dose groups	All dose groups

3. Diet preparation and analysis

The formulated pre-study diets at 40 and 1600 ppm were found to be homogenously and accurately prepared. Reanalysis following storage for 14 and 28 days at ambient temperature showed the formulation to be stable in the diet for at least 28 days. After 28 days storage the concentrations at 40 and 1600 ppm was within 5% of target concentrations. During the study the diet was freshly prepared at intervals of 7 – 21 days and stored at room temperature.

The diet was analysed at the start of the study and subsequently every three months. The 200-ppm-concentration was 9.6-19.5 % below target concentrations indicating this particular dose was not as well prepared (or that there were limitations with the method of measurement). The 40 ppm dose concentrations were not more than 11.8% below target, and at 1600 ppm no more than 7.8% below target. Despite these deficiencies it was concluded the diet concentrations were satisfactorily prepared.

4. In-life observations

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Food consumption and body weight of the animals in the main group and satellite group I were determined weekly during the first 14 weeks of the study and then every 4 weeks.

The state of health of all rats was checked daily; furthermore, the animals were subjected to additional inspection and palpation once a week.

At the beginning of the study and then about every 6 months, the first 10 animals of each dose and control group of the main groups were examined ophthalmologically.

5. Clinical chemistry, haematology and urinalysis

Clinical chemical and haematological examinations of the animals in satellite group II were carried out approximately 13, 26, 52, 78 and 103 weeks and urinalyses about 12, 25, 51, 77 and 102 weeks after the start of the administration period.

Haematology: Erythrocytes, haemoglobin, haematocrit, mean cell volume, mean haemoglobin content per erythrocyte, mean corpuscular haemoglobin concentration, platelet count, prothrombin time, leukocytes.

Clinical chemistry: Sodium, potassium, calcium, chloride, inorganic phosphorus, total bilirubin, glucose, creatinine, urea nitrogen, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactic dehydrogenase, total protein, albumin, total cholesterol.

The hormones triiodothyronine (T3) and thyroxine (T4) were also measured using enzyme immunoassays.

Urinalysis: Appearance, colour, pH, urinary volume, urinary refractive index, Glucose, bilirubin, ketone bodies, occult blood, protein, 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.

6. Investigations at necropsy

The rats of satellite group I were sacrificed after 12 months of the study. The rats in the main group and satellite group II were sacrificed at 24 months. Rats in all groups were subjected to gross-pathological and histopathological examination and the following organs were weighed:

Liver, kidneys, spleen, testes/ ovaries, heart, adrenals, pituitary, brain, thyroids from all animals sacrificed after 52 and 106 weeks and the thymus (if present) of the animals sacrificed after 52 weeks.

An extensive list of tissues were fixed and examined for histopathological findings.

7. Statistics

For food consumption and body weight statistical the tests used were ANOVA with subsequent Dunnett's test, or according to a t-test generalised by Williams for the simultaneous comparison of several dose groups with the control mean. Haematology and clinical chemistry were corrected using Nalimov criterion, and the t-test used. Urine analysis was conducted using the chi-squared test. For the main carcinogenicity study and satellite group II, histopathology findings were analysed with Fisher exact test ($p<0.05$ and $p<0.01$) and organ weights analysed using Student's t-test ($p<0.01$).

II. RESULTS AND DISCUSSION

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1. General observation/clinical signs/ Mortality

Mortality of the male and female animals was not adversely influenced by the administration of aclonifen, in fact survival was better in treated groups. The only clinical sign considered to be treatment-related was a yellow staining of the urine and yellow staining of fur on the abdominal side of the body and yellow-brownish staining of the tails with rats of the 1600 and 200 ppm dose groups. The staining of the urine, tails and fur was attributed to the intense colour of the test compound and of no toxicological significance.

The ophthalmoscopy examination revealed no findings that could be attributed to test substance administration.

Table 5.5- 2: Aclonifen carcinogenicity study in rats – Mortality and survival

	Males				Females			
Dietary concentration (ppm)	**0**	**40**	**200**	**1600**	**0**	**40**	**200**	**1600**
No of animals examined	60	60	60	60	60	60	60	60
Death wks 1-52	2	1	1	0	1	0	0	0
Death wks 53-78	3	1	2	3	2	2	3	0
Death wks 79-88	4	3	0	4	9	5	5	2
Death wks 89-96	8	5	7	1	6	6	5	7
Death after wk 97	6	8	5	5	4	6	7	6
Survival at necropsy	37	42	45	47	38	41	40	45

2. Body weight / food consumption / Substance intake

There was a reduction of food consumption and a statistically significant reduction in body weight in the females of the 1600 ppm dose group.

Table 5.5- 3: Aclonifen - carcinogenicity study in rats – Bodyweight and food consumption

	Males				Females			
Diet concentration (ppm)	**0**	**40**	**200**	**1600**	**0**	**40**	**200**	**1600**
	Carcinogenicity study (main group)							
Bodyweight – necropsy at 104 weeks (g)	681	708	695	669	373	370	379	**314** (↓16%)**
Food consumption – week 104 (g/day)	29.2	30.2	30.7	29.7	22.5	22.6	23.5	21.0 (↓7%)
	Chronic study (satellite group I)							
Bodyweight – necropsy at 52 weeks (g) (interim kill)	648	623	639	617 (↓10%)	318	315	317	**272** (↓14%)**
Food consumption – week 52 (g/day)	29.5	28.8	29.2	27.5 (↓7%)	21.4	19.8	20.2	18.4 (↓14%)

**statistically significant ($p<0.01$).
bold : considered to be treatment-related

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Male rats of all dose groups of the main (24 month) and satellite group I (12 month) study showed no treatment-related effects regarding food consumption. In contrast, food consumption of female rats in the 1600 ppm main group was slightly (maximum 13%) below the values of the control group. There was also a slight reduction in food consumption (maximum 15%) in females in satellite group I of test group 3 (1600 ppm). There was no effect on food consumption in females in other dose groups.

The body weight of females in the 1600 ppm main group were statistically significantly reduced compared to controls. Mean body weight was about 3% lower than controls after the 1st week and dropped continuously as the study proceeded to about 16% below controls by the end of the study. Body weight of females at 1600 ppm in satellite group I were also below the control values from about 3% after the first week to about 14% at the end of the study (at 12 months).

There was no effect on bodyweight in females from the other treated groups, and no effect on bodyweight in males in any dose group.

The achieved test material intake is given in the following table:

Table 5.5- 4: Aclonifen carcinogenicity study in rats – Achieved intake*

	Males			**Females**		
Diet concentration (ppm)	**40**	**200**	**1600**	**40**	**200**	**1600**
Substance-intake after 12 months (mg/kg bw/day)	1.7	8.5	67.1	2.3	11.6	98.2
Substance-intake after 24 months (mg/kg bw/day)	1.6	8.1	66.9	2.2	11.4	97.5
*Note that the achieved intake in the DAR from the previous renewal was incorrect						

3. Haematology / Clinical chemistry / Urinalysis

There were no haematology findings that could be attributed to test-substance intake. Clinical chemistry analysis of blood samples revealed a slight increase in total protein and albumin at 1600 ppm in both sexes and albumin was also increased at 200 ppm in males only. The toxicological significance of this finding is unclear but it is considered to be treatment-related. Reduced triglyceride values were noted in both sexes at 1600 ppm.

At 1600 ppm T4 was significantly reduced in males at 18 months, and in both sexes after 24 months. T3 was marginally reduced in males at 18 months. In males at 1600 ppm there was a slight increase (not statistically significant) in glutamate pyruvate transaminase activity at 24 months which may be a sign of slight liver cell damage.

Urine analysis revealed no pathological changes in either sex. The only finding was an intense yellow colouration of the urine, which can be attributed to the yellow colour of the test substance. The colouration was seen in males at all doses, and in females at the top two doses. However this effect diminished over time and by the end of the study urine discolouration was not evident in either sex.

Table 5.5- 5: Aclonifen - carcinogenicity study in rats – Clinical chemistry

	Males	**Females**

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Diet concentration (ppm)	**0**	**40**	**200**	**1600**	**0**	**40**	**200**	**1600**
Triglycerides (mmol/L)								
Week 13	2.783	4.989** (↑79%)	2.868	2.666	2.927[9]	2.587[9]	2.223[9]	1.922** (↓34%)
Week 26	3.189	5.200** (↑63%)	3.562	2.599[9]	4.365	3.148[9]	3.425[9]	2.464** (↓ 44%)
Week 52	3.705[9]	6.128** (↑65%)	3.488[9]	2.333*[9] (↓37%)	5.616	5.602	5.318	3.431* (↓39%)
Week 78	4.705[8]	6.630* (↑41%)	5.518	2.964**[9] (↓37%)	6.696	6.952	5.719	2.248**[9] (↓66%)
Week 103	4.883	5.197	4.739	2.491**[9] (↓49%)	6.044	6.789	6.069	2.954**[9] (↓51%)
Albumin (g/L)								
Week 13	38.500	40.180	**41.470** (↑8%)**	**44.080** (↑14%)**	41.100	42.730	41.767[9]	**44.510** (↑8%)**
Week 26	36.810	37.050	**39.290** (↑7%)**	**42.020** (↑14%)**	43.150	43.240	43.220	45.690
Week 52	37.356[9]	37.130	37.740	**40.970** (↑10%)**	42.640	43.790	45.660	**48.560** (↑14%)**
Week 78	37.075[8]	36.944[9]	36.450	**39.600** (↑7%)**	39.778[9]	40.350	40.450	41.780
Week 103	33.420	33.730	33.090	34.540	40.210	41.420	38.160	**43.867*[9] (↑9%)**
Albumin/globulin ratio								
Week 13	1.404	1.386	1.466	**1.688** (↑20%)**	1.624[9]	1.724	1.690	**1.825** (↑12%)**
Week 26	1.374	1.288	1.360	**1.622** (↑18%)**	1.603	1.566	1.572[9]	1.706
Week 52	1.232[9]	1.186	1.164[9]	**1.353* (↑10%)**	1.499	1.567[9]	1.501	**1.690** (↑13%)**
Week 78	1.140[8]	1.051	1.079	**1.141[9]**	1.224	1.206[9]	1.194	1.174
Week 103	0.935[9]	0.983	0.990[9]	**1.004**	1.238[9]	1.284	1.116	**2.884**[9] (↑x2)**
Total protein (g/L)								
Week 13	66.150	69.269	69.835	**70.290* (↑6%)**	66.129	67.851	66.734[9]	68.993
Week 26	63.792	66.156	68.254	**67.946** (↑7%)**	70.072	70.941	70.084	72.605
Week 52	67.774[9]	68.580	69.502	**71.482* (↑5%)**	71.237	72.639	76.178	**77.308* (↑8%)**
Week 78	71.308[8]	72.385[9]	70.748	73.506	73.307	74.978	74.710	**77.594* (↑6%)**
Week 103	68.370	68.208	67.858	69.397	73.526[9]	74.285	74.374[9]	75.130
T4 (µg/dL)								
Week 78	2.79[8]	2.59	2.70	**1.80* (↓35%)**	2.75	2.70	2.35	2.55
Week 103	1.50	1.44	1.27	**1.06*[9] (↓29%)**	3.28	2.89* (↓12%)	3.20	**2.87* (↓13%)**
T3 (ng/mL)								
Week 78	0.87[7]	0.97	0.95	**0.74* (↓15%)**	1.47	1.43	1.38	1.38
Week 103	1.41	1.46	1.46	1.28	0.47	0.41* (↓13%)	0.54	0.61* (↑49%)

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statistically significant: * $p < 0.05$, ** $p < 0.01$;
bold : considered to be treatment-related
mean of 10 animals, where fewer than 10 animals the number used is stated in superscript
T3 and T4 were only measured at 18 and 24 months

4. Gross pathology / Organ weights / Histopathology

12-month study (satellite group I)

The only treatment-related effect noted at necropsy after 12 months of treatment was a significant decrease in body weight of female rats of the 1600 ppm dose group. There was a slight decrease in body weight in males at 1600 ppm but it did not reach statistical significance. There were some statistically significant changes in absolute organ weights at 1600 ppm in both sexes. The reduction in heart and adrenal weight can be attributed to reduced body weight as the relative weights of these organs was not affected. Increased relative liver weight in females is not clearly treatment-related as absolute liver weight was not affected.

An increase in absolute and relative thyroid weight in both sexes (though more marked in males) did not correlate with any treatment-related histopathological findings in the thyroid. C-cell hyperplasia was seen in 2 animals, but the lack of dose response and low incidence suggests this is an incidental finding. The structure of the thyroid gland, the size of the follicles, the height of the follicular epithelium and the number and distribution of the C-cells also corresponded to the control. Likewise in the liver focal necrosis with haemorrhage in three males at 1600 ppm is not clearly treatment-related as the same finding was seen in 2 females of the control group and one female in the low dose group.

Table 5.5- 6: Aclonifen - carcinogenicity study in rats – Organ weights and non-neoplastic histopathological findings at 12 months

	Males				Females			
Diet concentration (ppm)	**0**	**40**	**200**	**1600**	**0**	**40**	**200**	**1600**
Bodyweight – necropsy at 12 months (g)	648	623	639	617 (↓10%)	318	315	317	**272** (↓14%)**
Organ weights								
Liver weight (g)	19.287	18.050	18.676	18.920	10.229	9.879	10.006	9.600
Liver weight % bw	2.976	2.889	2.943	3.061	3.207	3.141	3.162	3.529** (↑10%)
Heart weight (g)	1.608	1.541	1.534	1.454** (↓10%)	1.049	1.020	1.032	0.948* (↓10%)
Heart weight % bw	0.249	0.249	0.241	0.237	0.331	0.327	0.327	0.351
Adrenal weight (g)	0.0915	0.0861[9]	0.0857	0.0799* (↓13%)	0.1427	0.1405	0.1442	0.1206* (↓15%)
Adrenal weight % bw	0.0142	0.0143	0.0135	0.0130	0.0451	0.0451	0.0456	0.0448
Thyroid weight (g)	0.0361	0.0383	0.0390	**0.0473** (↑31%)**	0.0327	0.0299	0.0314	0.0372 (↑14%)
Thyroid weight % bw	0.0056	0.0062	0.0062	**0.0077** (↑38%)**	0.0103	0.0095	0.0100	0.0137** (↑33%)
Histopathology								
Liver focal necrosis with haemorrhage	0/10	0/10	0/10	3/10	2/10	1/10	0/10	0/10
Thyroid focal C-cell hyperplasia	0/10	1/10	0/10	0/10	0/10	0/10	1/10	0/10

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statistically significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$;
bold : considered to be treatment-related
where fewer than 10 organs were weight the number used is stated in superscript

At 12 months there were no malignant tumours. Benign tumours in the brain, pituitary, adrenals and mammary gland have lack any dose response and are considered to be incidental findings (see table below).

Table 5.5- 7: Aclonifen - carcinogenicity study in rats – tumours at 12 months

	Males				Females			
Diet concentration (ppm)	**0**	**40**	**200**	**1600**	**0**	**40**	**200**	**1600**
Number of organs examined	10	10	10	10	10	10	10	10
Brain – haemangioma in meninx	0	1	0	0	0	0	0	0
Pituitary - adenoma	0	0	0	0	0	3	1	1
Adrenals - ganglioneuroma	0	1	0	0	0	0	0	0
Mammary gland - fibroadenoma					1	1	1	0

Main 24-month study

Necropsy after 24 months of treatment revealed a slight decrease in absolute liver and spleen weight, for females at 1600 ppm, which are likely to be secondary to a statistically significant decrease in bodyweight as relative organ weights were not affected. There were no effects on any other absolute or relative organ weights including thyroid, spleen, and heart in either sex.

Microscopic examination revealed some lesions that were statistically significantly increased in treated animals compared to control animals (see table below). The lesions were of a broad spectrum and typical of rats of this strain and age in the liver, lymph nodes, spleen, uterus/vagina and mammary gland. Generally these lesions showed no dose-response and are considered to be spontaneous findings. In the thyroid slightly increased incidence of colloid cysts in females at 1600 ppm did not reach statistical significance and were considered to be incidental as a similar effect was not seen in males.

Table 5.5- 8: Aclonifen - carcinogenicity study in rats – Organ weights and non-neoplastic histopathological findings at 24 months

	Males				Females			
Diet concentration (ppm)	**0**	**40**	**200**	**1600**	**0**	**40**	**200**	**1600**
Bodyweight – necropsy at 24 months (g)	681	708	695	669	373	370	379	**314** (↓16%)**
	Organ weights							
Liver weight (g)	19.34	20.55	20.03	21.16	13.22	13.21	14.05	11.82** (↓11%)
Liver weight % bw	2.88	2.94	2.88	3.17	3.56	3.57	3.75	3.77
Spleen weight (g)	1.16	1.20	1.29	1.21	0.74	0.84	0.79	0.64** (↓14%)
Spleen weight % bw	0.17	0.17	0.18	0.18	0.20	0.23	0.22	0.20
Heart weight (g)	1.84	1.97	1.99	1.84	1.40	1.44	1.53	1.29
Heart weight % bw	0.28	0.28	0.29	0.28	0.38	0.39	0.41	0.41

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Adrenal weight (g)	0.106	0.105	0.098	0.098	0.140	0.148	0.1	0.125
Adrenal weight % bw	0.016	0.015	0.014	0.015	0.038	0.040	0.037	0.041
Thyroid weight (g)	0.049	0.049	0.054	0.057	0.038	0.038	0.041	0.040
Thyroid weight % bw	0.007	0.007	0.008	0.009	0.010	0.010	0.011	0.013
	Histopathology (non-neoplastic findings)							
	Liver							
Liver – number of organs examined	49	48	50	50	50	50	50	50
Liver – bile duct proliferation	1	7*	7*	6	2	7	5	2
Liver – mononuclear cell proliferation	0	0	4	7*	6	7	5	3
Liver – clear cell foci	5	11	13*	12	0	0	0	0
	Lymph nodes							
Lymph node – number of organs examined	50	50	50	50	50	50	50	50
Lymph node – lymphadenitis	6	16**	13	11	16	11	7	8
Lymph node - siderosis of the sinus/pulp	3	1	8*	8	5	11*	5	24**
	Spleen							
Spleen – number of organs examined	50	49	50	50	50	50	50	50
Spleen – siderosis of the red pulp	10	20**	20**	16*	17	26**	20	34**
	Uterus/vagina							
Uterus/vagina – number of organs examined					49	49	49	49
Uterus/vagina – pachydermia					3	7	6	18**
Uterus/vagina – hyperplasia in the cervix and/or corpus					2	4	2	11*
	Mammary gland							
Mammary gland – number of organs examined					51	53	52	50
Mammary gland – nodular glandular hyperplasia					15	17	28**	19
	Thyroid							
Thyroid – number of organs examined	49	50	50	50	50	50	50	49
Thyroid – colloid cysts	2	1	3	0	2	1	1	6

statistically significant: * p < 0.05, ** p < 0.01, *** p < 0.001;
bold : considered to be treatment-related
Organ weights 50 m/f per dose except for those that died, and organ weights which differed considerably from the normal values due to detected or likely tumours or other pathological changes were also excluded.

24-month study – neoplastic findings

Overall tumour incidence was not affected by test substance administration. The malignant/benign tumour ratio was generally he same for both treated and untreated animals (see table below).

Table 5.5- 9: Aclonifen carcinogenicity study in rats – tumour incidence

	Males				**Females**			
Dietary concentration (ppm)	**0**	**40**	**200**	**1600**	**0**	**40**	**200**	**1600**
No of animals examined	60	60	60	60	60	60	60	60

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Total number of tumour-bearing animals	48	46	48	48	56	52	55	56
Benign tumours only	28	30	37	33	44	39	38	40
Malignant tumours only	8	4	6	6	1	1	3	3
Benign and malignant tumours	12	12	5	9	11	12	14	13

A few tumours give the impression that according to their frequency they might be test-substance related. Tumours in the uterus are typical for old rats of this strain and age. Significant differences from the control were not found (see table below) and lack dose response so are considered to be incidental.

Table 5.5- 10: **Aclonifen carcinogenicity study in rats – Uterine tumours**

	Females			
Dietary concentration (ppm)	**0**	**40**	**200**	**1600**
No of animals examined	60	60	60	60
Fibroadenoma	0	0	2	0
Polyp (different types)	6	2	12	12
Corpus adenocarcinoma	0	0	0	2
Concroid carcinoma	0	0	2	0
Cervical carcinoma	2	0	0	0
Endometrial sarcoma	4	6	2	2
Leimyoma	0	1	0	1

In the thyroid a significant increase in C-cell carcinoma in the females of the 200 and 1600 ppm dose group (see table below).

Table 5.5- 11: **Aclonifen carcinogenicity study in rats – Thyroid tumours**

	Males				**Females**			
Dietary concentration (ppm)	**0**	**40**	**200**	**1600**	**0**	**40**	**200**	**1600**
No of animals examined	60	60	60	60	60	60	60	60
C-cell nests of foci	0	2	2	3	1	1	1	1
C-cell adenoma	1	2	4	2	2	2	0	3
C-cell carcinoma	1	4	5	2	1	2	7*	6*
Follicular/papillary adenoma	1	3	4	5	1	0	2	3
Follicular/papillary carcinoma	0	1	0	0	0	1	1	1
statistically significant: * $p < 0.05$,								

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The overall conclusion from all the reviews was that in the absence of any significant dose-related trend, coupled with the biological finding of the susceptibility of this strain of rat to develop both hyperplasia and neoplasia in the C-cell population, the statistical significance in tumour incidence was considered a chance phenomenon and not related to aclonifen administration.

III. CONCLUSION

Based on changes in some clinical chemistry parameters, the NOAEL was 40 ppm (equating to approximately 1.6 and 2.2 mg/kg/day in males and females, respectively).

A slight increase in C-cell carcinoma of the thyroid in females at 200 and 1600 ppm was considered to be within the normal background incidence for this strain of rat. Therefore under these test conditions aclonifen displayed no oncogenic potential following 24 months administration in the Wistar rat.

Assessment and conclusion by applicant:

The study was performed to GLP and OECD 435 (although there are some minor omissions overall the study is deemed to meet the current 2018 test guidelines).

The NOAEL in the study report is proposed as 40 ppm (equating to approximately 1.6 and 2.2 mg/kg/day in males and females, respectively) based in increased serum albumin in males at 200 ppm (8/11 mg/kg bw/day in males/females respectively).

However this NOAEL based on a single minimal clinical chemistry change, though treatment-related is not evidence of an adverse effect.

Therefore the overall NOAEL if this study is 200 ppm (8/11 mg/kg bw/day in males/females). The NOAEL of 200 ppm is supported by the NOAEL of the second carcinogenicity study (2004, M-234946-01-1

A slight increase in C-cell carcinoma of the thyroid in females at 200 and 1600 ppm is considered to be within the normal background incidence for this strain of rat. Therefore under these test conditions aclonifen displayed no oncogenic potential following 24 months administration in the Wistar rat up to a dose of 1600 ppm (67/98 mg/kg bw/day in males/females).

The histological sections of the thyroids from the aclonifen rat carcinogenicity study were subsequently reviewed by a consultant pathologist, Professor P. Grasso (and the results from this and the original histopathology report (performed by the pathologist were analysed using appropriate statistics (Peto analysis) to adjust for increased mortality in the control animals which may affect tumour incidence levels in comparison to surviving animals in the treated groups. Peto analysis was carried out by For re-evaluation of the slides see M-175852-01-1 by (1009), KCA 5.5/07.

The C-cell tumours were reviewed again by (see KCA 5.5/09, 1995, M-174790-01-1).

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Assessment and conclusion by RMS:

Data Point:	KCA 5.5/02
Report Author:	███
Report Year:	1989
Report Title:	PATHOLOGY REPORT OF THE SATELLITE GROUPS I on the study of the chronic toxicity and oncogenic potential of ACLONIFEN (CME 127) in rats after 24-month administration in the diet Volume II
Report No:	R007114
Document No:	M-174243-01-1
Guideline(s) followed in study:	MAFF: (1985); OECD: 453; USEPA (=EPA): F 83-5
Deviations from current test guideline:	Current Guideline: OECD 453, 2018 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Organ weights and histopathological findings in Satellite group I (chronic rat study).

Assessment and conclusion by applicant:

This document is part of the chronic and carcinogenicity study reported under KCA 5.5/01 by ███ (1989) M-174241-01-1.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.5/03
Report Author:	[redacted]
Report Year:	1988
Report Title:	Report on the study of the chronic toxicity and oncogenic potential of Aclonifen (CME l27) in rats after 24-month administration in the diet - Volume III (Pathology report)
Report No:	C033028
Document No:	M-232479-01-1
Guideline(s) followed in study:	OECD: 453; USEPA (=EPA): F,83-5
Deviations from current test guideline:	Current Guideline: OECD 453, 2018 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Organ weights and histopathological findings in carcinogenicity study and Satellite group II.

Assessment and conclusion by applicant:

This document is part of the chronic and carcinogenicity study reported under KCA 5.5/01 by [redacted] (1989) M-174241-01-1.

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Data Point:	KCA 5.5/04
Report Author:	;
Report Year:	1986
Report Title:	Report on the study of the chronic toxicity and oncogenic potential of ACLONIFEN (CME l27) in rats after 24-month administration in the diet - Volume IV (Test substance characterisation and analysis of CME 127 in test diets)
Report No:	C033029
Document No:	M-232482-01-1
Guideline(s) followed in study:	OECD: 453; USEPA (=EPA): F,83-5
Deviations from current test guideline:	Current Guideline: OECD 453, 2018 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

This document reports on the Test substance characterisation and analysis of aclonifen (CME 127) in test diets used in the rat chronic and carcinogenicity study (1989, document number M-174241-01-1). The findings are summarised under KCA 5.5/01.

Assessment and conclusion by applicant:

This document is part of the chronic and carcinogenicity study reported under KCA 5.5/01 by (1989) M-174241-01-1.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.5/05
Report Author:	██████
Report Year:	1989
Report Title:	Report on the study of the chronic toxicity and oncogenic potential of ACLONIFEN (CME l27) in rats after 24-month administration in the diet - Volume V (Clinical examinations / Clinical chemistry and hematology)
Report No:	C033030
Document No:	M-232485-01-1
Guideline(s) followed in study:	OECD: 453; USEPA (=EPA): F,83-5
Deviations from current test guideline:	Current Guideline: OECD 453, 2018 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Assessment and conclusion by applicant:

This document is part of the chronic and carcinogenicity study reported under KCA 5.5/01 by ██ ███ (1989) M-174241-01-1.

Assessment and conclusion by RMS:

Data Point:	KCA 5.5/06
Report Author:	██████
Report Year:	1989
Report Title:	Report on the study of the chronic toxicity and oncogenic potential of ACLONIFEN (CME 127) in rats after 24-month administration in the diet - Volume VI (Historical data / Conversion tables)
Report No:	C033031
Document No:	M-232487-01-1
Guideline(s) followed in study:	OECD: 453; USEPA (=EPA): F,83-5
Deviations from current test guideline:	Current Guideline: OECD 453, 2018 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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This report contains reference values for haematology and clinical chemistry findings in the Wistar ChbbTHOM rat.

Assessment and conclusion by applicant:

This document is part of the chronic and carcinogenicity study reported under KCA 5.5/01 by [redacted] (1989) M-174241-01-1 and gives reference values for haematology and clinical chemistry parameters.

Assessment and conclusion by RMS:

Data Point:	KCA 5.5/07
Report Author:	[redacted]
Report Year:	1990
Report Title:	Carcinogenicity study on CME127 (aclonifen) - Report of review of thyroid C-cell tumours.
Report No:	R007908
Document No:	M-175852-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The histological sections of the thyroid in the rat carcinogenicity study (KCA 5.5/01 by [redacted] (1989) M-174241-01-1) were re-examined for C-cell tumours and hyperplasia using clearly defined criteria for tumour classification. Statistical analysis was conducted using Peto analysis which adjusts for mortality during the course of the study and time of lesion onset. The results indicated that there was no dose related increase in tumour incidence and that overall the C-cell tumours were concluded to be not related to test substance administration.

I. MATERIALS AND METHODS

The histological sections of the thyroid C-cell lesions from the rat carcinogenicity study (KCA 5.5/01 by [redacted] (1989) M-174241-01-1) were reviewed by a consultant pathologist [redacted]. Prior to

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reviewing the slides clear criteria were set for classification of lesions as hyperplasia and adenoma because the dividing line between hyperplasia and adenoma, and sometimes between adenoma and carcinoma can be arbitrary. Combining the incidence of benign and malignant tumours was advocated for assessing tumourigenic potential as a better indicator or carcinogenic potential than analysing benign and malignant tumours separately.

In addition an age-adjusted analysis based on the IARC Peto method was conducted to take account of the mortalities during the study, because fewer animals survived to the end of the study in the control groups compared to those receiving the test substance. This analysis was conduced to take account of the time and mode of death and was conducted on the C-cell tumour classification in the original study, and using the C-cell tumour classification following re-analysis by ██████ in the current report. In the reclassification system used in this review C-cell 'nests' is not used as a classification term.

Table 5.5- 12 Tumour classification criteria used for the review of thyroid C-cell tumours

Lesion	Criteria
Hyperplasia	An increase in C-cells which is diffuse but may have focal collections (nodules). The diffuse hyperplasia may affect a small or large area of the thyroid gland.
Adenoma	Well-differentiated nodular proliferations with distortion and compression of thyroid parenchyma evident. Lesion greater than 3-4 average follicles.
Carcinoma	Large tumours - destruction of thyroid tissue, abnormal cytology, invasion beyond capsule.

Peto analysis divides the study into time intervals and within each time interval the numbers of animals observed to have the lesion is compared to the number expected to have the lesion assuming that there is no difference in tumour incidence rate between groups. The total observed and expected tumour incidence for each test group is summed over each the time period in the study, and for a total observed and expected for each group over the whole study. Chi-squared statistics are used to determine statistical significance, and dose-related trend. The method distinguishes between tumours that are fatal (to have cause the death of the animal) and tumours that are incidental (occurred in an animal that died of another cause). Time interval for incidental deaths were weeks 1-52, weeks 53-78, weeks 79-88, weeks 89-96, and week 97 until termination. For fatal tumours one-week intervals were used.

II. RESULTS AND DISCUSSION

Re-examination and classification of the C-cell hyperplastic lesions is shown in the table below. A statistically significant increase in tumours in males in the mid dose group, and in the low dose group for all neoplastic and pre-neoplastic lesions combined is considered unlikely to be treatment-related, as overall there is no dose-response in tumour incidence.

Table 5.5- 13: Aclonifen carcinogenicity study in rats – Frequency of C-cell lesions according to reanalysis by Dr Gasso

	Males	Females

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Dietary concentration (ppm)	0	40	200	1600	Total lesions	0	40	200	1600	Total lesions
No of animals per group	60	60	60	60		60	60	60	60	
Not accessible	0	2	2	2	6	0	1	0	2	3
Too autolysed	4	6	3	1	14	5	0	4	2	11
None	21	9	13	11	54	5	7	8	2	22
Hyperplasia	33	35	30	37	135	39	40	36	44	159
Adenoma	1	5	11**	7	24	10	9	8	7	34
Carcinoma	1	3	1	2	7	1	3	4	3	11
Carcinoma and adenoma combined	**2**	**8**	**12***	**9**	**31**	**11**	**12**	**12**	**10**	**44**
Carcinoma, adenoma and hyperplasia combined	**35**	**43***	**42**	**46**	**166**	**50**	**52**	**48**	**54**	**204**
statistically significant: * $p < 0.05$; ** $p < 0.01$ (time adjusted statistical test using Peto analysis)										

The table below shows the results using the histopathological classification system undertaken by in the main study, but with Peto analysis to take account mortalities during the study and time of tumour onset. Although there are some slight differences in classification the findings are broadly in agreement with the classification system by (above).

Table 5.5- 14 Aclonifen carcinogenicity study in rats – Frequency of C-cell lesions classified by with Peto analysis

	Males					Females				
Dietary concentration (ppm)	**0**	**40**	**200**	**1600**	**Total lesions**	**0**	**40**	**200**	**1600**	**Total lesions**
No of animals per group	60	60	60	60		60	60	60	60	
None	58	52	49	53	212	56	55	52	50	213
C-cell nests	0	2	2	3	7	1	1	1	1	4
Benign tumour	1	2	4	2	9	2	2	0	3	7
Malignant tumour	1	3	5	1	10	1	1	6	6	14
Malignant tumour (fatal)	0	1	0	1	2	0	1	1	0	2
Benign and malignant tumours combined	**2**	**6**	**9**	**4**	**21**	**3**	**4**	**7**	**9**	**23**

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Benign and malignant tumours and nests combined	**2**	**8**	**11***	**7**	**28**	**4**	**5**	**8**	**10**	**27**
statistically significant: * $p < 0.05$, ** $p < 0.01$ (time adjusted statistical test using Peto analysis)										

III. CONCLUSION

There was no evidence of any dose-response relationship in the increase of the C-cell lesions described and statistically there was no significant dose related trend.

The absence of any significant dose-related trend in both reviews and analyses is important and demonstrated that the statistical significance in tumour incidence at the low and mid dose levels is a chance phenomenon and not related to treatment.

Assessment and conclusion by applicant:

The histological sections of the thyroids from the aclonifen rat carcinogenicity study KCA 5.5/01 by (1989) M-174241-01-1, were subsequently reviewed by a consultant pathologist, and the results from this and the original histopathology report (performed by the pathologist were analysed using appropriate statistics (Peto analysis) to adjust for increased mortality in the control animals which may affect tumour incidence levels in comparison to surviving animals in the treated groups. Peto analysis was carried out by

The re-assessment with age-adjustment for onset of tumours and mortality concluded there was a statistically significant increase in adenomas in males in the mid-dose group, and in the low-dose group a statistically significant increase in males for all neoplastic and pre-neoplastic lesions combined (carcinoma, adenoma and hyperplasia). These findings are considered unlikely to be treatment-related, as overall there is no dose-response in tumour incidence.

Overall it is concluded there is carcinogenic potential in the Wistar rat following 24 months administration of aclonifen in the diet up to a dose of 1600 ppm (67/98 mg/kg bw/day in males/females).

Assessment and conclusion by RMS:

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Data Point:	KCA 5.5/09
Report Author:	[redacted]
Report Year:	1995
Report Title:	24-month Dietary Study of ACLONIFEN (CME 127) in the Rat - BASF Project No. 7150001/8401 LPT Project No. 3790/86 - Report of Review of Thyroid C-cell Lesions
Report No:	R007378
Document No:	M-174790-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Assessment and conclusion by applicant:

This is the pathology report of the re-examination of the histopathology slides of the thyroid in the rat carcinogenicity study (KCA 5.5/01 by [redacted] (1989) M-174241-01-1). This report is also contained in Annex A of M1174788-01-1. Please refer to the summary under KCA 5.5/07.

Assessment and conclusion by RMS:

Data Point:	KCA 5.5/09
Report Author:	[redacted]
Report Year:	1995
Report Title:	24-month Dietary Study of ACLONIFEN (CME 127) in the Rat - BASF Project No. 7150001/8401 LPT Project No. 3790/86 - Report of Review of Thyroid C-cell Lesions
Report No:	R007378
Document No:	M-174790-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

The histological sections of the thyroids from the aclonifen rat carcinogenicity study KCA 5.5/01 by (1989) M-174241-01-1, were subsequently reviewed using WHO classification of rodent tumours. The re-evaluation of the slides broadly is in agreement with the previous two assessments by (1988) M-232479-01-1 reported in the main study (KCA 5.5/01), and by (1990) M-175852-01-1 (reported under KCA 5.5/07).

The analysis shows a statistically significant increase in C-cell adenoma in males in the low (40 ppm) mid dose level (200 ppm) but the lack of dose response suggests this is an incidental finding. C-cell carcinoma was increased in treated animals but did not achieve statistical significance and shows no dose-response.

Overall it is concluded there is carcinogenic potential in the Wistar rat following 24 months administration of aclonifen in the diet up to a dose of 1600 ppm (67/98 mg/kg bw/day in males/females).

I. MATERIALS AND METHODS

Following a request from the German authorities another review was conducted on thyroid C-cell lesions from the 24-month dietary study of aclonifen (study KCA 5.5/01 by (1989) M-174241-01-1), by group at the .

The review was conducted on histological sections of the thyroid gland for the rats in the main study and satellite group II (a total of 60 animals per sex/dose group) which had been administered aclonifen in the diet for a period of 24 months. For most animals 2 histological slides of the thyroid, with a total of 5 tissue sections of each thyroid were available.

Diagnostic criteria were according to WHO International Classification of Rodent Tumours, Part 1, The Rat, Vol. 6: Endocrine System, IARC Scientific Publications No 122, Lyon, 1994.

More than one different type of C-cell lesion in the same animal was reported, however multiplicity of the same type of lesion in the same thyroid gland was not recoded.

Statistical significance of differences in incidences between each treated group and the control group was assessed using the Fisher's exact test (one-tailed).

II. RESULTS AND DISCUSSION

Diffuse C-cell hyperplasias are not regarded as pre-neoplastic lesions. The incidence in treated animals in both sexes and all dose groups was lower than in the controls (statistically significant only in females at 200 ppm).

Focal and multifocal C-cell hyperplasia are considered to be pre-neoplastic lesions. In female rats the incidence was significantly increased at 40 ppm. There was no statistically significant increase in this lesion in males or in females in the higher dose groups.

For C-cell tumours, a statistically significant increase was only observed for the incidence of C-cell adenomas in the 40 ppm and the 200 ppm dose level groups in male rats. There was no increase in C-cell adenomas in males at 1600 ppm.

In females, the frequency of C-cell adenomas was not increased significantly in any dose group.

For C-cell carcinomas no statistically significant increase could be detected in rats of either sex in any dose group.

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Table 5.5- 15: Aclonifen carcinogenicity study in rats ██████ review of thyroid C-cell tumours and lesions

	Males				Females			
Dietary concentration (ppm)	**0**	**40**	**200**	**1600**	**0**	**40**	**200**	**1600**
No of animals examined	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**
C-cell diffuse hyperplasia	53	52	46	51	56	54	48*	52
C-cell focal/multifocal hyperplasia	15	15	17	10	15	25*	23	14
C-cell adenoma	2	11**	12**	7	6	7	6	10
C-cell carcinoma	1	3	1	2	1	2	5	2

statistically significant: * $p < 0.05$, ** $p < 0.01$

Where several categories of lesion were present in a single thyroid gland each category of lesion has been counted (however multiple lesions of the same category in a single tissue are counted as one lesion)

██████ concluded that there was a relatively good agreement between his diagnoses of C-cell tumours with those of ██████ and suggested that discrepancies observed were likely to have been due to the different diagnostic criteria used.

III. CONCLUSION

Based on the histopathological review, it can be concluded that in the male rats in the 40 ppm and the 200 ppm dose level groups the incidences of C-cell adenomas was statistically significantly increased. All other tumour incidences (in males and females) are in most cases numerically higher than in the corresponding controls, but not statistically significantly increased. Since there was no increase in tumours with increasing dose it has to be concluded that there was no treatment-related effect on thyroid C-cell tumours.

Assessment and conclusion by applicant:

The histological sections of the thyroids from the aclonifen rat carcinogenicity study KCA 5.5/01 by ██████ (1989) M-174241-01-1, were subsequently reviewed ██████
The classification categories for the assessment of C-cell hyperplasia and tumours used by ██████ in the current review and those used in the two previous reviews are detailed in the table below.

Aclonifen carcinogenicity study in rats – Classification criteria in assessing C-cell thyroid lesions

Pathologist	██████ (1988) M-232479-01-1	██████ (1990) M-175852-01-1	██████ (1995) M-174790-01-1
Lesion	normal C-cells	normal C-cells	normal C-cells
	C-cell nests	C-cell hyperplasia	diffuse C-cell hyperplasia
			focal/multifocal C-cell hyperplasia
	C-cell adenoma	C-cell adenoma	C-cell adenoma

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	C-cell carcinoma	C-cell carcinoma	C-cell carcinoma
Classification system reference	Histological examinations carried out according to international regulations considering OECD and EPA/USA. Sections of the different groups were reviewed using an un-coded procedure, i.e. 'not blind'.	STP Criteria Document on Proliferative lesions of the thyroid and parathyroid glands. (1989).	WHO in "International Classification of Rodent Tumours, Part I: The Rat, Volume 6: Endocrine Syste IARC Scientific Publicatio N° 122, Lyon, 1994, pp 15

The re-evaluation of the slides is in agreement with the previous assessments.

The analysis shows a statistically significant increase in C-cell adenoma in males in the low (40 ppm) mid dose level (200 ppm) but the lack of dose response suggests this is an incidental finding. C-cell carcinoma was increased in treated animals but did not achieve statistical significance and shows no dose-response.

Overall it is concluded there is carcinogenic potential in the Wistar rat following 24 months administration of aclonifen in the diet up to a dose of 1600 ppm (67/98 mg/kg bw/day in males/females).

Assessment and conclusion by RMS:

Data Point:	KCA 5.5/10
Report Author:	
Report Year:	2001
Report Title:	24-MONTH DIETARY STUDY IN THE RAT BASF PROJECT 71S001/8401 LPT PROJECT STATISTICAL EVALUATION OF THYROID C-CELL TUMORS FOR AUSTRIAN OFFICIAL STATISTICAL ANALYSIS REPORT ACLONIFEN
Report No:	C013814
Document No:	M-205316-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

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The incidence of tumours and pre-neoplastic lesions in the C-cells of thyroid glands in the rat carcinogenicity study (study KCA 5.5/01 by [redacted] (1989) M-174241-01-1) were reviewed by [redacted] group at the [redacted] (reported in KCA 5.5/09 by [redacted] (1995) M-174790-01-1). Statistical analysis of the tumour incidence reported by [redacted] was conducted for carcinomas, combined carcinomas and adenomas, and combined carcinomas, adenomas and hyperplasia.

For carcinomas there were no statistically significant differences compared to controls. Statistical analysis of carcinoma and adenoma combined including animals in the main and satellite group II found no statistically significant increase in females, and in males a statistically significant increase in the low and mid dose group only. For combined carcinoma, adenoma and hyperplasia there was a statistically significant increase in lesions in females in the low and mid dose group for the main and satellite groups combined.

Overall where statistical analysis was conducted including all animals in the main and satellite groups statistically significant increases in tumours are not seen in the top dose groups in either sex.

I. MATERIALS AND METHODS

[redacted], from [redacted] performed a histopathological review of C-cell lesions in thyroid glands of Wistar rats from the 24-month dietary study of Aclonifen using the WHO/IARC classification of cell lesions. Statistical analysis was conducted on selected groups of data from this report to compare incidences of thyroid C-Cell lesions by
considering:
- Carcinoma;
- Combined Adenoma and Carcinoma;
- Combined Adenoma, Carcinoma and Hyperplasia (focal and multifocal C-cell hyperplasia).

Two analyses were conducted; the first one on the main group of the study, the second one on the combined main and satellite groups of the study. Males and females were analysed separately. Statistical significance of differences in incidences between each treated group and the control group was assessed using the Fisher's exact test (one-sided). Dose related trends was assessed using the Cochran-Armitage trend test (one-sided).

Trend tests were conducted firstly including all the four groups. When the trend test including all the 4 doses was significant, a second trend test excluding the high dose group was also done.

Statistical comparisons were performed using SAS programs and the STATXACT3 package.

II. RESULTS AND DISCUSSION

Table 5.5- 16: Aclonifen carcinogenicity study in rats – Prof. Mohr review of thyroid C-cell tumours and lesions

	Males				Females			
Dietary concentration (ppm)	**0**	**40**	**200**	**1600**	**0**	**40**	**200**	**1600**
No of animals examined main group	**48**	**47**	**45**	**48**	**48**	**49**	**47**	**47**
No of animals examined (including satellite group II)	**58**	**57**	**54**	**58**	**58**	**59**	**56**	**57**

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	C-cell focal/multifocal hyperplasia[A]							
Main group	15	7	10	7	11	19	21	7
Total all groups combined	15	8	12	10	13	23	22	10
	C-cell adenoma[A]							
Main group	0	8	10	6	4	6	5	9
Total all groups combined	2	11	11	7	6	7	6	9
	C-cell carcinoma							
Main group	1	3	1	2	0	2	3	2
Satellite group	0	1	0	0	1	0	2	0
Total all groups combined	1	4[B]	1	2	1	2	5	2
	C-cell adenoma & carcinoma combined							
Main group	1# §§	11**	11**	8*	4#	8	8	11*
Total all groups combined	3	15**	12**	9	7	9	11	11
	C-cell hyperplasia, adenoma & carcinoma combined							
Main group	16	18	21	15	15	27*	29**	18
Total all groups combined	18	23	24	19	20	32*	33**	21

statistically significant: * $p < 0.05$, ** $p < 0.01$

one-sided trend test including all 4 groups: # $p < 0.05$

one-sided trend test excluding the high dose group §§ $p < 0.01$

[A] Statistical analysis not conducted

[B] This value differs from the report by [redacted] where total incidence of C-cell carcinoma at 40 ppm is reported to be 3.

Where more than one type of lesion is present in a single thyroid gland only the most severe category of lesion has been counted.

III. CONCLUSION

Carcinoma

Incidence of carcinoma was not significantly different when the treated groups were compared to the control group on both sexes. Trend tests which were not significant confirmed that there was no dose related relationship.

Carcinoma and adenoma combined

In males, pairwise comparisons and trend test were significant for the main group analysis but only the low and middle doses were significant when the combined main + satellite groups was analysed.

In females of the main group, pairwise comparisons indicated that only the high dose was significantly different from the control group and the significance of the trend test disappeared when the high dose was excluded. When the combined main + satellite groups was analysed, neither pairwise comparisons nor trend test was significant.

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Hyperplasia, adenoma and carcinoma combined

On males, pairwise comparisons and trend test were not significant.
On females, only the low and middle doses were significant compared to the control group.
The high dose was not significant and the trend test which was not significant confirmed that there was no dose related relationship.

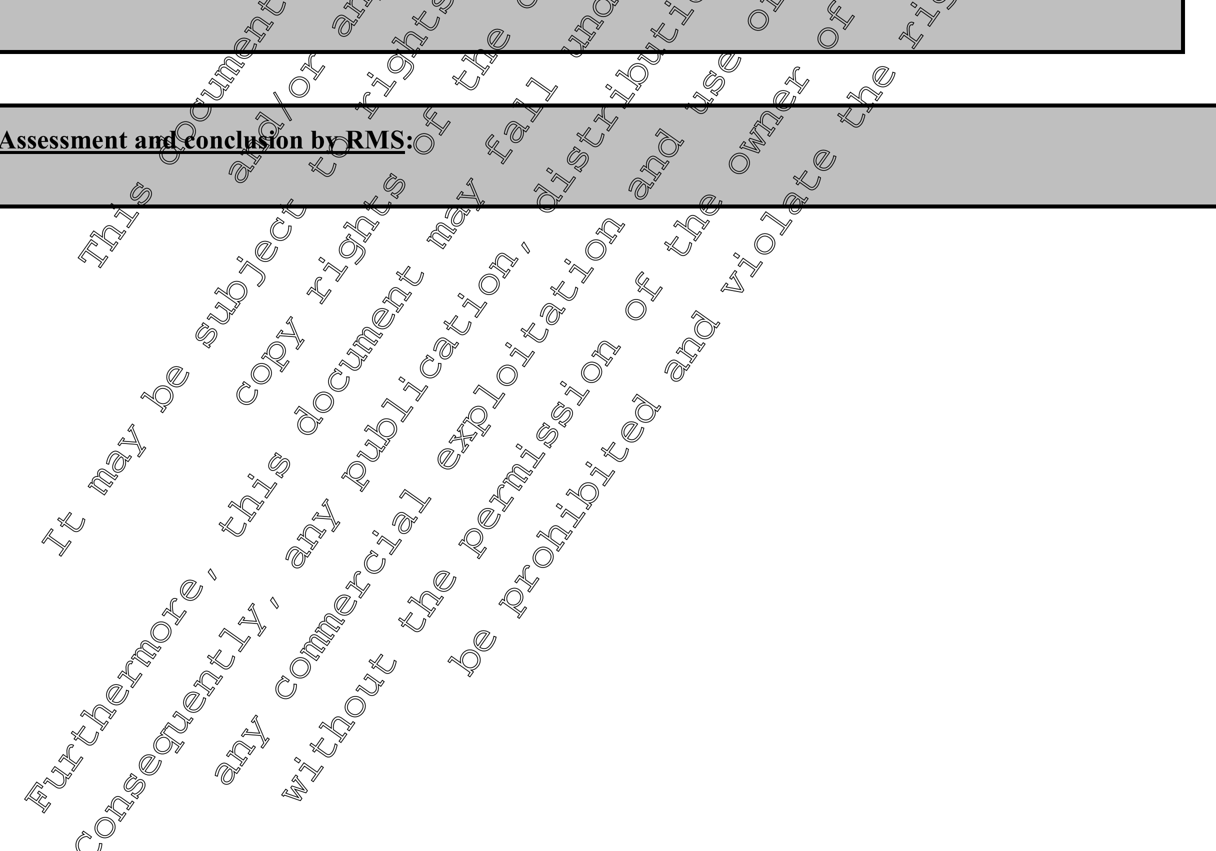
Overall where statistical analysis is conducted including all animals in the main and satellite groups statistically significant increases in tumours are not seen in the top dose groups in either sex.

Assessment and conclusion by applicant:

A statistical analysis was conducted on the incidence of tumours and pre-neoplastic lesions in the C-cells of thyroid glands in the rat carcinogenicity study (study KCA 5.5/01 by (1989) M-174241-01-1) The analysis was on lesions as categorised by group at the (reported in KCA 5.5/09 by (1995) M-174790-01-1) for carcinomas, combined carcinomas and adenomas, and combined carcinomas, adenomas and hyperplasia.

Statistical analysis using all animals in the main and satellite groups found no statistically significant increase in tumours in the top dose groups in either sex. Increases in the low and mid dose groups therefore show no dose response and confirm that the C-cell tumours are likely to be incidental.

Assessment and conclusion by RMS:



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Data Point:	KCA 5.5/11
Report Author:	
Report Year:	2004
Report Title:	Chronic toxicity and carcinogenicity study of aclonifen in the Wistar rat by dietary administration (24-month report)
Report No:	C034454
Document No:	M-234946-01-1
Guideline(s) followed in study:	EU (=EEC): 92/69, Method B33; MAFF: in Japan, 12 Nousan No. 8147; OECD: 453; USEPA (=EPA): 870.4300
Deviations from current test guideline:	Current guideline: OECD 453, 2018 It is not clear if a histological examination was conducted on the coagulating gland. This minor deviation does not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In a combined chronic and carcinogenicity study in Wistar rats (60 animals/sex/dose in main 24 month study, 10/sex/dose in the 12 month study) were administered aclonifen (purity 99.5%) in the diet at doses of 0, 20, 40, 200, 1600 ppm (equivalent to 0, 0.8/1.1, 1.6/2.1, 7.6/10.6, 61/86 mg/kg bw/day in males/females respectively).

At the top dose of 1600 ppm food consumption and body weight gain was reduced in females, increased aspartate aminotransferase and alanine aminotransferase activities in males, increased cholesterol in both sexes, increased absolute and relative liver weight in females, and increased hepatocellular hypertrophy in both sexes.

The NOAEL was 200 ppm in both sexes (equating to approximately 9/12 mg/kg/day in males and females, respectively).

There was a slight in increase in C-cell adenomas in the thyroid in females at 1600 ppm but with no clear dose-response overall these lesions were considered to be incidental. An increased incidence of astrocytoma in females at 1600 ppm was possibly treatment-related. There were no neoplastic changes in males that could be related to treatment.

The study was performed to GLP and OECD 435 (although there are some minor omissions in meeting the current 2018 these omissions to not affect the overall adequacy of the study).

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description: Aclonifen

Lot/Batch: 97013/03

Purity: 99.5%

Appearance:	Yellow powder
Stability of test compound:	Fresh batches of aclonifen incorporated into the diet were prepared every 4 weeks. The stability of the test substance in the diet at room temperature was demonstrated in a pre-study mix at 20 ppm for up to 70 days and at 5000 ppm for up to 79 days.

2. Vehicle and /or positive control Aclonifen was incorporated into pulverised rodent diet

3. Test animals

Species and strain:	Rat – WI-IOPS AF Wistar
Source:	
Number of animals:	60 per sex/dose group for main study (plus satellite group for interim sacrifice of 10 per sex/dose)
Sex:	Male + Female
Age:	6 weeks at the start of treatment
Weight at treatment:	Males: 213 - 273g; females: 153 - 199g (day of first treatment)
Acclimation period:	16 days
Diet:	Ground and irradiated U.A.R. Certified Rodent Meal A04C-10 P1 *ad libitum*
Water:	Filtered and softened tap water, *ad libitum*
Cage type:	Suspended stainless steel and wire mesh with gridded bottoms.
Housing:	5 per sex and per cage.
Environmental conditions:	Temperature: 20 - 24°C Humidity: 40 - 70 % Air changes: 10 – 15 per hour Photoperiod: 12 hour light/dark cycles

B. STUDY DESIGN AND METHODS

1. In life dates

23 February 2001 to 12 March 2003

2. Animal assignment and treatment

Aclonifen (batch 97013/03, purity 99.5%) was orally administered, via the diet, at levels of 0, 20, 40, 200 and 1600 ppm to groups of 70 male and 70 female Wistar rats for up to 24 months.

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In each dose group, the rats were divided into a main group (60 animals/sex) and a satellite group (10 animals/sex). The satellite group was terminated after 52 weeks. The rats of the main group were sacrificed after 24 months.

3. Diet preparation and analysis

Homogeneity and concentration of aclonifen in diet were found to be acceptable (as they were within the in-house target range of 85 to 115%), except for one homogeneity sample (out of nine samples) at 40 ppm (84%), four concentration samples at 20 ppm (83, 84, 117 and 119%), one concentration sample at 40 ppm (83%) and one concentration sample at 200 ppm (84%) (out of 44 samples at each dose level). Since these samples were only slightly outside the target range and occurred in isolated instances, they were considered acceptable for use. Preparations in a pre-study mix at 20 and 5 000 ppm were found to be stable over 70 and 79 days, respectively, at ambient temperature.

4. In-life observations

Food consumption and body weight of the animals were determined weekly during the first 13 weeks of the study and then every 4 weeks.

The state of health of all rats was checked daily; furthermore, the animals were subjected to detailed physical examinations including palpation were conducted twice monthly during the first 13 weeks of the study and once a week thereafter.

Prior to the start of the study and then 12 and 24 months all animals were give an ophthalmological examination.

5. Clinical chemistry, haematology and urinalysis

Clinical chemistry and haematology determinations and urinalysis were performed during months 6, 12, 18 and 24 on 10 animals per sex/dose from the main and chronic dose groups. Blood and urine was collected following overnight fasting. In addition, blood samples were taken at weeks 2, 9, 24 and 52/53 for possible T_3, T_4 and TSH determinations in animals allocated to the chronic phase. However, after the assessment of histopathological examination at the interim sacrifice, it was decided that hormonal analysis was not necessary.

Haematology: Erythrocytes, haemoglobin, haematocrit, mean cell volume, mean haemoglobin content per erythrocyte, mean corpuscular haemoglobin concentration, platelet count, prothrombin time, total and differential leucocyte count. Blood smears were also prepared and examined where blood counts were abnormal. Reticulocytes were counted at 18 and 24 months only.

Clinical chemistry Sodium, potassium, calcium, chloride, inorganic phosphorus, total bilirubin, glucose, creatinine, urea, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, total protein, albumin, total cholesterol.

Urinalysis: Appearance, colour, pH, urinary volume, urinary refractive index, Glucose, bilirubin, ketone bodies, occult blood, protein, 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.

6. Investigations at necropsy

All surviving animals allocated to the chronic and carcinogenicity phases were subjected to necropsy after a minimum of 52 weeks and a minimum of 104 weeks of treatment, respectively.

The following selected organs were weighed and designated tissues sampled and examined microscopically:

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Organ weights: Adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, pituitary gland, prostate gland, spleen, testes, thymus, thyroid gland (with parathyroid glands) and uterus (with cervix).

Histology: An extensive list of tissues were fixed and examined for histopathological findings.

For the chronic phase of the study histological examination was conducted on all gross lesions and all organs and tissues from animals that died during the treatment period, all organs and tissues from animals in the control and high dose groups, the liver, lung, kidney and thyroid glands of animals in the mid and low dose groups. For the carcinogenicity phase of the study histopathological examination was conducted on all tissues in all dose groups.

7. Statistics

Bartett's test was used to test for homogeneity of group variances. For groups with homogenous variances ANOVA was used followed by Dunnett's test. Log transformed data and square root transformed data was used where appropriate followed by ANOVA and Dunnett's test. For groups with non-homogenous variances Kruskal-Wallis test was used followed by the Dunn test.

Adjusted mortality rates were estimated using ███ estimation procedures for each sex and treatment group. Animals that died following accidents or at scheduled sacrifice were excluded. Differences in survival rates were assessed using Cox's test.

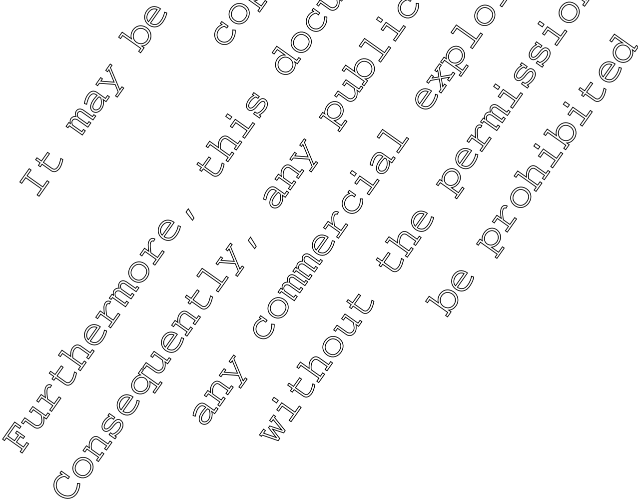
Not all histopathological lesions were statistically analysed, but only those lesions from the carcinogenicity phase where a positive trend was suspected. For these lesions Fisher's exact test and the Cochran-Armitage trend test was used. This was followed by logistic regression analyses using the logistic prevalence method to adjust for differential mortality. Finally trend tests were conducted.

II. RESULTS AND DISCUSSION

1. General observation/clinical signs/ Mortality

Over the entire 24 months of the study, mortality rate was unaffected by treatment. Statistical analysis showed no differential mortality between the treated groups and control groups for either sex when adjusted for survival. At the end of the carcinogenicity phase, mortality rate was higher in males than in females including in control animals (see table below).

Table 5.5- 17: **Aclonifen - carcinogenicity study in rats – Survival analysis**



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	Number of unscheduled deaths									
	Males					Females				
Diet concentration (ppm)	**0**	**20**	**40**	**200**	**1600**	**0**	**20**	**40**	**200**	**1600**
Total mortalities at 18 months	18/60	21/60	20/60	23/60	18/60	19/60	15/60	15/60	18/60	15/60
Total mortalities during study (24 months)	39/60	38/60	42/60	41/60	37/60	24/60	30/60	27/60	24/60	22/60
Total mortality during study (crude rates %)[1]	65	63.3	70	68.3	61.7	40	50	45	40	36.7
Total mortality during study (adjusted rates %)[2]	65.4	63.3	70	68.3	61.7	40	50	45	40.5	38.7

[1] Crude mortality defined as the number of animals dying (found dead, humane kills) during the study, over the total number of animals per group.

[2] ████████ estimated mortality rates at the end of the study after adjusting for censored animals (i.e. animals that died due to accidents).

Treatment-related clinical signs consisted of abnormal coloured (dark yellow) urine with yellow stained fur in all animals of both sexes at 1600 ppm. At 200 ppm, 2/70 males had abnormal coloured (dark yellow) urine, whilst 8/70 males and 11/70 females had yellow staining of the fur.

There were no treatment-related ophthalmological findings at the 12-month examination. At the 24-month examination there was an increase in densified suture lines in the lens in males at 1600 ppm. In addition, there was a slight increase in the incidence of chromodacryorrhea in males at 1600 ppm and 200 ppm. However in most cases this finding was associated with abnormal dentition and in the absence of a treatment-related effect in females, is considered to be incidental.

No treatment-related clinical signs were observed at 40 or 20 ppm.

Table 5.5- 18: **Aclonifen - 24-month oncogenicity study in rats – Ophthalmological findings (number of animals with findings)**

	Males					Females				
Diet concentration (ppm)	**0**	**20**	**40**	**200**	**1600**	**0**	**20**	**40**	**200**	**1600**
No ocular abnormalities	63	59	62	63	62	61	62	61	63	65
Densified suture lines in lens	1	6	8	6	19	9	15	11	16	13
Chromodacryorrhea	2	4	1	6	8	2	1	3	3	1

2. Body weight / food consumption / Substance intake

At 1600 ppm in females, absolute body weight was marginally lower than the controls during the first year of treatment, with an overall reduction of 5% at Week 50, whilst during the second year of

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treatment the effect was more pronounced, with a reduction of 11% at Week 102. Body weight gain was reduced by 12% (p<0.01) after 1 week of treatment, compared with the control group. Throughout the remainder of the first year of treatment, cumulative body weight gain was reduced from 6 to 10% and from 13 to 17% during the second year of treatment. The effect was statistically significant (p<0.05 or 0.01) at most time points for both absolute body weight and body weight gain.

There was no effect on body weight or body weight gain in males at 1600 ppm, or in either sex at 200, 40 or 20 ppm.

Food consumption was slightly reduced throughout treatment in females at 1600 ppm, the effect being statistically significant (p<0.05 or 0.01) on several occasions. The effect was most pronounced during the first four weeks of treatment, where food consumption was reduced by 10%, compared with the control group.

Food consumption was comparable with the controls in males at 1600 ppm and both sexes at 200, 40 and 20 ppm.

Table 5.5- 19: Aclonifen – carcinogenicity study in rats – Bodyweight and food consumption

	Males					**Females**				
Diet concentration (ppm)	**0**	**20**	**40**	**200**	**1600**	**0**	**20**	**40**	**200**	**1600**
Body weight – week 50 (g)	695	708	693	694	686	357	361	366	361	**339** (↓5%)**
Bodyweight gain – week 50 (g)	456	466	454	455	448	181	183	190	184	**163* (↓10%)**
Body weight - week 102 (g)	672	681	679	663	656	442	421	464	444	**395* (↓11%)**
Bodyweight gain – week 102 (g)	436	442	443	427	421	267	245	288	268	**221* (↓17%)**
Food consumption : weeks 1-4 (g/animal/day)	27.8	28.0	27.7	27.4	28.0	21.8	21.4	20.8	21.4	**19.7* (↓10%)**
Food consumption : weeks 53-104 (g/animal/day)	24.3	23.8	23.3	22.95	23.0	19.6	19.3	19.6	19.6	**18.2 (↓7%)**

statistically significant: * p < 0.05, ** p < 0.01
bold : considered to be treatment-related

The mean achieved dosage intake per group was as follows:

Table 5.5- 20: Aclonifen – carcinogenicity study in rats – Achieved dosage (mg/kg/ bw/day)

	Males				**Females**			
Diet concentration (ppm)	**20**	**40**	**200**	**1600**	**20**	**40**	**200**	**1600**
Weeks 1-13	1.22	2.44	12.02	97.5	1.54	3.04	15.51	120.5
Weeks 1-52	0.87	1.75	8.64	70.6	1.19	2.38	11.82	93.3
Weeks 1-104	0.76	1.52	7.56	61.4	1.07	2.11	10.61	85.5

3. Haematology / Clinical chemistry / Urinalysis

There were no haematology findings that could be attributed to test-substance intake. Clinical chemistry analysis of blood samples revealed statistically significantly higher mean aspartate aminotransferase and alanine aminotransferase activities and mean total cholesterol concentrations in

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males at 1600 ppm, at 6 and 12 months. At 1600 ppm in females, a higher mean total cholesterol concentration was noted at 6 months only.

No toxicologically relevant changes were seen at 18 or 24 months, in either sex at the dose levels tested.

Table 5.5- 21: Aclonifen - Carcinogenicity study in rats – Clinical chemistry

	Males					Females				
Diet concentration (ppm)	**0**	**20**	**40**	**200**	**1600**	**0**	**20**	**40**	**200**	**1600**
	Aspartate aminotransferase (IU/L)									
6 months	60	57	69	70	**108** (↑80)**	59[19]	65	106	115	65[19]
12 months	61[19]	62	53[19]	60	**129**[19] (↑x2.1)**	66[18]	69[19]	68	79[19]	65[19]
	Alanine aminotransferase (IU/L)									
6 months	37	35	41	44	**61* (↑65%)**	26[19]	29	48	53	32[19]
12 months	32[19]	35	29[19]	38	**74**[19] (↑x2.3)**	29[18]	31[19]	31	34[19]	31[19]
	Total cholesterol (µmol/L)									
6 months	2.14	2.18	2.15	2.16	**2.54 (↑19%)**	2.01[19]	1.98	20.3	2.21	**2.39*[19] (↑19%)**
12 months	2.37[19]	2.46	2.35[19]	2.37	**2.91*[19] (↑23%)**	2.15[18]	1.99[19]	2.13	2.14[19]	2.27[19]

xstatistically significant, * p<0.05, ** p<0.01
bold : considered to be treatment-related
Number of animals = 20 per sex/dose, where fewer than 20 animals the number used is stated in superscript

Dark yellow coloured urine was observed at 1600 ppm in both sexes throughout the study. In addition, at 200 ppm, isolated instances of coloured urine were observed in both sexes. At 1600 ppm in males, a slight tendency towards a higher number of normally observed crystals was seen at 18 months, compared with the control group.

No other treatment-related effect was seen. The only statistically significant difference, lower mean volume noted in females at 12 months and in males at 18 months (associated with higher mean refractive index), at 200 ppm, was considered to be incidental in the absence of any effect at 1600 ppm.

4. Gross pathology / Organ weights / Histopathology

In the 12-month chronic phase of the study here was no difference in mean terminal body weights of treated males and females, when compared to controls. Mean liver weights were statistically significantly higher in females at 1600 ppm.

In the 24-month carcinogenicity phase of the study there was no change in mean terminal body weights of treated males. In the high dose females there was a 10% reduction in terminal body weight when compared with the controls, though the effect was not statistically significant. Mean terminal body weights of females at the lower dose levels were comparable with the controls.

Table 5.5- 22: Aclonifen - carcinogenicity study in rats – organ weights and histopathology

	Males					Females				
Diet concentration (ppm)	**0**	**20**	**40**	**600**	**1600**	**0**	**20**	**40**	**200**	**1600**
Interim sacrifice (12 month)										
Number of animals	10	10	9	10	9	8	9	10	10	9
Bodyweight – necropsy at 12 months (g)	660.1	655.7	630.8	682.9	658.8	322.8	329.0	350.2	336.6	344.8
Liver weight (g)	13.0	12.1	12.0	13.2	13.3	7.0	7.1	7.8	7.2	**8.4** (↑20%)**
Liver weight % bw	1.99	1.84	1.90	1.93	2.02	2.18	2.15	2.22	2.14	**2.45** (↑12%)**
Hepatocellular hypertrophy (at week 52 sacrifice)	0/10	0/10	1/9	0/10	0/9	0/10	0/10	0/10	0/10	**4/9**
Final sacrifice (24 month)										
Number of animals	21	22	18	19	23	36	30	33	36	38
Bodyweight – necropsy at 24 months (g)	642.7	629.7	646.6	617.5	633.2	422.0	408.9	431.5	426.8	379.8
Liver weight (g)	12.9	12.7	12.4	12.2	13.7	9.8	9.8	10.0	10.1	9.4
Liver weight % bw	2.03	2.02	1.93	2.00	2.16	2.33	2.39	2.33	2.37	2.48
Hepatocellular hypertrophy	0/60	0/60	0/60	4/60	**28/60**	0/60	0/60	0/60	2/60	**17/60**

statistically significant: * p < 0.05, ** p < 0.01;
bold : considered to be treatment-related

For histopathological findings values are expressed as number of lesions/number of animals examined

All gross pathology changes observed at interim sacrifice, at final sacrifice and on all decedent animals were considered as incidental and not treatment-related. At the 12-month sacrifice a treatment-related change was found in the liver of females at 1 600 ppm and consisted of a slight diffuse centrilobular hepatocellular hypertrophy in the liver of 4/9 females. This finding correlated to the higher mean liver weights observed in this group. At the 24-month terminal sacrifice centrilobular hepatocellular hypertrophy was found in 28/60 males and 17/60 females, the severity ranging from slight to mild in males and slight to moderate in females.

5. Neoplastic findings

The overall incidence of neoplastic findings observed at the interim sacrifice was very low and did not indicate any relationship to treatment.

In the carcinogenicity phase of the study there were four malignant astrocytomas of the brain in females at 1600 ppm. There was a positive trend for increasing incidences of brain/astrocytoma malignant which was statistically significant (p<0.01) but the pairwise comparison with controls was not statistically significant. A malignant astrocytoma occurred at 200 ppm in isolation but the incidence was not statistically significantly different to the controls. These tumours were found to occur in the cerebrum, often in periventricular areas.

One anaplastic glioma and two malignant oligodendrogliomas were seen in females at 1600 ppm. Since these tumours were not statistically significant and occurred at a low incidence, they were considered to be incidental.

In males, all these tumours were randomly distributed amongst the controls and different treated groups and were not statistically significant. In addition, one male at 40 ppm had a benign oligodendroglioma, in isolation, this finding was considered to be incidental.

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Table 5.5- 23: Aclonifen - carcinogenicity study in rats – Incidence of brain tumours

Sex	Male					Female				
Dose level of aclonifen (ppm)	**0**	**20**	**40**	**200**	**1600**	**0**	**20**	**40**	**200**	**1600**
Number examined	60	60	60	59	60	60	60	60	60	60
Astrocytoma, malignant	1	0	1	1	2	0	0	0	1	4**
Anaplastic glioma	0	0	0	0	0	0	0	0	0	1
Oligodendroglioma, malignant	0	1	0	1	0	0	0	0	0	2
Oligodendroglioma, benign	0	0	1	0	0	0	0	0	0	0
Trend test **p<0.01										

In the thyroid gland, there was a tendency towards a higher incidence of C-cell adenomas in females. This change was considered not to be toxicologically relevant since it was not dose-related. In addition, the incidence of C-cell adenomas in the concurrent control group was reported to be very low compared with in-house historical control data (1/60 on the current study, compared with 4/60 for study number SA 00551). When the treated female groups on the current study were compared with the control data from the SA 00551 study, there were no statistically significant differences concerning the incidence of C-cell adenomas. There was no evidence of an effect of treatment on the incidence of C-cell carcinoma. The differential diagnosis of C-cell adenoma and focal hyperplasia was based solely on the size of the lesion detected. It was considered appropriate, therefore to consider the incidence of focal hyperplasia with regard to the effect of treatment on focal proliferative lesions. When the incidence of focal hyperplasia and C-cell adenoma were combined, there was no evidence of an effect of treatment.

Table 5.5- 24: Aclonifen – carcinogenicity study in rats – Incidence of C-cell lesions

Sex	Male					Female				
Dose level of aclonifen (ppm)	**0**	**20**	**40**	**200**	**1600**	**0**	**20**	**40**	**200**	**1600**
Number examined	60	60	60	59	60	60	60	60	60	60
C-cell adenoma	5	2	5	3	5	1	9	9	7	6
C-cell hyperplasia, focal	12	15	7	7	5	15	7	15	11	7
C-cell hyperplasia and adenoma combined	17	17	10	10	10	16	16	24	18	13

All other neoplastic changes were considered to be incidental in origin and corresponded to those routinely observed in laboratory animals of this strain and age, kept under monitored environmental conditions.

III. CONCLUSION

The NOAEL over a 24-month period of dietary administration with aclonifen to the Wistar rat was 200 ppm in both sexes (equivalent to 7.6 mg/kg body weight/day in males and 11 mg/kg body weight /day in females).

Neoplastic findings observed at the 24-month terminal sacrifice were confined to the high dose

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females and consisted of 4/60 malignant astrocytoma, the toxicological relevance of which is unclear.

Assessment and conclusion by applicant:

The study was performed to GLP and OECD 435 (some minor omissions but overall the study is deemed to meet the current 2018 test guidelines).

The NOAEL over a 24-month period of dietary administration with aclonifen to the Wistar rat was 200 ppm in both sexes (equivalent to 7.6/11 mg/kg body weight/day in males/females) based on reduced food consumption and reduced body weight gain in females, increased aspartate aminotransferase and alanine aminotransferase activities in males, increased cholesterol in both sexes, increased absolute and relative liver weight in females, and increased hepatocellular hypertrophy in both sexes at 1600 ppm (61/86 mg/kg bw/day in males/females).

There was a slight in increase in C-cell adenomas in the thyroid in females at 1600 ppm but with no clear dose-response overall these lesions were considered to be incidental. An increased incidence of astrocytoma in females at 1600 ppm was possibly treatment-related. There were no neoplastic changes in males that could be related to treatment.

Aclonifen has a harmonised classification under (EC) No. 1272/2008 Carcinogenicity Category 2 H351, based on a low incidence of unusual brain tumours in female rats, due to the rarity of this tumour type and the absence of a mechanistic explanation, the finding in female rats was considered as limited evidence of carcinogenicity (RAC Opinion ECHA/RAC/ CLH-O-0000001543-79-03/A1 September 2011).

Assessment and conclusion by RMS:

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Data Point:	KCA 5.5/12
Report Author:	███
Report Year:	1991
Report Title:	CME 127: Toxicity and oncogenicity study by dietary administration to CD-1 mice Final report Volume 1
Report No:	R007159
Document No:	M-174334-01-1
Guideline(s) followed in study:	USEPA (=EPA): 83-5
Deviations from current test guideline:	Current guideline: OECD 453, 2018 Minor deviations. Ophthalmological examination not conducted. Haematology not measured at 3, 6 or 12 months, but only at 9 months and at termination. Clinical chemistry only measured at 9 months. Cholesterol and creatinine not measured. Urinalysis not conducted. Epididymides and ovaries not weighed. Histopathological examination not conducted on coagulating gland, upper respiratory tract, peripheral nerve, rectum, vagina. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

In a combined chronic and carcinogenicity study in CD-1 mice (52 animals/sex/dose in main 18 month study, 12/sex/dose in the 9 month study, and 12 animals/sex/dose in the 9 month study with 6 week recover period) were administered aclonifen (purity 97.6%) in the diet at doses of 0, 70, 700, 7000 ppm (equivalent to 0, 7.1/8.3, 76/80, 892/984 mg/kg bw/day in males/females respectively).

Body weight gain was reduced in both sexes at 7000 ppm and in males at 700 ppm however there were no changes in food consumption. There were no adverse haematology findings. Clinical chemistry investigations revealed increases in alanine amino transferase and aspartate aminotransferase in males at 9 months, though these findings were reversible in the recovery period. T4 was decreased in both sexes at 7000 and 700 ppm, but was also reversible in the recovery group. Levels of T3 showed no clear treatment-related effects. Absolute and relative liver weight was markedly increased in both sexes at 7000 ppm but there were no adverse histopathological findings in the liver. The only adverse histopathological findings were seen in the urinary bladder. Chronic inflammation was evident at 7000 ppm in both sexes at 9 months and 18 month sacrifice, and transitional cell hyperplasia was seen at 9 and 18 months in both sexes at 700 and 70000 ppm although the finding was much more prevalent in males. These findings were largely absent from animals in the recovery group. A low incidence of bladder tumours at 7000 ppm (one transitional cell papilloma in males, one transitional cell carcinoma in males, and one sarcoma in the bladder in females) are potentially treatment-related as these tumours are rare and may be a consequence of pre-neoplastic changes and chronic irritation in the bladder.

The NOAEL was 70 ppm in both sexes (equating to approximately 7.1/8.3 mg/kg/day in males and females, respectively) based on histopathological findings in the bladder in both sexes, and changes in thyroid hormone levels.

The study was performed to GLP and OECD 435 (although there are some omissions in meeting the current 2018 these omissions to not affect the overall adequacy of the study to investigate carcinogenic potential in the mouse).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description:	CME 127 (Aclonifen)
Lot/Batch:	DA535
Purity:	97.6%
Appearance:	Yellow powder
Stability of test compound:	Fresh batches of aclonifen incorporated into the diet were prepared weekly. Aclonifen was demonstrated to be stable in the mixtures at room temperature for at least 14 days.

2. Vehicle and /or positive control Aclonifen was incorporated into pulverised rodent diet

3. Test animals

Species and strain:	Mice – CD-1
Source:	[redacted]
Number of animals:	52 per sex/dose group for the main study (plus 3 satellite groups of 12 per sex/dose)
Sex:	Male + Female
Age:	21-28 days of age at start of acclimatisation period
Weight at treatment:	Males: 23-27 g; Females: 19-23 g (6 days after arrival)
Acclimation period:	13 days
Diet:	Powdered rodent diet, Laboratory Animal Diet No. 2 (LAD 2), was available to the mice *ad libitum*. This diet was marketed by [redacted]. It was an expanded diet, which was subsequently ground.
Water:	Tap water, *ad libitum*
Cage type:	Type M2 cages (polycarbonate, 33x15x13 cm)
Housing:	Groups of 4 mice per cage / sex (except if the number was reduced by mortality)
Environmental conditions:	Temperature: 21 ± 2°C Humidity: 55 (40-70) % Air changes: yes, but number of changes/hour not mentioned

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Photoperiod: 12 hours light and 12 hours darkness

B. STUDY DESIGN AND METHODS

1. In life dates

29 March 1988 to 16 October 1989.

2. Animal assignment and treatment

Aclonifen was administered via the diet at dietary levels of 0 (control), 70, 700 and 7000 ppm to groups of CD-1 mice. For the oncogenicity study 52 males and 52 females were assigned to each group and received treatment for a minimum of 80 weeks. In addition, groups of 12 male and 12 female mice were assigned to each of the following investigations: the toxicity study (sacrificed after 39 weeks of treatment); the reversibility study (sacrificed after 39 weeks of treatment and a 6 week period of withdrawal from treatment); and the satellite study for thyroid hormone investigation (used solely to provide blood for T3 and T4 assays), subjected to a similar schedule to that of the mice in the reversibility study.

3. Diet preparation and analysis

The test material was incorporated into the ground rodent diet at weekly intervals. The test material was stored at room temperature. The stability of aclonifen in the diet (doses 780 to 50000 ppm) for up to 14 days had been demonstrated in the 28-day mouse study (KCA 5.3.1/01 1988, M-174234-01-1). For this carcinogenicity study stability at the lowest dose 70 ppm were assayed after 8, 14 and 16 days storage at room temperature and deemed to have acceptable stability for at least 14 days at room temperature.

An aliquot of the test material was returned to the Sponsor for analysis at six monthly intervals and at completion of the treatment period, these results confirmed the stability of the test material. The homogeneity and concentration of the test material in the diet was determined before commencement of treatment. The concentrations of test material in mixes prepared for Weeks 1, 13, 26, 39, 52, 65 and 78 of treatment were also determined from an analytical procedure developed from information provided by the Sponsor. The method of analysis does not fulfil SANCO 3029, but can be regarded as fit for purpose.

Acceptable homogeneity and stability of the test material in the diet at room temperature were demonstrated in the pre-treatment investigations. The concentration in the mixes analysed at 13-week intervals during the study averaged 101, 95 and 98% of the intended concentration for the low, intermediate and high treatment levels respectively and were considered satisfactory.

4. In-life observations

Animals were observed twice daily for clinical signs and mortality. All animals were handled and palpated weekly throughout the treatment period. Food consumption was recorded weekly. Bodyweight was recorded weekly for the first 14 weeks of the study and every two weeks thereafter.

Haematological examinations included erythrocyte count, haemoglobin, haematocrit, platelets, leucocytes and differential blood count. MCV, MVH and MCHC were determined by calculation. The investigations were performed on all animals (12 m/f) of the interim sacrifice at week 38, on males in the reversibility group four weeks after the start of the reversibility period at week 42, and at study termination in the main carcinogenicity cohort (in week 79) on 10 male and 10 female animals per group. Animals were not faster prior to blood sampling.

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Clinical chemistry examinations were carried from blood samples taken after 39 weeks of treatment on animals in the interim sacrifice group (12 m/f). The following parameters were investigated: total bilirubin, glucose, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, total protein, electrophoretic protein fraction, urea concentration, sodium, potassium, calcium, and inorganic phosphorous.

Males from the reversibility group were examined for aspartate amino-transferase, alanine amino-transferase 5 weeks following the start of the reversibility period (at week 43).

For animals in the thyroid hormone cohort, T3 and T4 were measured at week 39, and then following withdrawal of test substance administration T3 was measured 5 weeks and T4 measured 7 weeks respectively after withdrawal of the test substance.

Blood samples for clinical chemistry measurements were taken following overnight withdrawal of food, however animals were not fasted prior to blood samples taken for T3 and T4 measurements.

5. Investigations at necropsy

The interim and terminal autopsy included a macroscopic examination of integuments, orifices, eyes and internal organs. Any abnormalities were recorded. The weights of selected organs were recorded:

adrenals, brain, heart, kidneys, liver, lungs, spleen, testes, thyroid (with parathyroids), uterus (with cervix).

An extensive range of tissues preserved. Bone marrow smears were also prepared and examined. Subsequently tissues from all animals, including decedents, were examined histopathologically.

6. Statistics

Cox's test was used to assess mortality rates, with Tarone's extension of Cox's test to examine linear trend. Data for females were also analysed using a Generalised Kruskall-Wallis test. For normally distributed data (body weight gain, and most haematology parameters) a series of Student's t-tests was performed using a pooled within-group error variance. Mann-Whitney test was used to analyse erythrocytes and haemoglobin concentration in males at 38 weeks. For organ weights Bartlett's test was used followed by Behrens-Fisher test or Dunnett's test. For histopathological findings where appropriate Fishers Exact Test was used.

II. RESULTS AND DISCUSSION

1. General observation/clinical signs/ Mortality

Mortality was low during the first 39 weeks of treatment and during the reversibility period. Subsequently, survival was about 60% in all groups, with the exception of males at 700 ppm (45%) and females at 7000 ppm, where survival remained higher than 80%.

Table 5.5- 25: Aclonifen carcinogenicity study in mice – Mortality

	Number of unscheduled deaths							
	Males				Females			
Diet concentration (ppm)	**0**	**70**	**700**	**7000**	**0**	**70**	**700**	**7000**
Week 1-39	5	1	4	3	4	4	3	0
Week 1-80 (Total)	23/52	23/52	28/52	21/52	19/52	19/52	19/52	10/52

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Mortality (%)	44.2	43.4	53.8	40.4	36.5	36.5	36.5	19.2

Treatment-related clinical signs were confined to yellow staining of the coat of animals of the 700 and 7000 ppm groups, which was attributable to the presence of yellow coloured test material in the diet and to the presence of test material in the urine.

2. Body weight / food consumption / Substance intake

Male and female mice receiving 7000 ppm in the diet showed lower bodyweight gains from weeks 2 or 5 respectively, which resulted in significantly reduced weight gains over the first 38 weeks for males and 80 weeks for females. Males receiving 700 ppm also showed impaired body growth over the first 38 weeks of treatment. Impaired growth showed some reversibility, following the withdrawal of the treatment.

Food consumption was not affected in any group.

Table 5.5- 26: Aclonifen oncogenicity study in mice – Bodyweight change and food consumption

	Males				Females			
Dietary concentration (ppm)	**0**	**70**	**700**	**7000**	**0**	**70**	**700**	**7000**
Bodyweight change weeks 0 – 38 (g)	22.6	20.7* (↓10%)	**20.1** (↓11%)**	**15.0*** (↓34%)**	18.3	17.0	17.5	**11.4*** (↓38%)**
Bodyweight change reversibility (g)	-0.7	-1.4	-0.1	1.2**	0.7	0.8	0.5	1.8
Bodyweight change weeks 38 – 80 (g)	-0.2	0.2	2.1*	0.3	4.5	5.7	4.2	3.5
Bodyweight changes weeks 0 – 80 (g)	23.2	22.3	22.1	**15.4*** (↓35%)**	22.7	22.4	22.7	**13.9*** (↓39%)**
Food consumption wk 1-80 (g/mouse)	2793	2779	2762	2914	2539	2492	2421	2559
statistically significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **bold** : considered to be treatment-related								

The achieved intake is given in the following table

Table 5.5- 27: Aclonifen carcinogenicity study in mice – Achieved intake

	Males			Females		
Diet concentration (ppm)	**70**	**700**	**7000**	**70**	**700**	**7000**
Substance-intake (mg/kg bw/day)	7.07	75.6	892	8.26	80.1	984

3. Haematology / Clinical chemistry / Urinalysis

Haematology and blood chemistry analysis revealed no changes considered to be of toxicological significance. Changes in haematology parameters, most notable reduced haemoglobin and increased mean cell volume in males at 7000 ppm are very slight and not toxicologically adverse. There were

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also some minor changes in clinical chemistry parameters, most notably increased alanine aminotransferase, and increased alanine aminotransferase. These findings were not seen in females and were reversible after 5 weeks in the reversibility group.

Plasma thyroxine (T4) estimation after 39 weeks of treatment revealed, relative to their respective controls, lower T4 concentration in males and females given 700 or 7000 ppm, although no evidence of a dosage-relationship was noted. After seven weeks respite from treatment, the T4 levels of control and formerly treated mice were similar.

Estimation of tri-iodothyronine T3 levels after 39 weeks of treatment or five weeks withdrawal from treatment revealed occasional minor inter-group differences which attained a level of statistical significance. These minor differences were clearly without a dose-related trend and were considered to have arisen by chance.

Table 5.5- 28: Aclonifen oncogenicity study in mice – Haematology and clinical chemistry

	Males				Females			
Dietary concentration (ppm)	**0**	**70**	**700**	**7000**	**0**	**70**	**700**	**7000**
Mean cell haemoglobin concentration – week 38 (%)	34	33	33	33* (↓3%)	33	32	31***	33
Mean cell volume – week 38 (Cμ)	52	52	52	54* (↑4%)	53	53	54	53
Alanine aminotransferase – week 38 (iu/L)	34	49	33	88** (↑x2.6)	40	53	47	61
Alanine aminotransferase – week 43 following 5 weeks reversibility (iu/L)	42	48	55	48	-		-	-
Aspartate aminotransferase – week 38 (iu/L)	64	74	70	94** (↑x1.5)	83	86	86	75
Aspartate aminotransferase – week 43 following 5 weeks reversibility (iu/L)	67	67	73	69	-	-	-	-
T3 – week 38 (ng/mL)	0.5	0.4	0.6	0.6* (↑20%)	0.2	0.2	0.2	0.4*** (↑x2)
T3 – week 43 following 5 weeks reversibility (ng/mL)	0.4	0.4	0.7*** (↑75%)	0.6* (↑50%)	0.5	0.4	0.3*	0.5
T4 – week 38 (ng/100 mL)	0.0046	0.0041	**0.0027*** (↓41%)**	**0.0026*** (↓43%)**	0.0041	0.0040	**0.0024*** (↓41%)**	**0.0029*** (↓29%)**
T4 – week 45 following 7 weeks reversibility (ng/100 mL)	0.0041	0.0041	0.0042	0.0044 (↑7%)	0.0042	0.0046	0.0042	0.0041
statistically significant: * p < 0.05, ** p < 0.01, *** p < 0.001; **bold** : considered to be treatment-related **N = 12**								

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4. Gross pathology / Organ weights / Histopathology

The only treatment-related change revealed during organ weight analysis was increased absolute and relative liver weight at 7000 ppm. This liver enlargement was reversible by the end of the six week recovery period.

Table 5.5- 29: Aclonifen oncogenicity study in mice – Organ weights

	Males				Females			
Diet concentration (ppm)	**0**	**70**	**700**	**7000**	**0**	**70**	**700**	**7000**
	Necropsy at week 39							
Number of animals	12	12	10	8	10	11	9	10
Bodyweight – necropsy (g)	48.5	44.8	45.7	**40.8** (↓16%)**	39.4	36.9	36.9	**34.3 (↓13%)**
Liver weight (g)	2.18	2.18	2.19	**3.13** (↑44%)**	1.56	1.63	1.82	**2.11** (↑35%)**
Liver weight (% bw)	4.492	4.905	4.795	**7.734** (↑72%)**	3.998	4.471	4.952* (↑24%)	**6.219** (↑56%)**
	Necropsy following 39 weeks treatment and 6 weeks reversibility							
Number of animals	11	12	10	11	10	9	11	12
Bodyweight – necropsy (g)	48.8	44.3	45.8	**42.3* (↓13%)**	42.2	39.7	42.5	36.6 (↓13%)
Liver weight (g)	2.41	2.27	2.26	2.37	1.97	1.79	1.73	1.76
Liver weight (% bw)	5.000	5.157	4.965	5.447	4.704	4.534	4.082*	4.835
	Necropsy at week 80							
Number of animals	29	29	24	31	33	33	33	42
Bodyweight – necropsy (g)	50.4	49.8	48.9	**41.0** (↓19%)**	45.0	44.5	44.1	**36.0** (↓20%)**
Liver weight (g)	3.22	2.89	2.67*	**3.58 (↑11%)**	1.91	1.80	1.91	**2.26** (↑18%)**
Liver weight (% bw)	6.388	5.870	5.523	**8.766** (↑38%)**	4.301	4.081	4.359	**6.331** (↑47%)**

statistically significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

bold : considered to be treatment-related

Histopathological examination revealed no treatment-related findings in the liver or thyroid. The only findings considered to be treatment-related were in the urinary bladder with male mice appearing to be more susceptible than females. These changes observed in the urinary bladder are summarised in the table below:

Table 5.5- 30: Aclonifen carcinogenicity study in mice - Histopathology of the urinary bladder in CD-1 mice treated with aclonifen for 39 and 80 weeks

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	Males				**Females**			
Dietary concentration (ppm)	**0**	**70**	**700**	**7000**	**0**	**70**	**700**	**7000**
	39 weeks (all animals killed after 39 weeks)							
No of animals examined	12	12	12	11	12	12	11	12
Chronic inflammation	0	0	0	2	0	0	0	4
Ulcer(s)/erosions(s)	0	0	0	1	0	0	0	2
Submucosal granulomatous reaction	0	1	0	0	0	0	0	1
Transitional hyperplasia	0	0	1	8**	0	0	0	2
	39 weeks + 6 weeks reversibility							
No of animals examined	11	12	10	11	9	9	11	12
Chronic inflammation	0	0	0	0	0	0	0	0
Transitional hyperplasia	0	1	0	2	0	0	0	0
	80 weeks (including animals that died during the study)							
No of animals examined	48	49	52	50	51	51	52	51
Chronic inflammation	0	0	0	4	1	2	1	6
Ulcer(s)/erosions(s)	0	0	0	1	0	0	0	1
Submucosal granulomatous reaction	0	0	0	1	1	0	0	4
Calculus	4	3	6	7	0	0	0	1
Transitional hyperplasia	2	3	10*	31**	1	0	2	2
Transitional cell papilloma	0	0	0	1	0	0	0	0
Transitional cell carcinoma	0	0	0	1	0	0	0	0
Sarcoma	0	0	0	0	0	0	0	1

statistically significant: * p < 0.05; ** p < 0.01; *** p < 0.001;

bold : considered to be treatment-related

A statistically significant higher incidence of transitional cell hyperplasia was observed in males from the 7000 ppm dosage group after 39 weeks of treatment and in males from both the 700 and 7000 ppm dosage group after 80 weeks of treatment, compared with their respective controls. After the six week reversibility period this change was only occasionally seen. Similar findings were infrequent or absent in females from all phases of the study.

A low incidence of chronic inflammation of the urinary bladder was observed among animals from the 7000 ppm dosage group after both 39 weeks and 80 weeks of treatment, with no inflammation observed after the 6 weeks of reversibility following the 39 weeks of treatment.

Urinary bladder tumours were noted in two males and one female in the carcinogenicity phase of the study. Although incidences of bladder tumours were low, bladder tumours in mice are rare and taken in conjunction with the pre-neoplastic lesions (hyperplasia) and irritation these tumours are considered unlikely to be spontaneous.

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It is concluded that the bladder is clearly a specific target organ for aclonifen, with the lesions found at high dosage levels suggesting an irritant effect.

III. CONCLUSION

The NOAEL for CD-1 mice receiving aclonifen continuously in the diet was 70 ppm equivalent to a daily intake of 7.1 and 8.3 mg/kg/day in males and females, respectively.

Assessment and conclusion by applicant:

This GLP chronic and carcinogenicity study has several deviations from the current test guideline, OECD 453 (2018) but overall is considered to be adequate to address the carcinogenic potential of aclonifen in the mouse. There was no urinalysis, and haematology and clinical chemistry analysis were only conducted at 9 and 18 months (not at 3, 6 or 12 suggested in the test guideline). A few organs were not weighed (epididymides and ovaries) or examined microscopically (coagulating gland, upper respiratory tract, peripheral nerve, rectum, vagina) however these omissions are considered minor and do not invalidate the study.

The NOAEL in CD-1 mice receiving aclonifen in the diet was 70 ppm in both sexes (equating to approximately 7.1/8.3 mg/kg/day in males and females, respectively) based on histopathological findings in the bladder in both sexes, and changes in thyroid hormone levels at 700 ppm (76/80 mg/kg bw/day in males/females respectively).

There was a slight increase in rare bladder tumours at 7000 ppm in both sexes (892/984 mg/kg bw/day in males/females) that are considered unlikely to be spontaneous.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.5/13
Report Author:	
Report Year:	1994
Report Title:	Aclonifen Evaluation of proliferative lesions in mouse urinary bladder in study CME 127: Toxicity and oncogenicity study by dietary administration to CD-1 mice
Report No:	R007367
Document No:	M-174769-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

A re-examination of the histology slides of the bladder in the mouse carcinogenicity study (KCA 5.5/12, 1991, M-174334-01-1) was conducted. The report concludes that the tumours observed in the bladder may be treatment related due to the observation of the presence of pre-neoplastic lesions. However only one malignant bladder tumour was observed. Therefore any carcinomic response in aclonifen is weak and even questionable.

I. MATERIALS AND METHODS

A review of the urinary bladder histologic lesions observed in the mice oncogenicity study on aclonifen (KCA 5.5/12, 1991, M-174334-01-1). was undertaken by a consultant pathologist (

II. RESULTS AND DISCUSSION

Re-examination confirmed the findings reported in the study report.

Only one malignant tumour (an invasive squamous cell carcinoma) and one papilloma (benign) were observed in the urinary bladder of male mice at 7000 ppm. No urothelial tumours were observed at lower dose levels in males and in any dose levels in females. The carcinoma occurred early (56 weeks) and was not followed by development of malignant tumours in any other animals. The papilloma was observed in an animal that survive to study termination (81 weeks).

This type of result is unusual in a positive carcinogenicity test where early appearance of a tumour is usually followed by a crop of similar tumours in subsequent weeks. However in the aclonifen study the early appearance of a malignant tumour does not herald the beginning of similar tumours later on in the study. Aclonifen is not mutagenic so if these tumours are induced by aclonifen administration then this would be by a non-genotoxic mechanism. An indication of a possible mechanism is the high incidence of hyperplasia particularly in male mice which bears a dose-response relationship. This hyperplastic reaction would appear to have been caused by the formation of calculi. No calculi were seen in the histopathology sections but angular outlines flanked by epithelial cells and macrophages seen in some lesions suggested their presence, as did crystal-like intrusions within granulomas. Thus the pathology suggests that at high doses crystalluria develops in both sexes and these crystals may conglomerate and form calculi or penetrate into the urothelium presumably through an erosion. The

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calculi and crystals within the lumen of the bladder would produce the hyperplastic response observed in the urothelium while the crystals in the submucosa would produce the granulominous foci observed.

The role of urothelial hyperplasia in the induction of urothelial tumours in mice has been studied in detail in the past in the scientific literature Solid bodies introduced surgically into the bladder of mice are known to produce hyperplasia, papillomas and carcinomas even though they do not contain any carcinogenic chemical, and persistent hyperplasia in the bladder induced by foreign bodies has been demonstrated as a precursor to tumours via the development of metaplasia that culminates in malignant growths.

In this mouse study hyperplasia was present, but no progression to metaplasia. The hyperplasia was graded mild to moderate, but was more prevalent and generally more severe in males. This low grade reaction and the fact that the lesions readily reversed on cessation of treatment may account for the paucity of tumours observed in males and their absence in females.

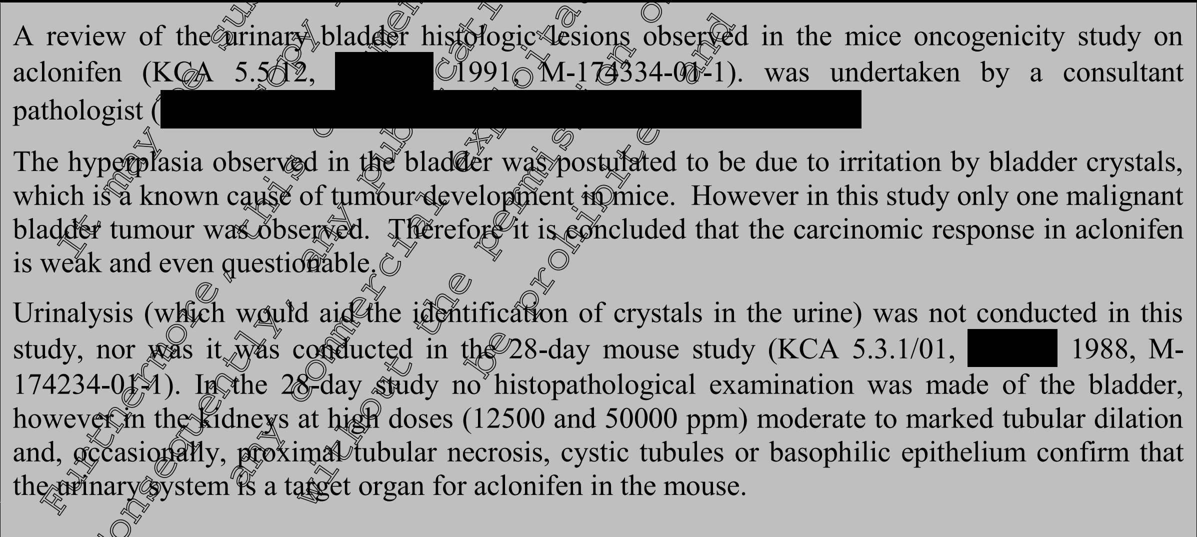
III. CONCLUSION

A dose-related incidence of mild to moderate urothelial hyperplasia was observed in males. In females the response was milder. There is evidence that this response was caused by crystals or calculi. The urothelial hyperplasia resolved after the 6-week reversibility period. In a few animals the hyperplasia persisted, likely due to residual crystals in the bladder. Persistent hyperplasia due to irritation by bladder crystals is a known mechanism of tumour development in mice.

In this study only one malignant tumour (squamous cell carcinoma) and one papilloma (benign) were observed in the urinary bladder of male mice at 7000 ppm aclonifen. No urothelial tumours were observed in males at lower dose levels or at any dose levels in females. Therefore it is concluded that the carcinomic response in aclonifen is weak and even questionable.

No adverse effects in the bladder was seen in the 70 ppm dose groups in males or 700 ppm in females.

The results of the study and the review confirmed that the dose level of 700 ppm was a clear NOAEL for tumour development and that aclonifen does not present a carcinogenic hazard for man.


A review of the urinary bladder histologic lesions observed in the mice oncogenicity study on aclonifen (KCA 5.5/12, [redacted] 1991, M-174334-01-1). was undertaken by a consultant pathologist ([redacted]

The hyperplasia observed in the bladder was postulated to be due to irritation by bladder crystals, which is a known cause of tumour development in mice. However in this study only one malignant bladder tumour was observed. Therefore it is concluded that the carcinomic response in aclonifen is weak and even questionable.

Urinalysis (which would aid the identification of crystals in the urine) was not conducted in this study, nor was it was conducted in the 28-day mouse study (KCA 5.3.1/01, [redacted] 1988, M-174234-01-1). In the 28-day study no histopathological examination was made of the bladder, however in the kidneys at high doses (12500 and 50000 ppm) moderate to marked tubular dilation and, occasionally, proximal tubular necrosis, cystic tubules or basophilic epithelium confirm that the urinary system is a target organ for aclonifen in the mouse.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.5/14
Report Author:	
Report Year:	1992
Report Title:	Incidence of naturally occuring C-cell hyperplasia and tumours in ageing rats
Report No:	R007373
Document No:	M-174781-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive summary

Historical control data is presented for C-cell hyperplasia in several strains of rats demonstrating that this lesion is a relatively common lesion spontaneous lesion in rats.

I. MATERIALS AND METHODS

The purpose of the document was to show that C-cell hyperplasia and tumours are common findings in the ageing Wistar rat. collected data from different laboratories in UK to demonstrate that the C-cell proliferative lesions observed in both control and treated rats of the aclonifen carcinogenicity study are within the expected range of variation.

Data were collected from control of 10 studies run at Hazelton laboratory, from 6 studies run at , from 4 studies run at the and from 3 studies run at

II. RESULTS AND DISCUSSION

Table 5.5-31: Historical control data on C-cell lesions in rats

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ACLONIFEN STUDY (Wistar rats)					HISTORICAL DATA			
Dose level	Control	40 ppm	200 ppm	1600 ppm	Hazelton	HRC	CMR	LSR
					(Sprague-Dawley rats)		(Charles River CD)	
% HYPERPLASIA								
Males	50	58.3	50	62	23 – 68	4 – 20	4.5 – 48	-
Females	65	66.5	60	73	13.3 – 85	6 – 20	3 – 78	-
% ADENOMA								
Males	5	8.3	18	11.5	6 – 27	0 – 4	1 – 19	4.73
Females	16.5	15	13	11.5	5 – 21	0 – 4	0 – 19	1.91
% CARCINOMA								
Males	1.7	5	1.7	3.3	0 - 5	4 - 12	1 - 19	2.78
Females	1.7	5	6.6	5	0 - 2	4 - 12	1 - 4	1.64

III. CONCLUSION

Collected historical control data provide an example of the high variation in the incidence of hyperplasia and thyroid C-cell lesions in ageing rats. More importantly the incidence of C-cell proliferative lesions found in the Wistar rat chronic study was within the historical control range.

Assessment and conclusion by applicant:

C-cell lesions were seen in the rat carcinogenicity study (KCA 5.5/01, 1989, M-174241-01-1) which was conducted using Wistar ChbbTHOM rat from January 1894 to February 1986 at

A second carcinogenicity study in the rat was also conducted (KCA 5.5/11, 2004, M-234946-01-1) using WI-IOPS AF Wistar rats from February 2001 to March 2003 at

The historical control data presented in this report is supplementary information as the data are from different strains, lab and time period to the rat carcinogenicity studies conducted using aclonifen, however it demonstrates that c-cell tumours are common spontaneous lesions in several strains of aging rat.

Assessment and conclusion by RMS:

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CA 5.6 Reproductive toxicity

A dietary two-generation study has been conducted, and developmental toxicity studies in rats and rabbits. A supplementary range-finding developmental study in rabbits, conducted more recently, is also provided, which gives support to the preceding rabbit study.

Aclonifen – summary of reproductive toxicity studies

Type of study, doses	NOAEL	Findings	Reference(s)
Generational studies			
Two-generation dietary reproduction study (Wistar rats) 0, 125, 500, 2000 ppm in diet (approximately equivalent to 9, 35 and 140 mg/kg bw/day)	NOAEL (systemic toxicity) 125 ppm (9 mg/kg bw/day) NOAEL (reproduction, fertility) ≥2000 ppm (140 mg/kg bw/day)	Based on reduced food consumption, body weight (-5% to -20%) in adults at ≥500 ppm, and reduced pup body weight (-10% to -20%) at 2000 ppm	KCA 5.6.1/01 1985 M-174748-01-1 KCA 5.6.1/02 1997 M-174922-01-1 (statistical report)
Developmental studies			
Teratogenicity in rats (Wistar) 0, 6, 60, 600 mg/kg bw/day by oral gavage on gestation days 7-21	NOAEL maternal and embryofoetal toxicity 60 mg/kg bw/day	Based on reduced maternal body weight gain (-10% bw at term) at 600 mg/kg bw/day. Reduced foetal weight (-8%) at 600 mg/kg bw/day, considered secondary to maternal effect.	CA 5.6.2/01 1982 M-174846-01-1
Embryofoetal toxicity in rabbits (Chinchilla rabbits) 0, 1, 5, 25 mg/kg bw/day by oral gavage on gestation days 6-18	NOAEL maternal and embryofoetal toxicity 25 mg/kg bw/day	No evidence of overt maternal toxicity. Treatment period restricted to major organogenetic period.	CA 5.6.2/02 1984 M-174853-01-1
Range-finding for developmental toxicity in rabbits (New Zealand White) 0, 5, 15, 30, 75, 150 mg/kg bw/day by oral gavage on gestation days 6-28	NOAEL maternal systemic toxicity 15 mg/kg bw/day NOAEL embryofoetal toxicity 150 mg/kg bw/day	Based on discoloured urine in some animals at ≥30 mg/kg bw/day, while at 150 mg/kg bw/day there was also transient slightly reduced food consumption with slight body weight effect in mid-late gestation. Dosing from implantation to one day prior to expected parturition.	CA 5.6.2/03 2003 M-233054-01-1

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CA 5.6.1 Generational studies

Data Point:	KCA 5.6.1/01
Report Author:	[redacted]
Report Year:	1985
Report Title:	Two-generation reproduction toxicity study with CME - 127 in the rat
Report No:	R007357
Document No:	M-174748-01-1
Guideline(s) followed in study:	OECD: 426
Deviations from current test guideline:	Current guideline: OECD 416, 2001 Minor deviations, No sperm analysis or related detailed histopathological examination of spermatogenesis or the ovary. No pre-mating monitoring of estrous cyclicity. No monitoring of anogenital distance or pubertal development. No retention and examination of organs from weanlings. No staining for implantation sites, study integrity not affected. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

CME 127 (aclonifen), 95.5% purity, was mixed with the diet and administered to groups of Wistar rats (25/sex/group) at fixed concentrations of 0 (control), 125, 500 or 2000 ppm. Beginning at 9-10 weeks of age, the F0 generation received test diets for 70 days prior to pairing for mating, then throughout mating, gestation and lactation for their first F1 litters. The F1 offspring continued on test diets for a 120-day pre-pairing period, then throughout mating, gestation and lactation for their first F2 litters.

The rats were monitored for mortality and clinical signs of toxicity, body weight and food consumption, abnormalities of the estrous cycle, pre-coital time, duration of gestation, pregnancy and delivery rates, litter size, live and still-births, any abnormalities of nursing/maternal care, gross anomalies in pups, pup sex ratio, pup body weight development and survival. F0 and F1 parents were sacrificed and necropsied, with preservation of the reproductive organs, and also the kidneys (target organs). Histopathology was conducted for all high dose and control adults. After weaning at day 21 post-partum, excess F1 pups not selected as parents were sacrificed and examined macroscopically, as were the excess pups removed at day 4 post-partum for standardisation of litter size to 4/sex, the F2 weanlings, and any pups found dead.

Average achieved dosages pre-pairing were approximately 9, 35 and 140 mg/kg/day, rising in females to approximately 20, 80 and 300 mg/kg/day during lactation, for the 125, 500 and 2000 ppm groups respectively.

There was decreased food consumption and body weight in F0 and F1 parents at 500 and 2000 ppm, and reduced body weight in F1 and F2 pups at 2000 ppm. There was no worsening from one generation to the next and no effect on any examined aspect of reproduction, or on offspring development apart from body weight.

It was concluded that the NOAEL for aclonifen for parent rats in this dietary two-generation reproduction study was 125 ppm (approx. 9 mg/kg bw/day average parental pre-mating exposure), based on slight decrease in food consumption and body weight of the parent animals at ≥500 ppm. A

NOAEL for the offspring was 500 ppm (approx. 35 mg/kg bw/day average parental pre-mating exposure), based on the reduced pup body weights at 2000 ppm. The NOAEL for reproductive performance and fertility of male and female rats was greater than 2000 ppm (approximately 140 mg/kg bw/day average parental pre-mating exposure).

I. MATERIALS AND METHODS

Study dates	Study start:	17 February 1984
	Completion:	09 July 1985

A. MATERIALS

1.	**Test Item**	CME 127 (aclonifen)
	Batch No.:	Ho 06/84/1
	Purity:	95.5%
	Appearance:	Brown powder
	Expiry:	Stated as stable for at least 2 years
2.	**Animals**	Male and female Wistar rats, KFM-HAN, from approximately 250 g (F0 males), 190 g (F0 females) at start of treatment, individually housed except for mating
	Diet:	Kliba 343 rat maintenance diet (and tap water *ad libitum*.

B. STUDY DESIGN AND METHODS

Dose levels were determined on the basis of previous studies. Aclonifen test diets were prepared at least every 2 weeks by admixture with the granulated diet, which was then pelleted and stored in paper bags at room temperature. Stability and homogeneity of aclonifen in the feed was determined by the study sponsor. Samples were collected in the first, 10th, 23rd and 37th weeks of the study for analysis.

The F0 animals were 9-10 weeks of age at the start of treatment. Following 10 weeks of exposure to the test diets, they were paired for mating, one male to one female, for a maximum of 12 days. Vaginal smears were taken daily and the day of detection of sperm and/or a vaginal plug was designated day 0 of gestation (GD 0), and the pre-coital time was determined. Any anomalies of the estrous cycle were also recorded. Positively mated females were removed immediately to individual housing.

The rats were monitored at least twice daily for mortality and clinical signs of toxicity. Body weights were generally recorded weekly, although for mated females weighing was on GD 0, 7, 14 and 21, and for littered females on days 1, 4, 7, 14 and 21 post partum. Food consumption was measured on the same schedule, although only until day 14 post partum for littered females.

The mated females were monitored twice daily towards the end of gestation for signs of parturition, and the duration of gestation was determined. Any females not littering, or losing their litters, were sacrificed and necropsied. Pregnancy and delivery rates were determined. The day on which pups were first delivered was designated lactation day (LD) 0. Litter size, live births, stillbirths, sex ratio and any gross pup anomalies were recorded. Individual pup weights were recorded on LD 1, 4, 7, 14 and 21. Survival, and any behavioural/maternal care abnormalities were recorded. Any dead offspring were necropsied, where practicable. At LD 4 each litter was culled by random selection to 4/sex as near as possible.

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All parental F0 and F1 rats were sacrificed when no longer required for breeding, at or soon after LD 21, and necropsied, with retention of the ovaries, cervix, vagina, testes, epididymides, seminal vesicles, prostate, coagulating gland, pituitary gland, and the kidneys (considered a target organ). These specimens were examined histopathologically for all high dose and control F0 and F1 animals that were paired for mating. Excess F1 and F2 pups at culling of litters were sacrificed on LD 4, while F1 pups not selected for pairing and all F2 pups were sacrificed after LD 21 weaning.

Statistical analysis was by univariate one-way analysis of variance for variables that could be assumed to follow a normal distribution. Test groups were compared with control using Dunnett's test. A one-way univariate analysis based on Wilcoxon ranks was applied to the numbers of pups. Fisher's exact test was applied for dichotomous data.

II. RESULTS AND DISCUSSION

There was no parental mortality and no clinical signs of toxicity.

Significantly reduced body weight and food consumption were observed in F0 males at 500 and 2000 ppm and in F1 males receiving 2000 ppm during the pre-pairing and post-pairing periods until necropsy. Significantly reduced body weight was observed in F0 females at 2000 ppm during pre-pairing, gestation and lactation until necropsy. Body weight of F1 females receiving 500 ppm was also reduced during all these periods, while food consumption was reduced only during the gestation period. The approximately 20% deficit in high dose F1 weight at commencement of their premating exposure period was only partly recovered by the time of termination.

No treatment-related effects on body weight or food consumption were noted at 125 ppm in either sex at any time-point.

Table 5.6.1-1: Aclonifen - 2-generation rat study – Mean bodyweights (g) of F0 and F1 parents ± Standard Deviation

	Periods	0 ppm	125 ppm	500 ppm	2000 ppm
F0					
Males	Pre-pairing (at day 1)	255 ± 10	253 ± 12	251 ± 8	253 ± 8
	Pre-pairing; At day 68	424 ± 33	412 ± 31	396* ± 24	386* ± 25
	(% diff. from control)	-	(-3%)	(-7%)	(-9%)
	Gain days 1-68 (%)	169 (66%)	159 (63%)	145 (58%)	133 (53%)
	Post-mating (at day 28)	463 ± 39	457 ± 33	439* ± 29	426* ± 26
	(% diff. from control)	-	(-1%)	(-5%)	(-8%)
Females	Pre-pairing (at day 1)	187 ± 8	187 ± 7	190 ± 8	187 ± 9
	Pre-pairing; At day 68	232 ± 14	230 ± 13	226 ± 10	203* ± 10
	(% diff. from control)	-	(-1%)	(-3%)	(-13%)
	Gain days 1-68 (%)	45 (24%)	43 (23%)	36 (19%)	16 (9%)
	Gestation period for F1 (at 21 days)	331 ± 21	324 ± 17	320 ± 17	270* ± 22
	(% diff. from control)	-	(-2%)	(-3%)	(-18%)
	Lactation period for F1 (at 21 days)	279 ± 19	272 ± 12	270 ± 12	243* ± 15
	(% diff. from control)	-	(-3%)	(-3%)	(-13%)
F1					
Males	Pre-pairing (at day 1)	102 ± 12	100 ± 17	98 ± 18	82 ± 15*
	(% diff. from control)	-	(-2%)	(-4%)	(-20%)
	Pre-pairing (at day 123)	432 ± 41	418 ± 34	406* ± 37	378* ± 34
	(% diff. from control)	-	(-3%)	(-6%)	(-13%)
	Gain days 1-123 (%)	330 (324%)	318 (318%)	308 (314%)	296 (361%)
	Post-mating (at day 22)	469 ± 50	456 ± 41	447 ± 42	418* ± 39
	(% diff. from control)	-	(-3%)	(-5%)	(-11%)

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Females	Pre-pairing (at day 1) (% diff. from control)	92 ± 10 -	91 ± 13 (-1%)	92 ± 17 (0)	75 ± 13* (-18%)
	Pre-pairing (at day 123) (% diff. from control)	249 ± 24 -	251 ± 19 (1%)	237 ± 19 (-5%)	209* ± 13 (-16%)
	Gain days 1-123 (%)	157 (171%)	160 (176%)	145 (158%)	134 (179%)
	Gestation period for F2 (at 21 days) (% diff. from control)	352 ± 29 -	352 ± 30 (0%)	343 ± 28 (-3%)	298* ± 22 (-15%)
	Lactation period for F2 (at 21 days) (% diff. from control)	286 ± 26 -	290 ± 19 (1%)	279 ± 17 (-2%)	246* ± 16 (-14%)
* statistically significant					

Table 5.6.1-2: Aclonifen - 2-generation rat study – Mean food consumption, F0 and F1 parents (g/animal/day)

	Periods	**0 ppm**	**125 ppm**	**500 ppm**	**2000 ppm**
F0					
Males	Pre-pairing (days 1-68) (% diff. from control)	25.8 -	25.1 (-3%)	24.2* (-6%)	23.8* (-8%)
	Post-mating for F1 (days 85-113)	25.2	24.8	23.6*	23.3*
Females	Pre-pairing (days 1-68) (% diff. from control)	17.0 -	17.1 (1%)	16.9 (-1%)	15.2* (-11%)
	Gestation period for F1 (days 0-21) (% diff. from control)	21.2 -	20.4 (-4%)	20.4 (-4%)	17.6* (-17%)
	Lactation period for F1 (days 1-14) (% diff. from control)	45.4 -	43.8 (-3%)	45.8 (1%)	37.0* (-19%)
F1					
Males	Pre-pairing (days 1-123) (% diff. from control)	23.0 -	22.9 (0)	22.4 (-3%)	21.7* (-6%)
	Post-mating for F2 (days 1-22)	23.4	22.7	22.6	22.1*
Females	Pre-pairing (days 1-123) (% diff. from control)	17.0 -	17.5 (3%)	16.5 (-3%)	15.0* (-12%)
	Gestation period for F2 (days 0-21) (% diff. from control)	21.5 -	21.4 (0)	20.0 (-7%)	17.9* (-17%)
	Lactation period for F2 (days 1-14) (% diff. from control)	43.2 -	42.9 (-1%)	44.6 (3%)	36.6* (-15%)
* statistically significant					

The achieved dosage based on the nominal concentrations for both generations is shown in the table below (compiled from the weekly values appended in the study report). Based on the results of the diet analyses (see dossier section CA 4.1.2/01, ████, 1984-85) the achieved concentrations averaged approximately 80% of nominal and showed acceptable homogeneity.

Table 5.6.1-3: Aclonifen - 2-Generation rat study - Mean nominal test substance intake as mg/kg/day (mean and range)

	Periods	**125 ppm**	**500 ppm**	**2000 ppm**
F0				
Males	Pre-pairing (week 1 – week 10)	9 (11 - 8)	35 (42 - 30)	144 (169 - 116)
Females	Pre-pairing (week 1 – week 10)	10 (11 - 9)	40 (40 - 37)	152 (133 - 145)
	Gestation period for breeding F1	10 (10 – 9)	40 (36 – 42)	153 (170 – 134)

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			125 ppm	500 ppm	2000 ppm
	Lactation period for breeding F1		19 (15 – 23)	83 (67 – 97)	305 (228 – 363)
F1					
Males	Pre-pairing (week 1 – week 17)		10 (19 - 7)	40 (80 - 27)	169 (323 - 115)
Females	Pre-pairing (week 1 – week 17)		11 (20 - 9)	44 (82 - 33)	182 (340 - 133)
	Gestation period for breeding F2		10 (10 – 9)	37 (34 – 38)	150 (146 – 155)
	Lactation period for breeding F2		17 (13 – 21)	75 (58 – 92)	295 (245 – 333)
Test diet analyses (mean % of nominal concentration, and range)		**Control, 0 ppm** None detected	78% 72 – 87%	81% 71 – 95%	84% 80 – 91%

Table 5.6.1-4: Aclonifen - 2-Generation rat study - Reproductive parameters for F0 and F1 parents

Parameter	0 ppm		125 ppm		500 ppm		2000 ppm	
	F0	**F1**	**F0**	**F1**	**F0**	**F1**	**F0**	**F1**
Number of females paired	25	25	25	24	25	25	25	25
Pre-coital time (days)	3.4	4.6	3.7	3.3	2.4	3.3	3.3	2.7
Percentage mating	100	100	100	100	100	100	100	100
Fertility index	96	92	96	95.8	92	96	88	100
Gestation index	100	100	95.8	100	100	100	100	100
Number of litters born	24	23	23	23	23	24	22	25
Number of pups at birth	267	224	247	250	236	268	166*	244
Surviving litters at LD 21	24	22	22	23	21	24	18	24
Litter size at birth	11.1 ± 2.0	10.2 ± 3.4	11.2 ± 2.0	10.9 ± 2.8	11.2 ± 1.3	11.2 ± 2.0	9.2* ± 2.3	10.2 ± 1.4
Litter size pre-cull at LD 4	10.8 ± 2.3	9.8 ± 3.6	10.4 ± 2.2	10.4 ± 3.1	11.1 ± 1.3	10.8 ± 2.4	9.2 ± 2.3	9.5 ± 2.0
Pups lost LD 0-4	7	9	18	11	3	10	1	17
Litter size post-cull	7.8	7.2	7.9	7.8	8.0	7.8	7.6	7.8
Pups lost LD 4-21	0	0	2	3	0	1	0	1
Mean M/F pups/litter LD 1	5.6/5.5	5.2/5.0	5.6/5.6	5.3/5.6	5.1/6.1	5.3/5.8	4.3/4.9	5.0/5.2
* = statistically significant								

Aclonifen had no effect on the parameters of reproduction measured. At 2000 ppm the mean number of pups per F0 dam was statistically reduced, and the number of total litter losses was slightly higher than in the other groups. However, this result was considered not to be biologically significant, as the number of pups per F1 dam and the number of litter losses at 2000 ppm were similar to that of control.

No treatment related external anomalies were observed among the pups of either generation.

Table 5.6.1-5: Aclonifen - 2-Generation rat study – Litter mean bodyweight (g) of F1 and F2 pups (combined sexes) during lactation ± Standard Deviation

Lactation Day	0 ppm	125 ppm	500 ppm	2000 ppm
F1				
Day 1 (% diff. from control)	5.8 ± 0.7	5.5** ± 0.6 (-5%)	5.6** ± 0.7 (-3%)	5.1** ± 0.5 (-12%)

Lactation Day	0 ppm	125 ppm	500 ppm	2000 ppm
Day 4 (% diff. from control)	8.3 ± 1.3	8.0** ± 1.0 (-4%)	8.0* ± 1.0 (-4%)	6.9** ± 1.0 (-17%)
Day 7	13.3 ± 1.8	12.8* ± 1.8	13.0 ± 1.8	10.6** ± 1.6
Day 14	28.6 ± 3.4	28.0 ± 3.4	27.0** ± 2.7	22.4** ± 2.9
Day 21 (% diff. from control)	45.6 ± 5.6	44.4 ± 5.1	43.3** ± 4.0 (-5%)	35.7** ± 3.9 (-22%)
F2				
Day 1 (% diff. from control)	6.0 ± 0.8	5.7** ± 0.6 (-5%)	5.7** ± 0.6 (-5%)	5.6** ± 0.6 (-7%)
Day 4 (% diff. from control)	9.0 ± 1.5	8.5** ± 1.2 (-6%)	8.5** ± 1.0 (-6%)	8.0** ± 0.9 (-11%)
Day 7	14.5 ± 2.1	13.8** ± 1.9	13.7** ± 1.5	12.1** ± 1.4
Day 14	30.1 ± 3.4	29.3* ± 3.4	29.0** ± 3.0	24.0** ± 2.8
Day 21 (% diff. from control)	48.4 ± 5.4	48.2 ± 4.9	47.0* ± 5.0 (-3%)	38.9** ± 4.6 (-20%)
Statistical significance: * p<0.05, ** p<0.01 (ANOVA and Dunnett's test; analyses as reported in CA 5.6.1/02, see also following summary)				

There was a significant reduction in pup weight at birth in both generations at 2000 ppm, 7-12% deficit from control, which increased to approximately -20% by the time of weaning. At 500 and 125 ppm there were very small differences from control at birth (approximately -5%) that were recovered by mid-late lactation and were considered neither adverse nor biologically significant.

There were no treatment related pathological findings in the parents of either generation.

Table 5.6.1-6: Aclonifen – 2-Generation rat study – Incidences of microscopic pathological findings in F0 and F1 parents

Finding[a] / Sex, number	F0 parents 0 ppm M 25	F0 parents 0 ppm F 25	F0 parents 2000 ppm M 25	F0 parents 2000 ppm F 25	F1 parents 0 ppm M 25	F1 parents 0 ppm F 25	F1 parents 2000 ppm M 25	F1 parents 2000 ppm F 25
Testes								
Tubular atrophy (minimal)	10		8		0		1	
Epididymides								
Azoospermia	0		0		0		1	
Seminal vesicles								
Alveolar distension	0		1		0		0	
Prostate								
Inflammation	2		4		1		3	
Uterus								
Dilation		3		1		8		1
Stromal polyp		1		0		0		0

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Finding[a] / Sex, number	F0 parents				F1 parents			
	0 ppm		2000 ppm		0 ppm		2000 ppm	
	M 25	F 25	M 25	F 25	M 25	F 25	M 25	F 25
Pituitary								
Cyst(s)	4	2	5	3	0	1	1	0
Kidneys								
Mineralisation					1	17	2	20
Tubular atrophy					1	0	11	1
Tubular dilation					4	0	3	1
Lymphoid infiltration					7	2	9	3
Pyelitis					2	1	1	0
Pelvic dilation					4	2	2	0

a Only organs with findings are listed

III. CONCLUSION

The NOAEL for parental rats in this study was 125 ppm aclonifen in the diet (equivalent to approximately 9 mg/kg bw/day average pre-mating exposure), based on reduced body weight and food consumption at ≥500 ppm.

The NOAEL for offspring was 500 ppm aclonifen in the diet (equivalent to approximately 35 mg/kg bw/day average parental pre-mating exposure), based on persisting reduction in body weight at 2000 ppm.

The NOAEL for fertility and reproductive performance in the study was 2000 ppm aclonifen in the diet, equivalent to approximately 140 mg/kg bw/day average pre-mating parental exposure.

Assessment and conclusion by applicant:

A valid and acceptable study that shows no concern for any effects on reproduction, with NOAELs based on evidence of general, systemic toxicity only.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.6.1/02
Report Author:	[redacted]
Report Year:	1997
Report Title:	Aclonifen - Two-generation reproduction toxicity study with CME - 127 in the rat Project 030644, Document No. 127AD-453-001 (R.C.C) - Statistical analysis report
Report No:	R007436
Document No:	M-174922-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current guideline: OECD 416, 2001 Minor deviations, No sperm analysis or related detailed histopathological examination of spermatogenesis or the ovary. No pre-mating monitoring of estrous cyclicity. No monitoring of anogenital distance or pubertal development. No retention and examination of organs from weanlings. No staining for implantation sites, study integrity not affected
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

Offspring body weights during lactation were analyzed statistically for the F1 and F2 litters in the two-generation study in rats with CME 127 (aclonifen). This separate report was added later, to expand the analyses for offspring body weight already shown in the two-generation study report.

The offspring body weight data were entered on a computer system by double-entry, to verify accuracy of transcription. They were analyzed by one-way analysis of variance (ANOVA), and if this was significant, the treated group means were compared with control using Dunnett's test, with alpha-levels of 0.05 and 0.01. The analyses were conducted using SAS programs.

Significantly reduced body weights were noted in the high dose group (2000 ppm) for both sexes during the entire lactation period, days 1-21 post partum, for both the F1 and F2 generations.

At 500 ppm there was significantly reduced pup weight during the whole lactation period for males of the F1 and F2 generations, while lesser significance for females occurred on days 1 and 4 post partum in the F2 generation only.

At the lowest dose (125 ppm), the effect in F1 male pups showed only a lesser significance ($p = 0.05$), then there was no significance by day 21 for the F2 males, showing that the early slight difference from control was resolved by late lactation and may be considered non-adverse overall. Meanwhile the female pups showed no significance for either the F1 or the F2 generations.

Table 5.6.1-7: Aclonifen - 2-Generation rat study – Litter mean bodyweight (g) of F1 and F2 male pups during lactation ± Standard Deviation

Lactation Day	0 ppm	125 ppm	500 ppm	2000 ppm
F1 males				
Day 1 (% diff. from control)	5.9 ± 0.8	5.7* ± 0.6 (-3%)	5.7** ± 0.7 (-3%)	5.2** ± 0.6 (-12%)
Day 4 (% diff. from control)	8.5 ± 1.3	8.1* ± 1.0 (-5%)	8.1* ± 0.9 (-5%)	7.0** ± 0.9 (-18%)

Lactation Day	0 ppm	125 ppm	500 ppm	2000 ppm
Day 7 (% diff. from control)	13.8 ± 1.8	13.1* ± 1.8 (-5%)	13.1* ± 1.7 (-5%)	10.6** ± 1.6 (-23%)
Day 14 (% diff. from control)	29.6 ± 3.1	28.6 ± 3.1 (-3%)	27.3** ± 2.6 (-8%)	22.3** ± 3.0 (-25%)
Day 21 (% diff. from control)	47.2 ± 5.5	45.1* ± 5.1 (-4%)	43.7** ± 3.9 (-5%)	36.0** ± 4.2 (-22%)
F2 males				
Day 1 (% diff. from control)	6.1 ± 0.9	5.8** ± 0.6 (-5%)	5.9** ± 0.5 (-3%)	5.7** ± 0.6 (-7%)
Day 4 (% diff. from control)	9.2 ± 1.6	8.6** ± 1.1 (-7%)	8.6** ± 1.0 (-7%)	8.1** ± 0.8 (-12%)
Day 7 (% diff. from control)	15.0 ± 2.0	13.9** ± 1.8 (-7%)	13.8** ± 1.5 (-8%)	12.2** ± 1.3 (-19%)
Day 14 (% diff. from control)	31.0 ± 3.1	29.4* ± 3.7 (-5%)	29.3** ± 2.9 (-5%)	24.2** ± 2.6 (-22%)
Day 21 (% diff. from control)	50.0 ± 5.1	48.9 ± 4.6 (-2%)	47.7* ± 5.3 (-5%)	39.0** ± 4.6 (-22%)
Statistical significance: * p<0.05, ** p<0.01 (ANOVA and Dunnett's test)				

Table 5.6.1-8: Aclonifen - 2-Generation rat study – Litter mean bodyweight (g) of F1 and F2 female pups during lactation ± Standard Deviation

Lactation Day	0 ppm	125 ppm	500 ppm	2000 ppm
F1 females				
Day 1 (% diff. from control)	5.6 ± 0.7	5.4 ± 0.7 (-4%)	5.5 ± 0.6 (-2%)	5.0** ± 0.5 (-11%)
Day 4 (% diff. from control)	8.0 ± 1.1	7.8 ± 0.9 (-3%)	7.9 ± 1.1 (-1%)	6.9** ± 1.0 (-14%)
Day 7 (% diff. from control)	12.9 ± 1.7	12.5 ± 1.8 (-3%)	12.8 ± 1.9 (-1%)	10.7** ± 1.6 (-17%)
Day 14 (% diff. from control)	27.0 ± 3.4	27.4 ± 3.5 (-1%)	26.7 ± 2.8 (-4%)	22.4** ± 2.9 (-19%)
Day 21 (% diff. from control)	44.0 ± 5.4	43.7 ± 5.1 (-1%)	42.8 ± 4.1 (-3%)	35.4** ± 3.7 (-20%)

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Lactation Day	0 ppm	125 ppm	500 ppm	2000 ppm
F2 females				
Day 1 (% diff. from control)	5.8 ± 0.7	5.7 ± 0.6 (-2%)	5.6* ± 0.6 (-3%)	5.6* ± 0.6 (-3%)
Day 4 (% diff. from control)	8.8 ± 1.4	8.5 ± 1.3 (-3%)	8.4* ± 1.1 (-5%)	8.0** ± 0.9 (-9%)
Day 7 (% diff. from control)	14.0 ± 2.1	13.7 ± 1.9 (-2%)	13.6 ± 1.5 (-3%)	11.9** ± 1.5 (-15%)
Day 14 (% diff. from control)	29.3 ± 3.5	29.2 ± 3.2 (0)	28.8 ± 3.0 (-2%)	23.9** ± 3.0 (-18%)
Day 21 (% diff. from control)	46.7 ± 5.2	47.6 ± 5.1 (2%)	46.3 ± 4.6 (-1%)	38.7** ± 4.6 (-17%)
Statistical significance: * p<0.05, ** p<0.01 (ANOVA and Dunnett's test)				

Table 5.6.1-9: Aclonifen - 2-Generation rat study – Litter mean bodyweight (g) of F1 and F2 pups (combined sexes) during lactation ± Standard Deviation

Lactation Day	0 ppm	125 ppm	500 ppm	2000 ppm
F1 males+females				
Day 1 (% diff. from control)	5.8 ± 0.7	5.5** ± 0.6 (-5%)	5.6** ± 0.7 (-3%)	5.1** ± 0.5 (-12%)
Day 4 (% diff. from control)	8.3 ± 1.3	8.0** ± 1.0 (-4%)	8.0** ± 1.0 (-4%)	6.9** ± 1.0 (-17%)
Day 7	13.3 ± 1.8	12.8* ± 1.8	13.0 ± 1.8	10.6** ± 1.6
Day 14	28.6 ± 3.4	28.0 ± 3.4	27.0** ± 2.7	22.4** ± 2.9
Day 21 (% diff. from control)	45.6 ± 5.6	44.4 ± 5.1	43.3** ± 4.0 (-5%)	35.7** ± 3.9 (-22%)
F2 males+females				
Day 1 (% diff. from control)	6.0 ± 0.8	5.7** ± 0.6 (-5%)	5.7** ± 0.6 (-5%)	5.6** ± 0.6 (-7%)
Day 4 (% diff. from control)	9.0 ± 1.5	8.5** ± 1.2 (-6%)	8.5** ± 1.0 (-6%)	8.0** ± 0.9 (-11%)
Day 7	14.5 ± 2.1	13.8** ± 1.9	13.7** ± 1.5	12.1** ± 1.4
Day 14	30.1 ± 3.4	29.3* ± 3.4	29.0** ± 3.0	24.0** ± 2.8
Day 21 (% diff. from control)	48.4 ± 5.4	48.2 ± 4.9	47.0* ± 5.0 (-3%)	38.9** ± 4.6 (-20%)
Statistical significance: * p<0.05, ** p<0.01 (ANOVA and Dunnett's test)				

<u>**Assessment and conclusion by applicant:**</u>

Valid and acceptable results, confirming the outcomes for offspring body weight development given in the two-generation study report.

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Assessment and conclusion by RMS:

CA 5.6.2 Developmental toxicity studies

Data Point:	KCA 5.6.2/01
Report Author:	■■■; ■■■; ■■■
Report Year:	1982
Report Title:	KUB 3359 - Teratogenicity study in rats (Trial No. B 81)
Report No:	R007400
Document No:	M-174846-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current guideline: OECD 414, 2018 Minor deviations. No additional endocrine disrupter endpoints. Food consumption not recorded. No analysis of dosing formulations, however overall study integrity not affected.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

KUB 3359 (aclonifen, purity not stated) was administered orally by gavage to groups of 25 mated female Wistar rats (25/group) at dosages of 0, 6, 60 or 600 mg/kg bw/day, once daily on days 7 to 21 of pregnancy (day 1 = day of confirmed positive mating). The vehicle was saline with 0.5% Tween 80. Each daily dose was determined by individual body weight on day 1 of dosing.

The rats were monitored daily for clinical signs of toxicity, and body weight was recorded on days 7-21 of pregnancy. They were killed on day 22 of pregnancy and subjected to a gross necropsy. Corpora lutea were counted and the gravid uterus weighed. The numbers of implantations, live or dead foetuses, early and late resorptions, and any externally visible malformations were recorded. Two-thirds of the viable foetuses from each pregnancy were examined for skeletal abnormalities by Alizarin staining, the remaining one-third being examined for visceral abnormalities by the serial slicing method of Wilson, following fixation in Bouin's solution. Foetuses were sexed by estimation of anogenital distance. Appropriate statistical analyses were applied as necessary.

There was no maternal mortality or any clinical signs of toxicity at any dosage. There was reduced maternal body weight gain at 600 mg/kg bw/day (10% reduced weight at termination, compared with control). There were no pathological findings in the dams at terminal necropsy.

There was no treatment effect on pre- or post-implantation loss, or on the incidence of foetal abnormalities or variants, but there was a reduction of mean foetal weight at 600 mg/kg bw/day (8.3% less than control). It was considered that this reduction in foetal weight was related to the reduced

dam body weight that resulted from general systemic toxicity, and was therefore a secondary effect of treatment.

It may be concluded that aclonifen was not teratogenic in rats, and that the NOAEL for maternal and embryotoxicity was 60 mg/kg bw/day.

I. MATERIALS AND METHODS

Study dates	Study start:	September 1981
	Completion:	02 April 1982

A. MATERIALS

1.	**Test Item**	KUB 3359 (aclonifen)
	Batch No.:	T5/81
	Purity:	Not stated
	Expiry:	Not stated
2.	**Animals**	Female Wistar rats, Chbb:THOM strain, from [redacted] [redacted] Approximately 11 weeks old at delivery, approximately 270 g at start of treatment. Mated with proven males of the same strain.
	Diet:	FUKO 8013 rat and mouse maintenance diet ([redacted] [redacted] and tap water *ad libitum*.

B. STUDY DESIGN AND METHODS

The dosages were selected on the basis of an earlier subacute feeding study in which reduced body weight gain and indications of renal damage were observed at 5000 ppm *(very approximately equivalent to 500 mg/kg bw/day)*.

Dosing suspensions of aclonifen in the vehicle were prepared each 3rd to 5th day of treatment. Analyses of the formulations was not conducted.

The females were mated overnight with proven males, at a ratio of 2 females to each male. The day of detection of a vaginal plug and/or sperm in a vaginal smear was considered day 1 of pregnancy. Groups of 25 positively mated females were administered dosing formulation of aclonifen at 6, 60 or 600 mg/kg bw/day once daily by oral gavage on days 7-21 inclusive of pregnancy. A vehicle control group received saline with Tween 80, and a further control group received saline only. The dose administered on each day was determined throughout by individual body weight on the first day of treatment, day 7 of pregnancy.

The females were monitored daily for physical condition, mortality and behaviour. Body weight was determined daily for days 7-22 of pregnancy. Food consumption was not measured.

The females were killed on day 22 of pregnancy and subjected to a gross necropsy. Corpora lutea were counted in each ovary and the gravid uterus was weighed. The numbers of implantations, live or dead foetuses, early and late resorptions, and any externally visible malformations were recorded. Two-thirds of the viable foetuses from each pregnancy (randomly selected) were examined for skeletal abnormalities by Alizarin staining, the remaining one-third being examined for visceral abnormalities by the serial slicing method of Wilson, following fixation in Bouin's solution. Foetuses were sexed by estimation of anogenital distance.

Statistical analysis was by parametric analysis of variance, followed by comparison with control using Dunnett's test. Percentages/ratios were arcsine transformed prior to analysis.

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II. RESULTS AND DISCUSSION

There was no maternal mortality and no clinical signs of toxicity were observed at any dosage.

Maternal body weight gain was significantly reduced at 600 mg/kg bw/day, resulting in a deficit of 10% in terminal body weight, as compared with control. There was no effect on body weight at the lower dosages.

Table 5.6.2-1: Aclonifen – Embryotoxicity in rats – Mean maternal body weight (g) ± Standard Deviation

Day of pregnancy	Dose (mg/kg bw/day)				
	0 (Saline)	0 (Saline+Tw80)	6	60	600
n	23	22	22	21	22
1	236 ± 11.5	241 ± 13.5	246 ± 18.6	243 ± 15.9	240 ± 16.7
7	261 ± 10.6	268 ± 14.2	271 ± 17.3	267 ± 14.3	265 ± 19.1
11	279 ± 12.4	287 ± 15.8	290 ± 19.4	285 ± 17.6	264 ± 17.7*
16	310 ± 13.3	319 ± 16.4	323 ± 21.7	316 ± 13.4	291 ± 16.6**
22	385 ± 24.5	400 ± 26.7	403 ± 39.9	398 ± 13.4	359 ± 28.3*
Gain corr.[a]	63 ± 10.8	62 ± 8.6	67 ± 8.9	53 ± 9.8	35 ± 13.0*

Statistical significance of difference from control: * p≤0.05, ** p≤0.01
a Body weight gain days 1-22 after subtraction of gravid uterine weight

There were no pathological findings in the dams at terminal necropsy.

There was no treatment effect on pre- or post-implantation loss, or on the incidence of foetal abnormalities or variants, but there was a reduction of mean foetal weight at 600 mg/kg bw/day (8.3% less than control). It was considered that this reduction in foetal weight was related to the reduced dam body weight that resulted from general systemic toxicity, and was therefore a secondary effect of treatment.

Table 5.6.2-2: Aclonifen – Embryotoxicity in rats – Pregnancy parameters (mean ± SD) and foetal findings

Parameter/Finding	Dose (mg/kg bw/day)				
	0 (Saline)	0 (Saline +Tw80)	6	60	600
Number of pregnancies	23	22	22	21	22
Corpora lutea	14.3 ± 1.4	15.1 ± 1.8	14.8 ± 2.7	15.3 ± 1.8	14.4 ± 1.9
Implantations	12.2 ± 3.5	14.0 ± 2.2	12.9 ± 4.4	14.9 ± 1.5*	13.4 ± 2.8
Live foetuses	11.7 ± 3.5	13.2 ± 2.7	12.0 ± 4.4	14.1 ± 1.6	12.1 ± 4.0
% male foetuses	50	50	49	53	54
Dead foetuses	0	0	0	0	0
Resorptions	0.6 ± 0.9	0.9 ± 1.2	0.8 ± 0.8	0.7 ± 0.8	1.3 ± 2.2
Number of resorptions (total)	13	19	18	15	28

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Parameter/Finding	Dose (mg/kg bw/day)				
Early resorptions (total number)	6	8	6	4	14
Late resorptions (total number)	7	11	12	11	14
Pre-implantation loss (%)	7.0 ± 12.0	2.2 ± 5.6	9.1 ± 11.1	0.6 ± 2.2	2.9 ± 5.2
Post-implantation loss (%)	1.6 ± 3.5	3.1 ± 3.9	4.4 ± 3.6	2.4 ± 2.6	4.7 ± 9.2
Background data (20 studies): Post-implantation loss (%) 95% confidence interval		3.8 0 – 9.3			
Placental weight (g)	0.44 ± 0.09	0.43 ± 0.05	0.48 ± 0.11	0.42 ± 0.03	0.45 ± 0.07
Foetal weight (g)	5.40 ± 0.22	5.35 ± 0.19	5.42 ± 0.35	5.30 ± 0.23	4.95 ± 0.34**
Malformation incidence:					
Multiple skeletal (vertebrae, ribs)	1	0	0	0	1
Brachygnathia	0	0	1	0	0
Internal hydrocephalus	0	0	0	0	1
Vertebral cleft, 12th thoracic	0	0	0	0	1
Variant incidence:					
Dilated renal pelvis	2	1	0	0	1
14th rib	1	0	0	0	0
Cleft, asymmetric sternebrae	0	0	1	0	2
Background control data (1399 foetuses, Sep 73 to Jan 77):					
Hydrocephalus		3			
Brachygnathia		1			
Vertebral cleft		18			
Vertebral + rib malformations		4			
Cleft/asymmetric sternebrae		NA			

Statistical significance of difference from control: * p≤0.05, ** p≤0.01
NA = Not available

III. CONCLUSION

It may be concluded that aclonifen was not embryotoxic or teratogenic in rats, and that the NOAEL for maternal and embryotoxicity was 60 mg/kg bw/day.

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Assessment and conclusion by applicant:

A valid and acceptable study that shows no concern for any selective embryofoetal toxicity of aclonifen in rats, the small reduction in foetal weight at 600 mg/kg bw/day being attributed as secondary to maternal toxicity. The NOAEL of 60 mg/kg bw/day for both maternal and embryotoxicity is supported.

Assessment and conclusion by RMS:

Data Point:	KCA 5.6.2/02
Report Author:	[redacted]
Report Year:	1984
Report Title:	Embryotoxicity (including teratogenicity) study with CME - 127 in the rabbit
Report No:	R007403
Document No:	M-174853-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current guideline: OECD 414, 2018 Minor deviations. Dosing during major organogenetic period only (days 6-18 of gestation), instead of continuing until day prior to expected delivery (sacrifice). No analysis of dosing formulations, however overall study integrity not affected.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Executive summary

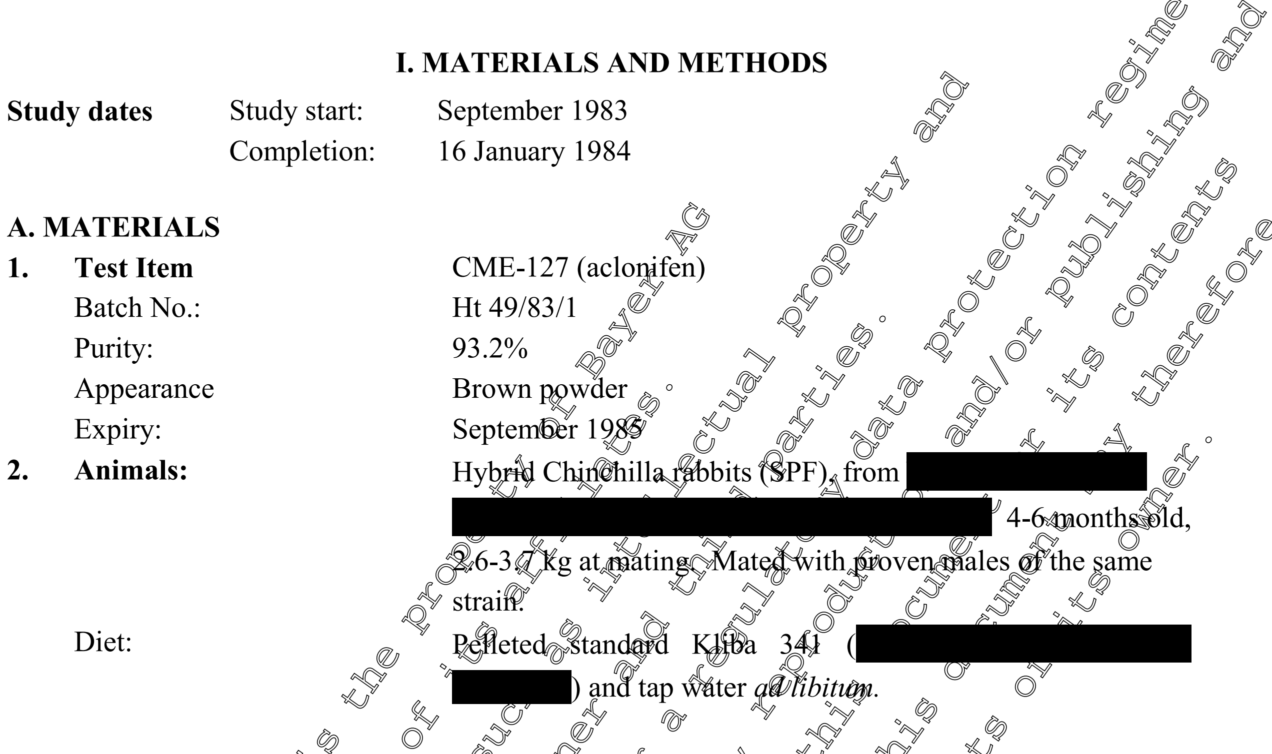
CME-127 (aclonifen, 93.2% purity) was administered by oral gavage once daily to groups of 16 mated female Chinchilla rabbits on days 6-18 inclusive of gestation (day 0 = day of mating), at dosages of 0 (vehicle control), 1, 5 or 25 mg/kg bw/day, formulated as a suspension in the vehicle, aqueous 2% carboxymethylcellulose. The dose was determined by individual body weight on each day of dosing.

Behaviour, general physical condition and body weight were assessed daily, food consumption over periods between days 6, 11, 15, 19, 24 and 28 of pregnancy. The females were killed and necropsied on day 29 of pregnancy, the uterus was opened and living or dead foetuses, embryonic resorptions, foetal intrauterine deaths, and corpora lutea in the ovaries were counted. All foetuses were examined for external abnormalities and the weights of the live foetuses were recorded. The foetuses were then killed and examined for visceral abnormalities, the heads being fixed separately and examined by serial slicing. The eviscerated carcasses, without heads, were then stained and examined for skeletal abnormalities. Foetal sex was determined at dissection.

There was no treatment related maternal mortality and no clinical signs of toxicity. There was no effect on maternal food consumption or body weight gain. There was no evidence of any embryotoxic/teratogenic effect in the study.

It was concluded that under the conditions of the study, aclonifen had no embryotoxic or teratogenic potential in the rabbit. The NOAEL was ≥25 mg/kg bw/day.

I. MATERIALS AND METHODS



Study dates	Study start:	September 1983
	Completion:	16 January 1984

A. MATERIALS

1.	**Test Item**	CME-127 (aclonifen)
	Batch No.:	Ht 49/83/1
	Purity:	93.2%
	Appearance	Brown powder
	Expiry:	September 1985
2.	**Animals:**	Hybrid Chinchilla rabbits (SPF), from [redacted] [redacted] 4-6 months old, 2.6-3.7 kg at mating. Mated with proven males of the same strain.
	Diet:	Pelleted standard Kliba 341 ([redacted] [redacted]) and tap water *ad libitum*.

B. STUDY DESIGN AND METHODS

Each female was mated with a proven male of the same strain, the day of mating being designated day 0 of gestation (GD 0). Four groups of 16 mated females, randomly allocated, then received aclonifen as a once-daily oral gavage dose at 0 (vehicle control), 1, 5 or 25 mg/kg bw/day at a dose volume of 4 ml/kg bw, on GD 6-18 inclusive, each dose being determined by the individual's body weight at the time of administration.

Suspensions of aclonifen in the vehicle, aqueous 2% carboxymethylcellulose, were prepared fresh daily using a homogeniser. Stability in this vehicle was stated as having been demonstrated by the study sponsor. Formulation analyses were not performed in the present study.

The rabbits were monitored twice daily for mortality and any clinical signs of toxicity. Body weight was recorded daily, food consumption over the periods GD 0-6, 6-11, 11-15, 15-19, 19-24 and 24-28.

The females were killed on GD 29, the uterus was opened and living or dead foetuses, embryonic resorptions, foetal intrauterine deaths, and corpora lutea in the ovaries were counted. All foetuses were examined for external abnormalities and the weights of the live foetuses were recorded. The foetuses were then killed and examined for visceral abnormalities, the heads being fixed separately and examined by serial slicing. The eviscerated carcasses, without heads, were then stained and examined for skeletal abnormalities. Foetal sex was determined at dissection.

Statistics were confined to calculation of group means and standard deviations, and use of a 2x2 Chi^2 test for foetal sex ratio.

II. RESULTS AND DISCUSSION

There was no treatment related maternal mortality and no clinical signs of toxicity. One high dose female died incidentally on GD 25 as a result of respiratory tract infection.

Maternal body weight performance and food consumption was unaffected by treatment.

Table 5.6.2-3: Aclonifen – Embryotoxicity in rabbits – Mean maternal body weight (g) ± Standard Deviation

Day of gestation	Dose (mg/kg bw/day)			
	0	1	5	25
n	16	14	16	15
0	3326 ± 208	3001 ± 147	2966 ± 231	3152 ± 267
6	3517 ± 197	3187 ± 173	3147 ± 225	3286 ± 280
9	3532 ± 205	3207 ± 168	3172 ± 228	3318 ± 283
19	3630 ± 216	3350 ± 193	3277 ± 219	3430 ± 307
28	3758 ± 249	3484 ± 222	3433 ± 210	3552 ± 291
Gain 6-19	113	163	130	144
Gain 6-28	241	297	286	266
Gain corr.[a]	-4.8 ± 2.9	-3.3 ± 4.0	-3.2 ± 3.6	-4.7 ± 2.7

a Body weight gain days 6-28 after subtraction of gravid uterine weight

Table 5.6.2-4: Aclonifen – Embryotoxicity in rabbits – Mean daily maternal food consumption (g)

Days of gestation	Dose (mg/kg bw/day)			
	0	1	5	25
n	16	14	16	15
0-6	200	194	194	196
6-11	190	193	181	196
6-19	181	181	182	183
19-28	144	144	165	139

There was no treatment effect on the numbers of implantations, pre- or post-implantation loss, live foetuses, or on foetal sex ratio. The statistically significant difference for the low dose sex ratio was incidental, related to the low value of 44% male for control (historical control value 52%). There was no meaningful effect on foetal weight, the -5% difference from control at the high dose being well within normally expected variation.

Table 5.6.2-5: Aclonifen – Embryotoxicity in rabbits – Pregnancy parameters (mean ± SD)

Parameter/Finding	Dose (mg/kg bw/day)			
	0	1	5	25
Number of pregnancies	16	14	16	15
Corpora lutea	10.3 ± 3.2	9.1 ± 1.6	8.7 ± 1.7	9.8 ± 1.8
Implantations	8.3 ± 3.6	7.3 ± 2.2	7.5 ± 2.0	8.7 ± 2.1
Live foetuses	7.3 ± 2.7	7.3 ± 2.2	7.0 ± 1.6	7.9 ± 2.5
% male foetuses	44	60*	53	56

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Parameter/Finding	Dose (mg/kg bw/day)			
Dead foetuses	0	0	0	0
Resorptions	1.0	0	0.5	0.7
Number of resorptions (total)	16	0	8	11
Early resorptions (total number)	4	0	1	5
Foetal resorptions (total number)	12	0	7	6
Pre-implantation loss (%)	20.0	20.3	13.7	11.6
Post-implantation loss (%)	12.1	0	6.7	8.5
Foetal weight (g) (% difference from control)	35.1 ± 4.5	35.5 ± 5.3	35.1 ± 4.6	33.2 ± 5.2 (-5%)
Statistical significance of difference from control: * $p \leq 0.05$ (Chi^2 test)				

There was no treatment effect on the incidence of foetal abnormalities or variants.

Table 5.6.2-6: Aclonifen – Embryotoxicity in rabbits – Foetal findings

Finding		Dose (mg/kg bw/day)			
		0	**1**	**5**	**25**
Malformation incidence:					
Kyphosis, most lumbar vertebrae unossified, rudimentary tail, rib fusions, agenesis left kidney+ureter, hydronephrosis right kidney		0	1	0	0
Agenesis left kidney+ureter		1	0	0	0
Rib fusions		0	1	1	0
Variant incidence:					
Sternebra 5 or 6 split		0	1	1	1
Sternebrae 4+5 fused		0	0	0	1
Ossification status indicators:					
Number of foetuses		116	102	112	119
Forelimbs:					
Proximal phalanx 1 unossified:	L	10	8	13	14
	R	10	9	12	13
Medial phalanx 5 unossified:	L	88	80	84	78
	R	84	76	82	75
Hindlimbs:					
Medial phalanx 4 unossified:	L	24	21	37	26
	R	24	19	30	22
Sternebrae:					
Sternebra 5 unossified		15	20	34	30
Sternebra 5 incomplete ossified		62	45	54	61

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Finding		Dose (mg/kg bw/day)			
		0	1	5	25
Ribs:					
Rib 13 unossified:	L	81	67	86	73
	R	87	73	90	75

III. CONCLUSION

It was concluded that under the conditions of the study, aclonifen was not embryotoxic or teratogenic in rabbits, and that the NOAEL for maternal and embryotoxicity was 25 mg/kg bw/day.

Assessment and conclusion by applicant:

A valid and acceptable study that shows no concern for any embryofoetal toxicity of aclonifen in rabbits at up to 25 mg/kg bw/day. There was no evidence of maternal toxicity at this, the highest dose. The NOAEL for both maternal and embryotoxicity from this study was 25 mg/kg bw/day.

Assessment and conclusion by RMS:

Data Point:	KCA 5.6.2/03
Report Author:	[redacted]
Report Year:	2003
Report Title:	Range-finding study for developmental toxicity in the rabbit by gavage Aclonifen
Report No:	C033372
Document No:	M-233054-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable (non-guideline range finder) although design reflects requirements of Current guideline: OECD 414 (pre-2018)
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

Aclonifen (99.2% purity) was administered to groups of 8 mated female New Zealand White rabbits by oral gavage at dosages of 0 (vehicle control), 5, 15, 30, 75 or 150 mg/kg bw/day, once daily on gestation days (GD) 6-28 (day of mating = GD 0). The vehicle was aqueous 0.5% methylcellulose 400 and the dosage volume was 4 ml/kg bw based on the most recent individual body weight.

Clinical observations were recorded daily from GD 2, body weights every 2 or 3 days, food consumption over 1- or 2-day periods from GD 4. At sacrifice on GD 29 the dams were necropsied,

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the number of ribs, gravid uterine weight, liver and kidney weights being recorded. Live foetuses were removed, counted and weighed, then killed and examined externally.

At 150 mg/kg bw/day, intensely yellow-coloured urine or dark urine was observed in 6/8 dams, mainly during the second half of gestation. Food consumption in this group was reduced between GD 14 and 18, and there was an accompanying slight effect on mean body weight (plateauing of weight gain) between GD 16 and 18. No litter parameters were affected at this dosage.

At 75 and 30 mg/kg bw/day, intensely yellow-coloured or dark urine was again observed during the second half of gestation, in 2/8 animals at each of these dosages. Litter parameters were again unaffected.

There were no observed treatment effects at 15 or 5 mg/kg bw/day.

A small number of external foetal abnormalities were noted among treated animals: Two foetuses in one litter at 150 mg/kg bw/day with median body wall defect (thoraco/gastroschisis); one of these also had anencephaly, cleft lip, anophthalmia and microtia. One foetus at 15 mg/kg bw/day had gastroschisis and spina bifida. One foetus at 5 mg/kg bw/day had an encephalocele and open eyes. Malrotated/flexed paws or limbs were also observed occasionally in these same foetuses. In the absence of a dose response, all these occurrences were considered unlikely to be a result of treatment.

I. MATERIALS AND METHODS

Study dates:	Study start:	22 October 2002
	Completion:	20 February 2003

A. MATERIALS

1.	**Test Item**	Aclonifen
	Batch No.:	97013/03
	Purity:	99.2%
	Appearance	Fine yellow powder
	Expiry:	Re-analysed 20 September 2002 and 16 January 2003
2.	**Animals**	Female New Zealand White rabbits, [redacted] [redacted] 17-19 weeks old at mating. Mated with proven males of the same strain, by the supplier. Received on GD 1 or 2.
	Diet:	Certified Rabbit Pellet diet 110 C-10 (UAR, France) and tap water *ad libitum*.

B. STUDY DESIGN AND METHODS

The timed-mated female NZW rabbits were allocated to groups of 8 using a bodyweight dependent procedure to ensure similar group mean body weight at the start.

Dosing formulations (suspensions) of aclonifen in the vehicle (aqueous 0.5% methylcellulose 400) were prepared on two occasions for the study and stored at approximately 4°C. Prior to study start, homogeneity of suspensions was evaluated for the first formulations at the highest and lowest concentrations, while the achieved concentration of all formulations was evaluated. Stability of the compound in suspension at 0.25 and 37.5 g/L (the highest concentration used in the study) over a 30-day period was determined in a pre-study. The formulations were continuously mixed with a magnetic stirrer prior to and during each dosing session.

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The dosing formulations were administered to groups of 8 mated females by oral gavage, once daily on GD 6-28 inclusive. Dosages were 0 (vehicle control), 5, 15, 30, 75 or 150 mg/kg bw/day, at a dose volume of 4 mL/kg bw determined by the most recent individual body weight.

Individual clinical signs were monitored daily from GD 2 until termination on GD 29. Body weight was recorded on GD 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29, food consumption from GD 3, on GD 4, 5, 6, then at the same intervals as for body weight.

The rabbits were killed on GD 29 and necropsied, with recording of the number of ribs and weights of the liver and kidneys. The gravid uterus was removed and weighed intact, then the following parameters were recorded: Numbers of corpora lutea, implantations, resorptions (early and late), live and dead foetuses, live foetal weight. Any uterus showing no implantations was stained with ammonium sulfide to reveal any non-apparent implantations. The maternal tissues and carcass were then discarded.

The live foetuses were killed and subjected to external examination for developmental abnormalities and variants (malformations, minor anomalies, or common variants).

Appropriate statistical analysis (analysis of variance and Dunnett's test) was applied for body weight change, food consumption and maternal liver and kidney weights. Equivalent non-parametric methods were applied if necessary.

II. RESULTS AND DISCUSSION

The results of homogeneity and concentration checks were within the range of 88-104% of nominal values. All values were within the target range of 90-110% of the nominal concentrations, apart from one homogeneity result at both 3.75 and 18.75 g/L, which were 89 and 88%, respectively, of the nominal concentration. These minor deviations were considered acceptable. Stability of aclonifen at concentrations of 0.25 and 37.50 g/L over a 30-day period was between 97 and 110% of the nominal concentrations.

There was no mortality or abortions of pregnancy in the study. In terms of clinical signs, intensely yellow-coloured or dark urine was observed in 6/8 dams at 150 mg/kg bw/day, and in 2/8 dams at both 75 and 30 mg/kg bw/day, mainly during the second half of gestation. There were no observed effects at 15 and 5 mg/kg bw/day.

At 150 mg/kg bw/day there was reduced food consumption for the period approximately GD 14-18, as compared with control. An accompanying slight effect on mean body weight (plateauing of weight gain) was observed for the period GD 16-18. Mean overall body weight change, corrected for gravid uterine weight, was unaffected.

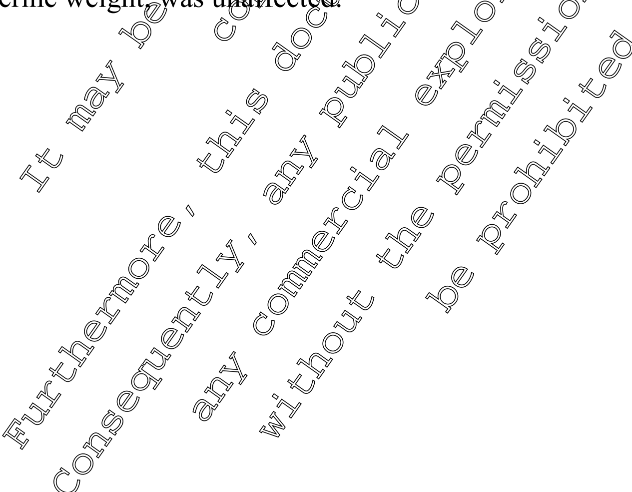

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Table 5.6.2-7: Aclonifen – Range finding embryotoxicity in rabbits – Mean maternal body weight (g) ± Standard Deviation

Day of gestation	Dose (mg/kg bw/day)					
	0	**5**	**15**	**30**	**75**	**150**
n	7	7	7	8	7	8
3	3.47 ± 0.27	3.38 ± 0.18	3.36 ± 0.14	3.54 ± 0.20	3.50 ± 0.21	3.41 ± 0.11
6	3.49 ± 0.27	3.38 ± 0.22	3.42 ± 0.13	3.54 ± 0.22	3.51 ± 0.21	3.49 ± 0.10
10	3.50 ± 0.26	3.42 ± 0.23	3.45 ± 0.11	3.57 ± 0.22	3.53 ± 0.16	3.51 ± 0.13
14	3.58 ± 0.25	3.45 ± 0.25	3.48 ± 0.11	3.61 ± 0.22	3.56 ± 0.20	3.54 ± 0.18
16	3.64 ± 0.23	3.49 ± 0.23	3.55 ± 0.14	3.65 ± 0.20	3.58 ± 0.17	3.57 ± 0.22
18	3.68 ± 0.27	3.52 ± 0.23	3.57 ± 0.16	3.66 ± 0.20	3.65 ± 0.18	3.54 ± 0.23
24	3.80 ± 0.28	3.59 ± 0.24	3.65 ± 0.17	3.76 ± 0.20	3.73 ± 0.18	3.69 ± 0.20
29	3.91 ± 0.27	3.69 ± 0.24	3.78 ± 0.23	3.87 ± 0.20	3.80 ± 0.22	3.80 ± 0.24
Gain 6-29	0.43 ± 0.17	0.31 ± 0.13	0.36 ± 0.12	0.33 ± 0.10	0.29 ± 0.17	0.31 ± 0.15
Gain corr.[a]	-0.09 ± 0.11	-0.22 ± 0.06	-0.11 ± 0.08	-0.17 ± 0.19	-0.19 ± 0.14	-0.12 ± 0.12

a Body weight gain days 6-29 after subtraction of gravid uterine weight

Table 5.6.2-8: Aclonifen – Range finding embryotoxicity in rabbits – Mean maternal daily food consumption (g) ± Standard Deviation

Days of gestation	Dose (mg/kg bw/day)					
	0	**5**	**15**	**30**	**75**	**150**
n	7	7	7	8	7	8
6 – 8	152 ± 31	162 ± 30	165 ± 7	173 ± 32	156 ± 15	170 ± 25
8 – 10	170 ± 22	175 ± 17	173 ± 15	170 ± 39	153 ± 32	166 ± 27
10 – 14	170 ± 13	138 ± 25	144 ± 29	142 ± 41	134 ± 41	144 ± 32
14 – 18	170 ± 26	140 ± 24	144 ± 27	133 ± 45	140 ± 49	117 ± 60
18 – 22	176 ± 25	144 ± 31	154 ± 25	162 ± 22	159 ± 15	146 ± 37
22 – 26	149 ± 17	114 ± 22	127 ± 11	131 ± 30	118 ± 33	137 ± 16
26 – 29	121 ± 28	109 ± 22	116 ± 12	128 ± 32	103 ± 39	127 ± 15

There were no treatment related findings in the dams at necropsy, and neither liver nor kidney weights were affected.

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Table 5.6.2-9: Aclonifen – Range finding embryotoxicity in rabbits – Mean absolute maternal organ weights (g) ± Standard Deviation

Organ	Dose (mg/kg bw/day)					
	0	**5**	**15**	**30**	**75**	**150**
n	7	7	7	8	7	8
Kidneys	15.2 ± 1.8	14.8 ± 2.0	14.6 ± 1.4	14.1 ± 2.0	14.9 ± 1.7	15.1 ± 1.2
Liver	93.0 ± 11.8	84.5 ± 9.1	85.5 ± 14.4	87.7 ± 9.9	95.7 ± 19.7	93.8 ± 13.5

The pregnancy/litter parameters, including mean litter and foetal weight, were also unaffected by treatment.

Table 5.6.2-10: Aclonifen – Range finding embryotoxicity in rabbits – Pregnancy parameters (mean ± SD)

Parameter/Finding	Dose (mg/kg bw/day)					
	0	**5**	**15**	**30**	**75**	**150**
Number of pregnancies	7	7	7	8	7	8
Corpora lutea	11.6 ± 1.4	12.0 ± 2.9	13.6 ± 2.3	12.0 ± 1.5	11.6 ± 1.7	11.8 ± 2.2
Implantations	10.6 ± 1.8	11.4 ± 2.6	12.0 ± 2.4	11.0 ± 2.3	10.1 ± 1.3	10.5 ± 1.9
Live foetuses	10.1 ± 1.7	10.3 ± 2.2	10.4 ± 1.8	10.0 ± 2.1	9.3 ± 1.1	9.4 ± 2.1
Early resorptions	0.1 ± 0.4	0.1 ± 0.4	0.7 ± 1.0	0.5 ± 0.8	0.3 ± 0.5	0.3 ± 0.5
Late resorptions	0.1 ± 0.4	0	0.1 ± 0.4	0.3 ± 0.7	0	0
Total resorptions	0.3 ± 0.5	0.1 ± 0.4	0.9 ± 0.9	0.8 ± 0.9	0.3 ± 0.5	0.3 ± 0.5
Number of live young	71	72	73	80	65	75
Litter weight (g)	367 ± 53	353 ± 67	361 ± 41	347 ± 69	331 ± 44	332 ± 71
Foetal weight (g)	36.4 ± 4.0	34.6 ± 2.8	35.0 ± 2.7	34.9 ± 3.4	35.8 ± 3.9	35.5 ± 2.8

There was a low incidence of externally visible foetal abnormalities. In the absence of a dose relationship, it was considered that these were unlikely to have been associated with treatment.

Table 5.6.2-11: Aclonifen – Range finding embryotoxicity in rabbits – Incidence of external foetal abnormalities: Number of foetuses (number of litters)

Finding	Dose (mg/kg bw/day)					
	0	**5**	**15**	**30**	**75**	**150**
Number of foetuses (litters)	71 (7)	72 (7)	73 (7)	80 (8)	65 (7)	75 (8)
Runt (bw <28 g)	6 (2)	7 (6)	13 (5)	11 (5)	5 (2)	5 (3)
Anencephaly, arrhinia, cleft lip, anophthalmia, microtia, thoracogastroschisis	0	0	0	0	0	1 (1)[a,b]
Gastroschisis	0	0	0	0	0	1 (1)[a]
Spina bifida occulta, acaudate,	0	0	1 (1)[b]	0	0	0

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Finding	Dose (mg/kg bw/day)					
gastroschisis						
Meningoencephalocele, open eyes	0	1 (1)[b]	0	0	0	0
Hyperflexion/hyperextension or malrotated limb(s)	0	1 (1)[b]	2 (2)[b]	1 (1)	0	1 (1)[b]

a Foetuses in the same litter
b Abnormalities in the same foetus

III. CONCLUSION

It was indicated that the NOAEL for maternal toxicity was 15 mg/kg bw/day, while a NOAEL for embryofetal toxicity was indicated to be 150 mg/kg bw/day.

Assessment and conclusion by applicant:

Considered a valid and acceptable supplementary study, dosed over the currently required period from implantation to the day before expected delivery, and providing limited evidence of maternal toxicity at ≥30 mg/kg bw/day. The small and transient effect on food consumption (and thereby on body weight gain) tended to coincide with the onset of the finding coloured/dark urine, but these effects did not involve all animals in the affected groups.

There was no effect on pre- or post-implantation loss, or on foetal weight. The various external foetal abnormalities that occurred in the study are not uncommon sporadic findings in rabbits, although no background data was given on their incidence in this particular strain of NZW rabbits (justifiably, for a preliminary study of this kind). The axial and ventral body wall abnormalities that occurred in one high dose litter were in a dam that did not show signs of systemic toxicity, while nothing occurred in those dams that did show such toxicity, which would support an interpretation they were incidental, not treatment related. Further, given the limited dimensions of the study and the absence of context from more detailed foetal examinations, conclusion of an association with treatment would not be appropriate.

The maternal and embryofetal NOAELs are considered appropriate within the limited context of this study. This supplementary study supports the preceding developmental toxicity study in rabbits (CA 5.6.2/02), which had a clear NOAEL for both maternal and embryofetal toxicity of 25 mg/kg bw/day.

Assessment and conclusion by RMS:

CA 5.7 Neurotoxicity studies

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CA 5.7.1 Neurotoxicity studies in rodents

Assessment and conclusion by applicant:
No neurotoxicity study has been conducted on aclonifen.

According to the Regulation EU 283/2013 section 5.7.1

'Neurotoxicity studies in rodents shall provide sufficient data to evaluate the potential neurotoxicity
of the active substance (neurobehavioural and neuropathological effects) after single and repeated exposure.

Such studies shall be performed for active substances with structures that are similar or related to those
capable of inducing neurotoxicity, and for active substances which induce specific indications of potential neurotoxicity, neurological signs or neuropathological lesions in toxicity studies at dose levels not associated with marked general toxicity. Performance of such studies shall also be considered for substances with a neurotoxic
mode of pesticidal action.'

No neurotoxicity study to be submitted. Waiver for neurotoxicity study.

Criteria triggering neurotoxicity test	Does aclonifen meet criteria?
Active substances with structures that are similar or related to those capable of inducing neurotoxicity	No. (Q)SAR on aclonifen show no alerts linking aclonifen to known neurotoxic agents
Active substances which induce specific indications of potential neurotoxicity, neurological signs or neuropathological lesions in toxicity studies	No. No indications of neurotoxicity in any studies. Astrocytomas were observed in the brains of one of the rat carcinogenicity studies but are not evidence of neurotoxicity.
Active substances with a neurotoxic mode of pesticidal action.	No. Aclonifen is a herbicide so a neurotoxic mode of action is not relevant for herbicides

In none of the short-term and subchronic studies in rats, in mice and dogs, in developmental toxicity studies (rats, rabbits), or in the reproductive study (rats) were there any clinical signs suggesting neurological dysfunction or neuropathological lesions either in adults or young animals.

There were no indications of neurotoxicity in the acute oral study in rats up to 2000 mg/kg bw/day.

The most recent rat subchronic (90-day) dietary toxicity study was carried out to O.E.C.D. guideline 408 (September, 1998) and U.S. E.P.A. OPPTS Series 870, Testing Guidelines, N°870.3100 (August, 1998). These guidelines place special emphasis on neurological endpoint investigations and allow for the identification of chemicals with the potential to cause neurotoxic

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effects, which may warrant further in-depth investigation. The study included a neurotoxicity screening consisting of grasping, righting, corneal, pupillary, auditory startle and head shaking reflexes was performed once during the acclimatisation phase and during Week 12. At necropsy, organs including brain, sciatic nerve, spinal cord, and optic nerves were taken, fixed and examined microscopically were taken, fixed and examined microscopically.

There was no evidence of neurotoxicity in the 90 day rat studies up to 5000 ppm (295 mg/kg bw/day)

Aclonifen does not meet the trigger for a neurotoxicity study according to EU Regulation 283/2013 section 5.7

Assessment and conclusion by RMS:

CA 5.7.2 Delayed polyneuropathy studies

Delayed polyneuropathy studies are not triggered (see 5.7.1 above).

CA 5.8 Other toxicological studies

Summary of toxicity studies of metabolites

(Q)SAR has been conducted using Derek Nexus, Leadscope and Toxtree software (expert and rules based predictive software respectively) on dietary metabolites of aclonifen and these have been compared to the (Q)SAR conducted on aclonifen. The aim was to group the metabolites according to similarity of (Q)SAR alerts to allow read-across. The lead metabolite in a group is the one with the most alerts so raises most concern. All other metabolites in the same group have the same (or fewer) alerts.

For aclonifen M-01 is designated the lead metabolite as it triggers the most (Q)SAR alerts of toxic (particularly genotoxic) concern. All other metabolites have the same (but fewer) alerts compared to M-01. There are some exceptions for minor alerts for skin sensitisation, nephrotoxicity or hepatotoxicity which are not considered relevant at low levels of exposure. Therefore all dietary metabolites form a single group where toxicity concerns can be addressed by read-across to M-01.

M-01 is the major intermediary metabolite in the rat metabolism studies with hydroxylation on the phenyl group being present in all other rat metabolites. As such the toxicity of M-01 is addressed in the toxicity studies conducted on rats because rats are exposed to significant levels of M-01.

It follows that all dietary metabolites have equivalent toxicity based on similarity of (Q)SAR alerts and can be read-across to M-01 so are also covered by the toxicity studies on rats.

The following (Q)SAR report has been conducted on dietary metabolites:

Report	Metabolites	Reference	New study

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Aclonifen – summary of *in silico* assessment of dietary metabolites	Aclonifen, M-01, M-02, M-05, M-07, M-09, M-12, M-17, M-18, M-19, M-20, M-21	KCA 5.8.1/ 01 [redacted] 2020 ~~M-676871-01-1~~ M-676871-03-1	**New**

Summary of supplementary studies of the active substance

This section of the dossier contains supplementary studies on the active substance. The table below lists the supplementary studies that have been conducted on the active substance. These comprise a unscheduled DNA synthesis assay in rat hepatocytes, a DNA binding assay in mice, an acute toxicity study in rats by the peritoneal all of which were included in the previous dossier submitted under Council Directive 91/414/EEC. Two papers from the literature are also included. These are *in vitro* studies used to screen a large number of industrial chemicals including aclonifen for binding to nuclear receptors, the constitutive androstane receptor (CAR) and the retinoic acid receptor (RAR).

The unscheduled DNA synthesis (UDS) is a well-conducted GLP and guideline compliant *ex vivo* genotoxicity assay conducted on primary rat hepatocytes. It was negative for unscheduled DNA synthesis up to cytotoxic concentrations and thus confirms the findings of the standard genotoxicity tests presented in the CA5.4. The UDS assay was deleted as an OECD test guideline in 2014 as it has been replaced by other tests deemed more reliable, but it can still contribute to a weight of evidence assessment of genotoxicity.

The DNA binding assay is a well-conducted GLP compliant non-standard study conducted in mice to investigate mechanisms for bladder tumours in the mouse carcinogenicity study (KCA 5.5/12, [redacted] 1991 M-174334-01-1), in particular whether tumours in mice is the result of *in vivo* covalent binding of aclonifen or its metabolites to DNA. Mice were administered a single oral dose of 900 mg/kg bw radiolabelled aclonifen (the same dose leading to tumours in the mouse carcinogenicity study). DNA and chromatin were isolated from the liver and bladder. Aclonifen did not bind to DNA though it did bind to chromatin. Binding to chromatin which contains DNA and proteins may be expected as aclonifen also was found to bind to plasma protein in a protein binding assay (KCA 5.1.1/10, M-569675-01-1, [redacted] 2016). The results of this study give no indication for a genotoxic potential of aclonifen mediated by DNA binding *in vivo*.

An acute toxicity by the peritoneal route in rats was a non-GLP study of limited relevance due to the route of exposure. The LD_{50} was 3742/3247 mg/kg bw in males/females respectively.

Two mechanistic studies are presented which were found in literature search conducted for the current renewal which screened a broad range of industrial chemicals for activation of the CAR and RAR receptor pathways. Both studies used yeast cells transduced with human forms of the receptors and activation was measured using a reporter gene. These data show that aclonifen does possess CAR and RAR agonistic activity comparable with a range of other compounds, but for both receptors the activation was only about 0.2% of the positive controls.

Test system, Concentration range	**Results**	**Remarks**	**Reference**	**New study**
Unscheduled DNA synthesis (UDS) *ex vivo* primary hepatocytes from male rats 0.079, 0.25, 0.79, 2.5, 7.9 µg/mL aclonifen in culture medium for 17 hours	Negative	Cytotoxicity at ≥7.9 µg/mL	KCA 5.8.2 / 01 [redacted] 1991 M-174373-01-1	-

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DNA binding assay CD1 Mice single oral gavage dose of 900 mg/kg bw of [Aniline-UL-14C]aclonifen. After 24 hrs DNA extracted from urinary bladder and liver.	No DNA binding.	Some interaction of aclonifen with chromatin protein in liver and bladder.	KCA 5.8.2 / 02 [redacted] 1995 M-174903-01-1	
Acute toxicity peritoneal route Wistar rat both sexes Single dose 2500, 3200, 4000, 5000 mg/kg bw	LD_{50} 3742/3247 mg/kg bw in males/females	Not a relevant route for human exposure	KCA 5.8.2 / 03 [redacted]; [redacted] 1981 M-174876-01-1	-
Constitutive androstane receptor (CAR) agonist assay *in vitro* recombinant yeast-cell transfected with human CAR receptor ligand binding domain, CAR DNA binding domain, and β-galactosidase reporter gene. 7 concentrations of aclonifen 10 µM and 156 nM for 4 hours	EC_{10} 6.3µM	Relatively low agonist activity (0.2% of the potent agonist 4-*tert*-octylphenol)	KCA 5.8.2 / 04 [redacted] et al 2018 M-669642-01-1	New
Retinoic acid receptor (RAR) agonist assay *in vitro* recombinant yeast-cell assay transfected with human RARγ receptor ligand binding domain, RARγ coactivator, and β-galactosidase reporter gene. 7 concentrations of aclonifen 10 µM and 156 nM for 4 hours	EC_{10} 2.74µM	Relatively low agonist activity (0.2% of the potent agonist *all-trans* retinoic acid)	KCA 5.8.2 / 05 [redacted] et al 2008 M-669423-01-1	New

Summary of endocrine disrupting properties

This section of the dossier contains new studies conducted for this renewal in order to investigate endocrine activity in accordance with the EFSA and ECHA Guidance for the identification of endocrine disruptors (2018). *In vitro* studies investigating EAS modalities are the androgen and estrogen agonist and antagonist transactivation assays (OECD 458 and 455 respectively), and the steroidogenesis assay (OECD 456). The T modality is investigated in a hog thyroid peroxidase (TPO) assay, a rat/human sodium iodine symporter inhibition assay (NIS), and liver enzyme induction is investigated in a gene expression assay in primary rat hepatocytes.

Three tests investigated the EAS modality. The androgen receptor agonist and antagonist assay was a well-conducted GLP and guideline compliant study in the AR-Ecocreen™ cell line, with no cytotoxicity limiting the response up to the highest concentration tested of 1 µM. No androgenic activity was seen in either of two valid independent experiments. However some AR antagonistic activity was observed in both valid independent experiments with a Log IC_{30} 6.4 meaning that aclonifen displayed antagonism for the AR receptor in this study. The estrogen receptor agonist and antagonist study was a well-conducted, GLP compliant study using the human hERα-HeLa-9903 cell line, with cytotoxicity limiting the concentrations tested to 3.16 µM. The majority of the acceptability criteria of the ER agonist and antagonist assays were met and therefore the assays were considered to be valid. Aclonifen did not show any estrogenic agonist or antagonist activity when tested at concentrations up to 3.16 µM in this study. Steroid hormone synthesis was investigated in a well-conducted GLP and guideline compliant steroidogenesis assay which investigated testosterone and

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estradiol synthesis in H295R adrenal cell line. The highest concentration tested was 80 µM based on slight changes in cell morphology and higher concentrations. Aclonifen had no clear effect on estradiol secretion. However, a treatment related statistically significant inhibition of testosterone secretion (-77.4%; $p \leq 0.01$) was observed at the highest concentration (80µM). Overall no endocrine activity is seen for the E modality in these tests, however inhibition of the androgen receptor and inhibited testosterone synthesis indicate effects on the A and S modalities *in vitro*, however these findings do not correlate with any A or S adversity in the *in vivo* studies. *In vivo* there are no adverse findings in the reproductive parameters in the 2-generation or developmental toxicity studies. The only finding was a reduction in pup weight at the top dose in the presence of body weight effects of similar magnitude in parental animals and accompanied by parental reduced food consumption as a non-specific sign of general toxicity. Likewise the only effects on male reproductive organs in the repeat dose studies are changes in organ weights which only occur at high doses and secondary to reduced overall body weight and without any adverse histopathology. The only effect on female reproductive organs was seen in a change in ovary weight at high doses and secondary to marked reduction in body weight. Therefore though there is evidence *in vitro* of endocrine activity on the A and S modalities there is no endocrine adversity on EAS modalities *in vivo*.

Three tests investigated the T modality. The hog thyroid peroxidase assay does not currently have a validated OECD test guideline but was a well-conducted GLP study. The highest concentration tested was 100 µM (due to limit of solubility). Aclonifen did not inhibit thyroid peroxidase up to the highest concentration tested therefore there was no indication that aclonifen inhibits thyroid hormone synthesis. A second test on the T modality was the sodium iodide symporter (NIS) assay. This study does not currently have a validated OECD test guideline but was a well-conducted non-GLP study that investigated the potential for aclonifen to inhibit iodine uptake into the thyrocytes via the sodium iodide symporter in rat and human cell lines at concentrations up to 500µM. Aclonifen showed a dose dependent inhibition of NIS activity in both species, with significant inhibition occurring at concentrations of 50 µM. A further study investigating the effect of metabolism on NIS inhibition found that pre-incubation of aclonifen in S9 mix almost totally abolished the NIS inhibition in both species, suggesting that once metabolized, aclonifen loses its ability to interfere with NIS activity. Finally a study investigated the gene transcription of phase I and II metabolising enzymes in primary rat hepatocytes found aclonifen caused a marked increase in transcription of liver enzymes of CYP 2B1, CYP 3A23, UGT 1A1, UGT 1A6, UGT 2B1 which are markers of CAR/PXR receptor activation and induction of metabolism. Increased liver enzyme induction is a known mechanism for reduction of thyroid hormones by leading to increased metabolism of thyroid hormones, particularly T4. Overall these studies indicate that *in vitro* aclonifen inhibits the NIS, whereas *in vitro* metabolization almost completely abolished the NIS inhibition. *In vivo* aclonifen is rapidly metabolised in the liver and unmetabolized aclonifen is not detected in rat metabolism studies in plasma, or in the urine, therefore inhibition of NIS is not predicted to occur *in vivo* as systemic exposure of the thyroid to unmetabolized aclonifen is not likely. The thyroid gland was a target for organ for aclonifen as *in vivo* signs of adversity/endocrine activity were a reduction in T4 in rats and mice, reduced T3 in rats, and increased TSH in rats (TSH not measured in mice). In rats follicular cell hypertrophy and increased thyroid weight also occurred. The induction of liver enzyme transcription in hepatocytes provides evidence of a mechanism for reduction of T4 *in vivo*. Enhanced hepatic clearance of thyroid hormones is considered to be the most likely MoA for the changes in thyroid hormones and thyroid hypertrophy, it is a well-documented MoA, and species differences in thyroid hormone metabolism mean this MoA is of limited relevance to humans.

The assessment of the potential for aclonifen to interact with mammals is described in Appendix I, Assessment of the endocrine disrupting properties of the active substance aclonifen in accordance with Commission Regulation (EU) 2018/605, (M-676736-01-1, [redacted] & [redacted], 2020). A summary of all relevant studies is provided in the excel spreadsheet Appendix E.

The following studies to investigate endocrine disrupting properties have been conducted on the active substance

Test system, Concentration range	Results	Remarks	Reference	New study
OECD 458. *In vitro* Androgen receptor agonism and antagonism assay. AR agonism assay: 7 concentrations of aclonifen 10 pM to 1 µM for 22 – 24 hrs AR antagonism assay: 6 concentrations 100 pM to 1 µM for 22 – 24 hrs	Negative for AR agonism Positive for AR antagonism Log IC_{30} 6.4		KCA 5.8.3 / 01 2019 M-664621-01-1	New
OECD 455. *In vitro* Estrogen receptor agonism and antagonism assay. ER agonism assay: 7 concentrations of aclonifen 10 pM to 3.16 µM for 21 hrs. ER antagonism assay: 6 concentrations of aclonifen 100 pM to 3.16 µM for 22 to 24 hrs.	Negative for ER agonism Negative for ER antagonism		KCA 5.8.3 / 02 2019 M-664998-01-1	New
OECD 456. *In vitro* H295R steroidogenesis assay. concentrations of 0.8nM to 80 µM aclonifen for 48 hrs.	No effect on estradiol secretion. Inhibition of testosterone secretion at highest concentration (80µM).	No cytotoxicity up to 100 µM	KCA 5.8.3 / 06 , H 2019 M-675903-01-1	New
Thyroid peroxidase (TPO) assay. *Ex vivo* pig microsomes incubated in 10, 30, 100 µM aclonifen.	No effect on thyroid peroxidase activity.		KCA 5.8.3 / 03 2019 M-670319-02-1	
Rat/human sodium/iodine symporter (NIS) assay. *in vitro* rat PCCL3 and human hNIS-HEK 293 cells incubated in 5, 16, 50 µM aclonifen for 45 minutes +/- pre-incubation in S9 mix.	NIS inhibition at 50 µM: Rat: without S9: 22% activity vs control with S9: 75% activity vs control Human: without S9: 26% inhibition vs control with S9: 73% inhibition vs control	Aclonifen was a potent inhibitor of rat and human NIS. Inhibition of NIS was almost totally abolished in both species following pre-incubation in S9 mix.	KCA 5.8.3 / 05 2019 M-676484-01-1	New
Gene expression profile assay. *ex vivo* primary rat hepatocytes. Incubated in aclonifen at 3, 10, 30 µM for 8 hrs.	Induction of Cyp 2B1, Cyp 3A23, UGT 1A1, UGT 1A6, UGT 2B1 gene transcription indicates activation of CAR/PXR receptors and increased liver enzyme transcription.	Cytotoxicity was noted at 100 µM	KCA 5.8.3 / 04 2019 M-675870-01-1	New

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Appendix I - Assessment of the endocrine disrupting properties of the active substance aclonifen in accordance with Commission Regulation (EU) 2018/605 and Appendix E excel spreadsheet	T modality: in vivo adversity/endocrine activity. *in vitro/in vivo* evidence indicates most plausible MoA is increased clearance of T4/T3 secondary to induction of hepatic metabolism. This MoA for T modality is not relevant to humans. E modality: no *in vivo* adversity or in vitro endocrine activity. A and S modalities: *in vitro* endocrine activity, but no *in vivo* adversity. Not ED by EAS modalities		KCA 5.8.3/07 [redacted]; [redacted] [redacted] 2020 M-676736-01-1	New

CA 5.8.1 Toxicity studies of metabolites

Data Point:	KCA 5.8.1/01
Report Author:	[redacted]
Report Year:	2020
Report Title:	Amendment 02: Aclonifen - In silico assessment of dietary metabolites
Report No:	VC/19/017
Document No:	M-676871-03-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	No deviations
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive summary

In silico assessment was conducted on metabolites with potential human dietary exposure using expert and rules-based predictive software (Derek Nexus, Leadscope and Toxtree). This assessment was conducted on the metabolites M-01, M-05, M-19, M-09, M-20, M-17, M-21, M-18, M-07 M-02, and M-12. The predictions for the metabolites were compared to the predictions for aclonifen.

The metabolite M-01 had the most alerts of concern. M-01 is a major intermediary metabolite in the rat and therefore is considered to be addressed by the toxicology studies on aclonifen. All other metabolites showed fewer alerts compared to M-01 and based on similar of alerting structures it is proposed to read-across to M-01 for all other metabolites.

1. MATERIALS AND METHODS

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In silico methods and tools used

The SMILES codes of the metabolites listed in the table below, were used to generate QSAR predictions. The code number or name used in the following discussion is shown in bold type. As the exact structure of dihydroxy aclonifen sulphate conjugate is not known so the possible variants were drawn and are shown here as well.

Table 5.8.1- 1 Identity of parent substance Aclonifen and of the dietary metabolites assessed by *in silico* means

Company code **Common name** **SMILES code**	**Structure**
AE F068300 **Aclonifen** Nc1c(Cl)c(Oc2ccccc2)ccc1N(=O)=O	
BCS-AX74959 Aclonifen-4-OH **M-01** Nc1c(Cl)c(Oc2ccc(O)cc2)ccc1[N+](=O)[O-]	
RPA 407074 malonyl glucosidyl conjugate Aclonifen-4-OH-malonylglucoside **M-05** c1(c(N)c(Cl)c(cc1)Oc2ccc(cc2)O[C@H]3 [C@@H]([C@H]([C@@H]([C@H](O3)CO C(CC(O)=O)=O)O)O)O)[N+]([O-])=O	
M-19 Aclonifen-4-OH glucoside c1(c(N)c(Cl)c(cc1)Oc3ccc(O[C@@H]2O[C@H](CO)[C@@H](O)[C@H](O)[C@H]2O) cc3)[N+](=O)[O-]	
RPA 407074 sulfate conjugate Aclonifen-4-OH-sulfate **M-09** Clc2c(N)c([N+](=O)[O-])ccc2Oc1ccc(OS(=O)(=O)O)cc1	

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Company code **Common name** **SMILES code**	**Structure**
BCS-BX26038 Aclonifen-aminophenol **M-20** [N+](=O)([O-])c1c(c(c(c(c1)O)Oc2ccccc2)Cl)N	
BCS-BX26038-malonyl-glucoside Aclonifen-aminophenol-malonylglucoside **M-17** c1(c(N)c(Cl)c(c(c1)O[C@H]2[C@@H]([C@H]([C@@H]([C@H](O2)COC(CC(O)=O)=O)O)O)O)Oc3ccccc3)[N+]([O-])=O	
BCS-BX26039 Aclonifen-ortho-OH **M-21** [N+](=O)([O-])c1c(c(c(cc1)Oc2c(cccc2)O)Cl)N	
BCS-BX26039-malonylglucoside Aclonifen-ortho-OH-malonylglucoside **M-18** c1(c(N)c(Cl)c(cc1)Oc2c(cccc2)O[C@H]3[C@@H]([C@H]([C@@H]([C@H](O3)COC(CC(O)=O)=O)O)O)O)[N+]([O-])=O	
AE 0561851 methylimidazole Aclonifen-diamino-acetyl-4-OH-dehydrate **M-07** C=1C(=CC=C(C=1)O)OC=2C(=C3C(=CC=2)N=C(N3)C)Cl	
AE 0561852 Aclonifen-des-phenyl **M-02** [N+](=O)([O-])c1c(c(c(cc1)O)Cl)N	
Aclonifen-dihydroxylated sulphate conjugates **M-12** (SMILES codes for 10 different positions of OH and SO_4 groups listed below)	

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Company code **Common name** **SMILES code**	**Structure**
M-12 Dihydroxy aclonifen sulphate conjugate 1 Nc1c(Cl)c(Oc2ccccc2OS(=O)(=O)O)cc(O)c1[N+](=O)[O-]	
M-12 Dihydroxy aclonifen sulphate conjugate 2 Nc1c(Cl)c(Oc2ccccc2OS(=O)(=O)O)c(O)cc1[N+](=O)[O-]	
M-12 Dihydroxy aclonifen sulphate conjugate 3 Nc1c(Cl)c(Oc2cccc(OS(=O)(=O)O)c2)cc(O)c1[N+](=O)[O-]	
M-12 Dihydroxy aclonifen sulphate conjugate 4 Nc1c(Cl)c(Oc2cccc(OS(=O)(=O)O)c2)c(O)cc1[N+](=O)[O-]	
M-12 Dihydroxy aclonifen sulphate conjugate 5 Nc1c(Cl)c(Oc2ccc(OS(=O)(=O)O)cc2)cc(O)c1[N+](=O)[O-]	
M-12 Dihydroxy aclonifen sulphate conjugate 6 Nc1c(Cl)c(Oc2ccc(OS(=O)(=O)O)cc2)c(O)cc1[N+](=O)[O-]	
M-12 Dihydroxy aclonifen sulphate conjugate 7 Nc1c(Cl)c(Oc2cccc(OS(=O)(=O)O)c2)cc(O)c1[N+](=O)[O-]	
M-12 Dihydroxy aclonifen sulphate conjugate 8 Nc1c(Cl)c(Oc2cccc(OS(=O)(=O)O)c2)c(O)cc1[N+](=O)[O-]	
M-12 Dihydroxy aclonifen sulphate conjugate 9 Nc1c(Cl)c(Oc2ccccc2OS(=O)(=O)O)cc(O)c1[N+](=O)[O-]	
M-12 Dihydroxy aclonifen sulphate conjugate 10 Nc1c(Cl)c(Oc2ccccc2OS(=O)(=O)O)c(O)cc1[N+](=O)[O-]	

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Based on previously conducted calibration of commercial *in silico* tools licensed within Bayer (, 2018), the tools chosen for genotoxicity evaluation were Derek Nexus, Leadscope and Toxtree.

Derek Nexus version 2.3.0 (with Nexus version 6.1.0) was used for all predictions. ~~The Nexus version used for most metabolites was 2.2.0, Derek Nexus version 6.0.0, but updated to Nexus version 2.2.2, Derek Nexus version 6.0.1 for metabolites M-07 and M-12.~~ The Leadscope version used for all predictions was Leadscope Model Applier version 2.4.6-6 (with Leadscope Enterprise version 3.7.6-6 and Leadscope Personal version 4.7.6-6). ~~version 2.3.2, updated to version 2.3.3 for M-07 and version 2.4.6 for M-12. Toxtree version 2.6.13, updated to version 3.1.0 for metabolite M-07.~~ All alerts contained within Derek Nexus were used; non-genotoxicity alerts from Derek Nexus are presented in the tables below for each substance, but the reliability of the predictions is not discussed. The Leadscope models for sister chromatid exchange were de-activated, as these studies are apparently no longer considered relevant for evaluation of genotoxicity on an OECD level and as insufficient data exists within Bayer archives with which to evaluate the predictivity of these models. Toxtree was used only to generate Cramer classification and predictions for Ames mutagenicity and *in vivo*

micronucleus clastogenicity. The pdf ~~(Derek Nexus and Leadscope) and xls (Toxtree)~~ reports produced from each tool are submitted with this document ~~along with the input file~~.

The above-mentioned calibration testing included examination of the impact of excluding or including those substances which were outside of the applicability domain in Leadscope. Generally, when predictions were included for out-of-domain substances, the positive predictivity of the model decreased slightly, but overall accuracy of the model increased markedly. Thus, the tables in the remainder of the document will denote whether the substances were within or outside of the applicability domain, but the domain coverage of the query substance will not be the first reason for invalidating a given alert.

An additional part of the *in silico* calibration work has involved structural alert (SA) 34 within the Toxtree *in vivo* mouse micronucleus model, which is triggered by substances which are H-acceptors. This particular alert was shown by the developers of the model to have low (34% true positives against the training set) predictivity ([redacted] 2010). Comparison of the Toxtree results with predictions from Derek Nexus and Leadscope for Bayer substances with positive *in vivo* micronucleus assays shows that there are no compounds where SA 34 is the only predictor of clastogenicity in this assay; in every case where SA 34 correctly predicted clastogenicity, at least one other structural alert was triggered within Toxtree. Thus, for substances where SA 34 is the only alert triggered in the Toxtree *in vivo* mouse micronucleus model, this alert can be disregarded.

The pdf (Derek Nexus, Leadscope and Toxtree) reports produced from each tool have been submitted.

QSAR model information for the main endpoints used in Derek Nexus are available in the QSAR model reporting format (QMRF) from the Lhasa website: https://www.lhasalimited.org/publications/derek-nexus-mutagenicity-qmrfpdf/3512 in addition information on these models and those of Toxtree are also from the JRC website at:

http://cidportal.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/QSARDB/LATEST/qsardb.html

QSAR model information for the endpoints used in Leadscope in the QSAR model reporting format (QMRF) have been submitted.

II RESULTS AND DISCUSSION

Aclonifen

The table below lists the alerts triggered by aclonifen in the *in silico* tools used.

Table 5.8.1- 2 *In silico* alerts triggered in Derek Nexus, Leadscope, and Toxtree by aclonifen

Package	Endpoint	Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide	Plausible Plausible
	Chromosome damage *in vitro* 329 aromatic nitro compound	Equivocal
	Chromosome damage *in vivo* 329 aromatic nitro compound	Equivocal
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound	Equivocal

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Model	Endpoint		Prediction
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine		~~Plausible~~ Equivocal
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide		Plausible Plausible
	Hepatotoxicity 544 aromatic nitro compound		Plausible
	Splenotoxicity Rapid prototype 152 aniline or precursor		Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal
Toxtree	Cramer class		III (high)
	Ames mutagenicity		SA 27, Nitro aromatic
	In vivo mouse micronucleus		SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive
		Chrom Ab CHO v. 2	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. ~~1~~ 2	Negative
		Chrom Ab Other v. 1	Negative
		Chrom Ab rat v. 1	Negative
		Mouse micronuc v. 2	Positive
	Gene mutation	HPRT Mut v. 1	Negative
		Mouse lymphoma Act v. 2	Positive
		Mouse lymphoma unact v. 2	Negative
		Rodent DL mut v. 1	Negative
		Rodent mut v. 1	Negative
		Bacterial Mut v. ~~1~~ 2	Positive
		E coli – Sal 102 A-T Mut v. ~~1~~ 2	Negative
		Salmonella mut v. ~~3~~ 4	Positive

[a] Cells colored gray in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

The genotoxicity studies conducted with aclonifen are listed in the table below. Aclonifen was negative in all *in vitro* and *in vivo* genotoxicity studies.

Table 5.8.1-3 Genotoxicity studies available for aclonifen

Study type	Reference	Results
Ames mutagenicity	1982; M-174849-01-1	Negative
	, 2006; M-266365-01-1	Negative
Mammalian mutagenicity	1984; M-174850-01-1	Negative
Chromabs *in vitro*	1992; M-174403-01-1	Negative
Micronucleus *in vivo*	, 1984; M-174855-01-1	Negative
Micronuclues *in vitro*	, 2019; M-664242-01-2	Negative
Mammalian mutagenicity	2019; M-664619-01-1	Negative

<u>Aclonifen - genotoxicity alerts</u>

All *in silico* systems provide at least one prediction of mutagenicity in the bacterial Ames assay for aclonifen. In Derek Nexus and Toxtree, this alert is based on the presence of an aromatic nitro group;

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the aromatic amine group also triggering a mutagenicty alert in Derek Nexus; in Leadscope, the nitro but not the aromatic nitro is also recognized as a contributer to the potential mutagenicity.

The figure below shows the aromatic nitro alerting group from Derek Nexus

Match with query compound

The figure below shows the alerting aromatic amine group from Derek Nexus

Match with query compound

For mammalian mutagenicity, most of the alerts in Leadscope do not predict activity; only the activated (S9 metabolism) mouse lymphoma model predicts a mutagenic outcome, with the nitro group as a contributor to the prediction. Derek Nexus has equivocal alerts for in vivo mutagenicity triggered by the aromatic nitro group and by the aromatic amine.

Both Derek Nexus and Leadscope predict clastogenicity *in vitro*, again due to the presence of the aromatic nitro group. Leadscope predicts clastogenicity *in vivo* in the mouse micronucleus assay due to the presence of the nitro group. The prediction in Derek Nexus only receives a rating of "equivocal" as the experimental evidence is not clear.

The genotoxicity studies conducted with aclonifen are shown in the Table 5.8.1-2 above. Based on these studies, the alerts resulting from *in silico* alerts for genotoxicity can be disregarded.

<u>Aclonifen – general toxicity alerts</u>

The aromatic nitro nature of aclonifen triggers alerts in Derek Nexus for carcinogenicity and hepatotoxicity. Skin sensitization ~~is predicted~~ prediction is based on the nature of the compound as an aromatic primary or secondary amine. A "rapid prototype" alert for splenotoxicity is triggered based on the presence of an aniline or precursor, although as this is based on undisclosed compounds its reliability and relevance are unknown. The relevance overall of these alerts to aclonifen can be judged by comparison to the effects observed in toxicology studies.

Aclonifen is classed as a skin sensitizer (Category 1A) which agrees with the prediction for skin sensitization.

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In the toxicity studies on aclonifen there was no evidence of splenotoxicity so the alert for splenotoxicity can be disregarded.

With regards to carcinogenicity aclonifen has a classification (Category 2) for carcinogenicity based on a very slight increase in astrocytomas in female rats at the highest dose in one of the two carcinogencity studies. Aclonfen was shown to be non-genotoxic, ~~though~~ and there is no mechanistic information or pre-neoplastic lesions that would suggest how these tumours might be formed. Although this finding seems unlikely to be related to the administration of aclonifen, based on the classification of aclonifen the alert for carcinogenicity cannot be disregarded.

M-01

The table below lists the alerts triggered by M-01 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1- 4 *In silico* alerts triggered in Derek Nexus, Leadscope, and Toxtree by M-01

Package	Endpoint	M-01 Prediction	Aclonifen Prediction
		[structure: HO-phenyl-O-phenyl with Cl, NH_2, NO_2]	[structure: phenyl-O-phenyl with Cl, NH_2, NO_2]
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide	Plausible Plausible	Plausible Plausible
	Chromosome damage *in vitro* ~~Both:~~ 329 aromatic nitro compound ~~M-01:~~ 470 phenol	Plausible Plausible	Equivocal No alert
	Chromosome damage *in vivo* ~~Both:~~ 329 aromatic nitro compound ~~M-01:~~ 470 phenol	Equivocal Equivocal	Equivocal No alert
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound 352 aromatic amine or amide	Equivocal Equivocal	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine	~~Plausible~~ Equivocal	~~Plausible~~ Equivocal
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative	Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide	Plausible Plausible	Plausible Plausible
	Hepatotoxicity	Plausible	Plausible

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	544 aromatic nitro compound			
	Splenotoxicity Rapidprototype 152 aniline or precursor		Equivocal	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, Nitro aromatic	SA 27, Nitro aromatic
	In vivo mouse micronucleus		SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. ~~1~~ 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Negative	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. ~~1~~ 2	Positive	Positive
		E coli - Sal 102 A-T Mut v. ~~1~~ 2	Negative	Negative
		Salmonella mut v. ~~3~~ 4	Positive	Positive

[a] Cells colored gray in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

M-01 is a major metabolite in animal feed. See Document MCA-6, Chapter 6.2.1 and Chapter 6.6.1 for levels in plant metabolism and CRC studies. See Document MCA-6, Chapter 6.3.3 and Chapter 6.6.2 for magnitude of residues in crop residue trials and rotational crops

Genotoxicity alerts: In large part, the genotoxicity alerts provided by Derek Nexus, Toxtree, and Leadscope for M-01 are based on the same alerting structures as those seen with aclonifen. However, the hydroxylation of the phenyl ring gives rise to a phenol-like or quinol-like structure, which triggers alerts in both Derek Nexus and Leadscope for genotoxicity (alert 470 for chromosome damage, both in vitro and in vivo in Derek Nexus, and in vitro gene mutation in the mouse lymphoma without metabolic activation in Leadscope ~~*in vitro* mammalian mutagenicity, *in vitro* and *in vivo* clastogenicity~~) which were not triggered for aclonifen.

The phenol alerting structure in Derek Nexus is shown in the figure below:

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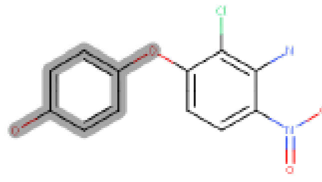
Match with query compound



Addition of the hydroxyl group also influences the positive prediction in Leadscope for mouse lymphoma without metabolic activation. The main driver of this positive prediction is the nitro group (also present in aclonifen) but an additional feature in M-01 triggering the positive prediction is the number of hydrogen bond donors which is higher in M01 compared to aclonifen.

Because these are plausible alerts and genotoxicity of M-01 cannot be ruled out by *in silico* methods, it is possible that genotoxicity studies should be conducted with M-01.

General toxicity alerts: Identical to aclonifen.

Conclusion on M-01
With regards to genotoxicity alerts, M-01 has alerts for chromosome damage and gene mutation *in vitro*, that are not present in aclonifen, however the *in vivo* alerts are identical to aclonifen. With regards to general toxicity M-01 shares the same alerts as aclonifen.

M-01 is a rat metabolite.

M-01 was found to be a major metabolite in plasma. In rats following a single oral dose at 2 mg/kg bw in rats M-01 was present at 10.2% and 10.7% of TRR in males and females respectively (see KCA 5.1.1/07 M-598008-01-1 [redacted], 2017). In urine M-01 is detected at fairly low levels (less than 1% of administered dose following single low dose of 2, 30, or 1000 mg/kg bw or a repeated dose of 30 mg/kg bw/day). However M-01 is a major intermediary in the metabolism of aclonifen as all rat metabolites of aclonifen contain a hydroxyl group on the phenyl ring which is then conjugated primarily by sulfation and glucuronidation (see Figure 5.1-01 in MCA5). Of particular note is the aclonifen-4-sulphate metabolite (M-09) as this is the major rat metabolite found in urine at over 20% of the administered dose (single oral dose of 2 mg/kg bw M-598008-01-1 [redacted], 2017, Tables 14 and 15 in the study report). M-09 was similarly identified as a major rat metabolite in urine in all other metabolites studies on aclonifen (at 30 mg/kg bw single dose present at 10% / 36% in males/females, at 30 mg/kg bw/day repeat dosing at 15% / 27% in males/females, at 1000 mg/kg bw/day present at 13% / 10 % in males/females).Therefore in rats exposure to M-01 also occurs during the formation of M09 as M-01 is an intermediary metabolite on the path of formation of M-09. As such the toxicity of M-01 is addressed in the toxicity studies conducted on rats because rats are exposed to M-01.

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M-05

The table below lists the alerts triggered by M-05 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1- 5 ***In silico*** **alerts triggered in Derek Nexus, Leadscope, and Toxtree by M-05**

Package	Endpoint		M-05 Prediction	Aclonifen Prediction
			[structure: HO, O, Cl, NH2, NO2, OH]	[structure: Cl, NH2, NO2, O]
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide		Plausible No alert	Plausible Plausible
	Chromosome damage *in vitro* 329 aromatic nitro compound		Equivocal	Equivocal
	Chromosome damage *in vivo* 329 aromatic nitro compound		Equivocal	Equivocal
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound 352 aromatic amine or amide		Equivocal No alert	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine		~~Plausible~~ Equivocal	~~Plausible~~ Equivocal
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide		Plausible No alert	Plausible Plausible
	Hepatotoxicity 544 aromatic nitro compound		Plausible	Plausible
	Splenotoxicity Rapidprototype 152 aniline or precursor		No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, Nitro aromatic	SA 27, Nitro aromatic
	In vivo mouse micronucleus		SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Negative	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. ~~1~~ 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative

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		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Negative	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. ~~1~~ 2	Indeterminate	Positive
		E coli – Sal 102 A-T Mut v. ~~1~~ 2	Negative	Negative
		Salmonella mut v. ~~3~~ 4	Indeterminate	Positive
[a] Cells colored gray in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.				

M-05 is a major metabolite in animal feed. See Document MCA-6, Chapter 6.2.1 and Chapter 6.6.1 for levels in plant metabolism and CRC studies.

Genotoxicity alerts: In large part, the genotoxicity alerts provided by Derek Nexus, Toxtree, and Leadscope for M-05 are based on the same alerting structures as those seen with aclonifen. The only alert triggered by M-05 that is not present in aclonifen is the Leadscope alert for gene mutation in vitro without S9 metabolic activation in the mouse lymphoma assay. The nitro group is the main contributor to this positive prediction but for aclonifen this group is not sufficient to trigger an overall positive prediction outcome in the software. The reason for this positive prediction for M-5 and not for aclonifen appears mainly to be due to structural features from the malonyl conjugate which are associated with positive predictions in the training set. Only two nearest neighbours were identified from the training set and these show less than 40% similarity to M-05, contain neither the nitro nor the malonyl group, and were negative in the mouse lymphoma assay without S9, indicating that this positive prediction is not reliable even though the software indicates it is within the applicability domain. Instead M-05 has closer similarity to aclonifen, therefore read-across to aclonifen is more appropriate considering structural similarity and overall similarity of all other QSAR predictions.

~~The Leadscope unactivated mouse lymphoma mutagenicity model predicts a positive outcome for M-05, while it predicts a negative or indeterminate outcome for aclonifen itself. The reason for this positive prediction is not clear, as the only structural feature with a majority of positive genotoxicity data, the nitro group, is present in both structures. In fact, that nitro group is identified by the Derek Nexus models in both substances leading to the same predictions for both aclonifen and the metabolite.~~

~~Thus, there are no meaningful differences in the alerts for genotoxicity between M-05 and aclonifen itself.~~

General toxicity alerts: Identical to aclonifen.

Conclusion on M-05

With regards to genotoxicity alerts that are not shared by aclonifen, M-05 as same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity. With regards to general toxicity, M-05 has a similar hazard to aclonifen except that it contains fewer alerts, so read-across to aclonifen may be applied.

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M-19

The table below lists the alerts triggered by M-19 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1- 6 ***In silico*** **alerts triggered in Derek Nexus, Leadscope, and Toxtree by M-19**

Package	Endpoint		M-19 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound		Plausible	Plausible
	352 aromatic amine or amide		No alert	Plausible
	Chromosome damage *in vitro* 329 aromatic nitro compound		Equivocal	Equivocal
	Chromosome damage *in vivo* 329 aromatic nitro compound		Equivocal	Equivocal
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound		Equivocal	Equivocal
	352 aromatic amine or amide		No alert	Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine		~~Plausible~~ Equivocal	~~Plausible~~ Equivocal
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound		Plausible	Plausible
	587 aromatic amide or amide		No alert	Plausible
	Hepatotoxicity 544 aromatic nitro compound		Plausible	Plausible
	Splenotoxicity Rapidprototype 152 aniline or precursor		No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, Nitro aromatic	SA 27, Nitro aromatic
	In vivo mouse micronucleus		SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Negative	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. ~~1~~ 2	Negative	Negative

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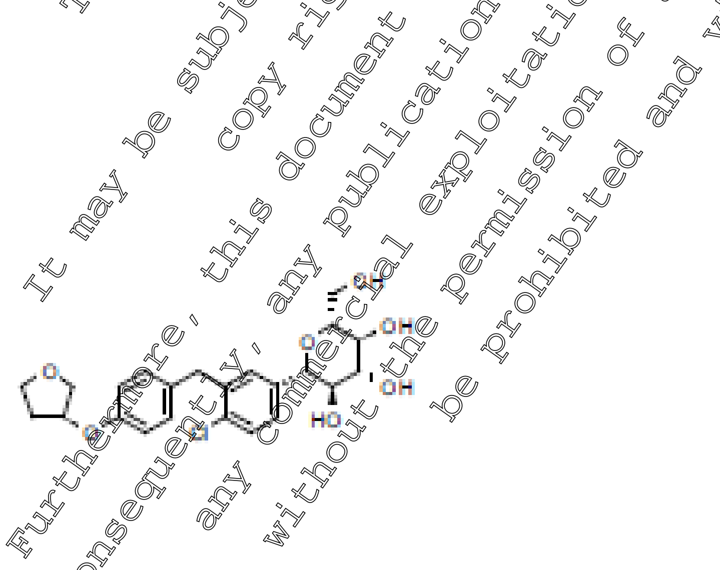
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. ~~1~~ 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. ~~1~~ 2	Negative	Negative
		Salmonella mut v. ~~3~~ 4	Positive	Positive

[a] Cells colored gray in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

M-19 is a minor metabolite.

Genotoxicity alerts: As for the malonyl glucoside conjugate (M-05) listed above, the glucoside conjugate M-19 has largely the same genotoxicity predictions as for aclonifen. The only prediction which differs is the Leadscope alert for gene mutation in vitro without S9 metabolic activation in the mouse lymphoma assay ~~unactivated mouse lymphoma model,~~ in which a genotoxic outcome is predicted for the glucoside ~~but~~ not for the active substance. ~~The reason for this prediction is again unclear as the only structural feature listed as contributing to the positive prediction was also the only structural feature with adverse genotoxicity data identified for aclonifen in this model. Thus, there are no meaningful differences between the predictions for M-19 and for aclonifen.~~ As noted under the assessment for M-05, the nitro group is the main contributor to this positive alert, with the number of hydrogen donors being the second most important feature, suggesting that the glucoside is contributing to the positive alert. Alpha-glucose is one of the nearest neighbours in the training set that tested positive in the mouse lymphoma assay without metabolic activation, although glucose is not generally considered to be mutagenic casting doubt on the reliability of the prediction. The other nearest neighbour in the training set is the type II diabetes drug Empagliflozin (structure shown below) which shows 46% similarity (i.e. strong similarity) to M-19 and tested negative in the same assay.



Based on consideration of the nearest neigbours the Leadscope alert for the mouse lymphoma assay can be dismissed as not reliable. Instead M-19 has closer similarity to aclonifen, therefore read-across

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to aclonifen is more appropriate considering structural similarity and overall similarity of all other QSAR predictions.

General toxicity alerts: Identical to aclonifen.

Conclusion on M-19

With regards to genotoxicity alerts that are not shared by aclonifen, M-19 as same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity.

M-19 is a minor metabolite therefore alerts for general toxicity are not a concern due to low exposure levels.

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M-09

The table below lists the alerts triggered by the sulphate conjugate M-09 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1- 7 ***In silico*** **alerts triggered in Derek Nexus, Leadscope, and Toxtree by M-09**

Package	Endpoint	M-09 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide	Plausible Plausible	Plausible Plausible
	Chromosome damage *in vitro* 329 aromatic nitro compound	Equivocal	Equivocal
	Chromosome damage *in vivo* 329 aromatic nitro compound	Equivocal	Equivocal
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound 352 aromatic amine or amide	Equivocal Equivocal	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine ~~837 ortho or para amino or hydroxy aniline~~	Equivocal ~~Plausible~~ ~~No alert~~	Equivocal ~~Plausible~~ ~~No alert~~
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative	Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide	Plausible Plausible	Plausible Plausible
	Hepatotoxicity 544 aromatic nitro compound	Plausible	Plausible
	Splenotoxicity Rapidprototype 152 aniline or precursor	No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation	Equivocal	Equivocal
Toxtree	Cramer class	III (high)	III (high)
	Ames mutagenicity	SA 27, Nitro aromatic	SA 27, Nitro aromatic
	In vivo mouse micronucleus	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene
Leadscope[a]	Clasto. *In* / Chrom Ab CHL v. 2	Positive	Positive

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	vitro	Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. ~~1~~ 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Negative	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Negative	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. ~~1~~ 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. ~~1~~ 2	Negative	Negative
		Salmonella mut v. ~~3~~ 4	Positive	Positive

[a] Cells colored gray in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

Genotoxicity alerts: Identical to aclonifen.

General toxicity alerts: Identical to aclonifen.

Conclusion in M-09

The alerts for the sulphate conjugate M-09 do not differ from those observed for aclonifen itself. Therefore M-09 is of equivalent toxicity to aclonifen. Therefore M-09 is of equivalent toxicity to aclonifen, and read-across to aclonifen may be applied for genotoxicity and general toxicity endpoints. In addition, M-09 is a major rat metabolite found in urine at >10% in all metabolism studies, so toxicity of M-09 is covered by toxicity studies with aclonifen.

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M-20

The table below lists the alerts triggered by M-20 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1- 8 ***In silico*** **alerts triggered in Derek Nexus, Leadscope, and Toxtree by M-20**

Package	Endpoint	M-20 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide	Plausible Plausible	Plausible Plausible
	Chromosome damage *in vitro* 329 aromatic nitro compound	Equivocal	Equivocal
	Chromosome damage *in vivo* 329 aromatic nitro compound	Equivocal	Equivocal
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound 352 aromatic amine or amide	Equivocal Equivocal	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization ~~Aclonifen:~~ 427 aromatic primary or secondary amine ~~M-20:~~ 837 ortho or para amino- or hydroxy- aniline	No alert Plausible	~~Plausible~~ Equivocal No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative	Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide	Plausible Plausible	Plausible Plausible
	Hepatotoxicity 544 aromatic nitro compound	Plausible	Plausible
	Splenotoxicity Rapidprototype 152 aniline or precursor	Equivocal	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation	Equivocal	Equivocal
	Nephrotoxicity 818 para-aminophenol or derivative	Plausible	No alert
Toxtree	Cramer class	III (high)	III (high)
	Ames mutagenicity	SA 27, Nitro aromatic	SA 27, Nitro aromatic
	In vivo mouse micronucleus	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-

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			benzene	benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. ~~1~~ 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. ~~1~~ 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. ~~1~~ 2	Negative	Negative
		Salmonella mut v. ~~3~~ 4	Positive	Positive

[a] Cells colored gray in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

M-20 is a minor metabolite.

~~Genotoxicity alerts: Mammalian mutagenicity is largely predicted to be negative, although the mouse lymphoma models in Leadscope predict a positive alert for mouse lymphoma (Mouse lymphoma unact v.2) that is not present in aclonifen. This prediction is largely based on the nitro group and the nitrobenzene scaffold. Aclonifen also has Leadscope alert for mouse lymphoma (mouse lymphoma Act v. 2) again triggered by the nitro group.~~

~~For clastogenicity, the *in vitro* alerts for chromosome damage in Derek Nexus and Leadscope predict M-20 will be clastogenic, again due to the nitro structure or the nitrobenzene scaffold. For *in vivo* clastogenicity, the alert in Derek Nexus is based on the presence of the aromatic nitro, but has a rating only of equivocal. In Leadscope, only the mouse micronucleus model predicts a positive response for clastogenicity. The alerts for clastogenicity are all the same as those triggered by aclonifen.~~

~~General toxicity alerts: M-20 has an alert for skin sensitization and nephron toxicity which are not present in aclonifen. The alert for nephrotoxicity (alert 818) is based on the presence of a para-aminophenol or derivative, an alert that is not shared with aclonifen. This is a human-relevant alert, however the nephrotoxic effects of para-aminophenols and similar compounds is a high-dose phenomenon that would not be seen after consumption of nearly negligible levels of metabolites such as these.~~

~~An alert for skin sensitisation (837) is based on ortho or para amino or hydroxy aniline structures. Aclonifen does not have this alert but is already classified as a Category 1A for skin sensitisation and the effects of these metabolites at low levels of exposure would be negligible.~~

Genotoxicity alerts: In large part, the genotoxicity alerts provided by Derek Nexus, Toxtree, and Leadscope for M-20 are based on the same alerting structures as those seen with aclonifen. The only alert triggered by M-20 that is not present in aclonifen is the Leadscope alert for gene mutation *in vitro* without S9 metabolic activation in the mouse lymphoma assay. The nitro group is a major contributor to this positive prediction but for aclonifen this group is not sufficient to trigger an overall positive prediction outcome in the software. The reason for a positive prediction for M-20 is that it contains an additional alerting feature (shown in the figure below) which is not present in aclonifen.

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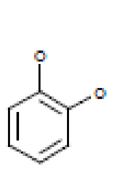

Five nearest neighbours were identified from the training set for this alert, four of which tested positive, but none have the alerting structure shown above, rather they all contain the nitro group, except for the nearest neighbour that tested negative. Overall, the relevance of this alerting structure to M-20 is questionable. Instead M-20 has closer similarity to aclonifen, therefore read-across to aclonifen is more appropriate considering structural similarity and overall similarity of all other QSAR predictions.

General toxicity alerts: M-20 has an alert for skin sensitization and nephron toxicity which are not present in aclonifen. The alert for nephrotoxicity (alert 818) is based on the presence of a para-aminophenol or derivative, an alert that is not shared with aclonifen. This is a human-relevant alert, however the nephrotoxic effects of para-aminophenols and similar compounds is a high-dose phenomenon that would not be seen after consumption of nearly negligible levels of metabolites such as these.

An alert for skin sensitisation (837) is based on amino or hydroxy aniline structures. Aclonifen does not have this alert but is already classified as a Category 1A for skin sensitisation and the effects of this metabolite would not increase the hazard compared to aclonifen.

Conclusion on M-20

With regards to genotoxicity alerts that are not shared by aclonifen, M-20 as same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity. M-20 is a minor metabolite therefore alerts for general toxicity are not a concern due to low exposure levels.

M-17

The table below lists the alerts triggered by the malonyl glucoside conjugate M-17 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1-9 *In silico* alerts triggered in Derek Nexus, Leadscope, and Toxtree by M-17

Package	Endpoint	M-17 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide	 Plausible No alert	 Plausible Plausible
	Chromosome damage *in vitro*	Equivocal	Equivocal

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	329 aromatic nitro compound			
	Chromosome damage *in vivo* 329 aromatic nitro compound		Equivocal	Equivocal
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound 352 aromatic amine or amide		Equivocal No alert	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine		~~Plausible~~ Equivocal	~~Plausible~~ Equivocal
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide		Plausible No alert	Plausible Plausible
	Hepatotoxicity 544 aromatic nitro compound		Plausible	Plausible
	Splenotoxicity Rapidprototype 152 aniline or precursor		No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, Nitro aromatic	SA 27, Nitro aromatic
	In vivo mouse micronucleus		SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. ~~1~~ 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Negative	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. ~~1~~ 2	Indeterminate	Positive
		E coli – Sal 102 A-T Mut v. ~~1~~ 2	Negative	Negative
		Salmonella mut v. ~~3~~ 4	~~Negative~~ Positive	Positive

[a] Cells colored gray in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

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M-17 is a minor metabolite.

Genotoxicity alerts: Like the unconjugated form M-20, the presence of the catechol structure in this conjugated metabolite M-17 leads to a prediction of a genotoxic outcome in the Leadscope mouse

lymphoma model without S9 activation. ~~unactivated mouse lymphoma model~~. The reasons for the alert are the same triggers described for M-20, and well as the malonyl group described under M-05. However the only nearest neighbour to M-20 identified in the training set was the type II diabetes drug Empagliflozin (structure shown under M-05) with 34% similarity to M-19 and tested negative in the same assay casting doubt on this positive prediction. Removal of the malonyl glucoside conjugate would release M-20, and thus the same assumptions can be drawn for the conjugate as for the aglycone.

General toxicity alerts: identical to aclonifen.

Conclusion on M-17

With regards to genotoxicity alerts that are not shared by aclonifen, M-17 as same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity. M-17 is a minor metabolite therefore alerts for general toxicity are not a concern due to low exposure levels.

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M-21

The table below lists the alerts triggered by M-21 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1- 10 ***In silico*** **alerts triggered in Derek Nexus, Leadscope, and Toxtree by M-21**

Package	Endpoint		M-21 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide		Plausible Plausible	Plausible Plausible
	Chromosome damage *in vitro* 329 aromatic nitro compound		Equivocal	Equivocal
	Chromosome damage *in vivo* 329 aromatic nitro compound		Equivocal	Equivocal
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound 352 aromatic amine or amide		Equivocal Equivocal	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine		~~Plausible~~ Equivocal	~~Plausible~~ Equivocal
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide		Plausible Plausible	Plausible Plausible
	Hepatotoxicity 544 aromatic nitro compound		Plausible	Plausible
	Splenotoxicity Rapidprototype 152 aniline or precursor		Equivocal	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, Nitro aromatic	SA 27, Nitro aromatic
	In vivo mouse micronucleus		SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. ~~1~~ 2	Negative	Negative

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		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. ~~1~~ 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. ~~1~~ 2	Negative	Negative
		Salmonella mut v. ~~3~~ 4	Positive	Positive

[a] Cells colored gray in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

M-21 is a minor metabolite.

~~Genotoxicity alerts: Like M-20, (and most other metabolites of aclonifen) this metabolite triggers an alert for mutagenicity in the Leadscope unactivated mouse lymphoma model, and like that substance M-21 has a catechol structure in addition to the nitro group which is shared with aclonifen itself. It is possible that the substitution on the catechol structure would reduce the potential for genotoxicity, but the assumption would need to be tested experimentally.~~

Genotoxicity alerts: In large part, the genotoxicity alerts provided by Derek Nexus, Toxtree, and Leadscope for M-21 are based on the same alerting structures as those seen with aclonifen. The only alert triggered that is not present in aclonifen is the Leadscope mouse lymphoma without metabolic activation. The reason for the positive prediction is the same as for M-20 in that an additional hydroxy group triggers an alert based on two oxygens attached to an aromatic ring, however like M-20 the nearest neighbours do not contain this structure, but only the nitro group, casting doubt on the relevance of the alert to M-21. Instead M-21 has closer similarity to aclonifen, therefore read-across to aclonifen is more appropriate considering structural similarity and overall similarity of all other QSAR predictions.

However, given the high similarity between M-21 and M-20, and the fact that the only alert which is not common with aclonifen is common to both of the metabolites, it should be possible (if needed) to conduct studies with only one of the two, and read across to the other metabolite.

All other alerts for genotoxicity are the same as those triggered by aclonifen.

General toxicity alerts: identical to aclonifen.

Conclusion on M-21

With regards to genotoxicity alerts that are not shared by aclonifen, M-21 as same (or fewer) alerts as M-01, therefore may be grouped with M-01 for read-across for genotoxicity. M-21 is a minor metabolite therefore alerts for general toxicity are not a concern due to low exposure levels.

M-18

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The table below lists the alerts triggered by the malonyl glucoside conjugate M-18 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1- 11 ***In silico* alerts triggered in Derek Nexus, Leadscope, and Toxtree by M-18**

Package	Endpoint	Metabolite Prediction	Aclonifen Prediction
		[structure of M-18: malonyl glucoside conjugate; labels O, OH, HO, Cl, NH_2, NO_2]	[structure of aclonifen; labels Cl, O, NH_2, NO_2]
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide	Plausible No alert	Plausible Plausible
	Chromosome damage *in vitro* 329 aromatic nitro compound	Equivocal	Equivocal
	Chromosome damage *in vivo* 329 aromatic nitro compound	Equivocal	Equivocal
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound 352 aromatic amine or amide	Equivocal No alert	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine	~~Plausible~~ Equivocal	~~Plausible~~ Equivocal
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative	Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide	Plausible No alert	Plausible Plausible
	Hepatotoxicity 544 aromatic nitro compound	Plausible	Plausible
	Splenotoxicity Rapidprototype 152 aniline or precursor	No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation	Equivocal	Equivocal
Toxtree	Cramer class	III (high)	III (high)
	Ames mutagenicity	SA 27, Nitro aromatic	SA 27, Nitro aromatic
	In vivo mouse micronucleus	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene
Leadscope[a]	Clasto. *In vitro* – Chrom Ab CHL v. 2	Negative	Positive
	Clasto. *In vitro* – Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo* – Chrom Ab Comp v. ~~1~~ 2	Negative	Negative
	Clasto. *In vivo* – Chrom Ab Other v. 1	Negative	Negative

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		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Negative	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. ~~1~~ 2	Indeterminate	Positive
		E coli – Sal 102 A-T Mut v. ~~1~~ 2	Negative	Negative
		Salmonella mut v. ~~3~~ 4	~~Negative~~ Indeterminate	Positive

[a] Cells colored gray in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

M-18 is a minor metabolite.

Genotoxicity alerts: Like the non-conjugated metabolite M-21, the only alert which differs in a significant manner between the malonyl glucoside conjugate and the parent compound aclonifen is for the Leadscope alert for mouse lymphoma without S9 activation ~~unactivated mouse lymphoma model~~, which again is triggered by the presence of a catechol structure as well as the common nitro group and the malonyl group (which also triggers this alert in M-05 and M17).

Deconjugation of the malonyl glucoside would liberate M-21, which also has the catechol structure in it. Thus, the potential for genotoxicity of M-18 can be read across to the conjugated form as well.

General toxicity alerts: identical to aclonifen.

Conclusion on M-18

With regards to genotoxicity alerts that are not shared by aclonifen, M-18 as same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity. M-18 is a minor metabolite therefore alerts for general toxicity are not a concern due to low exposure levels.

M-07

The table below lists the alerts triggered by M-07 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1-12 ***In silico* alerts triggered in Derek Nexus, Leadscope, and Toxtree by M-07**

Package	Endpoint	M-07 Prediction	Aclonifen Prediction

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Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide		~~No alert~~ Inactive, no misclassified or unclassified features	Plausible Plausible
	Chromosome damage *in vitro* 329 aromatic nitro compound ~~(mammal)~~ 470 phenol		No alert Plausible	Equivocal No alert
	Chromosome damage *in vivo* 329 aromatic nitro compound ~~(mammal)~~ 470 phenol		No alert Equivocal	Equivocal No alert
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound 352 aromatic amine or amide		No alert No alert	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine		~~No alert~~ Non-sensitiser, no misclassified or unclassified features	~~Plausible~~ Equivocal
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		No alert	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide		No alert No alert	Plausible Plausible
	Hepatotoxicity 544 aromatic nitro compound		No alert	Plausible
	Hepatotoxicity 544 aromatic nitro compound 694 Benzimidazole or derivative		No alert Plausible	Plausible No alert
	Splenotoxicity Rapid prototype 152 aniline or precursor		No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		No alert	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		No alerts	SA 27, Nitro aromatic
	In vivo mouse micronucleus		SA 34, H acceptor SA 33, 1-phenoxy-benzene	SA 27, Nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy-benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive

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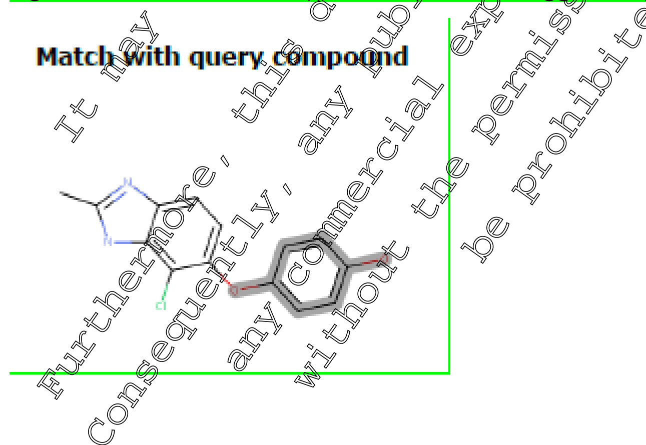
	Clasto. *In vivo*	Chrom Ab Comp v. ~~1~~ 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Negative	Positive
		Mouse lymphoma unact v. 2	Negative	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. ~~1~~ 2	Negative	Positive
		E coli – Sal 102 A-T Mut v. ~~1~~ 2	Negative	Negative
		Salmonella ~~mut~~ v. ~~3~~ 4	Negative	Positive

[a] Cells colored gray in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

M-07 is a major metabolite in human food of animal origin. See Document MCA-6, Chapter 6.2.3 for levels in goat metabolism study.

Genotoxicity alerts: There is an absence of mammalian alerts for metabolite M-07 in most tested (Q)SAR models provided by Derek Nexus, Toxtree, and Leadscope. In large part, the genotoxicity alerts provided by Derek Nexus, Toxtree, and Leadscope for M-07 are based on the same alerting structures as those seen with aclonifen. However, the hydroxylation of the benzene ring gives rise to a phenol or quinone-like structure, which triggers alerts in Derek Nexus (alerts 470 for phenol) for mutagenicity in mammals *in vitro* (plausible) and mutagenicity in mammals *in vivo* (equivocal). The alert is for *in vitro* chromosome aberration, and *in vitro* micronucleus test, an *in vitro* gene mutation all in mammalian cells, but only equivocal for genotoxicity *in vivo*.

The alerting phenol structure for chromosome damage (alert 470) from Derek Nexus is shown in the figure below. These same alerts were also triggered by the rat metabolite M-01.



In Leadscope and Toxtree the alerts for M-07 are the same as those triggered by aclonifen.

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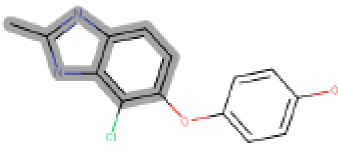
General toxicity alerts: There is a plausible alert for hepatoxicity triggered by M-07 (alert 694 for Benzimidazole or derivative). Aclonifen also has a plausible alert for hepatotoxicity although triggered by a different alert (544 for aromatic nitro compound). The benzimidazole derivate alerting structure for hepatoxicity from Derek Nexus is shown in the figure below.

Match with query compound



M-07 a major metabolite in goat kidney, but all tissue residues <0.01 mg eq/kg in goat dosed at 7X maximum dietary burden, therefore this alert is not a concern due to low exposure levels.

Conclusions on M-07

The plausible alert for genotoxicity of M-07 (*in vitro* mammalian cells) cannot be ruled out by *in silico* methods therefore testing or further information on the genotoxicity of this metabolite may be required. ~~The alert for hepatoxicity is not likely to be a concern provided exposure levels are low. M-07 a major metabolite in goat kidney, but all tissue residues <0.01 mg eq/kg in goat dosed at 7X maximum dietary burden, therefore this alert is not a concern due to low exposure levels.~~

Apart for the alert for hepatotoxicity, M-07 has the same alerts as the metabolite M-01, which are triggered in both metabolites by the hydroxylation of the benzene ring, therefore read-across from M-01 (a rat metabolite) can be adopted in order to address the genotoxicity of M-07.

With regards to general toxicity M-07, the alert for hepatoxicity is not likely to be a concern provided exposure levels are low. M-07 a major metabolite in goat kidney, but all tissue residues <0.01 mg eq/kg in goat dosed at 7X maximum dietary burden, therefore this alert is not a concern due to low exposure levels.

M-02

The table below lists the alerts triggered by M-02 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1- 13 ***In silico*** **predictions for M-02**

Package	**Endpoint**	**M-02 Prediction**	**Aclonifen Prediction**
		HO, Cl, NH_2, NO_2	O, Cl, NH_2, NO_2
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide	Plausible Plausible	Plausible Plausible

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	Chromosome damage *in vitro* in mammal 329 aromatic nitro compound		Equivocal	Plausible
	Chromosome damage *in vivo* in mammal 329 aromatic nitro compound		Equivocal	Equivocal
	Mutagenicity *in vivo* in mammal 329 aromatic nitro compound 352 aromatic amine or amide		Equivocal Equivocal	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine 837 amino- or hydroxy-aniline		No alert Plausible	Equivocal No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide		Plausible Plausible	Plausible Plausible
	Hepatotoxicity in mammal 544 aromatic nitro compound		Plausible	Plausible
	Splenotoxicity in mammal Rapidprototype152 aniline or precursor		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic (Q)SAR6, aromatic amines	SA 27, nitro aromatic (Q)SAR6, aromatic amines
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H-acceptor	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope [a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative

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		Salmonella mut v. 4	Positive	Positive
[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.				

~~Package~~	~~Endpoint~~		~~M-02 Prediction~~	~~Aclonifen Prediction~~
~~Derek Nexus genotoxicity~~	~~Ames mutagenicity 329 aromatic nitro compound~~		~~Plausible~~	~~Plausible~~
	~~Chromosome damage *in vitro* in mammal 329 aromatic nitro compound~~		~~Equivocal~~	~~Plausible~~
	~~Chromosome damage *in vivo* in mammal 329 aromatic nitro compound~~		~~Equivocal~~	~~Equivocal~~
~~Derek Nexus general toxicity~~	~~Skin sensitization 427 aromatic primary or secondary amine~~		~~Plausible~~	~~Plausible~~
	~~Carcinogenicity in mammal 105 aromatic nitro compound~~		~~Plausible~~	~~Plausible~~
	~~Hepatotoxicity in mammal 544 aromatic nitro compound~~		~~Plausible~~	~~Plausible~~
	~~Splenotoxicity in mammal Rapidprototype152 aniline or precursor~~		~~Equivocal~~	~~Equivocal~~
~~Toxtree~~	~~Cramer class~~		~~III (high)~~	~~III (high)~~
	~~Ames mutagenicity~~		~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~	~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~
	~~*In vivo* mouse micronucleus~~		~~SA 27, nitro aromatic SA 34, H-acceptor~~	~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~
~~Leadscope[a]~~	~~Clasto. *In vitro*~~	~~Chrom Ab CHL v. 2~~	~~Positive~~	~~Positive~~
		~~Chrom Ab CHO v. 2~~	~~Positive~~	~~Positive~~
		~~SCE CHO v. 1~~	~~Positive~~	~~Positive~~
		~~SCE Comp v. 1~~	~~Positive~~	~~Positive~~
		~~SCE Other v. 1~~	~~Positive~~	~~Positive~~
	~~Clasto. *In vivo*~~	~~Chrom Ab Comp v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab Other v. 1~~	~~Negative~~	~~Negative~~

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		~~Chrom Ab rat v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse micronuc v. 2~~	~~Positive~~	~~Positive~~
	~~Gene mutation~~	~~HPRT Mut v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse lymphoma Act v. 2~~	~~Positive~~	~~Positive~~
		~~Mouse lymphoma unact v. 2~~	~~Positive~~	~~Negative~~
		~~Rodent DL mut v. 1~~	~~Negative~~	~~Negative~~
		~~Rodent mut v. 1~~	~~Negative~~	~~Negative~~
		~~E coli – Sal 102 A-T Mut v. 1~~	~~Negative~~	~~Negative~~
		~~Salmonella mut v. 3~~	~~Positive~~	~~Positive~~
~~a Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.~~				

M-02 is a major metabolite in human food of animal origin. See Document MCA-6, Chapter 6.2.1 and Chapter 6.6.1 for levels in plant metabolism and CRC studies. See Document MCA-6, Chapter 6.3.3 and Chapter 6.6.2 for magnitude of residues in crop residue trials and rotational crops.

Genotoxicity alerts: ~~Using Leadscope M-02 has an additional alert for mouse lymphoma that is triggered by the nitro group (mouse lymphoma unact v.2) but is not triggered by aclonifen. Cleavage of the diphenol ether bond is not necessarily the trigger for this alert as most of the metabolites of aclonifen including M-01 have the same alert. Compared to M-01, cleavage of the molecule to M-02 triggers fewer alerts. As the genotoxicity alerts triggered by M-02 are also present in M-01 it is proposed to read-across from M-01 for genotoxicity.~~

~~Most of these alerts are for the same structures as those seen with aclonifen, and thus their relevance in this case is doubted. The alerts for genotoxicity are the same as those triggered by rat metabolite M-01 thus read across from M-01 is proposed.~~

In large part, the genotoxicity alerts provided by Derek Nexus, Toxtree, and Leadscope for M-02 are based on the same alerting structures as those seen with aclonifen. The only alert in M-02 that is not also seen in aclonifen is the Leadscope alert for mouse lymphoma without S9 activation that is triggered by the nitro group (mouse lymphoma unact v.2). Cleavage of the diphenol ether bond is not the trigger for this alert in M-02 as most of the metabolites of aclonifen including M-01 have the same alert. However, the aromatic ring in aclonifen (cleaved off in M-02) was associated with negative predictions, so counteracted the positive feature of the nitro group, and contributed to an overall negative prediction for aclonifen. An additional feature of M-02 triggering the positive prediction in is the number of hydrogen bond donors, which is higher in M-02 compared to aclonifen, probably due to the hydroxyl group (a feature also present in other metabolites with OH groups such as M-01).

The nearest neighbour to M-02 in the training set with a similarity score of 49% (which is a relatively high score) is 4-chloro-2-nitroaniline which tested positive in this assay. Therefore, this alert in M-02 for *in vitro* gene mutation in mammalian cells cannot readily be discounted.

The alerts for genotoxicity are the same as those triggered by rat metabolite M-01 thus read-across from M-01 is proposed. Read-across is appropriate with regards to the Leadscope alert for mouse lymphoma (without S9) which is being triggered by similar structural features in both metabolites,

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firstly the nitro group, but secondly by the number of hydrogen bond donors which is increased in both by the addition of the OH group.

General toxicity alerts: ~~identical to aclonifen~~. M-02 has the same alerts as aclonifen, the exception being an alert for skin sensitisation (837) based on amino or hydroxy aniline structures. Aclonifen does not have this alert but is already classified as a Category 1A for skin sensitisation and the effects of this metabolite would not increase the hazard compared to aclonifen

Conclusions on M-02

With regards to genotoxicity alerts that are not shared by aclonifen, M-02 as same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity. With regards to general toxicity, M-02 has a similar hazard to aclonifen therefore read-across to aclonifen may be applied.

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M-12

The tables below list the alerts triggered by M-12 in the *in silico* tools used, in comparison to those triggered by aclonifen.

Table 5.8.1- 14 *In silico* predictions for M-12 di-hydroxy aclonifen sulphate conjugate 1

Package	Endpoint	M12 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound	Plausible	Plausible
	352 aromatic amine or amide	Plausible	Plausible
	Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Mutagenicity *in vivo* 329 aromatic nitro	Equivocal	Equivocal
	352 aromatic amine or amide	Equivocal	Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine	No alert	Equivocal
	837 amino- or hydroxy-aniline	Plausible	No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative	Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound	Plausible	Plausible
	587 aromatic amide or amide	Plausible	Plausible
	Hepatotoxicity in mammal 544 aromatic nitro compound	Plausible	Plausible
	Splenotoxicity Rapid prototype 152 aniline or precursor	No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation	Equivocal	Equivocal
Toxtree	Cramer class	III (high)	III (high)
	Ames mutagenicity	SA 27, nitro aromatic	SA 27, nitro aromatic
	In vivo mouse micronucleus	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope [a]	Clasto. – Chrom Ab CHL v. 2	Positive	Positive
	In vitro – Chrom Ab CHO v. 2	Positive	Positive
	Clasto. – Chrom Ab Comp v. 2	Negative	Negative

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	In vivo	Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Indeterminate	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative
		Salmonella mut v. 4	Positive	Positive
[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.				

~~Package~~	~~Endpoint~~		~~M12 Prediction~~	~~Aclonifen Prediction~~
~~Derek Nexus genotoxicity~~	~~Ames mutagenicity 329 aromatic nitro compound~~		~~Plausible~~	~~Plausible~~
	~~Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound~~		~~Equivocal~~	~~Plausible~~
	~~Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound~~		~~Equivocal~~	~~Equivocal~~
~~Derek Nexus general toxicity~~	~~Skin sensitization 427 aromatic primary or secondary amine~~		~~Plausible~~	~~Plausible~~
	~~Carcinogenicity in mammal 105 aromatic nitro compound~~		~~Plausible~~	~~Plausible~~
	~~Hepatotoxicity in mammal 544 aromatic nitro compound~~		~~Plausible~~	~~Plausible~~
	~~Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative~~		~~Plausible~~	~~Plausible~~
	~~Teratogenicity Extrapolation from androgen receptor modulation~~		~~Equivocal~~	~~Equivocal~~
~~Toxtree~~	~~Cramer class~~		~~III (high)~~	~~III (high)~~
	~~Ames mutagenicity~~		~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~	~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~
	~~*In vivo* mouse micronucleus~~		~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~	~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~
~~Leadscope[a]~~	~~Clasto. *In vitro*~~	~~Chrom Ab CHL v. 2~~	~~Positive~~	~~Positive~~
		~~Chrom Ab CHO v. 2~~	~~Positive~~	~~Positive~~

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		~~SCE CHO v. 1~~	~~Positive~~	~~Positive~~
		~~SCE Comp v. 1~~	~~Negative~~	~~Positive~~
		~~SCE Other v. 1~~	~~Positive~~	~~Positive~~
	~~Clasto. *In vivo*~~	~~Chrom Ab Comp v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab Other v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab rat v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse micronuc v. 2~~	~~Positive~~	~~Positive~~
	~~Gene mutation~~	~~HPRT Mut v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse lymphoma Act v. 2~~	~~Positive~~	~~Positive~~
		~~Mouse lymphoma unact v. 2~~	~~Positive~~	~~Negative~~
		~~Rodent DL mut v. 1~~	~~Negative~~	~~Negative~~
		~~Rodent mut v. 1~~	~~Negative~~	~~Negative~~
		~~E coli – Sal 102 A-T Mut v. 1~~	~~Negative~~	~~Negative~~
		~~Salmonella mut v. 3~~	~~Positive~~	~~Positive~~
~~[a] Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.~~				

Table 5.8.1- 15 *In silico* predictions for Di-hydroxy aclonifen sulphate conjugate 2

Package	Endpoint	M12 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide	Plausible Plausible	Plausible Plausible
	Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Mutagenicity *in vivo* 329 aromatic nitro 352 aromatic amine or amide	Equivocal Equivocal	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine 837 ortho- or para amino or hydroxy aniline	No alert Plausible	Equivocal No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative	Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide	Plausible Plausible	Plausible Plausible

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Package	Endpoint		M12 Prediction	Aclonifen Prediction
	Hepatotoxicity in mammal 544 aromatic nitro compound		Plausible	Plausible
	Splenotoxicity Rapid prototype 152 aniline or precursor		No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
	Nephrotoxicity in mammal 818 para-aminophenol or derivative		Plausible	No alert
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic	SA 27, nitro aromatic
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope [a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative
		Salmonella mut v. 4	Positive	Positive

[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model

~~Package~~	~~Endpoint~~	~~M12 Prediction~~	~~Aclonifen Prediction~~
~~Derek Nexus genotoxicity~~	~~Ames mutagenicity~~ ~~329 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Chromosome damage *in vitro* (mammal)~~ ~~329 aromatic nitro compound~~	~~Equivocal~~	~~Plausible~~
	~~Chromosome damage *in vivo* (mammal)~~ ~~329 aromatic nitro compound~~	~~Equivocal~~	~~Equivocal~~
~~Derek Nexus~~	~~Carcinogenicity in mammal~~ ~~105 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Skin sensitization~~ ~~837 ortho- or para amino or~~	~~Plausible~~	~~No alert~~

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~~general toxicity~~	~~hydroxy aniline~~			
	~~Skin sensitization 427 aromatic primary or secondary amine~~		~~No alert~~	~~Plausible~~
	~~Hepatotoxicity in mammal 544 aromatic nitro compound~~		~~Plausible~~	~~Plausible~~
	~~Nephrotoxicity in mammal 818 para-aminophenol or derivative~~		~~Plausible~~	~~No alert~~
	~~Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative~~		~~Plausible~~	~~Plausible~~
	~~Teratogenicity Extrapolation from androgen receptor modulation~~		~~Equivocal~~	~~Equivocal~~
~~Toxtree~~	~~Cramer class~~		~~III (high)~~	~~III (high)~~
	~~Ames mutagenicity~~		~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~	~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~
	~~*In vivo* mouse micronucleus~~		~~SA 27, nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy benzene~~	~~SA 27, nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy benzene~~
~~Leadscope[a]~~	~~Clasto. *In vitro*~~	~~Chrom Ab CHL v. 2~~	~~Positive~~	~~Positive~~
		~~Chrom Ab CHO v. 2~~	~~Positive~~	~~Positive~~
		~~SCE CHO v. 1~~	~~Positive~~	~~Positive~~
		~~SCE Comp v. 1~~	~~Negative~~	~~Positive~~
		~~SCE Other v. 1~~	~~Positive~~	~~Positive~~
	~~Clasto. *In vivo*~~	~~Chrom Ab Comp v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab Other v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab rat v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse micronuc v. 2~~	~~Positive~~	~~Positive~~
	~~Gene mutation~~	~~HPRT Mut v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse lymphoma Act v. 2~~	~~Positive~~	~~Positive~~
		~~Mouse lymphoma unact v. 2~~	~~Positive~~	~~Negative~~
		~~Rodent DL mut v. 1~~	~~Negative~~	~~Negative~~
		~~Rodent mut v. 1~~	~~Negative~~	~~Negative~~
		~~E coli Sal 102 A-T Mut v. 1~~	~~Negative~~	~~Negative~~
		~~Salmonella mut v. 3~~	~~Positive~~	~~Positive~~

~~[a] Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.~~

Table 5.8.1- 16 *In silico* predictions for di-hydroxyl aclonifen sulphate conjugate 3

Package	Endpoint	M12 Prediction	Aclonifen Prediction
Derek Nexus	Ames mutagenicity 329 aromatic nitro compound	Plausible	Plausible

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genotoxicity		352 aromatic amine or amide	Plausible	Plausible
		Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
		Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
		Mutagenicity *in vivo* 329 aromatic nitro	Equivocal	Equivocal
		352 aromatic amine or amide	Equivocal	Equivocal
Derek Nexus general toxicity		Skin sensitization 427 aromatic primary or secondary amine	No alert	Equivocal
		837 Amino- or hydroxy-aniline	Plausible	No alert
		Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative	Plausible	Plausible
		Carcinogenicity 105 aromatic nitro compound	Plausible	Plausible
		587 aromatic amide or amide	No alert	Plausible
		Hepatotoxicity in mammal 544 aromatic nitro compound	Plausible	Plausible
		Splenotoxicity Rapid prototype 152 aniline or precursor	No alert	Equivocal
		Teratogenicity Extrapolation from androgen receptor modulation	Equivocal	Equivocal
Toxtree		Cramer class	III (high)	III (high)
		Ames mutagenicity	SA 27, nitro aromatic	SA 27, nitro aromatic
		In vivo mouse micronucleus	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope [a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Indeterminate	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative
		Salmonella mut v. 4	Positive	Positive

[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

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Package	Endpoint		M12 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound		Plausible	Plausible
	Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound		Equivocal	Plausible
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound		Equivocal	Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine		Plausible	Plausible
	Carcinogenicity in mammal 105 aromatic nitro compound		Plausible	Plausible
	Hepatotoxicity in mammal 544 aromatic nitro compound		Plausible	Plausible
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic (Q)SAR6, aromatic amines	SA 27, nitro aromatic (Q)SAR6, aromatic amines
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope[a]	Clasto, *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
		SCE CHO v. 1	Positive	Positive
		SCE Comp v. 1	Negative	Positive
		SCE Other v. 1	Positive	Positive
	Clasto, *In vivo*	Chrom Ab Comp v. 1	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 1	Indeterminate	Positive
		E coli – Sal 102 A-T Mut v. 1	Negative	Negative
		Salmonella mut v. 3	Positive	Positive

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~~[a] Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.~~

Table 5.8.1- 17 ***In silico*** **predictions for Di-hydroxy aclonifen sulphate conjugate 4**

Package	Endpoint	M12 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound	Plausible	Plausible
	352 aromatic amine or amide	Plausible	Plausible
	Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Mutagenicity *in vivo* 329 aromatic nitro	Equivocal	Equivocal
	352 aromatic amine or amide	Equivocal	Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine	No alert	Equivocal
	837 ortho or para amino or hydroxy aniline	Plausible	No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative	Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound	Plausible	Plausible
	587 aromatic amide or amide	No alert	Plausible
	Hepatotoxicity in mammal 544 aromatic nitro compound	Plausible	Plausible
	Splenotoxicity Rapid prototype 152 aniline or precursor	No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation	Equivocal	Equivocal
	Nephrotoxicity in mammal 818 para-aminophenol or derivative	Plausible	No alert
Toxtree	Cramer class	III (high)	III (high)
	Ames mutagenicity	SA 27, nitro aromatic	SA 27, nitro aromatic
	In vivo mouse micronucleus	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene

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Leadscope [a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative
		Salmonella mut v. 4	Positive	Positive

[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

~~Package~~	~~Endpoint~~	~~M12 Prediction~~	~~Aclonifen Prediction~~
~~Derek Nexus genotoxicity~~	~~Ames mutagenicity 329 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound~~	~~Equivocal~~	~~Plausible~~
	~~Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound~~	~~Equivocal~~	~~Equivocal~~
~~Derek Nexus general toxicity~~	~~Skin sensitization 837 ortho or para amino or hydroxy aniline~~	~~Plausible~~	~~No alert~~
	~~Skin sensitization 427 aromatic primary or secondary amine~~	~~No alert~~	~~Plausible~~
	~~Carcinogenicity in mammal 105 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Hepatotoxicity in mammal 544 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Nephrotoxicity in mammal 818 para-aminophenol or derivative~~	~~plausible~~	~~No alert~~
	~~Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative~~	~~Plausible~~	~~Plausible~~
	~~Teratogenicity Extrapolation from androgen receptor modulation~~	~~Equivocal~~	~~Equivocal~~
~~Toxtree~~	~~Cramer class~~	~~III (high)~~	~~III (high)~~
	~~Ames mutagenicity~~	~~SA 27, nitro aromatic~~	~~SA 27, nitro aromatic~~

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			~~(Q)SAR6, aromatic amines~~	~~(Q)SAR6, aromatic amines~~
	~~*In vivo* mouse micronucleus~~		~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~	~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~
~~Leadscope[a]~~	~~Clasto. *In vitro*~~	~~Chrom Ab CHL v. 2~~	~~Positive~~	~~Positive~~
		~~Chrom Ab CHO v. 2~~	~~Positive~~	~~Positive~~
		~~SCE CHO v. 1~~	~~Positive~~	~~Positive~~
		~~SCE Comp v. 1~~	~~Negative~~	~~Positive~~
		~~SCE Other v. 1~~	~~Positive~~	~~Positive~~
	~~Clasto. *In vivo*~~	~~Chrom Ab Comp v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab Other v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab rat v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse micronuc v. 2~~	~~Positive~~	~~Positive~~
	~~Gene mutation~~	~~HPRT Mut v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse lymphoma Act v. 2~~	~~Positive~~	~~Positive~~
		~~Mouse lymphoma unact v. 2~~	~~Positive~~	~~Negative~~
		~~Rodent DL mut v. 1~~	~~Negative~~	~~Negative~~
		~~Rodent mut v. 1~~	~~Negative~~	~~Negative~~
		~~Bacterial Mut v. 1~~	~~Negative~~	~~Negative~~
		~~E coli / Sal 102 A-T Mut v. 1~~	~~Positive~~	~~Positive~~

~~[a] Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.~~

Table 5.8.1- 18 *In silico* predictions for Di-hydroxy aclonifen sulphate conjugate 5

Package	Endpoint	M12 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide	Plausible Plausible	Plausible Plausible
	Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Mutagenicity *in vivo* 329 aromatic nitro 352 aromatic amine or amide	Equivocal Equivocal	Equivocal Equivocal
Derek Nexus general	Skin sensitization 427 aromatic primary or secondary amine 837 ortho or para amino or	No alert	Equivocal

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toxicity	hydroxy aniline		Plausible	No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide		Plausible No alert	Plausible Plausible
	Hepatotoxicity in mammal 544 aromatic nitro compound		Plausible	Plausible
	Splenotoxicity Rapid prototype 152 aniline or precursor		No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic	SA 27, nitro aromatic
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope [a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Indeterminate	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative
		Salmonella mut v. 4	Positive	Positive

[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

~~Package~~	~~Endpoint~~	~~M12 Prediction~~	~~Aclonifen Prediction~~
~~Derek Nexus genotoxicity~~	~~Ames mutagenicity~~ ~~329 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Chromosome damage *in vitro* (mammal)~~ ~~329 aromatic nitro compound~~	~~Equivocal~~	~~Plausible~~
	~~Chromosome damage *in vivo*~~	~~Equivocal~~	~~Equivocal~~

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	(mammal) 329 aromatic nitro compound			
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine		Plausible	Plausible
	Carcinogenicity in mammal 105 aromatic nitro compound		Plausible	Plausible
	Hepatotoxicity in mammal 544 aromatic nitro compound		Plausible	Plausible
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic (Q)SAR6, aromatic amines	SA 27, nitro aromatic (Q)SAR6, aromatic amines
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
		SCE CHO v. 1	Positive	Positive
		SCE Comp v. 1	Negative	Positive
		SCE Other v. 1	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 1	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 1	Negative	Negative
		E coli - Sal 102 A-T Mut v. 1	Positive	Positive

[a] Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

Table 5.8.1- 19 *In silico* predictions for Di-hydroxy aclonifen sulphate conjugate 6

Package	Endpoint	M12 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound	Plausible	Plausible

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	352 aromatic amine or amide		Plausible	Plausible
	Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound		Equivocal	Equivocal
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound		Equivocal	Equivocal
	Mutagenicity *in vivo* 329 aromatic nitro		Equivocal	Equivocal
	352 aromatic amine or amide		Equivocal	Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine		No alert	Equivocal
	837 ortho- or para amino or hydroxy aniline		Plausible	No alert
	Androgen receptor modulation 852 [1,1’-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound		Plausible	Plausible
	587 aromatic amide or amide		No alert	Plausible
	Hepatotoxicity in mammal 544 aromatic nitro compound		Plausible	Plausible
	Splenotoxicity Rapid prototype 152 aniline or precursor		No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic	SA 27, nitro aromatic
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope [a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative
		Salmonella mut v. 4	Positive	Positive

[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

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~~**Package**~~	~~**Endpoint**~~	~~**M12 Prediction**~~	~~**Aclonifen Prediction**~~
~~Derek Nexus genotoxicity~~	~~Ames mutagenicity 329 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound~~	~~Equivocal~~	~~Plausible~~
	~~Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound~~	~~Equivocal~~	~~Equivocal~~
~~Derek Nexus general toxicity~~	~~Skin sensitization 837 ortho- or para amino or hydroxy aniline~~	~~Plausible~~	~~No alert~~
	~~Skin sensitization 427 aromatic primary or secondary amine~~	~~No alert~~	~~Plausible~~
	~~Carcinogenicity in mammal 105 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Hepatotoxicity in mammal 544 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Nephrotoxicity in mammal 813 para-aminophenol or derivative~~	~~Plausible~~	~~No alert~~
	~~Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative~~	~~Plausible~~	~~Plausible~~
	~~Teratogenicity Extrapolation from androgen receptor modulation~~	~~Equivocal~~	~~Equivocal~~
~~Toxtree~~	~~Cramer class~~	~~III (high)~~	~~III (high)~~
	~~Ames mutagenicity~~	~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~	~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~
	~~*In vivo* mouse micronucleus~~	~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~	~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~
~~Leadscope[a]~~	~~Clasto, *In vitro*: Chrom Ab CHL v. 2~~	~~Positive~~	~~Positive~~
	~~Clasto, *In vitro*: Chrom Ab CHO v. 2~~	~~Positive~~	~~Positive~~
	~~Clasto, *In vitro*: SCE CHO v. 1~~	~~Positive~~	~~Positive~~
	~~Clasto, *In vitro*: SCE Comp v. 1~~	~~Negative~~	~~Positive~~
	~~Clasto, *In vitro*: SCE Other v. 1~~	~~Positive~~	~~Positive~~
	~~Clasto, *In vivo*: Chrom Ab Comp v. 1~~	~~Negative~~	~~Negative~~
	~~Clasto, *In vivo*: Chrom Ab Other v. 1~~	~~Negative~~	~~Negative~~
	~~Clasto, *In vivo*: Chrom Ab rat v. 1~~	~~Negative~~	~~Negative~~
	~~Clasto, *In vivo*: Mouse micronuc v. 2~~	~~Positive~~	~~Positive~~
	~~Gene mutation: HPRT Mut v. 1~~	~~Negative~~	~~Negative~~
	~~Gene mutation: Mouse lymphoma Act v. 2~~	~~Positive~~	~~Positive~~

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		~~Mouse lymphoma unact v. 2~~	~~Positive~~	~~Negative~~
		~~Rodent DL mut v. 1~~	~~Negative~~	~~Negative~~
		~~Rodent mut v. 1~~	~~Negative~~	~~Negative~~
		~~Bacterial Mut v. 1~~	~~Negative~~	~~Negative~~
		~~E coli – Sal 102 A-T Mut v. 1~~	~~Positive~~	~~Positive~~
~~[a] Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.~~				

Table 5.8.1- 20 ***In silico*** **predictions for Di-hydroxy aclonifen sulphate conjugate 7**

Package	**Endpoint**	**M12 Prediction**	**Aclonifen Prediction**
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound	Plausible	Plausible
	352 aromatic amine or amide	Plausible	Plausible
	Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Mutagenicity *in vivo* 329 aromatic nitro	Equivocal	Equivocal
	352 aromatic amine or amide	Equivocal	Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine	No alert	Equivocal
	837 ortho- or para amino or hydroxy aniline	Plausible	No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative	Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound	Plausible	Plausible
	587 aromatic amide or amide	No alert	Plausible
	Hepatotoxicity in mammal 544 aromatic nitro compound	Plausible	Plausible
	Splenotoxicity Rapid prototype 152 aniline or precursor	No alert	Equivocal
	Teratogenicity Extrapolation from androgen	Equivocal	Equivocal

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	receptor modulation			
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic	SA 27, nitro aromatic
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope [a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Indeterminate	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative
		Salmonella mut v. 4	Positive	Positive

[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

~~Package~~	~~Endpoint~~	~~M12 Prediction~~	~~Aclonifen Prediction~~
~~Derek Nexus genotoxicity~~	~~Ames mutagenicity 329 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound~~	~~Equivocal~~	~~Plausible~~
	~~Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound~~	~~Equivocal~~	~~Equivocal~~
~~Derek Nexus general toxicity~~	~~Skin sensitization 427 aromatic primary or secondary amine~~	~~Plausible~~	~~Plausible~~
	~~Carcinogenicity in mammal 105 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Hepatotoxicity in mammal 544 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative~~	~~Plausible~~	~~Plausible~~
	~~Teratogenicity Extrapolation from androgen receptor modulation~~	~~Equivocal~~	~~Equivocal~~

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~~Toxtree~~	~~Cramer class~~		~~III (high)~~	~~III (high)~~
	~~Ames mutagenicity~~		~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~	~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~
	~~*In vivo* mouse micronucleus~~		~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~	~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~
~~Leadscope~~[a]	~~Clasto. *In vitro*~~	~~Chrom Ab CHL v. 2~~	~~Positive~~	~~Positive~~
		~~Chrom Ab CHO v. 2~~	~~Positive~~	~~Positive~~
		~~SCE CHO v. 1~~	~~Positive~~	~~Positive~~
		~~SCE Comp v. 1~~	~~Negative~~	~~Positive~~
		~~SCE Other v. 1~~	~~Positive~~	~~Positive~~
	~~Clasto. *In vivo*~~	~~Chrom Ab Comp v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab Other v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab rat v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse micronuc v. 2~~	~~Positive~~	~~Positive~~
	~~Gene mutation~~	~~HPRT Mut v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse lymphoma Act v. 2~~	~~Positive~~	~~Positive~~
		~~Mouse lymphoma unact v. 2~~	~~Positive~~	~~Negative~~
		~~Rodent DL mut v. 1~~	~~Negative~~	~~Negative~~
		~~Rodent mut v. 1~~	~~Negative~~	~~Negative~~
		~~Bacterial Mut v. 1~~	~~Negative~~	~~Negative~~
		~~E coli – Sal 102 A-T Mut v. 1~~	~~Positive~~	~~Positive~~

~~[a] Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.~~

Table 5.8.1- 21 *In silico* predictions for Di-hydroxy aclonifen sulphate conjugate 8

Package	Endpoint	M12 Prediction	Aclonifen Prediction
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide	Plausible Plausible	Plausible Plausible
	Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Mutagenicity *in vivo* 329 aromatic nitro 352 aromatic amine or amide	Equivocal Equivocal	Equivocal Equivocal
Derek Nexus	Skin sensitization 427 aromatic primary or secondary amine	No alert	Equivocal

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Package	Endpoint		M12 Prediction	Aclonifen Prediction
general toxicity	837 ortho or para amino or hydroxy aniline		Plausible	No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound		Plausible	Plausible
	587 aromatic amide or amide		No alert	Plausible
	Hepatotoxicity in mammal 329 aromatic nitro compound		Plausible	Plausible
	Splenotoxicity Rapid prototype 152 aniline or precursor		No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
	Nephrotoxicity in mammal 818 para-aminophenol or derivative		Plausible	No alert
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic	SA 27, nitro aromatic
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative
		Salmonella mut v. 4	Positive	Positive

[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

~~Package~~	~~Endpoint~~	~~M12 Prediction~~	~~Aclonifen Prediction~~
~~Derek Nexus~~	~~Ames mutagenicity~~ ~~329 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Chromosome damage *in vitro*~~	~~Equivocal~~	~~Plausible~~

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genotoxicity	(mammal) 329 aromatic nitro compound			
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound		Equivocal	Equivocal
Derek Nexus general toxicity	Skin sensitization 837 ortho or para amino or hydroxy aniline		Plausible	Plausible
	Skin sensitization 427 aromatic primary or secondary amine		No alert	Plausible
	Carcinogenicity in mammal 105 aromatic nitro compound		Plausible	Plausible
	Hepatotoxicity in mammal 329 aromatic nitro compound		Plausible	Plausible
	Nephrotoxicity in mammal 818 para-aminophenol or derivative		Plausible	No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic (Q)SAR6, aromatic amines	SA 27, nitro aromatic (Q)SAR6, aromatic amines
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H acceptor SA 33, 1-phenoxy benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
		SCE CHO v. 1	Positive	Positive
		SCE Comp v. 1	Negative	Positive
		SCE Other v. 1	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 1	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 1	Negative	Negative
		E coli – Sal 102 A-T Mut v. 1	Positive	Positive

[a] Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

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Table 5.8.1- 22 *In silico* predictions for Dihydroxy aclonifen sulphate conjugate 9

Package	Endpoint	M12 Prediction	Aclonifen Prediction

Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound 352 aromatic amine or amide		Plausible Plausible	Plausible Plausible
	Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound		Equivocal	Equivocal
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound		Equivocal	Equivocal
	Mutagenicity *in vivo* 329 aromatic nitro 352 aromatic amine or amide		Equivocal Equivocal	Equivocal Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine 837 ortho or para amino or hydroxy aniline		No alert Plausible	Equivocal No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative		Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound 587 aromatic amide or amide		Plausible No alert	Plausible Plausible
	Hepatotoxicity in mammal 544 aromatic nitro compound		Plausible	Plausible
	Splenotoxicity Rapid prototype 152 aniline or precursor		No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation		Equivocal	Equivocal
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic	SA 27, nitro aromatic
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope [a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Indeterminate	Negative

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		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative
		Salmonella mut v. 4	Positive	Positive

[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

~~Package~~	~~Endpoint~~		~~M12 Prediction~~	~~Aclonifen Prediction~~
~~Derek Nexus genotoxicity~~	~~Ames mutagenicity 329 aromatic nitro compound~~		~~Plausible~~	~~Plausible~~
	~~Chromosome damage invitro (mammal) 329 aromatic nitro compound~~		~~Equivocal~~	~~Plausible~~
	~~Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound~~		~~Equivocal~~	~~Equivocal~~
~~Derek Nexus general toxicity~~	~~Skin sensitization 427 aromatic primary or secondary amine~~		~~Plausible~~	~~Plausible~~
	~~Carcinogenicity in mammal 105 aromatic nitro compound~~		~~Plausible~~	~~Plausible~~
	~~Hepatotoxicity in mammal 544 aromatic nitro compound~~		~~Plausible~~	~~Plausible~~
	~~Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative~~		~~Plausible~~	~~Plausible~~
	~~Teratogenicity Extrapolation from androgen receptor modulation~~		~~Equivocal~~	~~Equivocal~~
~~Toxtree~~	~~Cramer class~~		~~III (high)~~	~~III (high)~~
	~~Ames mutagenicity~~		~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~	~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~
	~~*In vivo* mouse micronucleus~~		~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~	~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~
~~Leadscope[a]~~	~~Clasto. *In vitro*~~	~~Chrom Ab CHL v. 2~~	~~Positive~~	~~Positive~~
		~~Chrom Ab CHO v. 2~~	~~Positive~~	~~Positive~~
		~~SCE CHO v. 1~~	~~Positive~~	~~Positive~~
		~~SCE Comp v. 1~~	~~Negative~~	~~Positive~~
		~~SCE Other v. 1~~	~~Positive~~	~~Positive~~
	~~Clasto. *In vivo*~~	~~Chrom Ab Comp v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab Other v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab rat v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse micronuc v. 2~~	~~Positive~~	~~Positive~~
	~~Gene mutation~~	~~HPRT Mut v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse lymphoma Act v. 2~~	~~Positive~~	~~Positive~~

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		~~Mouse lymphoma unact v. 2~~	~~Positive~~	~~Negative~~
		~~Rodent DL mut v. 1~~	~~Negative~~	~~Negative~~
		~~Rodent mut v. 1~~	~~Negative~~	~~Negative~~
		~~Bacterial Mut v. 1~~	~~Negative~~	~~Negative~~
		~~E coli – Sal 102 A-T Mut v.~~	~~Positive~~	~~Positive~~

~~[a] Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.~~

Table 5.8.1- 23 ***In silico*** **predictions for Di-hydroxy aclonifen sulphate conjugate 10**

Package	**Endpoint**	**M12 Prediction**	**Aclonifen Prediction**
Derek Nexus genotoxicity	Ames mutagenicity 329 aromatic nitro compound	Plausible	Plausible
	352 aromatic amine or amide	Plausible	Plausible
	Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound	Equivocal	Equivocal
	Mutagenicity *in vivo* 329 aromatic nitro	Equivocal	Equivocal
	352 aromatic amine or amide	Equivocal	Equivocal
Derek Nexus general toxicity	Skin sensitization 427 aromatic primary or secondary amine	No alert	Equivocal
	837 ortho or para amino or hydroxy aniline	Plausible	No alert
	Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile or derivative	Plausible	Plausible
	Carcinogenicity 105 aromatic nitro compound	Plausible	Plausible
	587 aromatic amide or amide	No alert	Plausible
	Hepatotoxicity in mammal 544 aromatic nitro compound	Plausible	Plausible
	Splenotoxicity Rapid prototype 152 aniline or precursor	No alert	Equivocal
	Teratogenicity Extrapolation from androgen receptor modulation	Equivocal	Equivocal
	Nephrotoxicity in mammal 818 para-aminophenol derivative	Plausible	No alert

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Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		SA 27, nitro aromatic	SA 27, nitro aromatic
	In vivo mouse micronucleus		SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene	SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene
Leadscope[a]	Clasto. *In vitro*	Chrom Ab CHL v. 2	Positive	Positive
		Chrom Ab CHO v. 2	Positive	Positive
	Clasto. *In vivo*	Chrom Ab Comp v. 2	Negative	Negative
		Chrom Ab Other v. 1	Negative	Negative
		Chrom Ab rat v. 1	Negative	Negative
		Mouse micronuc v. 2	Positive	Positive
	Gene mutation	HPRT Mut v. 1	Negative	Negative
		Mouse lymphoma Act v. 2	Positive	Positive
		Mouse lymphoma unact v. 2	Positive	Negative
		Rodent DL mut v. 1	Negative	Negative
		Rodent mut v. 1	Negative	Negative
		Bacterial Mut v. 2	Positive	Positive
		E coli – Sal 102 A-T Mut v. 2	Negative	Negative
		Salmonella mut v. 4	Positive	Positive

[a] Cells coloured grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.

~~Package~~	~~Endpoint~~	~~M12 Prediction~~	~~Aclonifen Prediction~~
~~Derek Nexus genotoxicity~~	~~Ames mutagenicity 329 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Chromosome damage *in vitro* (mammal) 329 aromatic nitro compound~~	~~Equivocal~~	~~Plausible~~
	~~Chromosome damage *in vivo* (mammal) 329 aromatic nitro compound~~	~~Equivocal~~	~~Equivocal~~
~~Derek Nexus general toxicity~~	~~Skin sensitization 837 ortho or para amino or hydroxy aniline~~	~~Plausible~~	~~Plausible~~
	~~Skin sensitization 427 aromatic primary or secondary amine~~	~~No alert~~	~~Plausible~~
	~~Carcinogenicity in mammal 105 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Hepatotoxicity in mammal 544 aromatic nitro compound~~	~~Plausible~~	~~Plausible~~
	~~Nephrotoxicity in mammal 818 para-aminophenol derivative~~	~~Plausible~~	~~Plausible~~
	~~Androgen receptor modulation 852 [1,1'-biphenyl]-4-carbonitrile~~	~~Plausible~~	~~Plausible~~

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	~~or derivative~~			
	~~Teratogenicity Extrapolation from androgen receptor modulation~~		~~Equivocal~~	~~Equivocal~~
~~Toxtree~~	~~Cramer class~~		~~III (high)~~	~~III (high)~~
~~Toxtree~~	~~Ames mutagenicity~~		~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~	~~SA 27, nitro aromatic (Q)SAR6, aromatic amines~~
~~Toxtree~~	~~*In vivo* mouse micronucleus~~		~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~	~~SA 27, nitro aromatic SA 34, H-acceptor SA 33, 1-phenoxy benzene~~
~~Leadscope[a]~~	~~Clasto. *In vitro*~~	~~Chrom Ab CHL v. 2~~	~~Positive~~	~~Positive~~
		~~Chrom Ab CHO v. 2~~	~~Positive~~	~~Positive~~
		~~SCE CHO v. 1~~	~~Positive~~	~~Positive~~
		~~SCE Comp v. 1~~	~~Negative~~	~~Positive~~
		~~SCE Other v. 1~~	~~Positive~~	~~Positive~~
	~~Clasto. *In vivo*~~	~~Chrom Ab Comp v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab Other v. 1~~	~~Negative~~	~~Negative~~
		~~Chrom Ab rat v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse micronuc v. 2~~	~~Positive~~	~~Positive~~
	~~Gene mutation~~	~~HPRT Mut v. 1~~	~~Negative~~	~~Negative~~
		~~Mouse lymphoma Act v. 2~~	~~Positive~~	~~Positive~~
		~~Mouse lymphoma unact v. 2~~	~~Positive~~	~~Negative~~
		~~Rodent DL mut v. 1~~	~~Negative~~	~~Negative~~
		~~Rodent mut v. 1~~	~~Negative~~	~~Negative~~
		~~Bacterial Mut v. 1~~	~~Negative~~	~~Negative~~
		~~E coli – Sal 102 A/T Mut v. 1~~	~~Positive~~	~~Positive~~
~~[a] Cells colored grey in the prediction column indicate that the evaluated substance is outside of the applicability domain for that particular model.~~				

M-12 is a major metabolite in human food of animal origin. See Document MCA-6, Chapter 6.2.2 for levels in hen metabolism study.

Genotoxicity alerts: All 10 variants of the hydroxy and sulphate groups show the same predictions for genotoxicity. The only genotoxicity alert triggered by M-12 that is not present in aclonifen is a Leadscope positive alert for gene mutation *in vitro* in the mouse lymphoma assay without S9 activation (Mouse lymphoma unact v.2) for all variants of this M-12 metabolite. This prediction is largely based on the nitro group and the nitrobenzene scaffold but for aclonifen this group is not sufficient to trigger an overall positive prediction outcome in the software. The reason for this positive prediction for variants of M-12 and not for aclonifen is mainly due to the additional hydroxy group on M-12 which triggers an alert based on two oxygens attached to an aromatic ring. This alerting structure is shown in the figure below:

In addition, the sulphate group is also acting as an alert based on the structure shown below

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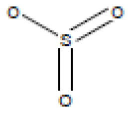

However none of the nearest neighbours in the training set contain either of these features casting doubt on the relevance of this prediction. Instead M-12 has closer similarity to aclonifen, therefore read-across to aclonifen is more appropriate considering structural similarity and overall similarity of all other QSAR predictions.

Because the alerting structures for genotoxicity and carcinogenicity are identical between aclonifen and the rat metabolite M-01, the genotoxicity data from aclonifen can be bridged to this metabolite.

General toxicity alerts: The varying structures of this metabolite have largely the same *in silico* alerts. All of them are predicted to be carcinogenic and hepatotoxic, with both alerts due to the presence of an aromatic nitro group. This same nitro group is present in aclonifen and thus the potential systemic toxicity of the metabolites can be drawn from the known systemic toxicity of aclonifen.

Four of the variants trigger an alert for nephrotoxicity (alert 818) based on the presence of a para-aminophenol or derivative, an alert that is not shared with aclonifen. This is a human-relevant alert, however the nephrotoxic effects of para-aminophenols and similar compounds is a high-dose phenomenon that would not be seen after consumption of negligible levels of metabolites such as these (see MCA-6, Chapter 6.2.2 for levels in hen metabolism study). At high doses the kidney was a target organ for aclonifen, so unless exposure to these metabolites is high, these alerts do not trigger concern and would not be expected to pose an increased hazard compared to the parent compound.

All variants of M-12 also have an alert for skin sensitisation (837) based on amino or hydroxy aniline structures. Aclonifen does not have this alert but is already classified as a Category 1A for skin sensitisation therefore the effects of these metabolites would not increase the hazard compared to aclonifen.

~~Genotoxicity alerts: For bacterial mutagenesis, nearly all *in silico* tools used predicted that these variant structures would be mutagenic in the Ames assay. This mutagenicity is primarily due to the presence of the aromatic nitro group which also trigger the same alerts in aclonifen.~~

~~Mammalian mutagenicity is largely predicted to be negative, although the mouse lymphoma models in Leadscope predict a positive alert for mouse lymphoma (Mouse lymphoma unact v.2) that is not present in aclonifen for all variants of this M-12 metabolite. This prediction is largely based on the nitro group and the nitrobenzene scaffold. Aclonifen also has Leadscope alert for mouse lymphoma (mouse lymphoma Act v. 2) again triggered by the nitro group.~~

~~For clastogenicity, the *in vitro* alerts for chromosome damage in Derek Nexus and Leadscope predict that these structures will be clastogenic, again due to the nitro structure or the nitrobenzene scaffold. For *in vivo* clastogenicity, the alert in Derek Nexus is based on the presence of the aromatic nitro, but has a rating only of equivocal. In Leadscope, only the mouse micronucleus model predicts a positive response for clastogenicity. The alerts for clastogenicity are all the same as those triggered by aclonifen.~~

~~Because the alerting structures for genotoxicity and carcinogenicity are identical between aclonifen and the rat metabolite M-01, the genotoxicity data from aclonifen can be bridged to this metabolite.~~

~~General toxicity alerts: The varying structures of this metabolite have largely the same *in silico* alerts. All of them are predicted to be carcinogenic and hepatotoxic, with both alerts due to the presence of an~~

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~~aromatic nitro group. This same nitro group is present in aclonifen and thus the potential systemic toxicity of the metabolites can be drawn from the known systemic toxicity of aclonifen.~~

~~Five of the variants trigger an alert for nephrotoxicity (alert 818) based on the presence of a para-aminophenol or derivative, an alert that is not shared with aclonifen. This is a human relevant alert, however the nephrotoxic effects of para-aminophenols and similar compounds is a high dose phenomenon that would not be seen after consumption of negligible levels of metabolites such as these (see MCA-6, Chapter 6.2.2 for levels in hen metabolism study). At high doses the kidney was a target organ for aclonifen, so unless exposure to these metabolites is high, these alerts do not trigger concern and would not be expected to pose an increased hazard compared to the parent compound.~~

~~The same variants that have the alert for nephrotoxicity also have an alert for skin sensitisation (837) based on ortho or para amino or hydroxy aniline structures. Aclonifen does not have this alert but is already classified as a Category 1A for skin sensitisation therefore the effects of these metabolites would not increase the hazard compared to aclonifen.~~

Conclusions on M-12

~~Aclonifen is itself already classified as a Category 1A skin sensitiser and has effects on the kidneys at high doses. Therefore general toxicity effects of M-12 are similar to aclonifen. Overall M-12 is not expected be more hazardous for general toxicity than the parent compound aclonifen.~~

~~With regards to genotoxicity alerts that are not shared by aclonifen, M-12 has same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity.~~

With regards to genotoxicity alerts that are not shared by aclonifen, M-12 has same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity.

With regards to general toxicity there is an alert for nephrotoxicity not triggered by aclonifen, but since aclonifen already has effects on the kidneys at high doses. Therefore general toxicity effects of M-12 are similar to aclonifen. Overall M-12 is not expected be more hazardous for general toxicity than the parent compound aclonifen.

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Grouping of metabolites

The table below summarises the (Q)SAR alerts that are triggered by dietary metabolites and not triggered by aclonifen. The aim was to group the metabolites according to similarity of (Q)SAR alerts to allow read-across. The lead metabolite in a group is the one with the most alerts so raises most concern. All other metabolites in the same group have the same (or fewer) alerts.

Table 5.8.1- 24 Summary of (Q)SAR alerts in dietary metabolism of aclonifen and proposals for read-across

Metabolite	(Q)SAR alerts present in metabolite but not in aclonifen	Comments	Group allocation
Aclonifen			
M-01 Major metabolite in animal feed	Genotoxicity alerts 1.Derek Nexus alert 329 (aromatic nitro compound) for chromosome damage *in vitro* – plausible. 2.Derek nexus alert 470 (phenol) for chromosome damage *in vitro* – plausible. 3.Derek nexus alert 470 (phenol) for chromosome damage *in vivo* – equivocal. 4.Leadscope mouse lymphoma alert (unact v.2) - triggered by the NO_2 group. General toxicity alerts No alerts for general toxicity	Genotoxicity alerts M-01 has four alerts for genotoxicity that are not present in aclonifen. M-01 is a major intermediary in metabolism of aclonifen therefore is covered by toxicity studies in rats. M-01 is a rat metabolite found at >10% TRR in plasma. It is a major intermediary in the metabolism of aclonifen with all rat metabolites being hydroxylated in the phenyl ring followed by conjugation. In particular the major metabolite in M-09, present at >10% administered dose in urine in all metabolism studies is formed via M-01.	Group 1 lead metabolite – a rat metabolite so can read across to studies conducted on aclonifen.
M-02 Major metabolite in human food of animal origin Not a rat metabolite	Genotoxicity alerts 1.Leadscope mouse lymphoma alert (unact v.2)- triggered by the NO_2 group. General toxicity alerts ~~No alerts for general toxicity~~ 1. Derek Nexus alert 837	Genotoxicity alerts ~~Although aclonifen also has the same NO_2 group the alert is not triggered. Aclonifen has a similar alert for mouse lymphoma (act v. 2) that is also triggered by the NO_2 group.~~ M-02 has one alert for genotoxicity not present in aclonifen. Compared to M-01, cleavage of the molecule to M-02 triggers fewer alerts. General toxicity alerts Aclonifen is itself already classified as a Category 1A skin	Group 1 For genotoxicity has same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity. For general toxicity M-2 can be compared to aclonifen.

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	(amino- or hydroxy-aniline) for skin sensitisation - plausible	sensitizer. Therefore general toxicity effects of M-2 are similar to aclonifen. Overall M-2 is not expected be more hazardous for general toxicity than the parent compound aclonifen.	
M-05 Major metabolite in animal feed	<u>Genotoxicity alerts</u> 1.Leadscope mouse lymphoma alert (unact v.2) triggered by the NO_2 group. <u>General toxicity alerts</u> No alerts for general toxicity	<u>Genotoxicity alerts</u> ~~See comment under M-02 regarding mouse lymphoma alert.~~ Mouse lymphoma alert is not reliable and can be dismissed (no close matches in training set). M-05 is a glucoside conjugate of M-01 with malonyl conjugated onto the glucoside. A glucoside is a type of glycoside where glucose is the conjoining sugar, with a malonyl group conjugated to the glycoside. Malonyl conjugates of glycosides are common plant metabolites as part of detoxification process. As such this metabolite is considered of minimal toxicological concern.	Group 1 Has same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity.
M-07 Major metabolite in human food of animal origin A rat metabolite at low levels in faeces.	<u>Genotoxicity alerts</u> 1. Derek Nexus alert 470 (phenol) for *in vitro* mutagenicity in mammals – plausible. 2. Derek nexus alert 470 (phenol) for *in vivo* chromosome damage equivocal. <u>General toxicity alerts</u> 1. Derek Nexus alert 694 (benzimidazole) for hepatoxicity - plausible	<u>Genotoxicity alerts</u> Hydroxylation of the benzene ring gives rise to phenol or quinone-like structure which triggers alerts 470. This alert is also triggered by M-01. <u>General toxicity alerts</u> Hepatoxicity alert 694 triggered by benzimidazole structure. This alert is not triggered by aclonifen or any other rat metabolites. Aclonifen does have an alert for hepatoxicity (544 but triggered by the aromatic nitro structure). M-07 a major metabolite in goat kidney, but all tissue residues <0.01 mg eq/kg in goat dosed at 7X maximum dietary burden, therefore this alert is not a concern due to low exposure levels.	Group 1 Has same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity. The exception is the alert for hepatoxicity however at low exposure levels this alert would not be a concern.
M-09 Minor metabolite Found in hen (exact position	No alerts except the same ones as aclonifen.	Major rat metabolite (>10% administered dose in urine in all metabolism studies) therefore covered by toxicity studies conducted on aclonifen.	Group 1 There are no toxicity concerns for this metabolite due to no alerts of concern and it being a major rat

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of hydroxyl group not fully established). Also a major rat metabolite in urine (>10% administered dose)			metabolite.
M-12 10 variants of this structure - Exact position of OH and SO_4 groups not known Major metabolite in human food of animal origin	Genotoxicity alerts 1 Leadscope mouse lymphoma alert (unact v.2) triggered by the NO_2 group. General toxicity alerts 1. Derek Nexus alert 818 for nephrotoxicity – plausible. 2. Derek Nexus alert 837 for skin sensitisation – plausible.	Genotoxicity alerts See comment under M-02 regarding mouse lymphoma alert. General toxicity alerts Aclonifen is itself already classified as a Category 1A skin sensitiser and has effects the kidneys at high doses. Therefore general toxicity effects of M-12 are similar to aclonifen. Overall M-12 is not expected be more hazardous for general toxicity than the parent compound aclonifen.	Group 1 Has same (or fewer) alerts for genotoxicity as M-01 therefore may be grouped with M-01 for read-across for genotoxicity. For general toxicity M-12 can be compared to aclonifen.
M-17 Minor metabolite	Genotoxicity alerts 1.Leadscope mouse lymphoma alert (unact v.2) triggered by the NO_2 group. General toxicity alerts No alerts for general toxicity.	Genotoxicity alerts ~~See comment under M-02 regarding mouse lymphoma alert.~~ Mouse lymphoma alert is not reliable and can be dismissed (nearest neighbour in training set tested negative). M-17 is a glucoside conjugate of M-20 with malonyl conjugated onto the glucoside. A glucoside is a type of glycoside where glucose is the conjoining sugar, with a malonyl group conjugated to the glycoside. Malonyl conjugates of glycosides are common plant metabolites as part of detoxification process. As such this metabolite is considered of minimal toxicological concern.	Group 1 Has same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity.
M-18 Minor metabolite	Genotoxicity alerts 1.Leadscope mouse lymphoma alert (unact v.2) triggered by the NO_2 group. General toxicity alerts No alerts for general toxicity.	Genotoxicity alerts ~~See comment under M-02 regarding mouse lymphoma alert.~~ Mouse lymphoma alert is not reliable and can be dismissed (nearest neighbours in training set tested negative). M-18 is a glucoside conjugate of M-21 with malonyl conjugated onto the glucoside. A glucoside is a type of glycoside where glucose is the conjoining sugar, with a malonyl group conjugated to the glycoside. Malonyl conjugates of glycosides	Group 1 Has same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity.

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		are common plant metabolites as part of detoxification process As such this metabolite is considered of minimal toxicological concern.	
M-19 Minor metabolite	Genotoxicity alerts 1.Leadscope mouse lymphoma alert (unact v.2) triggered by the NO_2 group. General toxicity alerts No alerts for general toxicity.	Genotoxicity alerts ~~See comment under M-02 regarding mouse lymphoma alert.~~ Mouse lymphoma alert is not reliable and can be dismissed (nearest neighbour in training set was glucose). This is a glucoside conjugate of the major rat metabolite M-01. Therefor e the toxicity of M-19 is characterized by M-01.	Group 1 Has same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity.
M-20 Minor metabolite	Genotoxicity alerts 1.Leadscope mouse lymphoma alert (unact v.2) triggered by the NO_2 group. General toxicity alerts 1. Derek Nexus alert 818 for nephrotoxicity – plausible. 2. Derek Nexus alert 837 for skin sensitisation – plausible.	Genotoxicity alerts ~~See comment under M-02 regarding mouse lymphoma alert.~~ Mouse lymphoma alert is not reliable and can be dismissed (no close matches in training set). General toxicity alerts Alerts for nephrotoxicity and skin sensitisation are not considered to pose any concern at the low levels of exposure. Aclonifen is itself already classified as a Category 1A skin sensitiser and effects the kidneys at high doses. This is a minor metabolite therefore general toxicity alerts are not a concern.	Group 1 Has same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity. Exception is M-01 does not carry same alerts for nephrotoxicity or skin sensitisation but these alerts are not a concern.
M-21 Minor metabolite	Genotoxicity alerts 1.Leadscope mouse lymphoma alert (unact v.2) triggered by the NO_2 group. General toxicity alerts No alerts for general toxicity.	Genotoxicity alerts ~~See comment under M-02 regarding mouse lymphoma alert.~~	Group 1 Has same (or fewer) alerts as M-01 therefore may be grouped with M-01 for read-across for genotoxicity.

For aclonifen M-01 is designated the lead metabolite as it triggers the most (Q)SAR alerts for genotoxicity that are not triggered by aclonifen. All other metabolites have the same (but fewer) alerts for genotoxicity compared to M-01. There are some exceptions for alerts for general toxicity (skin sensitisation, nephrotoxicity or hepatotoxicity) but these alerts for general toxicity are not considered relevant at low levels of exposure – all general toxicity alerts present in metabolites but not triggered by aclonifen are also listed in the table below. It is proposed to use M-01 to read-across to the other metabolites. Therefore all dietary metabolites form a single group covered by M-01.

M-01 is the major intermediary metabolite in the rat metabolism studies with hydroxylation on the phenyl group being present in all other rat metabolites.

M-01 was found to be a major metabolite in plasma. In rats following a single oral dose at 2 mg/kg bw in rats M-01 was present at 10.2% and 10.7% of TRR in males and females respectively (see KCA 5.1.1/07 M-598008-01-1 [redacted], 2017). In urine M-01 is detected at fairly low levels (less than 1% of administered dose following single low dose of 2, 30, or 1000 mg/kg bw or a repeated

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dose of 30 mg/kg bw/day). However as M-01 is a major intermediary in the metabolism of aclonifen as all rat metabolites of aclonifen contain a hydroxyl group on the phenyl ring which is then conjugated primarily by sulfation and glucuronidation (see Figure 5.1-01 in MCA5). Of particular note is the aclonifen-4-sulphate metabolite (M-09) as this is the major rat metabolite found in urine at over 20% of the administered dose (single oral dose of 2 mg/kg bw M-598008-01-1 [redacted], 2017, Tables 14 and 15 in the study report). M-09 was similarly identified as a major rat metabolite in urine in all other metabolites studies on aclonifen (at 30 mg/kg bw single dose present at 10% / 36% in males/females, at 30 mg/kg bw/day repeat dosing at 15% / 27% in males/females, at 1000 mg/kg bw/day present at 13% / 10 % in males/females).Therefore in rats exposure to M-01 also occurs during the formation of M09 as M-01 is an intermediary metabolite on the path of formation of M-09. As such the toxicity of M-01 is addressed in the toxicity studies conducted on rats because rats are exposed to M-01.

It follows that all dietary metabolites have equivalent toxicity based on similarity of (Q)SAR alerts and can be read-across to M-01 so are also covered by the toxicity studies on rats.

III CONCLUSION

An *in silico* assessment has been conducted using Derek Nexus and Leadscope software (expert and rules based predictive software respectively) on dietary metabolites of aclonifen and these have been compared to the *in silico* assessment conducted on aclonifen.

The aim was to group the metabolites according to similarity of (Q)SAR alerts to allow read-across. The lead metabolite in a group is the one with the most alerts so raises most concern. All other metabolites in the same group have the same (or fewer) alerts.

For aclonifen M-01 is designated the lead metabolite as it triggers the most (Q)SAR alerts for genotoxicity that are not triggered by aclonifen. All other metabolites have the same (but fewer) alerts for genotoxicity compared to M-01. It is proposed to use M-01 to read-across to the other metabolites. Therefore all dietary metabolites form a single group covered by M-01.

M-01 is the major intermediary metabolite in the rat metabolism studies with hydroxylation on the phenyl group being present in all other rat metabolites.

Assessment and conclusion by applicant:

An *in silico* assessment has been conducted using Derek Nexus and Leadscope software (expert and rules based predictive software respectively) on dietary metabolites of aclonifen and these have been compared to the *in silico* assessment conducted on aclonifen.

The aim was to group the metabolites according to similarity of (Q)SAR alerts to allow read-across. The lead metabolite in a group is the one with the most alerts so raises most concern. All other metabolites in the same group have the same (or fewer) alerts.

For aclonifen M-01 is designated the lead metabolite as it triggers the most (Q)SAR alerts for genotoxicity that are not triggered by aclonifen. All other metabolites have the same (but fewer) alerts for genotoxicity compared to M-01. It is proposed to use M-01 to read-across to the other metabolites. Therefore all dietary metabolites form a single group covered by M-01.

M-01 is the major intermediary metabolite in the rat metabolism studies with hydroxylation on the phenyl group being present in all other rat metabolites.

Assessment and conclusion by RMS:

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CA 5.8.2 Supplementary studies on the active substance

Data Point:	KCA 5.8.2/01
Report Author:	
Report Year:	1991
Report Title:	Unscheduled DNA synthesis (UDS) in primary rat hepatocytes (Autoradiographic method)
Report No:	R007175
Document No:	M-174373-01-1
Guideline(s) followed in study:	OECD: 482; USEPA (=EPA): F,84
Deviations from current test guideline:	Current guideline: OECD 482, 1986 No deviations
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

Aclonifen (purity ≥93%) was tested for its potential to induce unscheduled DNA synthesis (UDS) in primary cultures of hepatocytes obtained from young adult male Sprague-Dawley rats.

Primary rat hepatocytes were prepared by *in situ* perfusion and monolayer cultures were initiated on cover-slips. The test cultures were treated with aclonifen in the presence of tritiated thymidine (^{3}H-thymidine). After 17 h, the cells were washed and fixed, coated with a radiosensitive emulsion and stored in the cold to yield silver grains in proportion to the amount of incorporated radiolabelled thymidine. The silver grains of intact nuclei and of cytoplasm were counted, the cytoplasmic background count being subtracted from the nuclear count to give net grains/nucleus (NG). Unscheduled DNA synthesis was assessed by comparing the NGs of the treated cultures with the untreated (control) cultures. 2-acetylaminofluorene (2-AAF) was used as positive control.

Two assays were conducted with concentrations of aclonifen ranging from 0.0079 to 25.0 µg/mL, six replicate cultures being prepared at each dose level.

At the highest concentration of 25 µg/mL (in DMSO), aclonifen was excessively toxic to hepatocytes and survival was reduced to 51-55%. At the next lower dose level of 7.9 µg/mL survival was 71-85% compared to vehicle control and this dose was selected as the highest level for scoring.

In both experiments the mean NGs of the treated cultures were all similar. In contrast, 2-AAF induced large increases in nuclear labelling that greatly exceeded all three criteria used to indicate UDS.

It was concluded that aclonifen did not induce unscheduled DNA synthesis, under the conditions of the study.

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I. MATERIALS AND METHODS

Study dates	Study start:	10 July 1991
	Completion:	31 October 1991

A. MATERIALS

1. Test Item — Aclonifen technical

Batch No.:	DA 633
Purity:	≥93%
Appearance:	Yellow crystalline powder
Expiry:	Not stated. (23 April 1994 retest date quoted for CA 5.4.1/05)
Structure:	

Vehicle:	DMSO (batch 289370/890, [redacted])

2. Positive control — 2-acetylaminofluorene (2-AAF), batch 58F0313, Sigma Chemical Co. In DMSO at 7.5 and 15 µg/mL.

3. Test system (cells) — Primary hepatocytes from young adult male Sprague-Dawley rats (Charles River Italia). One animal used for each experiment.

Perfusion solutions:

PBS/EGTA:

Phosphate buffered saline (Ca/Mg free)	500 mL
Glucose (40%)	1.25 mL
EGTA (0.1 M)	2.5 mL
Gentamycin (50 mg/mL)	0.5 mL

Williams E perfusion medium:

Williams E medium	489 mL
Glutamine (200 mM)	5.0 mL
Gentamycin (50 mg/mL)	0.5 mL
HEPES (1 M)	5.0 mL
Collagenase	to *circa* 111 U/mL

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Culture medium:	Williams E medium complete (WEC):	
	Williams E medium	494.5 mL
	Glutamine (200 mM)	5.0 mL
	Gentamycin (50 mg/mL)	0.5 mL
	Fetal calf serum (FCS)	55.0 mL
	During treatment, Williams E medium incomplete (WEI):	
	Williams E medium	489.5 mL
	Glutamine (200 mM)	5.0 mL
	Gentamycin (50 mg/mL)	0.5 mL
	HEPES (1 M)	5.0 mL
Tritiated thymidine:	Specific activity 40-60 Ci/mM	

B. STUDY DESIGN AND METHODS

Each of the two independent experiments conducted included negative and positive controls, with 8 dose levels of the test item. Six cultures were prepared for each test point.

The highest concentration of test item was selected from preliminary solubility/precipitation work, as not more than double the lowest concentration that produced precipitation on addition of culture medium.

Following treatment the slides were coded and scoring was conducted in blind. Three slides were evaluated per treatment, 50 cells per slide being scored for nuclear and cytoplasmic grains.

Following extraction from perfused liver, hepatocytes were separated by slow centrifugation and their viability determined by the Trypan blue method. They were seeded onto plastic coverslips and incubated at 37°C in 5% CO_2 and maximum humidity for at least 90 min to permit cell attachment. The coverslips were then washed with WEI, leaving on attached viable cells.

Immediately after washing, the test item at concentrations of 0.0079, 0.025, 0.079, 0.25, 0.79, 2.5, 7.9 and 25 µg/mL, and tritiated thymidine at a final concentration of 10 µCi/mL were added in fresh WEI. The cultures were treated for 17 h ± 30 min at 37°C.

After treatment the cultures were rinsed several times. One culture per treatment was used to monitor toxicity, viable cell counts (Trypan blue exclusion) being used to estimate cell survival relative to control. Cell cultures for autoradiography were fixed using 1:3 acetic acid/absolute ethanol and the coverslips were then air dried, before being dipped in autoradiographic emulsion, left to dry and stored for 7 days. They were then developed and fixed, then stained with haematoxylin/eosin.

Counting of nuclear and cytoplasmic grains was conducted manually under high magnification, on coded slides, in blind. UDS was measured by counting nuclear grains and subtracting the average number of cytoplasmic grains in three nuclear-sized areas adjacent to each nucleus, to derive the net nuclear grain count (NG). This was determined for 50 randomly selected cells on each slide. The average NG was recorded for 3 cultures per treatment.

Acceptance criteria

Cells with abnormal morphology, such as those with pyknotic or lysed nuclei, were not counted.

Isolated cells not surrounded with cytoplasm were not counted.

Cells with unusual staining artefacts or in the presence of debris were not counted.

Heavily labelled cells in S-phase were not counted.

If the count for any nucleus was less than zero (cytoplasmic count greater than the nuclear count), the negative value was used in calculation of the mean value.

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In addition to cultures for medium, vehicle and positive control, the five highest assessable concentrations of test item were assessed (including one concentration showing evidence of some toxic effect).

Evaluation criteria

The test item would be considered active in the UDS assay if it caused an increase in the mean net nuclear grain count to at least 5 grains/nucleus in excess of the concurrent vehicle control, and the percentage of nuclei with 5 or more net grains increased above 20% of the examined cells, in excess of concurrent vehicle control.

An increase between 0-5 net grains would be considered a marginal response, and a dose response would be required in both net grains and the percentage of cells in repair, as additional confirmation of a positive response.

If vehicle control or medium control showed an average of 5 net grains per nucleus, or 20% of the cells have ≥5 net grains per nucleus, the assay would be considered invalid.

The test item would be considered inactive in the assay if none of the above conditions are met, and if the assay included concentrations of the test item that either extended into the toxic range, or reached the limit of solubility in the test system.

Positive control had to be positive for the assay to be considered acceptable.

II. RESULTS AND DISCUSSION

Aclonifen was soluble in DMSO, but addition to the culture medium caused precipitation at ≥12.5 µg/mL, while a clear solution was obtained at 6.25 µg/mL. Thus, 25 µg/mL was selected as the highest final concentration of test item for the study.

In both of the independent experiments, toxicity was indicated by reduction in cell survival at the highest dose level, to 51% and 55% relative to vehicle control. At 7.9 µg/mL, survival was slightly reduced, to 71% and 85%. Accordingly, slides generated for the dosages 7.9, 2.5, 0.79, 0.25 and 0.079 µg/mL were selected for scoring.

In both experiments, mean NG of the treated and the vehicle control cultures were similar. The means of the treated cultures were in the range of -4.34 to -16.21 at all test points and no NG value of 5 or greater was observed.

Good responses were observed with 2-AAF in both experiments, indicating both the metabolic capability of the hepatocytes and the correct response of the unscheduled DNA synthesis test systems. A sterility test was performed with all cultures immediately after the treatment in each experiment. All cultures were found to be sterile.

Table 5.8.2- 1: USD assay in rat hepatocytes ex vivo, Experiment I: Mean ± SE

Dose level (µg/mL)	Total cells scored	Nuclear grains	Cytoplasmic grains	Net nuclear grains	% cells in repair
WEI	150	7.55 ± 0.94	15.95 ± 0.71	-8.40 ± 1.07	0
DMSO 1%	150	7.89 ± 0.69	16.33 ± 0.36	-8.45 ± 0.99	0
0.079	150	11.00 ± 1.34	18.99 ± 2.62	-7.99 ± 1.31	0
0.25	150	17.43 ± 2.97	24.49 ± 1.83	-7.06 ± 1.24	1.33 ± 0.67
0.79	150	9.25 ± 3.22	18.80 ± 4.92	-9.55 ± 3.43	0
2.5	150	16.84 ± 2.81	24.27 ± 2.51	-7.43 ± 1.00	1.33 ± 1.33

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Dose level (µg/mL)	Total cells scored	Nuclear grains	Cytoplasmic grains	Net nuclear grains	% cells in repair
7.9	150	11.29 ± 1.97	18.61 ± 3.33	-7.32 ± 1.37	0
2-AAF 7.5	150	57.09 ± 6.93	25.86 ± 1.56	31.23 ± 5.51	96.67 ± 2.40
2-AAF 15	150	33.3 ± 8.30	17.10 ± 4.38	16.20 ± 3.92	90.0 ± 7.02
Historical control[a]	DMSO 1% 2-AAF 7.5 2-AAF 15			-19.3 to -0.81 10.2 – 38.8 12.7 – 41.5	0 62 – 100 58 – 100

a Historical ranges from 8 experiments with 3 to 6 cultures per test point

Table 5.8.2- 2 USD assay in rat hepatocytes ex vivo, Experiment II: Mean ± SE

Dose level (µg/mL)	Total cells scored	Nuclear grains	Cytoplasmic grains	Net nuclear grains	% cells in repair
WEI	150	10.02 ± 1.31	16.72 ± 0.66	-6.70 ± 1.02	0
DMSO 1%	150	14.67 ± 4.01	25.02 ± 1.61	-10.35 ± 3.81	0
0.079	150	10.25 ± 3.95	17.54 ± 5.14	-7.30 ± 1.19	0
0.25	150	15.83 ± 3.18	22.54 ± 4.60	-6.71 ± 1.60	0.67 ± 0.67
0.79	150	7.86 ± 1.18	14.08 ± 1.30	-6.22 ± 0.21	0
2.5	150	12.88 ± 2.71	18.53 ± 3.05	-5.65 ± 0.34	0
7.9	150	10.67 ± 3.83	17.04 ± 4.81	-6.37 ± 1.12	0
2-AAF 7.5	150	37.47 ± 12.85	23.46 ± 7.80	14.01 ± 5.06	82.00 ± 8.08
2-AAF 15	150	30.07 ± 3.72	18.21 ± 1.78	11.86 ± 1.96	84.00 ± 6.00
Historical control[a]	DMSO 1% 2-AAF 7.5 2-AAF 15			-19.3 to -0.81 10.2 – 38.8 12.7 – 41.5	0 62 – 100 58 – 100

a Historical ranges from 8 experiments with 3 to 6 cultures per test point

III. CONCLUSION

None of the conditions for a positive response were met for aclonifen at any test point in either independent experiment.

Aclonifen technical did not induce unscheduled DNA synthesis in rat primary hepatocytes under the conditions of the study.

Assessment and conclusion by applicant:

The study is valid and reliable, showing that aclonifen does not induce unscheduled DNA synthesis in cultured rat hepatocytes. Although the study is no longer a requirement, it provides acceptable

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supplementary information as part of a weight of evidence assessment for genotoxicity.

Assessment and conclusion by RMS:

Data Point:	KCA 5.8.2/02
Report Author:	██████
Report Year:	1995
Report Title:	Investigation of the Potential for DNA-Binding of Aclonifen
Report No:	R007427
Document No:	M-174903-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable (non-guideline study)
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In the combined chronic/carcinogenicity study performed in CD-1 mice (KCA 5.5/12, ██████ 1991, M-174334-01-1), bladder tumours were observed in 2/50 male and 1/50 female mice after feeding with a diet containing 7000 ppm aclonifen, equivalent to 892 and 984 mg/kg body weight/day in males and females respectively, over the 80-week dosing period. The DNA binding assay summarised here was performed to investigate whether a possible mechanism for tumourigenesis in mice is the *in vivo* covalent binding of aclonifen or its metabolites to DNA.

[Aniline-UL-^{14}C]aclonifen was administered by oral gavage to male CD1 mice at a single dose level of approximatively 900 mg/kg body weight (25 mCi/kg body weight), equivalent to the highest dose rate tested in the mouse carcinogenicity study. Within 24 hours, the animals had excreted about 34% of the administered radioactivity in urine indicating a high bioavailability of the radiolabel after oral administration. Exhaled CO_2 collected during the 24 hours after treatment amounted to less than 0.01% of the administered dose, indicating that aclonifen was metabolically stable over this period and that little radioactivity in the extracted DNA could have been derived from the biosynthetic incorporation of radiolabelled breakdown products of the test item. The radioactivity in the faeces or animal tissues was not determined.

After 24 hours, the animals were sacrificed and the liver and urinary bladder chromatin and DNA were isolated, purified and assayed for radioactivity. Some interaction of the radiolabelled test item and its metabolites with chromatin protein in the liver and the bladder was observed, resulting in specific radioactivities of 3189 – 4957 dpm/mg (177 – 275 pmol/mg) and 5751 dpm/mg (319 pmol/mg) in the

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liver and bladder chromatin respectively. No radioactivity above the historical lab limit of detection of 3.5 cpm per vial was detectable in the purified liver and bladder DNA samples, corresponding to specific activities of <4.6 and <17.5 dpm/mg DNA, for the liver and bladder respectively, after correction for counting efficiency and the amount of DNA per vial. This limit of detection, when converted to the units of the Covalent Binding Index (CBI = µmol chemical bound per mol nucleotides/mmol chemical applied per kg body weight) shows that the CBI for aclonifen in mice is <0.03 in liver and <0.1 in bladder. This maximum possible DNA-binding ability of aclonifen is more than five orders of magnitude lower than the corresponding value for the strong hepatocarcinogen aflatoxin B_1 and more than 4000 times lower than the CBI for the moderate hepatocarcinogen 2-acetylaminofluorene.

In conclusion, the results of this study give no indication for a genotoxic potential of aclonifen mediated by DNA binding *in vivo*.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material

Labelled test item

Chemical structure	Cl, O, NH_2, NO_2, * — *Position of the radiolabel
Common name	Aclonifen (code name RPA 099795)
IUPAC name	2-Chloro-6-nitro-3-phenoxy-[U-^{14}C]aniline
Radiolabelled test material	[Aniline-UL-^{14}C]aclonifen
Lot No.	GHS 854
Specific radioactivity	2.77 MBq/mg (74.8 µCi/mg)
Purity	Radiochemical purity >99% (TLC)

Unlabelled test item

Chemical structure	Cl, O, NH_2, NO_2
Common name	Aclonifen (code name RPA 099795)
IUPAC name	2-Chloro-6-nitro-3-phenoxyaniline
Lot No.	BES1572
Purity	99.6%

2. Vehicle and/or positive control

15% dimethylsulfoxide in sesame oil.

3. Test animals

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Species and strain:	Mouse – CD1
Source:	[redacted]
Number of animals:	16
Sex:	Male
Age:	8 – 10 weeks
Weight at treatment:	Not mentioned
Acclimation period:	12 days
Diet:	Nafag 890 ([redacted]) *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually in macrolone cages for the acclimatisation period and after the administration of the test compound except for animals 1, 2 and 9, which were held individually in all-glass metabolic cages after the administration of the test compound.
Identification of the animals	Tail painting
Environmental conditions:	Temperature: 22±2°C Humidity: 30 – 70% Air changes: Not mentioned Photoperiod: 12 hours light/dark-cycle

4. Preparation of dosing solution

The radiolabelled test item was dissolved in dimethylsulfoxide, mixed with the unlabelled test compound in dimethylsulfoxide and with sesame oil to a final concentration of about 90 mg/ml (2.5 mCi, 92.5 MBq/ml, final DMSO concentration 15% by volume). Two application solutions were prepared, solution 'A' and solution 'B'. The radioactivity of the dosing solutions was determined by liquid scintillation counting (LSC) and was found to be 2.50 ± 0.07 mCi/ml and 2.71 ± 0.16 mCi/ml, respectively.

B. STUDY DESIGN AND METHODS

1. In life dates
19 June to 4 July 1995.

2. Animal assignment and treatment
16 male mice were involved in the study, randomly assigned to three groups. Animals 1 – 8 received dosing solution at a dose level of 900 mg/kg body weight (25 mCi/kg body weight). The animals received about 10 ml of the radiolabelled dosing suspension per kg body weight by oral gavage (animals 1 – 7 received dosing solution 'A' whilst animal 8 received dosing solution 'B'). Animals 9 – 12 were untreated and were used for the determination of DNA binding *in vitro* during the DNA isolation process. Animals 13 – 16 were untreated controls.

3. Urinary excretion of radioactivity and exhalation of $^{14}CO_2$
After administration of the radiolabelled test item, animals 1, 2 and 9 were placed individually in all-glass metabolic cages. The excreted urine was collected and assayed for radioactivity by LSC whilst the exhaled radioactivity was absorbed in ethanolamine:2-methoxyethanol (1:2 v/v) and assayed by LSC.

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4. Sacrifice and sampling

24 hours after administration of the radiolabelled test item, the animals were killed with an overdose of CO_2/O_2 and exsanguinated by open heart puncture. The livers and bladders were excised, washed in ice cold saline, minced into small pieces and frozen in liquid nitrogen. The frozen tissue samples were stored at -80°C until processing.

5. Isolation of DNA and chromatin protein

The livers of the mice were pooled into four samples - treated mice 1 – 4, treated mice 5 – 8, untreated mice 9 – 12 for *in vitro* DNA binding and control mice 13 – 16. The liver samples were homogenised, and the DNA was isolated according to the method of Sagelsdorff et al. [*Carcinogenesis* 4, 1267 – 1273, 1983]. Briefly, crude chromatin was precipitated with the non-ionic detergent Nonident P40. The chromatin pellet was blended in a denaturing lysing medium, de-proteinated with chloroform/isoamyl alcohol/phenol, extracted with diethyl ether and the DNA was further purified by adsorption on a hydroxyapatite column, dialysis and precipitation with ethanol. The highly purified DNA (protein contamination less than 0.05%; RNA contamination less than 1%) was re-suspended in nucleotide buffer and quantified on the basis of UV absorption. The chromatin proteins were isolated from the chloroform/isoamyl alcohol/phenol extract by acetone precipitation. The pellet was re-suspended in 1% sodium dodecylsulfate and subject to further rounds of acetone precipitation and 1% sodium dodecylsulfate re-suspension. The purified chromatin protein in the final 1% sodium dodecylsulfate solution was quantified colourimetrically using a bicinchoninic acid/Cu^{2+} assay according to Smith et al. [*Anal. Biochem.* 150, 76 – 85, 1985]. The radioactivity in the purified chromatin protein and DNA in each sample was determined by LSC.

The bladder DNA was extracted in a slightly modified procedure. The bladders of the mice were pooled into three samples - all treated mice 1 – 8, untreated mice 9 – 12 for *in vitro* DNA binding and control mice 13 – 16. The bladders were minced and homogenised with 3 volumes of homogenising medium using a potter and the potter was washed with homogenising medium. The chromatin and DNA were then purified, quantified and radioassayed as described for liver samples.

***6. In vitro* incubation**

To demonstrate that the DNA workup procedure was sufficient to remove non-covalently bound radioactivity from the DNA, the purified liver DNA isolated from the pooled sample from untreated animals 9 – 12 was incubated *in vitro* with the radiolabelled supernatant from the first chromatin precipitation step of the pooled liver sample from treated animals 1 – 4. Likewise, the purified bladder DNA isolated from the pooled sample from untreated animals 9 – 12 was incubated *in vitro* with the radiolabelled supernatant from the first chromatin precipitation step of the pooled bladder sample from treated animals 1 – 8. After incubation, these DNA samples were subject to the standard work up procedure, quantified and radioassayed.

7. Background radioactivity

DNA was isolated from the pooled livers and bladders of the control mice 13 – 16. The obtained radioassays were compared to historical lab controls to demonstrate that the work-up procedure was performed without external contamination.

8. Calculation of the Covalent Binding Index (CBI)

The covalent binding index (that is the amount of radioactivity associated with the DNA normalised to the applied dose to the animal) was calculated for each sample according to the following equation, following the procedure of [redacted]. [*Mutation Res.* 65, 289 – 356, 1979]:

$$\mathrm{CBI} = \frac{\mu\text{mol chemical bound/mol DNA nucleotide}}{\text{mmol chemical applied/kg body weight}} = \frac{\text{dpm/mg DNA}}{\text{dpm/kg body weight}} \times 309 \times \mathbf{10^8}$$

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Where the average weight of 1 mol of DNA nucleotides is 309 g.

II. RESULTS AND DISCUSSION

Radioassay of the urine excreted from animals 1 and 2 indicated that 33.1% and 34.9% respectively of the administered radioactivity had been excreted in the urine within 24 hours of treatment, this suggests a high bioavailability of the radiolabel after oral administration. Radioassay of the exhaled CO_2 collected during the 24 hours period indicated that less than 0.01% of the administered radioactivity had been exhaled, this suggests that only minimal amounts of the radiolabel were metabolically degraded and released as $^{14}CO_2$. These results are summarised in the table below.

Table 5.8.2-03: Urinary excretion and exhalation of the radioactivity within 24 hours following oral administration of a single dose of [Aniline-UL-^{14}C]aclonifen to male CD1 mice

Animal number	Administered dose (mg/kg body weight)	Recovery of radioactivity (% administered dose)	
		Urine	Exhaled
1	867	33.1	0.0016
2	887	34.9	0.0015
9	0	-	-

The background radioactivity in the chromatin samples isolated from the pooled livers and bladder of the untreated mice was 276 dpm/mg and 391 dpm/mg respectively. In the treated mice, interaction of the radiolabel with chromatin protein resulted in specific radioactivities of 3189 – 4957 dpm/mg (177 – 275 pmol/mg) and 5751 dpm/mg (319 pmol/mg) in the pooled liver and the bladder chromatin samples respectively.

Based upon historical lab control data from 25 control DNA samples isolated in the course of DNA-binding studies over the preceding 2 years, the observed background radioactivity level in DNA was 11.6 cpm with a standard deviation of 1.23 cpm. This data was used to set the detection limit for radioactivity in a vial to 3.5 cpm over this background based upon 2 sigmas. The counting efficiency was 75.9 – 77% and this was used, along with the mass of DNA in each of the samples, to calculate the limit of detection for the specific radioactivity in the liver and bladder DNA samples. Upon analysis, no radioactivity above the limit of detection was detected in any of the liver and bladder DNA samples from the treated animals, the untreated animals in which the DNA had been subject to *in vitro* incubation or the untreated control animals. These respective limits of detection were used to derive upper limits for the DNA covalent binding indexes. The obtained results are summarised in the table below.

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Table 5.8.2-04: Radioactivity in chromatin and DNA extracted from livers and bladders of male CD1 mice following oral administration of a single dose of [Aniline-UL-^{14}C]aclonifen

		Aclonifen treated		Untreated – *in vitro* binding	Untreated control
Tissue		**Liver**			
Animal number		1 – 4	5 – 8	9 – 12	13 – 16
Mean dose	mg/kg bw	871.8	658.4	-	-
	$\times 10^{-10}$ dpm/kg bw	5.925	4.775		
Chromatin protein	Radioactivity (dpm/mg)	4957	3189	276	-
DNA	Amount of DNA in vial (mg)	1.009	1.016	1.036	0.998
	Gross radioactivity (cpm)	13.6	12.4	10.9	10.1
	Net radioactivity (cpm)*	<3.5	<3.5	<3.5	-
	Specific radioactivity (dpm/mg)**	<4.6	<4.6	-	-
	Covalent Binding Index	<0.02	<0.03	-	-
Tissue		**Bladder**			
Animal number		1 – 8		9 – 12	13 – 16
Mean dose	mg/kg bw	764.7		-	-
	$\times 10^{-10}$ dpm/kg bw	5.348			
Chromatin protein	Radioactivity (dpm/mg)	5751		391	-
DNA	Amount of DNA in vial (mg)	0.264		0.086	0.115
	Gross radioactivity (cpm)	11.1		10.8	10.9
	Net radioactivity (cpm)*	<3.5		<3.5	-
	Specific radioactivity (dpm/mg)**	<17.5		-	-
	Covalent Binding Index	<0.1		-	-

*Net radioactivity = minus lab historical control background of 11.6 cpm in extracted DNA samples.
**Corrected for the counting efficiency of 76% and mass of DNA in the sample.

The lack of detectable radioactivity in the control samples shows that there was no contamination of the DNA during extraction and workup. The lack of radioactivity in the DNA from the untreated samples subject to *in vitro* binding shows that the DNA work-up procedures were adequate to remove any non-covalently bound radiolabel from the DNA. Finally, the lack of detectable radioactivity in the treated DNA samples are evidence that there was no significant covalent binding of the radiolabelled aclonifen and its metabolites to the liver and bladder DNA.

III. CONCLUSIONS

[Aniline-UL-^{14}C]aclonifen was administered by oral gavage to male CD1 mice at a single dose level of approximatively 900 mg/kg body weight (25 mCi/kg body weight), equivalent to the highest dose rate tested in the mouse carcinogenicity study. Within 24 hours, the animals had excreted about 34% of the administered radioactivity in urine indicating a high bioavailability of the radiolabel after oral administration. Exhaled CO_2 collected during the 24 hours after treatment amounted to less than 0.01% of the administered dose, indicating that aclonifen was metabolically stable over this period and that little radioactivity in the extracted DNA could have been derived from the biosynthetic incorporation of radiolabelled breakdown products of the test item.

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After 24 hours, the animals were sacrificed and the liver and urinary bladder chromatin and DNA were isolated, purified and assayed for radioactivity. Some interaction of the radiolabelled test item and its metabolites with chromatin protein in the liver and the bladder was observed, resulting in specific radioactivities of 3189 – 4957 dpm/mg (177 – 275 pmol/mg) and 5751 dpm/mg (319 pmol/mg) in the liver and bladder chromatin respectively. No radioactivity above the historical lab limit of detection of 3.5 cpm per vial was detectable in the purified liver and bladder DNA samples, corresponding to specific activities of <4.6 and <17.5 dpm/mg DNA, for the liver and bladder respectively, after correction for counting efficiency and the amount of DNA per vial. This limit of detection, when converted to the units of the Covalent Binding Index (CBI = µmol chemical bound per mol nucleotides/mmol chemical applied per kg body weight) shows that the CBI for aclonifen in mice is <0.03 in liver and <0.1 in bladder. This maximum possible DNA-binding ability of aclonifen is more than five orders of magnitude lower than the corresponding value for the strong hepatocarcinogen aflatoxin B_1 and more than 4000 times lower than the CBI for the moderate hepatocarcinogen 2-acetylaminofluorene.

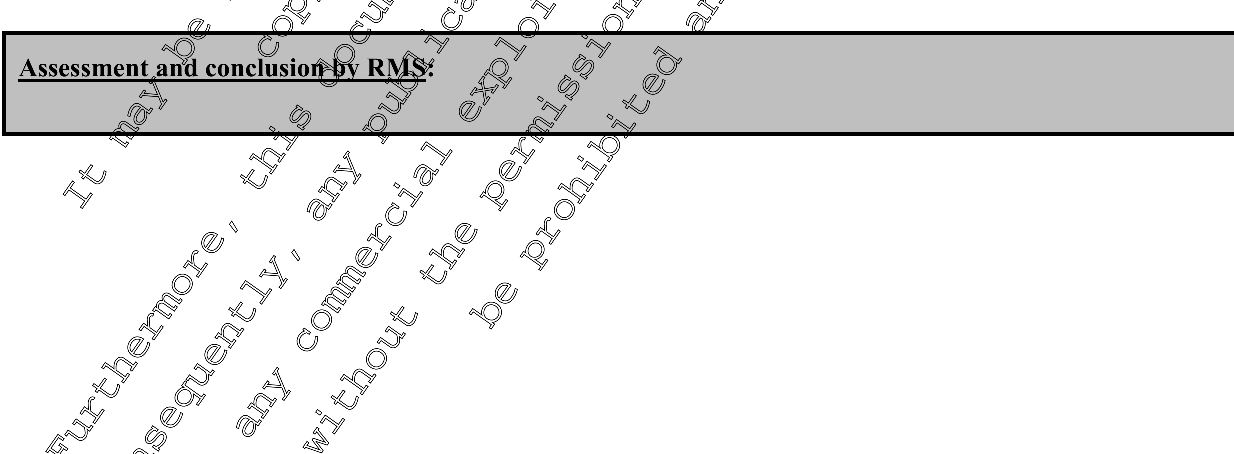
In conclusion, the results of this study give no indication for a genotoxic potential of aclonifen mediated by DNA binding *in vivo*.

Assessment and conclusion by applicant:

This study was conducted to GLP, there is no relevant OECD test guideline. This study was deemed to be acceptable in the DAR (2006).

The DNA Covalent Binding Index (CBI = µmol chemical bound per mol nucleotides/mmol chemical applied per kg body weight) for aclonifen in male CD1 mice in liver and bladder was determined to be <0.03 and <0.1 respectively, 24 hours following the oral administration of a single dose of [aniline-UL-14C]aclonifen at 900 mg/kg body weight (25 mCi/kg body weight). This maximum possible DNA-binding ability of aclonifen is more than five orders of magnitude lower than the corresponding value for the strong hepatocarcinogen aflatoxin B1 and more than 4000 times lower than the CBI for the moderate hepatocarcinogen 2-acetylaminofluorene. In conclusion, the results of this study give no indication for a genotoxic potential of aclonifen mediated by DNA binding *in vivo*.

Assessment and conclusion by RMS:



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Data Point:	KCA 5.8.2/03
Report Author:	;
Report Year:	1981
Report Title:	KUB 3359 - Studies for acute toxicity in mice with oral, and in rats with oral, intraperitoneal and dermal treatment, as well as for primary skin and eye irritation in rabbits
Report No:	R007415
Document No:	M-174876-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 423, 2001 Dose volume for rats in acute oral study exceeded recommended levels. These minor deviations do not affect the overall adequacy of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In an acute intraperitoneal toxicity limit test, groups of fasted Wistar-AF/HAN-EMD (SPF) rats, 5/sex/dose were given a single dose (intraperitoneal) of aclonifen (lot T5/81 – purity 96.2%) at 2500, 3200, 4000 or 5000 mg/kg bw in 0.5% aqueous carboxymethyl cellulose mucilage (CMC) vehicle and were observed for 14 days.

Clinical signs of intoxication were observed within 5 -15 minutes of dosing in all dose groups including reduced spontaneous activity, ataxia, dyspnea, piloerection and conspicuously yellow urine. Up to three days after dosing there was a clear inhibition of body weight development. Mortality was dose related. After 14 days there were no mortalities in the 2500 mg/kg bw dose group, 5 mortalities in the 3200 mg/kg bw dose group and 6 mortalities in the 4000 mg/kg bw dose group. In the 5000 mg/kg bw dose group, all 10 rats had died by day 2 after dosing. Necropsy of deceased animals indicated depositions of the test material in the abdominal cavity, mucosal haemorrhages and erosions in the stomach, as well as lung edema in some of the animals. Necropsy of animals that were sacrificed at the end of the study indicated small depositions of the test material on the liver capsule, adhesion of individual liver lobes as well as focal spleen capsular fibrosis.

Based upon the mortality data, LD_{50} values were determined by probit analysis separately and jointly for each sex.

In rats the intraperitoneal LD_{50} is (95% confidence intervals):
3742 (3246 – 4312) mg/kg bw in males
3247 (2811 – 3751) mg/kg bw in females
3486 (3139 – 3871) mg/kg bw in both sexes

In conclusion, aclonifen was found to not be of low acute toxicity following intraperitoneal exposure in rats. There was no significant gender bias to the toxicity.

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There were some limitations to the study as it does not strictly follow OECD test guidelines and is not to GLP, but the study integrity is not affected.

Table 5.8.2-05 Aclonifen – intraperitoneal toxicity study in rats – summary of findings

Dose (mg/kg bw)	Males	Females	Combined
2500	5/5/0	5/5/0	10/10/0
3200	5/5/2	5/5/3	10/10/5
4000	5/5/2	5/5/4	10/10/6
5000	5/5/5	5/5/5	10/10/10

Number of animals dosed/clinical signs/mortality

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Description: Aclonifen (KUB 3359)

Lot/Batch: Lot T5/81E

Purity: 96.2%

Appearance: Not mentioned

Stability of test compound: Not mentioned

2. Vehicle and /or positive control: Vehicle: 0.5% aqueous carboxymethyl cellulose mucilage (CMC)

3. Test animals:

Species and strain: Rat – Wistar AF/HAN-EMD (SPF)

Source: Not mentioned

Number of animals: 5 per sex/dose

Sex: Male and female

Age: Not mentioned

Weight at treatment: 161 g (range 143 – 173 g).

Acclimation period: Not mentioned

Diet: Altromin Standard, Diet TPF(R) N 1324, Lot 1442 (6 July, 1981)

Water: Not mentioned, ad libitum access

Cage type: Makrolon cages type III

Housing: Grouping per sex; ████████

Environmental conditions: Temperature: 23 – 28°C

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Humidity: 43 – 56%

Air changes: Not mentioned

Photoperiod: Not mentioned

B. STUDY DESIGN AND METHODS

1. In life dates

26 August to 15 September 1981

2. Animal assignment and treatment

Aclonifen was tested for acute intraperitoneal toxicity by dosing 5 male and 5 female rats with a single dose of 2500, 3200, 4000 or 5000 mg/kg bw in 0.5% aqueous CMC (5.0, 6.4, 8.0 and 10.0 g to 100 mL respectively). The volume of administration was 2.0 mL/100 g bw and this was administered by injection (needle No 1) into the intraperitoneal cavity.

For clinical examinations, the behaviour and general condition of all rats were monitored in the 6 hours after treatment and then checked daily during the follow-up period after dosing. All animals were weighed before treatment and on days 1, 3, 5, 7, 10, 12 and 14 after treatment. Pathologico-anatomical examinations were carried out on all animals which died during the study. Animals that survived to the end of the study were sacrificed and subjected to a gross pathological examination..

II. RESULTS AND DISCUSSION

1. Mortality

Mortality was dose-related. All animals receiving 5000 mg/kg body weight died. Two of the five males and four of the five females died after injection of 4000 mg/kg body weight. Two of the five males and three of the five females died after receiving 3200 mg/kg body weight. There was no mortality with a dose of 2500 mg/kg body weight in either sex. Mortalities occurred up to 3 days after administration. The mortality data are presented in the table below.

Table 5.8.2-06 Aclonifen – acute intraperitoneal toxicity study in rats – mortality data

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Dose (mg/kg bw)	Males	Females	Combined
2500	0/5	0/5	0/10
3200	2/5 1 on day 1 and 1 on day 3	3/5 3 on day 3	5/10
4000	2/5 1 on day 1 and 1 on day 3	4/5 1 on day 1 and 3 on day 3	6/10
5000	5/5 1 on day 1 and 4 on day 3	5/5 2 on day 1 and 3 on day 3	10/10

Number of animals which died (out of 10 animals 5 m/5 f)

Based upon the mortality data, LD_{50} values along with 95% confidence intervals were determined by probit analysis separately and jointly for each sex after the 14-day recovery period.

In males the intraperitoneal LD_{50} is 3742 (3246 – 4312) mg/kg bw

In females the intraperitoneal LD_{50} is 3247 (2811 – 3751) mg/kg bw

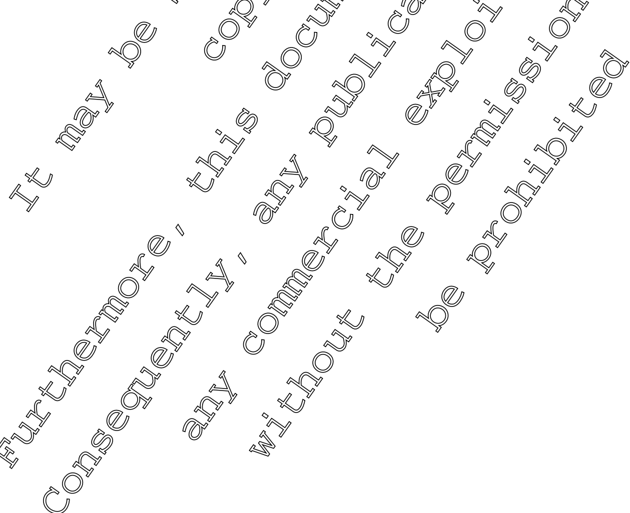
In both sexes combined the intraperitoneal LD_{50} is 3486 (3139 – 3871 mg/kg bw

The determined LD_{50} value does not trigger classification for acute toxicity. The potency ratio between males and females was estimated to be 1.15 indicating no significant gender bias to the toxicity.

2. Clinical observations

Clinical signs appeared 5 – 15 minutes after injection and consisted of reduced activity, ataxia, dyspnea and piloerection. Yellow staining of the urine was noted immediately after dosing and persisted up to day 6. The clinical observations are summarised in the table below.

Table 5.8.2- 3 Aclonifen – acute intraperitoneal toxicity study in rats – clinical signs



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Time after dosing	0 – 1 hours	1 – 2 hours	2 – 6 hours	6 - 24 hours	2 – 3 days	4 – 5 days	6 – 14 days
2500 mg/kg body weight							
Reduced activity	10/10	10/10	10/10	0/10	0/10	0/10	0/10
Ataxia	0/10	10/10	10/10	8/10	0/10	0/10	0/10
Yellow urine	0/10	10/10	10/10	10/10	10/10	10/10	0/10
3200 mg/kg body weight							
Reduced activity	10/10	10/10	10/10	0/8	0/6	0/5	0/5
Ataxia	0/10	0/10	10/10	8/8	0/6	0/5	0/5
Piloerection	0/10	0/10	0/10	1/8	1/6	0/5	0/5
Yellow urine	0/10	0/10	10/10	8/8	6/6	0/5	0/5
4000 mg/kg body weight							
Reduced activity	10/10	10/10	10/10	0/8	0/4	0/4	0/4
Ataxia	0/10	0/10	10/10	8/8	0/4	0/4	0/4
Dyspnea	0/10	0/10	0/10	1/8	0/4	0/4	0/4
Piloerection	0/10	0/10	0/10	0/8	1/4	0/4	0/4
Yellow urine	0/10	10/10	10/10	8/8	4/4	4/4	0/4
5000 mg/kg body weight							
Reduced activity	10/10	10/10	10/10	4/4	-	-	-
Prone position	0/10	3/10	4/10	4/4	-	-	-
Ataxia	3/10	10/10	10/10	4/4	-	-	-
Dyspnea	0/10	3/10	10/10	4/4	-	-	-
Piloerection	0/10	4/10	4/10	4/4	-	-	-
Yellow urine	3/10	10/10	10/10	4/4	-	-	-
Number of animals with clinical signs (out of the surviving animals, 5 m/5 f to start)							

3. Body weight

The rats displayed a clear inhibition of body weight development up to the third day of the trial.

4. Necropsy

Gross necropsy of the rats that had died indicated depositions of the test material in the abdominal cavity, mucosal haemorrhages and erosions in the stomach, as well as lung edema in some of the animals. Gross necropsy of the sacrificed animals at the end of the study indicated small depositions of the test material on the liver capsule, adhesion of individual liver lobes as well as focal spleen capsular fibrosis.

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III. CONCLUSION

Under these test conditions the intraperitoneal LD_{50} of aclonifen in rats is 3742 (3246 – 4312) mg/kg bw in males, 3247 (2811 – 3751) mg/kg bw in females with an LD_{50} of 3486 (3139 – 3871) mg/kg bw in both sexes combined (95% confidence interval). The determined LD_{50} value does not trigger classification for acute toxicity. The potency ratio between males and females was estimated to be 1.15 indicating no significant gender bias to the toxicity.

Assessment and conclusion by applicant:

Supplementary study. This study was not conducted to GLP, and it does not follow OECD test guidelines. This study was deemed to be acceptable in the DAR (2006).

Under these test conditions the intraperitoneal LD50 of aclonifen in rats is 3742 (3246 – 4312) mg/kg bw in males, 3247 (2811 – 3751) mg/kg bw in females with an LD50 of 3486 (3139 – 3871) mg/kg bw in both sexes combined (95% confidence interval). The determined LD50 value does not trigger classification for acute toxicity. The potency ratio between males and females was estimated to be 1.15 indicating no significant gender bias to the toxicity.

Assessment and conclusion by RMS:

Data Point:	KCA 5.8.2/04
Report Author:	[redacted]; [redacted]; [redacted]
Report Year:	2018
Report Title:	Agonistic effects of diverse xenobiotics on the constitutive androstane receptor as detected in a recombinant yeast-cell assay
Report No:	M-669642-01-1
Document No:	M-669642-01-1
Guideline(s) followed in study:	-
Deviations from current test guideline:	Not applicable (non-guideline study)
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Abstract

The constitutive androstane receptor (CAR) is a nuclear receptor and transcription factor regulating proteins involved in xenobiotic metab. Agonist activation of the CAR can trigger metabolic activation and toxification as well as detoxification and clearance; accordingly, xenobiotic substances acting as CAR ligands may pose a threat to human and animal health. The authors used yeast cells transduced with the human CAR and the response pathway to measure the CAR-agonistic activities of 549 synthetic or natural compds.: 216 of the tested compds. exhibited CARagonistic effects. Eighty-four percent of CAR-activating compds. were arom. compds., and greater than 65 percentof these active compds. were arom. hydrocarbons, bisphenols, monoalkyl phenols, phthalates, styrene dimers, di-Ph ethers, organochlorines, and organophosphates. The ten most potent compds. were 4-tert-octylphenol (4tOP; ref. substance), 4-nonylphenol, diethylstilbestrol, benzyl Bu phthalate, 2-(4-hydroxyphenyl)-2,4,4-trimethylchroman, o,p-DDT, methoxychlor, di-Pr phthalate, hexestrol, and octachlorostyrene. The activities of these nine non-ref. compds. exceeded 10 percent of the 4tOP activity. Anal. of para-monoalkyl phenols suggests that branching of the alkyl group and chlorination at the ortho position raises potency. This study provides crit. information for identifying the potential of CAR-mediated toxic hazards and for understanding the relevant mechanism.

Summary of relevant parts of this publication

I. MATERIALS AND METHODS

Yeast cells transduced with the human constitutive androgen receptor (CAR) and the response pathway were used to measure the CAR-agonistic activities of 549 synthetic or natural compounds: 216 of the tested compounds exhibited CAR-agonistic eff ects. Eighty-four percent of CAR-activating compounds were aromatic compounds, and >65% of these active compounds were aromatic hydrocarbons, bisphenols, monoalkyl phenols, phthalates, styrene dimers, diphenyl ethers (including aclonifen), organochlorines, and organophosphates.

The compound 4-*tert*-octylphenol (4tOP), a potent agonist for the CAR, was used as a reference compound, against which the relative potency of the other compounds was measured. For each compound an EC x 10 value was obtained in the assay (the test solution concentration producing 10 x the value for blank control), which approximated reasonably well to reported EC_{50} values obtained with mammalian cells transfected with human CAR plasmid.

The assay used yeast cells (*Saccharomyces cervisiae* Y190) carrying the β-galactosidase reporter gene. The cells were transfected with two expression plasmids: pGBT9-CARLBD carrying the ligand-binding domain of the human CAR and the DNA-binding domain of transcriptional activator GAL4, and pGAAD424-TIF-2 carrying the co-activator NCOA2 (aka TIF2) and the transcriptional activation domain of GAL4.

The assay was conducted using a chemiluminescent detection method for β-galactosidase in 96-well plates. Yeast cells were pre-incubated for 24 h at 30°C in medium lacking tryptophan and leucine, and cell density was adjusted to an absorbance of 1.65 to 1.80 at 595 nm. Test solutions were prepared in dimethylsulfoxide (DMSO), of which a series of two-fold dilutions in medium were set out on the plate, and to which an aliquot of yeast suspension was added to prepare a solution containing 1% DMSO in each well. Each compound was tested at a minimum of 7 concentrations in 2-fold series between 10 µM and 156 nM (lower concentrations were used for some chemical showing strong CAR agonism). No precipitated materials were observed in any final mixture. The plate was incubated at 30°C under high humidity for 4 h. Reaction buffer containing GalactLux substrate (Aurora GAL-XE, MP Biochemicals, USA) was added for detection of chemiluminescent activity of released β-galactosidase, and zymolyase for enzymic digestion. The plate was then incubated for 1 h at 37°C and then assayed in a luminometer with added light-emission accelerator solution. All compounds were evaluated in duplicate, then those showing agonistic activity were subjected to a minimum of 2 further assays of 2 replicates each. A blank control excluding only the test item was also analyzed.

A dose response curve was plotted for luminescence intensity, and activity values were derived by linear regression. The EC x 10 (ten-fold effective concentration) was defined as the test concentration producing 10x the luminescence of the blank control. Relative activity was calculated by dividing the EC x 10 value for the reference compound 4tOP by that of the test compound.

II. RESULTS

For the reference substance 4tOP, no plateau of luminescence intensity was achieved, even at high concentrations, owing to the nature of the response and the sensitivity of the method. Consequently it was decided to evaluate based on the EC x 10, instead of the EC_{50} conventionally used in *in vitro* assays with mammalian cells.

Selected results for a range of the chemicals tested are shown below, including examples of the most active compounds and a series of diphenyl ethers, including aclonifen. *(Although the inducer phenobarbital was tested, its results were not reported in the publication).*

Table 5.8.2/04-1: CAR activity of aclonifen, other diphenyl ethers, and other agonists

Compound	EC x 10 (nM) Mean ± SD (n = 3)	Relative to 4tOP Mean ± SD (n = 3)
4-*tert*-octylphenol (reference)	13 ± 4	1
4-*n*-octylphenol	1200 ± 960	0.023 ± 0.019
4-*n*-butylphenol	4800 ± 3300	0.0036 ± 0.0011
Bisphenol-A	370 ± 79	0.038 ± 0.014
Benzyl *n*-butyl phthalate	52 ± 25	0.31 ± 0.12
Dicyclohexyl phthalate	3900 ± 900	0.0034 ± 0.00050
5α-dihydrotestosterone	4100 ± 1700	0.0035 ± 0.00088
Diethylstilbestrol	45 ± 21	0.32 ± 0.059
Clofibrate	220 ± 34	0.059 ± 0.0093
Benfuracarb	2200 ± 330	0.0059 ± 0.0011
Fenoxycarb	7600 ± 1100	0.0017 ± 0.00041
Edifenphos	140 ± 8	0.094 ± 0.023
Parathion	2700 ± 780	0.0050 ± 0.00048
Dimethametryn	7300 ± 7600	0.0045 ± 0.0030
Pencycuron	5800 ± 1100	0.0023 ± 0.00035
Diphenyl ethers:		
Chlornitrofen	1900 ± 1200	0.0059 ± 0.0011
Nitrofen	2400 ± 250	0.0054 ± 0.0012
Chlomethoxynil	5800 ± 2800	0.0026 ± 0.00085
Aclonifen	**6300 ± 800**	**0.0021 ± 0.00047**
Bifenox	6800 ± 3800	0.0023 ± 0.00064

These data show that aclonifen does possess CAR agonistic activity comparable with a range of other compounds. Together with other diphenyl ethers, its activity in this assay was relatively low, at 0.2%

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of the very potent reference material (4tOP), while the most potent of the example chemicals listed above were mostly in the range 1 to 10% of the reference activity.

III. CONCLUSION

Aclonifen possesses constitutive androstane receptor (CAR) agonistic activity comparable with a range of other agonistic compounds.

Assessment and conclusion by applicant:

A valid, peer-reviewed publication showing that aclonifen has CAR agonistic activity comparable with other agonists. Comparison with phenobarbital was not possible here, as these results were not reported.

Assessment and conclusion by RMS:

Data Point:	KCA 5.8.2/05
Report Author:	[redacted]; [redacted]; [redacted]; [redacted]; [redacted]
Report Year:	2008
Report Title:	Screening and detection of the in vitro agonistic activity of xenobiotics on the retinoic acid receptor
Report No:	M-669423-01-1
Document No:	M-669423-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	Not applicable (non-guideline study)
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

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Abstract

The retinoic acid receptors (RARs) play key roles in various biol. processes in response to endogenous retinoic acids. However, excessive embryonic exposure to specific ligands for each subtype of the RAR was reported to induce specific developmental abnormalities. The authors measured the RAR agonistic activity of 543 chems. using an assay system adopting yeast cells transfected with the human RAR.gamma. and a coactivator. Eighty-five of the 543 chems., including 16 organochlorine pesticides, 14 styrene dimers, 9 monoalkylphenols and 6 parabens, exhibited RAR.gamma. agonistic effects in this assay. In particular, monoalkylphenols having a 6-9 carbon alkyl group para to the phenolic hydroxyl group possessed high affinity for the RAR.gamma., and their activities were 1.363-

0.446 percent of that of all-trans RA. Para-Alkylphenols chlorinated at the ortho position also were about as active or more active than their unchlorinated analogs. In addn., all tested styrene dimers showed pos. effects, and the activity of 1-phenyltetralin, the strongest in this category, was 1.169 percent that of all-trans RA. A no. of chems. having binding affinity for the RAR.gamma. were revealed in this study (both newly identified and confirmed), further comprehensive studies of *in vitro* and *in vivo* effects via the RARs are required for the reliable risk assessment of chems. *In vitro* receptor binding studies represent an important step in hazard identification and suggest a potential mechanism of action, which can be an important step in risk assessment and in particular for screening studies to identify potential toxicity and inform mechanistic studies.

Summary of relevant parts of this publication

I. MATERIALS AND METHODS

The retinoic acid receptor (RAR) agonistic activity of 543 chemicals, including aclonifen, was measured using an assay system of yeast cells transfected with the human RARγ and a coactivator.

The transcriptional agonistic activities were measured with a reporter assay using yeast cells (*Saccharomyces cervisiae* Y190). An expression plasmid for the ligand binding domain of the human RARγ and the coactivator pGAAD424-TIF-2 was introduced into yeast cells carrying the β-galactosidase reporter gene.

The assay was performed using a chemiluminescent reporter gene method in a 96-well culture plate, based on a yeast two-hybrid estrogenicity assay. Yeast cells were pre-incubated for 24 h at 30°C in medium lacking tryptophan and leucine, and cell density was adjusted to an absorbance of 1.65 to 1.80 at 595 nm. Test solutions were prepared in dimethylsulfoxide (DMSO), of which a series of two-fold dilutions in medium were set out on the plate, and to which an aliquot of yeast suspension was added to prepare a solution containing 1% DMSO in each well. Each compound was tested at a minimum of 7 concentrations in 2-fold series between 10 µM and 156 nM (lower concentrations were used for some chemical showing high RAR agonism). The plate was incubated at 30°C under high humidity for 4 h. Reaction buffer containing GalactLux substrate (Aurora GAL-XE, MP Biochemicals, USA) was added for detection of chemiluminescent activity of released β-galactosidase, and zymolyase for enzymic digestion. The plate was then incubated for 1 h at 37°C and then assayed in a luminometer with added light-emission accelerator solution. All compounds were evaluated in duplicate in a minimum of three separate assays. For comparative purposes, *all-trans* RA, an endogenous agonist of RAR, was used as a standard.

A dose response curve was plotted for luminescence intensity, and two activity values were derived by linear regression: The EC x 10 (ten-fold effective concentration) was the test concentration producing 10x the luminescence of the blank control, and the REC20 (20% relative effective concentration) was the concentration showing 20% of the activity of 10^{-7} M *all-trans* RA. Activity relative to RA was calculated by dividing the REC20 value for the reference *all-trans* RA by that of the test compound.

II. RESULTS

In the chemiluminescence method an artificial substrate for β-galactosidase was added to detect transcriptional activation via the human RAR, and the large amount of substrate did not allow the luminescence intensity to reach a plateau, even at high concentrations of *all-trans* RA. Consequently, the EC x 10 value for *all-trans* RA (5.41 nM, a mean from 22 experiments) was substituted for the EC_{50} that would normally be evaluated in assays of this kind. Chemiluminescence intensity at 10^{-8} M *all-trans* RA was 21.2 x that of the blank control.

Eighty-five of the 543 tested compounds, at their highest concentrations (10 µM), exhibited transcriptional agonistic activity via the human RARγ of at least 20% that of 10^{-8} M *all-trans* RA. Selected results for a range of the chemicals tested are shown below, including examples of the active compounds and those diphenyl ethers tested, including aclonifen. *(Phenobarbital was not tested).*

Table 5.8.2/04-1: RAR activity of aclonifen, other diphenyl ethers, and other agonists

Compound	EC x 10 (µM) Mean ± SD (n = 3)	REC20 (µM) Mean ± SD (n = 3)	REC20 relative to RA (Mean%, n = 3)
all-trans retinoic acid (reference)	0.00541 ± 0.00173	0.00219 ± 0.00020	100
4-t-octylphenol	0.78 ± 0.41	0.29 ± 0.13	0.997
4-*n*-octylphenol	1.70 ± 0.56	0.66 ± 0.41	0.446
2-chloro-4-octylphenol	0.61 ± 0.37	0.23 ± 0.05	1.286
4-benzylphenol	9.39 ± 3.55	4.74 ± 1.07	0.062
2,2-dichlorobisphenol-A	18.95 ± 7.05	6.15 ± 1.61	0.048
Isobutyl-4-hydroxybenzoate	5.54 ± 0.93	4.08 ± 1.36	0.072
Butyl-4-hydroxybenzoate	10.35 ± 2.66	6.37 ± 1.80	0.046
all-trans retinol	2.81 ± 0.17	1.67 ± 0.77	0.175
4-chloro-17β-estradiol	16.23 ± 3.77	4.35 ± 1.75	0.067
Thiobencarb	16.74 ± 1.64	5.48 ± 0.39	0.054
Cyanofenphos	8.83 ± 1.51	3.42 ± 1.89	0.086
Chlorpyrifosmethyl	13.81 ± 3.47	7.08 ± 2.11	0.041
Endrin	1.22 ± 0.79	0.85 ± 0.21	0.346
β-endosulfan	9.06 ± 0.87	4.36 ± 2.71	0.067
Diphenyl ethers:			
Nitrofen	4.45 ± 1.31	2.48 ± 0.61	0.134
Chlornitrofen	7.77 ± 3.31	2.82 ± 1.22	0.104
Aclonifen	**2.74 ± 0.62**	**1.33 ± 0.82**	**0.220**

These data show that aclonifen does possess retinoic acid receptor (RAR) agonistic activity broadly comparable with a range of other agonistic compounds. Together with other diphenyl ethers, its activity in this assay was moderate, at 0.2% of the potent reference material.

III. CONCLUSION

Aclonifen possesses retinoic acid receptor (RAR) agonistic activity comparable with a range of other agonistic compounds.

Assessment and conclusion by applicant:

A valid, peer-reviewed publication showing that aclonifen has RAR agonistic activity broadly comparable with other agonists. Comparison with phenobarbital was not possible, as it was not tested.

Note: In the publication, it is not fully clear how the presented relative activities as percent of all trans retinoic acid was calculated, and it is assumed here that each is a mean of the relative activities from the three separate assays conducted for that compound. In the later publication from

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the same authors (CA 5.8.2/04 above), the relative activities are shown as mean ratios, which would support this interpretation.

Assessment and conclusion by RMS:

CA 5.8.3 Endocrine disrupting properties

Data Point:	KCA 5.8.3/01
Report Author:	███
Report Year:	2019
Report Title:	Evaluation of the androgenic agonist and antagonist activity of aclonifen technical (AE F068300) using the stably transfected human androgen receptor transcriptional activation assay (AR-EcoScreen™)
Report No:	20184671
Document No:	M-664621-01-1
Guideline(s) followed in study:	OECD Test guideline No. 456 (July 2016)
Deviations from current test guideline:	Current guideline: OECD 458, 2016 No deviation
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 ability of aclonifen technical to act as an agonist or antagonist of androgen receptor (AR) signalling was investigated using the AR EcoScreen™ cell line (cell based reporter assay expressing the human AR and an AR inducible luciferase reporter gene which results in androgen-dependent luminescence). Two independent experiments were performed for both the AR agonist and AR antagonist assays. Concentrations were selected following solubility analysis (no cytotoxicity was noted up to and including the highest concentration tested).

In the AR agonist assay (2 independent experiments), aclonifen was tested at 7 concentrations ranging from 10 pM to 1 µm, alongside vehicle and positive controls and the reference items DHT and DEHP. The RPC_{max} (maximum response level induced by aclonifen in comparison with the response induced by 10 nM DEHP) of Aclonifen was less than 10% (-0.7%) in both independent experiments, therefore aclonifen technical was considered negative in the AR agonist assay. All of the acceptability criteria were met thus confirming the validity of the assay to detect AR agonists.

Aclonifen was tested at 6 concentrations (100 pM to 1 µM) in each independent experiment of the AR antagonist assay. Vehicle controls, positive controls and the reference items HF, BPA and DEHP were also included. In both experiments a log IC_{30} could be determined for aclonifen (-6.31 in experiment 1

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and -6.39 in experiment 2); therefore, aclonifen technical was considered to be positive in the AR antagonist assay. With regard to the acceptability criteria, the RTA values for the PC_{ATG} were slightly outside the acceptability criteria in both experiments; however, as the $logIC_{30}$ and the $logIC_{50}$ values of the reference items HF and BFA were within acceptable ranges the ability of the test system to detect AR antagonistic activity was demonstrated. All other acceptability criteria were met and therefore the assay was considered valid.

Overall, both independent experiments of the AR agonist and antagonistic assays were considered valid. Aclonifen technical showed no androgenic agonist activity but did show some androgen antagonist activity in the AR-EcoScreen™ cell line.

I. MATERIALS AND METHODS

Study dates: Experimental: 07 to 20 February 2019
Completion: 06 June 2019

A. MATERIALS

1. Test Item Aclonifen technical

Batch No.: AE F068300-01-28

Purity: 99.9%

Appearance: Yellow powder

Expiry: 13 November 2020

Structure:

Vehicle: DMSO

2. Positive controls: 5α-Dihydrotestosterone (DHT)
Mestanolone (17α-Methylandrostan)
Bis-(2-ethylhexyl)-phthalate (DEHP)
Hydroxyflutamide (HF)
Bisphenol A (BPA)
Cycloheximide

3. Test system: AR-EcoScreen™ cell line

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Medium:	Cell culture medium Dulbecco's Modified Eagle Medium/Ham's F12 nutrient mix (DMEM/F12) without phenol red, supplemented with 5% foetal bovine serum (FBS), 100 U/mL Penicillin, 100 µg/mL Streptomycin, 100 µg/mL Hygromycin B and 200 µg/mL Zeocin Seeding medium DMEM/F12 without phenol red, supplemented with 5% charcoal stripped FBS, 100 U/mL Penicillin and 100 µg/mL Streptomycin Exposure medium DMEM/F12 without phenol red

B. METHODS

Two independent experiments each were performed which investigated the AR agonist and AR antagonist potential of aclonifen. Aclonifen was tested at seven concentrations in the AR agonist assay and six concentrations in the AR antagonist assay. Vehicle controls, positive controls and positive controls for cytotoxicity were included on each plate.

1. Preliminary solubility test

The solubility of the test item in the vehicle and culture medium was investigated in a preliminary test by the assessment of cloudiness or precipitate (examined microscopically). To test solubility in the vehicle, the test item was initially dissolved in DMSO at a concentration of 1 M and then further diluted to the highest soluble concentration. To test solubility in the culture medium, the stock solution of 1 M test item in DMSO was diluted 10-fold in the exposure medium.

2. Plating of cells

Both assays were performed in 96-well plates (for luminescence detection) at a seeding density of $1x10^5$ cells/mL, 90 µL/well. Cells were seeded once they had reached 80% confluency and had grown 11 or 13 passages from the stock solution. Prior to exposure, the cells were pre-incubated in the dark at 37±1˚C, with 80-100% humidity and 5±0.5% CO_2. Cells were sub-cultured upon reaching 75-90% confluency and were cultured for a minimum of one and a maximum of thirteen passages.

3. Exposure of cells

AR agonist assay:

For each experiment, appropriate spiking solutions of test item and controls were prepared in vehicle and finally into 10x dosing solutions in the exposure media. 10 µL of the dosing solutions were then added to appropriate wells of the 96-well plate containing the test cells (AR-EcoScreen™) and 90µL of seeding media. Vehicle controls wells treated with DMSO (0.1% v/v), positive control wells treated with 10 nM DHT and positive control wells treated with 10 µg/mL cycloheximide were also included. Exposure medium was added to each well and the plates were incubated under the conditions described above for 23.5 hours or 22 hours in experiments 1 and 2 respectively.

AR Antagonist assay:

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For each experiment, 10x dosing solutions were prepared as before in exposure media containing 56 nM DHT. 10 µL of the dosing solutions were added to wells of the 96-well plate containing the test cells and 90 µL of seeding media. Positive agonist control wells treated with 10 nM DHT (in triplicate), positive antagonist control wells treated with 0.1 µM HF and 500 pM DHT (in triplicate), positive cytotoxicity control wells treated with 10 µg/mL cycloheximide and 500 pM DHT (in triplicate) and AG agonist reference control wells with 500 pM DHT and 0.1% DMSO were also included. Exposure medium was added to each well and the plates were incubated under the described conditions for 23.5 or 22 hours for experiments 1 and 2 respectively.

4. Luciferase measurements

AR agonist assay:

Luciferase activity was determined using the Steady-Glo Luciferase assay system. 40 µL of Steady-Glo was added to each well following exposure, and the plate was incubated for 5 minutes at room temperature in the dark. The luminescence intensity was determined by reading each well for 2 seconds using a luminometer.

AR antagonist assay:

Luciferase activity was determined using the Dual-Glo luciferase assay system to simultaneously evaluate AR-mediated transcriptional activity and cytotoxicity. 40 µL of Dual-Glo reagent was added to each well following exposure. The plate was shaken and incubated for 10 minutes at room temperature in the dark. The firefly luminescence intensity was then measured with a luminometer and 60 µL of the medium removed from each well for the addition of 40 µL of Stop-Glo luciferase reagent. The plate was shaken and incubated for a further 10 minutes after which the *Renilla* luminescence intensity was measured

In all cases the luminosity data was presented as Relative Light Units (RLU).

5. Acceptability criteria

The test item is considered positive in the AR agonist assay if the maximum response induced (RPC_{max}) was ≥ 10% that of the positive control in two/two or two/three experiments. The test item is considered negative in the AR agonist assay if the (RPC_{max}) is < 10% that of the positive control in two/two or two/three experiments.

The AR agonist assay is considered acceptable if the following criteria are met:

a) The mean luciferase activity of the positive control (DHT) is ≥ 6.4-fold that of the mean vehicle control on each plate

b) The fold induction corresponding to the PC_{10} value of the concurrent positive control is greater than 1+2SD of the fold induction value of the vehicle control (set at 1)

c) The % CV of the raw data of triplicate wells (luminescence intensity data) is < 20%

d) The shape of the concentration-response curve of positive controls is sigmoidal

e) The results of the three reference items are within the acceptable range (see table below) and classified in the correct class (negative or positive for androgenicity)

f) The results are reproducible in at least one other independent experiment

The test item is considered positive in the AR antagonist assay if the log IC_{30} can be calculated in two/two or two/three experiments. The test item is considered negative if the log IC_{30} cannot be calculated in two/two or two/three experiments.

The AR antagonist assay is considered acceptable if the following criteria are met:

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a) The fold induction of the antagonist reference control (DHT) is ≥ 5

b) The RTA of the positive antagonist controls (DHT and HF) is less than 46%

c) The %CV of the raw data of triplicate wells (luminescence intensity data) is < 20%

d) The shape of the concentration curve of the positive reference controls is sigmoidal

e) The results of the three reference items are within the acceptable range (see table below) and classified in the correct class (negative or positive for anti-androgenicity)

f) The results are reproducible in at least one other independent experiment

Acceptable range values for the three reference values used in the AR agonist assay

Reference item	Log PC_{10} (M)	Log PC_{50} (M)	Judgement
5α-dihydrotestosterone (DHT)	-12.08 □ -9.87	-11.03 □ -9.00	Positive
Mestanolone	-10.92 □ -10.41	-10.15 □ -9.26	Positive
Di-(2-ethylhexyl) phthalate (DEHP)	-	-	Negative

Acceptable range values for the three reference values used in the AR antagonist assay

Reference item	Log [linear IC_{30}] (M)	Log [linear IC_{50}] (M)	Judgement
Hydroxyflutamide (HF)	-8.37 □ -6.41	-7.80 □ -6.17	Positive
Bisphenol A	-7.52 □ -4.48	-7.05 □ -4.29	Positive
Di-(2-ethylhexyl) phthalate (DEHP)	-	-	Negative

6. Calculations and abbreviations:

AR agonist assay

Mean relative light unit (RLU)	Calculated for the vehicle controls (VC) of each pate
Normalised RLU	All RLU values were normalised by subtracting the mean RLU of the vehicle control of a specific plate from the data from each well of the same plate
PC_{AGO}	The mean (normalised RLU values for the positive control
Relative transcriptional Activity (RTA)	RTA compared to positive control (set at 100%): The normalised value for each well divided by the PC_{AGO}
Fold induction of PC_{AGO}	Mean RLU of PC_{AGO} divided by the mean RLU of VC
RPC_{max}	Maximum level of response induced by a test chemical expressed as a percentage against the response by PC_{AGO} on the same plate.
Log PC_{10}	Concentrations that induce a response corresponding to that of a 10% effect of the positive control
Log PC_{50}	Concentrations corresponding to a 50% effect for the positive control

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AR antagonist assay

Mean relative light unit (RLU)	Calculated for the vehicle controls (VC) of each pate
Normalised RLU	All RLU values were normalised by subtracting the mean RLU of the vehicle control of a specific plate from the data from each well of the same plate
Normalised AG ref	Mean, normalised RLU values for the spiked-in (500 pM DHT). For each well of the plate the normalised value was divided by the mean value of the normalised AG ref (set to 100%)
Fold induction of AG ref	Mean RLU of AG ref divided by the mean RLU of VC
PC_{ATG}	Positive antagonist control
RTA of PC_{ATG}	RTA of PC_{ATG}(%)=(Mean RLU PC_{ATG} – Mean RLU VC/Mean RLU of AG ref – Mean RLU VC) x 100
Log IC_{30}	Concentrations corresponding to 30% inhibition of transcriptional activity induced by 500 pM DHT
Log IC_{50}	Concentrations corresponding to 50% inhibition of activity of 500 pM DHT
Log [IC_x]	Log [IC_x] = 1-[(b-(100-x))/(b-d)] (a-c)
Cytotoxicity (%)	Determined by reduction of the *Renilla* luciferase activity and calculated as follows: Cytotoxicity (%) = 100 – (RLU each well – Mean RLU of PC_{CT})/(Mean RLU AG ref – Mean RLU PC_{CT}) x 100

II. RESULTS AND DISCUSSION

1. Solubility test

The test item was soluble in 1 M DMSO; however, a 10-fold dilution of the solution in the exposure medium resulted in precipitation of the test item. A clear solution was achieved by the addition of a 10-fold dilution of a 1 mM solution of the test item in the exposure medium. Therefore, this concentration was selected as the highest test item spiking concentration in the first main experiments.

2. AR agonist assays

The fold-induction of the positive control compared with the vehicle control was above the requisite 6.4-fold induction and the fold induction of DHT (corresponding to the PC_{10} value) was higher than the fold induction of VC+2SD in both experiments, therefore the acceptance criteria are met.

The results for the positive control vehicle control obtained in both AR agonist experiments are presented in the table below:

Table 5.8.3-01: Results for the positive control (10 nM DHT) in the AR agonist assays

Experiment number	Passage number AR-EcoScreen™ cell line	Fold induction		
		PC_{AGO} [a)]	PC_{10}	VC + 2SD
1	11	8.43	1.74	1.10
2	13	8.06	1.71	1.05

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[a)]Compared to mean vehicle control, VC-vehicle control, PC=positive control (10nM DHT), SD= standard deviation

The reference items DHT, mestanolone and DEHP were included in each AR agonist experiment to demonstrate the stability of the cell line. The log PC_{50} and log PC_{10} values for DHT, Mestanolone and DEHP were all within the acceptability criteria and the reference items were correctively classified as positive (DHT and Mestanolone) or negative (DEHP). All coefficients of variation (CV) for the luminescence data obtained for Aclonifen and the reference items DHT, Mestanolone, DEHP, VC, PC_{AGO} and PC_{CT} were within the acceptability criteria (≤ 20%) for both experiments. Since all of the acceptability criteria were met, both of the AR agonist assays were considered valid.

The values for the Log PC_{10}, Log PC_{50} and the RPC_{max} for aclonifen and the refence items are presented in the table below.

Table 5.8.3-02 AR agonism assay - Values obtained for Aclonifen and the reference items DHT, Mestanolone and DEHP

Reference item	Experiment number	Log PC_{10} (M)	Log PC_{50} (M)	RPC_{max} (%)	Judgement
DHT	1	-10.74	-9.79	103.3	Positive
	2	-10.78	-9.81	98.0	Positive
Mestanolone	1	-10.73	-9.75	96.1	Positive
	2	-10.83	-9.87	96.3	Positive
DEHP	1	-	-	-0.5	Negative
	2	-	-	-0.2	Negative
Aclonifen technical	1	-	-	-0.7	Negative
	2	-	-	-0.7	Negative

The maximum level of response induced by the test item compared with the response induced by 10 nM DHT (the RPC_{max}) was -0.7% in both experiments. Since these values were below 10% for both independent experiments, Aclonifen was considered to be negative in the AR agonist assay.

3. AR antagonist assays

No cytotoxicity (>20% reduction of *Renilla* luciferase activity) was observed for aclonifen at any concentration or for any reference substance.

The RTA value for the positive antagonistic control (PC_{ATG}) was >46% in both experiments and so therefore did not meet the acceptability criteria (see table below).

Table 5.8.3-03 Results for the positive control (10 nM DHT and 0.1 µM HF) in the AR antagonist assays

Experiment number	Passage number AR-EcoScreen™ cell line	RTA (%) of PC_{ATG}	Fold induction AG ref
1	11	47.0	7.4
2	13	49.6	6.5

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The RTA value for the PC_{ATG} determines whether there is a large enough window to measure AR antagonist activity and should be ≤46%. However, the Log IC_{30} and the Log IC_{50} values for the reference items HF and BPA were within the acceptability criteria, thus demonstrating that sufficient AR antagonistic activity could be detected. Therefore, the RT values of PC_{ATG} were acceptable in the context of this assay, despite being slightly outside the normal criteria. The fold induction of the AG_{ref} met the acceptability criteria, being ≥ 5-fold for both experiments. The CVs for the luminescence data for aclonifen and for the reference items (HF, BPA, DEHP) and other controls (AG ref, VC, PC_{AGO}, PC_{ATG} and PC_{CT}) were within the acceptability criteria of ≤ 20% for both experiments. The log IC_{50} and log IC_{30} values for HF, BPA and DEHP were within the acceptability criteria and were correctly classified as positive or negative in the AR antagonist assay. Therefore, both assays were considered valid. The log IC_{50} and logIC_{30} values for Aclonifen and reference items HF, BPA and DEHP are presented in the table below.

Table 5.8.3-04 AR antagonism assay - Log IC_{50} and log IC_{30} values for aclonifen, HF, BPA and DEHP

Reference item	Experiment number	Log IC_{30} (M)	Log IC_{50} (M)	Judgement
Aclonifen technical	1	-6.31	-6.07	Positive
	2	-6.39	-6.13	Positive
HF	1	-7.35	-6.88	Positive
	2	-7.55	-7.07	Positive
BPA	1	-5.84	-5.54	Positive
	2	-5.93	-5.63	Positive
DEHP	1	-	-	Negative
	2	-	-	Negative

A log IC_{30} and a log IC_{50} could be determined for aclonifen in both independent experiments. Because a log IC_{30} could be determined, Aclonifen technical was considered to be positive in the AR antagonist assay.

III. CONCLUSION

The majority of the acceptability criteria were met and therefore the assay was considered valid to determine the AR agonist and AR antagonist activity of aclonifen technical.

No androgenic activity was seen in either of two valid independent experiments in the AR-Ecocreen™ cell line.

AR antagonistic activity was observed in both valid independent experiments in the AR-Ecoscreen™ cell line.

Assessment and conclusion by applicant:

The study is valid and acceptable, AR antagonistic, but not agonistic activity was observed in the AR-EcocreenTM cell line.

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Assessment and conclusion by RMS:

Data Point:	KCA 5.8.3/02
Report Author:	[redacted]
Report Year:	2019
Report Title:	Evaluation of the estrogen receptor agonist and antagonist activity of aclonifen technical (AE F068300) using the stably transfected human estrogen receptor-α transactivation assay (hERα-HeLa-9903 cell line)
Report No:	20184672
Document No:	M-664998-01-1
Guideline(s) followed in study:	OECD Test guideline No. 455 (July 2016)
Deviations from current test guideline:	Current guideline: OECD 455, 2016 No deviation
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 ability of aclonifen technical to activate or block the oestrogen receptor alpha (ERα) was investigated using the human hERα-HeLa-9903 cell line (a cell-based reporter assay, expressing human ERα and an ERα inducible luciferase reporter gene resulting in oestrogen dependent luminescence). Two valid independent experiments were performed for the ER agonist assay and three for the ER antagonistic assay. Based on solubility analysis a top concentration of 10 µM aclonifen was selected for cytotoxicity analysis. Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test under agonistic and antagonistic conditions. At 10 µM aclonifen caused cytotoxicity of 22.5% and 18.5% under antagonistic and agonistic conditions respectively, therefore, 3.16 µM was selected as the high concentration in the main assays.

In the ER agonist assay (two independent experiments) the test substance was tested at seven concentrations ranging from 10 pM to 3.16 µm, vehicle controls, positive controls and complete concentration response curved of the reference items (17ß-estradiol (E2), 17α-estradiol, 17α-methyltestosterone and corticosterone) were included. Both ER agonists met the acceptability criteria and were therefore considered valid. Aclonifen technical dd not show an oestrogenic agonist response up to a concentration of 3.16 µM in both experiments (RPC_{max} 4.7% and 2.8% respectively). Therefore, aclonifen was considered negative in the ER agonist assay.

In the ER antagonistic assay (three independent experiments) aclonifen was tested at six concentrations ranging from 100 pM to 3.16 µM, together with complete concentration response curves for the reference items tamoxifen and flutamide. Log IC_{50} and log IC_{30} values could be determined for tamoxifen, although they were slightly outside the acceptability criteria. Nonetheless, as the positive control for antagonistic activity demonstrated the ability of the assay to detect ER antagonists the data was accepted. All other acceptability criteria were met and therefore the assay was considered valid. Aclonifen technical did not show an oestrogenic antagonistic potential (no IC_{50}

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could be determined) up to a concentration of 3.16 µM. Therefore, aclonifen was considered negative in the ER antagonistic assay.

Overall, aclonifen technical did not show any oestrogenic agonist or antagonist potential when tested at concentrations up to 3.16 µM in the hERα-HeLa-9903 cell line.

I. MATERIALS AND METHODS

Study dates: Experimental: 07 to 26 February 2019
Completion: 06 June 2019

A. MATERIALS

1. Test Item Aclonifen technical

Batch No.:	AE F068300-01-28
Purity:	99.9%
Appearance:	Yellow powder
Expiry:	13 November 2020
Structure:	
Vehicle:	DMSO

2. Positive/negative controls:

<u>Positive controls</u>
17β-Estradiol (E2)
17α-Estradiol
17α-Methyltestosterone
Tamoxifen
4-Hydroxytamoxifen (OHT)
Digitonin

<u>Negative controls</u>
Corticosterone
Flutamide

3. Test system: Stably transfected human hERα-HeLa-9903 cell line.

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Medium: Cell culture and seeding media

Eagle's essential medium (EMEM) without phenol red, supplemented with 2 mM L-Glutamine, 60 mg/L Kanamycine and 10% charcoal stripped foetal bovine serum (FBS).

Exposure medium

Eagle's essential medium (EMEM) without phenol red, supplemented with 2 mM L-Glutamine and 60 mg/L Kanamycine. With 10% charcoal stripped foetal bovine serum (FBS) for the ER antagonist assay and without for the AR agonist assay.

B. METHODS

1. Solubility and cytotoxicity tests

The cloudiness and/or precipitate of the test item in the vehicle was assessed under the microscope in a preliminary solubility test. The test item initially dissolved in DMSO at 1 M and was further diluted to ascertain the highest soluble concentration of the test item in the vehicle. The solubility of the test item in the exposure media was also determined by diluting 1 M test item stock solution (in DMSO) 10-fold in the exposure medium.

To determine the cytotoxicity of aclonifen in the ER agonist and antagonist assays a separate experiment was performed in which the hERα HeLa 9903 cells were plated in 96-well plates and exposed to aclonifen technical at concentrations of 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM and 10 pM under ER agonist culture conditions and at 10 µM, 1 µM, 100 nM, 10 nM, 1 nM and 100 pM under ER antagonistic culture conditions. After 24 hours a cell viability test was performed using MTT.

2. Plating and exposure of cells

Cells were grown for at least one passage until they reached 75-90% confluency and were then seeded in 96-well plates at a seeding density of $1x10^4$ cells/100 µL/well ($1x10^5$ cells/mL) for the ER agonist assay or $1.5x10^4$ cells/100µL/well ($1.5x10^5$ cells/mL) for the ER antagonist assay.

An appropriate range of concentrations for the main experiments were selected based on the solubility and MTT assays. In cases where cytotoxicity was observed, the highest concentration was chosen to reach a maximum of 20% cytotoxicity.

ER agonist assay:

Prior to exposure of the cells, the exposure medium was obtained by preparing 1000x concentrated spiking solutions for the test and reference items and then adding 1.5 µL of the appropriate spike to 498.5 µL medium. Cells were then exposed by adding 50 µL of the exposure medium to the appropriate wells of a 96-well plate containing the cells to obtain a final volume of media in each cell of 150 µL. Positive control wells treated with 1 nM E2 (in 6-fold) and vehicle control wells treated with DMSO (0.1% v/v in 6-fold) were included on each plate. The plates were then incubated in the dark for 21 or 21.5 hours in experiments 1 and 2 respectively, under appropriate conditions (37±1˚C, 80-100% humidity and 5±0.5% CO_2).

ER antagonist assay:

Appropriate spiking solutions of the test and reference items were prepared on a 96-well plate and further diluted in a 96-deep-well plate in exposure medium. The hERαHeLa-9903 cells were exposed by the addition of 50 µL exposure medium to the 96-well plate containing the cells (to a final volume of 150 µL). Positive agonist controls (PC) wells treated with 1 nM E2(in 6-fold), vehicle controls (VC) treated with DMSO (0.1% v/v in 6-fold), positive antagonistic control wells treated with 1 µM

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OHT, positive cytotoxicity control wells treated with 100 µM digitonin and 25 pM E2 (in triplicate) and spike-in control wells treated with 25 pM E2 and DMSO (0.1% v/v in 6-fold) were included. After the addition of the exposure medium to each well the plates were incubated under appropriate conditions (37±1˚C, 80-100% humidity and 5±0.5% CO_2) for 22 hours (experiment 1), 23.5 hours (experiment 2) or 21.5 hours (experiment 3).

3. Luciferase measurements

ER agonist assay:

Following incubation, the exposure medium was removed, and the cells washed twice with 100 µL PBS and subsequently lysed for 10 minutes at room temperature using 20 µL lysis buffer. The plates were then frozen at ≤ 75˚C for 30 minutes to 72 hours until analysis (plates were thawed and equilibrated at room temperature on an orbital shaker in the dark). Luciferase activity was determined at ambient temperature using a standard Promega (E1501) luciferase assay system; the 96-well plate was placed into the luminometer and 100 µL luciferase assay reagent/well was injected before immediate reading of the well.

ER antagonistic assay:

Luciferase activity was determined using the Steady-Glo Luciferase assay system from Promega (E2510). Following incubation, 50µL of the exposure medium was removed and 100 µL of Steady-Glo reagent added to each well, the plates were further incubated for 7 to17 minutes at room temperature in the dark before each well was read for 2 minutes using a luminometer to determine the luminescence intensity.

In all cases the luminescence data was presented as Relative Light Units (RLU).

4. Acceptability criteria

The test item is considered positive in the ER agonist assay if the maximum response induced (RPC_{max}) was ≥ 10% that of the positive control in at least two/two independent experiments. The test item is considered negative in the ER agonist assay if the (RPC_{max}) is < 10% that of the response of the positive control in at least two/two independent experiments.

The ER agonist assay is considered acceptable if the following criteria are met:

a) The mean luciferase activity of the positive control (1 nM E2) is ≥ 4-fold that of the mean vehicle control on each plate

b) The fold induction corresponding to the PC_{10} value of the concurrent positive control is greater than 1+2SD of the fold induction value of the vehicle control (set at 1)

c) The coefficient of variation (CV) in triplicate luminescence intensity data is < 20%

d) The results of the three reference items are within the acceptable range (see table below)

e) The results are reproducible in at least one other independent experiment

The test item is considered positive in the ER antagonist assay if the log IC_{30} can be calculated in two/two experiments. The test item is considered negative if the log IC_{30} cannot be calculated in two/two experiments.

The AR antagonist assay is considered acceptable if the following criteria are met:

a) The mean luciferase activity of the spike-in control (25 pM E2) is ≥ 4-fold that of the mean vehicle control on each plate

b) The relative transcriptional activation (RTA) of 1 nM E2 is > 100%

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c) The RTA of 1 µM OHT is < 40.6%

d) The RTA of 100 µM Digitonin is <0%

e) The results of the three reference items are within the acceptable range (see table below) and judged correctly as negative or positive

f) The results are reproducible in at least one other independent experiment

Acceptable range values for the reference values used in the ER agonist assay

Reference item	Log PC_{50}	Log PC_{10}	Log EC_{50}	Hill slope
17ß-estradiol (E2)	-11.4 □ -10.1	< -11	-11.3 □ -10.1	0.7 □ 1.5
17α-estradiol	-9.6 □ -8.1	-10.7 □ -9.3	-9.6 □ -8.4	0.9 □ 2.0
17α-methyltestosterone	-6.0 □ -5.1	-8.0 □ -6.2	-	-
Corticosterone	-	-	-	-

Acceptable range values for the reference values used in the ER antagonist assay

Reference item	Criteria	Log IC_{50}
Tamoxifen	Positive: IC_{50} could be calculated	-5.942 □ -7.596
Flutamide	Negative: IC_{50} could not be calculated	-

5. Calculations and abbreviations

ER agonist assay

Mean relative light unit (RLU)	Calculated for the vehicle controls (VC) of each pate
Normalised RLU	All RLU values were normalised by subtracting the mean RLU of the vehicle control of a specific plate from the data from each well of the same plate
The mean (normalised) RLU	Calculated for the positive control for each plate
Relative transcriptional Activity (RTA)	RTA of each well compared to positive control (set at 100%): The normalised RLU value for each well divided by the mean normalised value for the positive control
Mean value of transcriptional activity	Calculated for each concentration group of test or reference item
EC_{50}	The half maximal effective concentration of a test chemical Calculated using Prism v. 4.03 using the following equation: $Y=B + (T-B)/1+10^{(log(EC50-x) \times Hillslope)}$ Where Y = response in RLU, B = bottom, T = top and X = logarithm of the concentration
RPC_{max}	Maximum level of response induced by a test chemical expressed as a percentage against the response induced by 1 nM E2 on the same plate.
PC_{max}	Concentration associated with the RPC_{max}

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PC_{50}	Concentration inducing 50% of the maximum level of the positive control
PC_{10}	Concentration inducing 10% of the maximum level of the positive control
ER antagonist assay	
Mean relative light unit (RLU)	Calculated for the vehicle controls (VC) of each pate
Normalised RLU	All RLU values were normalised by subtracting the mean RLU of the vehicle control of a specific plate from the data from each well of the same plate
The mean (normalised) RLU	Mean, normalised RLU values for the spike-in (25 pM E2).
Relative transcriptional activity (compared to the spike-in control response)	For each well of the plate the normalised values were divided by the mean value of the normalised spike-in control (set to 100%)
Concentrations that induced the IC_{30} and/or the IC_{50}	Calculate for positive substances. The IC_x value was calculated by interpolating between 2 points on X-Y co-ordinate, one immediately above and one immediately below an IC_x value. The IC_x value was calculated in excel using the following equation (where the points above and below have the co-ordinates (c, d) and (a, b): Lin IC_x = a-(b-(100-x)) x (a-c)/(b-d)
PC_{ATG}	Positive control for agonism
PC_{AGO}	Positive control for antagonism
PC_{CT}	Positive control for cytotoxicity

II. RESULTS AND DISCUSSION

1. Solubility and cytotoxicity tests

In the solubility test, aclonifen was soluble at a concentration of 1 M in DMSO. Precipitation of the test item resulted from a 333-fold dilution of the 1 M solution in the exposure medium. However, a 333-fold dilution of a 10mM solution of the test item in the exposure medium resulted in a clear solution and therefore, this concentration was used as the highest concentration in the MMT test for cytotoxicity (final concentration in well of 10 µM).

In the MTT test, aclonifen technical caused cytotoxicity (22.5%) under antagonistic conditions and was almost cytotoxic under agonist conditions. Therefore, 3.16 µM was used as the highest concentration in the subsequent experiments.

2. ER agonist assay

Two independent agonistic assays were performed. The fold induction of the vehicle control + 2SD and the PC_{10} of the positive control obtained for both experiments for the reference item and test item plate are presented below.

Table 5.8.3-05 Performance criteria for the ER agonist assay

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Experiment No.	Passage No.	Plate ID	Fold induction		
			PC* (1 nM E2)	PC_{10} (1 nM E2)	VC (mean + 2SD)
1	P14	Reference	13.25	2.23	1.40
		Test	14.08	2.31	1.61
2	P15	Reference	12.31	2.13	1.29
		Test	10.34	1.93	1.23

*compared to vehicle control, VC = vehicle control, PC = positive control, SD = standard deviation, PC_{10} = concentration inducing 10% of the maximum level of the positive control

The fold induction of 1nM E2 compared to the vehicle control was greater than the required 4-fold induction in both experiments. The fold induction of 1nM E2 compared with vehicle control was 13.25 and 12.31 in experiments 1 and 2 respectively for the reference plate, and 14.08 and 10.34 respectively for the test item plate. Therefore, the acceptance criteria are met. Some variation was observed in the CVs of triplicate luminescence data points of aclonifen technical and for the raw data obtained for E2, 17α-estradiol, corticosterone and 17α-methyltestosterone, with some of the CV's being outside the acceptability criteria of ≤20%, however this did not preclude the construction of a dose-response curve for aclonifen or for the reference items (averages of luminescence data were used to construct the curves). Therefore, as all other ER agonist acceptability criteria were met, this was not considered to affect the validity of the study and the data were accepted.

Within each AR agonist assay, the reference items E2, 17α-estradiol, corticosterone and 17α-methyltestosterone were included to monitor the stability of the cell line. The log PC_{50}, log PC_{10}, log EC_{50}, Hill slope and RPC_{max} values for aclonifen and the reference items are presented in the table below:

Table 5.8.3-06 Values obtained for aclonifen and reference items in the ER agonist assay

Test/reference item	Experiment number	Log PC_{50}	Log PC_{10}	Log EC_{10}	Hill slope	RPC_{max} (%)	Judgement
17ß-estradiol (E2)	1	-10.68	-12.45	-10.56	0.66	110.6	Positive
	2	-10.59	-11.71	-10.66	1.34	102.3	Positive
17α-estradiol	1	-8.57	-9.88	-8.67	0.59	101.8	Positive
	2	-8.74	-9.70	-8.86	2.31	100.7	Positive
17α-methyltestosterone	1	-	-6.85[1)]	-	-	35.5	Positive
	2	-	-5.91[1)]	-	-	40.2	Positive
Corticosterone	1	-	-	-	-	0.8	Negative
	2	-	-	-	-	-1.3	Negative
Aclonifen technical	1	-	-	-	-	4.7	Negative
	2	-	-	-	-	2.8	Negative

[1)]Historical control range Log PC_{10} = -7.23 □ 5.58; Log PC_{50} = -5.05 (a log PC_{50} value was obtained in only one experiment, no value was obtained in other HC experiments)

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Some criteria were outside the acceptability ranges for the reference substances. The Hillslope for E2 in experiment 1 was outside the acceptability criteria, however, the dose response curve yielded a similar (acceptable) log EC_{50} as that obtained in experiment 2, in which the Hill slope was acceptable. Similarly, for 17α-estradiol, the Hill slopes in both experiments were variable and were outside the acceptability criteria, however, the EC_{50} value was similar for both experiments and was within the acceptability range. The log PC_{50} values in both experiments and the log PC_{10} value in experiment 2 for 17α-methyltestosterone were outside the acceptability criteria. However, the values are within the laboratories historical control ranges and the RPC_{max} values were above 10% (correctly classified as positive). Overall, since all other acceptability criteria were met and all reference items were correctly classified as positive or negative, the assays were considered to be valid to detect ER agonistic activity.

No log PC_{50} or log PC_{10} could be calculated for aclonifen technical. The maximum level of response induced by the test item compared with the response induced by the 1 nM E2 (the RPC_{max}) was 4.7% and 2.8% in experiments 1 and 2 respectively. Since the RPC_{max} values were below 10% in both independent experiments, Aclonifen technical was considered to be negative in the ER agonistic assay.

3. ER antagonistic assay

Three independent ER antagonistic assays were performed; as the log IC_{50} values for tamoxifen were just outside the acceptability criteria in experiments 1 and 2, a third experiment was performed to confirm the negative results. The PC_{AGO}, PC_{ATG}, PC_{TG} and the fold induction of the spike-in control are presented below:

Table 5.8.3-07 Performance criteria in the ER antagonistic assay

Experiment No.	Passage No.	RTA %			Fold induction
		PC_{ATG}	PC_{AGO}	PC_{CT}	Spike-in control*
1	P14	26.7	224	-20.7	5.6
2	P15	30.3	224	-21.9	5.5
3	P10	23.0	214	-15.9	7.1

*compared with mean vehicle control

For all experiments, the RTA values of the PC_{ATG} was <40.6%, the RTA of the PC_{AGO} was above 100%, the RTA for the PC_{CT} was below 0% and the fold-induction of the spike-in control was >4.0%, therefore the acceptability criteria were met.

The reference items tamoxifen and flutamide were included in all experiments. The log IC_{50} and log IC_{30} values for aclonifen technical and the reference items are presented below:

Table 5.8.3-08 Log IC_{50} and log IC_{30} values for tamoxifen and flutamide in the ER antagonistic assay

Test substance	Experiment No.	Log IC_{50} (M)	Log IC_{30} (M)	Judgement
Tamoxifen	1	-5.89	-6.27	Positive
	2	-5.53	-6.36	Positive
	3	-5.93	-6.36	Positive

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Flutamide	1	-	-	Negative
	2	-	-	Negative
	3	-	-	Negative
Aclonifen technical	1	-	-	Negative
	2	-	-	Negative
	3	-	-	Negative

No IC_{50} or IC_{30} values could be obtained for flutamide, thus meeting the acceptability criteria. Values could be obtained for tamoxifen in all three experiments but the log IC_{50} values were just outside the acceptability criteria (-5.942 □ -7.596). However, in all three assays the positive control for antagonistic activity was acceptable at ≤ 40.6% (26.7%, 30.3% and 23% in experiments 1, 2 and 3 respectively). Therefore, it has been shown that the assay is able to detect ER antagonistic activity and so the results were accepted. Since all other acceptability criteria were met the assay was considered valid.

A log IC_{50} and log IC_{30} could not be calculated for aclonifen technical. Therefore, aclonifen is considered to be negative in the ER antagonistic assay.

III. CONCLUSION

The majority of the acceptability criteria of the ER agonist and antagonist assays were met and therefore the assays were considered to be valid.

Aclonifen technical did not show any estrogenic agonist or antagonist activity when tested at concentrations up to 3.16 µM in the hERα-HeLa-9903 cell line.

Assessment and conclusion by applicant:

The study is valid and acceptable. Aclonifen did not show ER agonistic or antagonistic activity in the hERα-HeLa-9903 cell line.

Assessment and conclusion by RMS:

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Data Point:	KCA 5.8.3/06
Report Author:	
Report Year:	2019
Report Title:	Evaluatin of aclonifen (AE F068300) in the H295R steroidogenesis assay
Report No:	SA 18235
Document No:	M-675903-01-1
Guideline(s) followed in study:	US-EPA, OCSPP Series 890, Endocrine Disruptor Screening Program test guidelines, No. 890.1550: Steroidogenesis (Human Cell line – H295R) (July 2011). OECD Guideline for the testing of chemicals, Test No. 456: H295R Steroidogenesis Assay (July 28, 2011).
Deviations from current test guideline:	Current guideline: OECD Guideline for the testing of chemicals, Test No. 456 No deviation
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 effect of aclonifen on steroidogenesis *in vitro* was investigated using H295R cells according to OECD TG 456. The cells were exposed to aclonifen for 48 hours at concentrations of 8 x 10^{-11} to 8x 10^{-5} M for each of three independent evaluations. The culture medium was then recovered, and the concentrations of testosterone and estradiol were determined using specific Enzyme Immunoassay (EIA) kits (a slight interference of 27% was noted at the high concentration for the estradiol kit only). The responsive of the H295R was investigated in a preliminary quality control evaluation (and concurrently in the main investigations) using two reference compounds (forskolin and prochloraz). The data obtained in the quality control study and in the concurrent positive control evaluations were within the guideline criteria, thus confirming the suitability of the cells to be used in the evaluations. The EC_{50} values of the reference compounds were within the guideline ranges.

No cytotoxicity was noted up to 100 µM; however, owing to a change in morphology at this concentration (indicative of some interference to the proliferative capacity of the cells), a high concentration of 80 µM was selected for the main evaluations

Aclonifen had no clear effect on estradiol secretion when tested in the H295R adrenal cell line. However, a treatment related statistically significant inhibition of testosterone secretion (-77.4%; $p \leq 0.01$) was observed at the highest concentration (80µM).

This *in vitro* screening assay is categorized as a tier 2 study of the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters (2012) and is intended for screening purposes only. Therefore, although the data indicate that aclonifen has a potential to interfere with steroid hormone synthesis in H295R cells when tested at high concentrations, such data should be put into context with all other scientifically relevant data, using a weight of evidence approach before concluding on whether this test item has a potential to interfere with steroidogenesis *in vivo*.

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I. MATERIALS AND METHODS

Study dates: Experimental: 04 to 21 February 2019
Completion: 25 February 2019

A. MATERIALS

1 Test Item Aclonifen technical

Batch No.: PTDF001324

Purity: 99.9%

Appearance: Yellow powder

Expiry: Not stated

Structure:

Vehicle: DMSO

2 Positive controls: Forskolin (batch: SLBP3308V; purity: 98%)
Prochloraz (batch: BCBW4694; purity: 98.7%)

3. Test system: H295R cells purchased from the American Type Culture Collections

Batch Number	58660579			
Date of freezing A/5.0/5 cells*	September 24, 2015			
Date of thawing	January 10, 2019			
	QC evaluation	Aclonifen and positive control evaluations		
		First	Second	Third
Passage numbers	B/3.5/8	B/5.5/10	B/6.5/11	B/7.5/12
Sample numbers	A1-A24	A25-A72	B25-B72	C25-C72

*Passage nomenclature: cells were grown from a commercial vial for a total of 5 passages (A1.0-A5.0) at passage A5.0 the cells were frozen and stored in liquid nitrogen. For all evaluations, already frozen passages 5.0 cells (A5.0) were thawed and used. B/X.5/Y where X= number of passages after thawing and Y = total number of passages for the cells.

Cell culture: H295R cells were grown and maintained at 37˚C±1˚C, 5% CO_2 in DMEM:F12 medium supplemented with 1% ITS+ premix, 2.5% Nu-Serum and 0.1% Penicillin-Streptomycin.

B. METHODS

1. Cytotoxicity

Aclonifen was assessed for interference with the testosterone and estradiol kits up to a maximum concentration of 8 x 10^{-5}M. Prior to the first evaluation and during the second evaluation, the

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cytotoxicity of aclonifen was assessed using the XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) method, which measures the mitochondrial activity of living cells. During the last 4 hours of incubation the cells were exposed to XTT/phenazine methosulphate, with the resulting orange formazan derivative measured at OD 450 nm. In addition, cell viability was assessed at the end of each evaluation (immediately following culture medium recovery) by staining cells with trypan blue.

2. Positive controls

Positive control stock solutions were prepared by dissolving the appropriate positive control substance in DMSO to give concentrations of 10^{-2} M and 10^{-3} M for forskolin and 10^{-3} M and 10^{-4} M for prochloraz. These stock solutions were placed in glass air-tight bottles and stored for up to 4 months at approximately +5°C ± 3°C when not in use. Immediately prior to cell treatment, positive control master mixes were prepared by diluting the appropriate stock solution in culture medium so as to achieve the appropriate positive control substance concentrations, whilst ensuring a final concentration of 0.1% v/v DMSO per well. For forskolin the final concentrations were 1 µM and 10 µM and for prochloraz they were 0.1 µM and 1 µM. Based on visual inspection forskolin and prochloraz appeared to be soluble in both DMSO and the culture medium.

A laboratory proficiency determination was conducted in which EC_{50} values were estimated for the positive controls forskolin and prochloraz, with identical conditions as the main study. Threshold values or lowest observable effect concentrations (LOEC) were established using changes in hormone secretions of greater than 20% compared with the vehicle control. The concentrations used, the resulting EC_{50} values and the LOECs are provided in the table below:

Table 5.8.3-09 Steroidogenesis assay - EC_{50} values and LOECs for forskolin and prochloraz

	Conc. Tested	**Estimated EC_{50} values *(OCSPP TG No. 1550 ranges)***		**LOEC (% changes)**	
		Testosterone	**Estradiol**	**Testosterone**	**Estradiol**
Forskolin	0.03, 0.1, 0.3, 1, 3, & 10 µM	□ 0.1 µM *(0.2 - 2µM)*	0.5 – 0.6 µM *(0.3 – 3 µM)*	0.1 µM *(+22%)*	0.03 µM *(+52%)*
Prochloraz	0.01, 0.03, 0.1, 0.3, 1 & 3 µM	□ 0.05 µM *(0.01 – 1 µM)*	□ 0.2 µM *(0.03 – 0.3 µM)*	0.01 µM *(-24%)*	0.3 µM *(-61%)*
OECD TG 456 indicates forskolin LOECs of ≤10 µM for testosterone and ≤0.1 µM for estradiol and prochloraz LOECs of ≤0.1 µM for testosterone and ≤1.0 µM for estradiol.					

3. Experimental design

Test substance stock solutions were prepared by dissolving aclonifen in DMSO to give concentrations of 10-1M to 10-7M. Immediately prior to cell treatment, test substance master mixes were prepared by diluting the appropriate stock solution in culture medium so as to achieve the final test substance concentrations of between 8 x10-5M and 8 x 10-11 M (as shown in the table below), whilst ensuring a final concentration of 0.1%v/v DMSO per well. Based on visual inspection, aclonifen appeared to be soluble in both DMSO and the culture medium.

Table 5.8.3-10 Steroidogenesis assay - Final concentrations of aclonifen in the test substance stock solutions (master mix)

Test substance		**Aclonifen**
Stock Solution (M)	Master Mix	Final Concentration (M)

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8 x 10^{-2}	1	8 x 10^{-5}
8 x 10^{-3}	2	8 x 10^{-6}
8 x 10^{-4}	3	8 x 10^{-7}
8 x 10^{-5}	4	8 x 10^{-8}
8 x 10^{-6}	5	8 x 10^{-9}
8 x 10^{-7}	6	8 x 10^{-10}
8 x 10^{-8}	7	8 x 10^{-11}

The H295R cells were isolated from flasks of ≥75% confluence and seeded into 24-well plates at a density of approximately 0.3×10^6 cells/ml and a final volume of 1ml/well. The cells were cultured at 37°C ± 1°C /5% CO_2 for approximately 24h prior to treatment. For each evaluation the cells were checked microscopically for good attachment and proper morphology approximately 24 hours after placement in the wells. The medium in each well was then removed and replaced with 1ml of the appropriate master mix (freshly prepared), the solvent control DMSO (0.1% v/v) in fresh culture medium or fresh culture medium only.

For purposes of quality control, the cells were exposed in triplicate to the appropriate concentration of positive control, DMSO or cell culture medium only. For the test substance and positive control evaluations the cells were exposed in triplicate to forskolin, prochloraz or aclonifen, at the appropriate concentrations, or at least in triplicate to DMSO or culture medium. All plates were exposed for 48h at 37°C ± 1°C /5% CO_2. Following treatment of the cells for 48-hours, three or four aliquots of culture medium/well were collected. The samples were frozen at -74˚C ± 10˚C until analysis. The concentrations of testosterone and estradiol were determined in the culture media from each well, using specific EIA kits. Based on internal validation the LOD for testosterone was 95.7 pg/mL and for estradiol was 16.6 pg/mL.

4. Statistical evaluations

For each evaluation the mean and standard deviation was calculated for each hormone and each group.

Quality control:

To assess the responsiveness of the H295R cells to reference positive controls (prior to the assessment of aclonifen), the data from the quality control plate were compared with the criteria below:

	Testosterone	Estradiol
Minimum Basal Production	500 pg/mL	40 pg/mL
Basal Production	≥ 5-times MDL	≥ 2.5-times MDL
Induction (10µM forskolin)	≥ 1.5-times SC	≥ 7.5-times SC
Inhibition (1µM prochloraz)	≤ 0.5-times SC	≤ 0.5-times SC
MDL=minimum detection limit, SC=solvent control		

No statistical analyses were performed for this data owing to the small sample size.

Reference positive controls:

In the evaluation of the reference positive controls, the mean and standard deviation were calculated for each hormone and each group for the first second and third evaluations. In addition, the overall mean and standard deviation were calculated (based on data from evaluations 1-3) for each hormone and each group (blank, solvent control, forskolin treatments and prochloraz treatments). A comparison of the data generated from the individual evaluations as well as the overall data, with the criteria above was carried out.

Changes relative to the solvent control were calculated for each positive control substance at each concentration and for each hormone, using the equation below:

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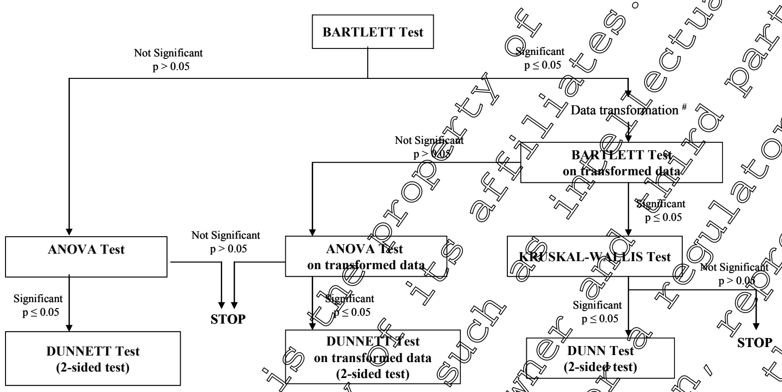
Relative change = (hormone concentration in each well) / (mean solvent hormone concentration)

Test item:

The mean and standard deviation were calculated for each hormone and each group for the three evaluations. In addition, the overall mean hormone concentration and standard deviation was calculated for each hormone and each group (based on data from evaluations 1-3). For each evaluation and the overall data, changes relative to the solvent control were calculated at each aclonifen concentration and for each hormone using the equation below:

Relative change = (hormone concentration in each well)/(mean solvent control hormone concentration)

Statistical analyses were conducted using the mean and standard deviations for the solvent control versus positive control or test substance as outlined below:



The levels of significance for each statistical comparison were performed at ≤ 0.05

II. RESULTS

1. Quality control plate

The quality control evaluation demonstrated that the data were aligned with the guideline criteria. The basal hormone proliferation was greater than the guideline values for both testosterone and for estradiol (see table below):

Table 5.8.3-11 Steroidogenesis assay - Results of the quality control plate

	Testosterone	Estradiol
Minimum Basal Production	4631 ± 270.6 pg/mL *(500 pg/mL)* [A]	154 ± 10.8 pg/mL *(40 pg/mL)* [A]
Basal Production	48-times MDL *(≥ 5-times MDL)* [A]	9.3-times MDL *(≥ 2.5-times MDL)* [A]
Solvent control hormone concentrations	4515 ± 169.6 pg/mL	138 ± 10.5 pg/mL
Induction (10 µM Forskolin)	8746 ± 538.8 pg/mL 1.9-times SC *(≥ 1.5-times SC)* [A]	4390 ± 88.6 pg/mL 28.5-times SC *(≥ 7.5-times SC)* [A]
Inhibition (1 µM Prochloraz)	222 ± 25.3 pg/mL 0.05-times SC *(≤ 0.5-times SC)* [A]	Complete inhibition[B] *(≤ 0.5-times SC)* [A]
Note: Data have been rounded-up. MDL: Minimum detection limit (testosterone: 95.7 pg/mL; estradiol: 16.6 pg/mL); SC: solvent control.		

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[A] guideline values are given in parentheses.
[B] estradiol concentration was <60pg/mL for all three samples.

The reference inhibitor (prochloraz) led to a marked inhibition in hormone production at both concentrations, meeting the guideline criteria at 1 µM. The reference stimulator (forskolin) markedly increased testosterone and estradiol production at both concentrations meeting the criteria at 10 µM. Therefore, it was considered that the responsiveness of the cells to the positive control substances was established and the cells were considered acceptable for use in the main study.

2. Interference evaluations

A slight interference of 27% at 80 µM aclonifen was observed with the EIA kit for estradiol. No interference with the testosterone EIA kit was observed at the concentrations tested.

3. Cytotoxicity evaluations

No cytotoxicity was noted up to the highest concentration of 100 µM; however, changes in morphology were noted at this concentration, indicating an effect on the proliferative capacity of the cells. Therefore, the high concentration was reduced to 80 µM.

4. Positive control evaluations

For each evaluation there was no difference in the hormone concentrations between the solvent control sample and the blank samples (basal levels), see tables below:

Table 5.8.3-12 Steroidogenesis assay - Basal and solvent control hormone measurements

	Mean testosterone concentration (pg/mL) ± SD			
	First Evaluation	**Second Evaluation**	**Third Evaluation**	**Overall**
DMSO	5059 ± 184.8	5891 ± 289.7	6359 ± 389.0	5769 ± 620.9
Blank†	4918 ± 284.9	6412 ± 256.3	6743 ± 415.4	6024 ±872.7
Note: Data have been rounded up. †: Blank wells contain cells and culture medium only.				

	Mean estradiol concentration (pg/mL) ± SD			
	First Evaluation	**Second Evaluation**	**Third Evaluation**	**Overall**
DMSO	170± 13.1	156 ± 10.8	187 ± 21.3	171 ± 19.7
Blank†	183 ± 20.0	191 ± 6.8	215 ± 10.2	196 ± 18.9***
Note: Data have been rounded up. †: Blank wells contain cells and culture medium only.; ***: p≤0.001.				

The overall mean basal production of testosterone was within the guideline recommendation (~10.8% compared to the recommended guideline of 30%), whilst the overall mean basal production of estradiol for the present study was also within the guideline recommendation (~11.5% compared to the recommended 30%).

Forskolin stimulated the secretion of testosterone and estradiol during each evaluation following 48h treatment of the H295R cells (see table below).

Table 5.8.3-13 Steroidogenesis assay - Forskolin treatment results

Forskolin conc.	Mean testosterone con. (pg/mL) ± SD (% change), mean fold change relative to DMSO	Mean estradiol con. (pg/mL) ± SD (% change), mean fold change relative to DMSO

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Evaluation	1	2	3	Overall	1	2	3	Overall
DMSO	5059 ± 184.8	5891 ± 289.7	6359 ± 389.0	5769 ± 620.9	170 ± 13.1	156 ± 10.8	187 ± 21.3	171 ± 19.7
1 µM	6277 ± 1214.4 (+24%)	8899 ± 897.1 (+51%)	8860 ± 176.4 (+39%)	8012 ± 1507.0** (+39%)	3135 ± 392.5 (+1744%)	3048 ± 668.7 (+1854%)	3591 ± 451.8 (+1820%)	3258 ± 514.9** (+1805%)
	1.24	1. 51	1.39	1.39	18.4	19.5	19.2	19.1
10 µM	9041 ± 814.4 (+79%)	11791 ± 953.0 (+100%)	11605 ± 163.2 (+82%)	10812 ± 1473.6** (+87%)	5299 ± 251.9 (+3017%)	5422 ± 136.7 (+3376%)	5524 ± 192.9 (+2854%)	5415 ± 198.2*** (+3067%)
	1.79	2.00	1.82	1.87	31.2	34.8	29.5	31.7

Note: Statistical evaluation conducted on overall data only; **: p≤0.01. ***: p≤0.001
Data have been rounded-up

Testosterone was increased by up to 51% and 100% at 1 µM and 10 µM forskolin respectively when compared to the DMSO controls. Overall, the increases in testosterone secretion were statistically significantly different (p≤0.01) from the controls at both concentrations. Similarly, estradiol was markedly increased by up to 1854% and 3376% at 1 µM and 10 µM forskolin respectively when compared to the DMSO controls. Overall the increases in estradiol were statistically significantly different from the controls at 1µM (p≤0.01) and 10 µM (p≤0.001).

Prochloraz induced a marked inhibition in testosterone and estradiol secretion during each evaluation following 48h treatment of the H295R cells (see table below):

Table 5.8.3-14 Steroidogenesis assay - Prochloraz treatment results

Prochloraz conc.	**Mean testosterone conc. (pg/mL) ± SD *(% change)*, mean fold change relative to DMSO**				**Mean estradiol conc. (pg/mL) ± SD (% change), mean fold change relative to DMSO**			
Evaluation	1	2	3	Overall	1	2	3	Overall
DMSO	5059 ± 184.8	5891 ± 289.7	6359 ± 389.0	5769 ± 620.9	170 ± 13.1	156 ± 10.8	187 ± 21.3	171 ± 19.7
0.1 µM	928 ± 154.5 (-82%)	1501 ± 202.0 (-75%)	1570 ± 87.1 (-75%)	1333 ± 333.6** (-77%)	100 ± 11.1 (-41%)	123 ± 16.6 (-21%)	114± 7.3 (-39%)	112± 14.6*** (-35%)
	0.18	0.25	0.25	0.23	0.59	0.79	0.61	0.65
1 µM	173 ± 12.8 (-97%)	330 ± 31.1 (-94%)	313 ± 6.4 (-95%)	272 ± 76.5*** (-95%)	Complete inhibition[A]	Complete inhibition[A]	Complete inhibition[A]	Complete inhibition[A]
	0.03	0.06	0.05	0.05	Complete inhibition[A]	Complete inhibition[A]	Complete inhibition[A]	Complete inhibition[A]

Note: Statistical evaluation conducted on overall data only; **: p≤0.01. ***: p≤0.001
Data have been rounded-up
[A]: estradiol concentration was <60pg/mL for all samples.

Prochloraz inhibited testosterone secretion by up to 82% and 97% following treatment with 0.1 µM and 1µM respectively compared to the DMSO controls. Overall the decreases in testosterone secretion were statistically significantly different from the controls for both concentrations (p≤0.01 for 0.1 µM and p≤0.001 for 1 µM). Estradiol secretion was reduced by up to 41% following treatment of the H295R cells with 0.1 µM prochloraz and was statistically significantly reduced overall (p≤0.001).

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Complete inhibition of estradiol secretion was recorded for all samples following treatment with 1 µM prochloraz.

The data for each evaluation as well as the overall data agreed with the guideline criteria, thus confirming the continued responsiveness of the cells during the study.

5. Aclonifen evaluations

The results for the testosterone measurements are summarised in the tables below:

Table 5.8.3-15 Steroidogenesis assay - Mean testosterone concentrations

Aclonifen Concentration (M)	Mean testosterone concentration (pg/mL) ± SD (% change)			
	First Evaluation	Second Evaluation	Third Evaluation	Overall
DMSO	4865 ± 505.0	6779 ± 358.4	6622 ± 359.5	6089 ± 986.7
8×10^{-11}	4351 ± 781.9 (-10.6%)	5019 ± 271.0 (-26%)	5298 ± 550.3 (-20%)	4889 ± 651.6** (-19.7%)
8×10^{-10}	4253 ± 254.4 (-12.6%)	5377 ± 214.4 (-20.7%)	5655 ± 228.3 (-14.7%)	5095 ± 673.5* (-16.3%)
8×10^{-9}	4042 ± 434.2 (-16.9%)	5187 ± 131.0 (-23.5%)	5127 ± 378.7 (-22.6%)	4785 ± 631.6** (-21.4%)
8×10^{-8}	4539 ± 36.1 (-6.7%)	6016 ± 90.1 (-11.3%)	5833 ± 523.3 (-11.9%)	5463 ± 746.4 (-10.3%)
8×10^{-7}	4578 ± 493.9 (-5.9%)	5751 ± 121.7 (-15.2%)	5885 ± 332.1 (-11.1%)	5405 ± 692.6 (-11.2%)
8×10^{-6}	4045 ± 435.7 (-16.9%)	5027 ± 123.8 (-25.8%)	5680 ± 214.1 (-14.2%)	4917 ± 755.3** (-19.2%)
8×10^{-5}	683 ± 165.7 (-86%)	2098 ± 152.1 (-69%)	1353 ± 162.0 (-80%)	1378 ± 628.7** (-77.4%)
Note: Data have been rounded-up. **: $p \leq 0.01$.				

Table 5.8.3-16 Steroidogenesis assay - Mean fold change in testosterone relative to DMSO

Aclonifen Concentration (M)	Mean fold change relative to DMSO controls for testosterone			
	First Evaluation	Second Evaluation	Third Evaluation	Overall
8×10^{-11}	0.89	0.74	0.80	0.80
8×10^{-10}	0.87	0.79	0.85	0.84
8×10^{-9}	0.83	0.77	0.77	0.79
8×10^{-8}	0.93	0.89	0.88	0.90
8×10^{-7}	0.94	0.85	0.89	0.89
8×10^{-6}	0.83	0.74	0.86	0.81
8×10^{-5}	0.14	0.31	0.20	0.23
Note: Data have been rounded-up.				

The variability (CV) for testosterone between the runs for the solvent controls of the aclonifen evaluations was within the guideline recommendation (~16% compared to the recommended guideline of 30%). Aclonifen induced a statistically significant inhibition of testosterone secretion (-77.4%; $p \leq 0.01$) at the highest concentration (80 µM) tested. The slight reductions of between -16.3% and -21.4% observed at the lowest three concentrations (8×10^{-11} to 8×10^{-9}M) were considered not to be relevant due to the absence of a dose response and the absence of effect at the higher concentrations of 8×10^{-8} and 8×10^{-7}M.

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The results for the estradiol measurements are summarised in the tables below:

Table 5.8.3-16 Steroidogenesis assay - Mean estradiol concentration

Aclonifen Concentration (M)	Mean estradiol concentration (pg/mL) ± SD (% change)			
	First Evaluation	Second Evaluation	Third Evaluation	Overall
DMSO	183 ± 11.8	187 ± 20.4	183 ± 14.9	184 ± 14.1
8×10^{-11}	172 ± 33.4 (-6%)	159 ± 20.1 (-15.0%)	146 ± 15.3 (-20.2%)	159 ± 23.7 (-13.6%)
8×10^{-10}	193 ± 14.0 (+5.5%)	177 ± 9.8 (-5.3%)	178 ± 5.6 (-2.7%)	183 ± 11.9 nc
8×10^{-9}	160 ± 12.8 (-12.6%)	146 ± 8.6 (-21.9%)	151 ± 2.9 (-17.5%)	152 ± 10.1** (-17.4%)
8×10^{-8}	174 ± 3.5 (-4.9%)	164 ± 9.1 (-12.3%)	171 ± 5.9 (-6.6%)	170 ± 7.3 (-7.6%)
8×10^{-7}	178 ± 7.0 (-2.7%)	171 ± 6.0 (-8.6%)	184 ± 5.8 nc	178 ± 7.8 (-3.2%)
8×10^{-6}	330 ± 26.7 (+80.3%)	312 ± 21.7 (+66.8%)	355 ± 30.6 (+94.0%)	332 ± 29.7 (+80.4%)
8×10^{-5}	173 ± 26.5 (-5.5%)	288 ± 9.6 (+54.0%)	176 ± 13.3 (-3.8%)	212 ± 58.8 (+15.2%)
Note: Data have been rounded-up. nc: no change; **: $p \leq 0.01$.				

Table 5.8.3-17 Steroidogenesis assay - Mean fold change in estradiol relative to DMSO

Aclonifen Concentration (M)	Mean fold change relative to DMSO controls for estradiol			
	First Evaluation	Second Evaluation	Third Evaluation	Overall
8×10^{-11}	0.94	0.85	0.80	0.86
8×10^{-10}	1.06	0.94	0.97	0.99 (nc)
8×10^{-9}	0.88	0.78	0.82	0.83
8×10^{-8}	0.95	0.88	0.93	0.92
8×10^{-7}	0.97	0.92	1.01 (nc)	0.96
8×10^{-6}	1.81	1.67	1.94	1.8
8×10^{-5}	0.95	1.54	0.96	1.15
Note: Data have been rounded-up. nc: no change				

The variability (CV) for estradiol between the runs for the solvent controls of the aclonifen evaluations was within the guideline recommendation (~7.7% compared to the recommended guideline of 30%). Overall, aclonifen had no clear effect on estradiol secretion. The statistically significant decrease of 17.4% observed at 8×10^{-9}M is not considered to be relevant due to the absence of a dose response. A non-statistically significant increase in estradiol secretion (overall fold increase of 1.8) was recorded at 8×10^{-6}M; however, the significance in this increase is unclear due to the absence of a dose response.

III. CONCLUSION

The data from the quality control evaluations indicated that the data were in agreement with the guideline criteria, and thus, the cells were considered suitable for the test substance evaluations.

The positive control evaluations, run concurrently with the aclonifen assessments, confirmed the continued responsiveness of the H295R cells to forskolin and prochloraz. All data from each run and overall agreed with the guideline criteria.

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Aclonifen had no clear effect on estradiol secretion when tested in the H295R adrenal cell line. However, a treatment related statistically significant inhibition of testosterone secretion (-77.4%; p≤0.01) was observed at the highest concentration (8 x 10^{-5}M).

These *in vitro* screening data indicate that aclonifen has a potential to interfere with the steroid hormone synthesis in H295R cells when tested at high concentrations. Such data should be considered in context with all other scientifically relevant data, using a weight of evidence approach before concluding on whether this test item has a potential to interfere with steroidogenesis *in vivo*.

Assessment and conclusion by applicant:

Aclonifen had no clear effect on estradiol secretion when tested in the H295R adrenal cell line. However, a treatment related statistically significant inhibition of testosterone secretion (-77.4%; p≤0.01) was observed at the highest concentration (8 x 10-5M).

These *in vitro* screening data indicate that aclonifen has a potential to interfere with the steroid hormone synthesis in H295R cells when tested at high concentrations. Such data should be considered in context with all other scientifically relevant data, using a weight of evidence approach before concluding on whether this test item has a potential to interfere with steroidogenesis *in vivo*.

Assessment and conclusion by RMS:

Data Point:	KCA 5.8.3/03
Report Author:	
Report Year:	2019
Report Title:	AE F068300 (aclonifen): Study on potential interactions with hog thyroid peroxidase using guaiacol and iodide as peroxidase substrates
Report No:	T104324-4
Document No:	M-670319-02-1
Guideline(s) followed in study:	There is currently no existing guideline for this assay.
Deviations from current test guideline:	Not applicable (non-guideline study)
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

To investigate potential interactions of Aclonifen with thyroid peroxidase (TPO), the influence of aclonifen on TPO-catalyzed reactions was studied *in vitro*. Solubilized thyroid microsomes from domestic pigs were used as an enzyme source, while guaiacol and potassium iodide served as TPO

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substrates. Positive control substances amitrole and ethylenethiourea (ETU) were included to demonstrate sensitivity of the test systems.

TPO-catalyzed oxidation of guaiacol results in the formation of an amber diphenoquinone, whereas TPO-catalyzed oxidation of iodide initially results in the generation of the so-called iodinating intermediate, which combines with iodide to form iodine (I_2). The formation of diphenoquinone and of iodine after combination with further iodide to the yellow tri-iodide ion (I_3^-) can readily be followed spectrophotometrically.

Owing to the limitation of solubility of aclonifen in aqueous solution for the assays, a concentration of 100 µM aclonifen was used as the highest concentration for testing, with additional concentrations of 30 and 10 µM. The TPO inhibitor amitrole was tested at 0.1 µM, while ETU was tested at 5 µM. Amitrole inhibited the initial rate of TPO-catalyzed guaiacol oxidation by more than 50% at a concentration of 1 µM and inhibited the initial rate of TPO-catalyzed iodine formation by more than 50% at a concentration of 0.1 µM. In contrast, ethylenethiourea, which is not an inhibitor of hog TPO enzyme, but a trap of the iodinating intermediate generated by TPO from iodide through oxidation and thus a trap of iodine formation, temporarily suppressed iodine formation.

Aclonifen did not affect TPO-catalyzed guaiacol oxidation up to 100 µM, the highest testable concentration. Similarly, TPO-catalyzed iodine formation was not affected. The observed TPO activity was always in the range 83% to 118% of the respective control activity and greater than 83% of the respective control activity and thus in line with the criterion ≥75% of control activity for no TPO inhibition.

It was concluded that aclonifen did not interfere with TPO-catalyzed reactions *in vitro* in a valid test system and was thus unable to interfere with thyroid hormone synthesis at the level of TPO.

I. MATERIALS AND METHODS

Study dates:	Study start:	11 February 2019
	Completion:	16 August 2019

A. MATERIALS

1.	**Test Item**	Aclonifen (AE F068300)
	Batch No.:	PTDF001324
	Purity:	99.9%
	Appearance:	Yellow powder
	Expiry:	13 November 2020
2.	**Reference compound**	Amitrole ( )
	Batch No.:	MKBW9117V
	Purity:	≥95%
	Expiry:	May 2022
3.	**Reference compound**	Ethylenethiourea (ETU),
	Batch No.:	1221889
	Expiry:	February 2020
4.	**Vehicle**	Dimethylsulfoxide (DMSO),
	Batch No.:	K4806982644
	Purity:	99.9%
	Expiry:	July 2019

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B. METHODS

The aclonifen and the reference compounds were dissolved in DMSO, then the highest concentration of aclonifen that could be tested in the aqueous environment of the test incubations was assayed in a dissolution test. Aclonifen could be dissolved in DMSO at 50 mM and it was then tested in conditions corresponding to the biochemical incubations, i.e., containing guaiacol or iodide as substrates, hydrogen peroxide and 2% DMSO, but no TPO.

The tested concentrations were then as follows:

Compound	Final Concentration	Corresponding concentration in DMSO
Amitrol (guaiacol-assay)	1.0 µM	50 µM
Amitrol (iodide-assay)	0.1 µM	5.0 µM
Ethylenethiourea (iodide-assay)	5 µM	250 µM
Aclonifen	100, 30 or 10 mM*	5, 1.5 or 0.5 mM

Solubilized hog thyroid microsomes, prepared from domestic pigs and stored at ≤-70°C, were used as the TPO source.

1. Guaiacol assay

Incubations were performed at room temperature in potassium phosphate buffer pH 7.4 (final concentration 0.1 M), in a total volume of 1000 µL. Solubilized hog thyroid microsomes (initial activity corresponding to about Δ0.1/min in the guaiacol assay), test item, reference compound or vehicle alone and guaiacol (final concentration 5 mM), were pre-incubated for 1 min, then the reaction was initiated by addition of hydrogen peroxide (final concentration 250 µM). Oxidation of guaiacol was followed spectrophotometrically at 470 nm. For each assay condition, three incubations were typically performed, except for control incubations for which four incubations were performed, evenly spread during the course of the study. All incubations contained 2% DMSO.

2. Iodide assay

Incubations were carried out as described above, except that guaiacol was replaced by potassium iodide (final concentration 10 mM), whereas the TPO concentration corresponded to that of the guaiacol-assay. The increase of absorption at 350 nm was used to follow iodide oxidation.

For the guaiacol assay the initial linear increase of the absorption at 470 nm was used to calculate the peroxidase activity, whereas for the iodide assay the initial linear increase of the absorption at 350 nm was used to calculate the peroxidase activity.

Only descriptive statistics (mean, standard deviation) were applied for the evaluation of TPO activity. Mean activity observed in the absence of compound (vehicle control) was set to 100%, then activity in the presence of test item or reference compound was expressed as percent activity relative to the vehicle control.

The following criteria were used to define TPO inhibition:

Negative result: TPO activity > 75%

Equivocal result: TPO activity ≤ 75%, but > 50%

Positive result: TPO activity ≤ 50%

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II. RESULTS

1. Solubility test

Aclonifen could readily be dissolved in DMSO at a concentration of 50 mM. Accordingly, in biochemical incubations containing 2% DMSO, a concentration of 1 mM Aclonifen would theoretically have been possible, however, the solubility in an aqueous environment was much lower than in DMSO. Under conditions corresponding to the guaiacol-assay or the iodide-assay, even at 200 µM aclonifen could not be dissolved, and turbidity and precipitation were observed. However, 100 µM aclonifen could be dissolved under conditions of the assays (corresponding to a concentration of 5 mM aclonifen in the DMSO stock solution) and therefore 100 µM aclonifen was used as the highest concentration when testing interactions with TPO.

2. Guaiacol assay

The TPO inhibitor amitrole at 1.0 µM inhibited guaiacol oxidation by about 56%. Mainly at 100 µM sclonifen the baseline for guaiacol oxidation showed a clearly higher extinction before start of the reaction, with a tendency to decrease during the pre-incubation period in comparison to the control baseline. This increase was likely to represent UV absorption of aclonifen at the wavelength used to follow guaiacol oxidation. Irrespective of the concentration, in the presence of aclonifen the slope of TPO-catalyzed guaiacol oxidation was similar to the corresponding control slope. No concentration dependency was observed, and no IC_{50} value could be derived. An overview of the results observed in the assay is given in the following Figures and Table.

Figure 5.8.3-1: **TPO-catalyzed guaiacol oxidation – Photometer tracings for control and in the presence of amitrole**

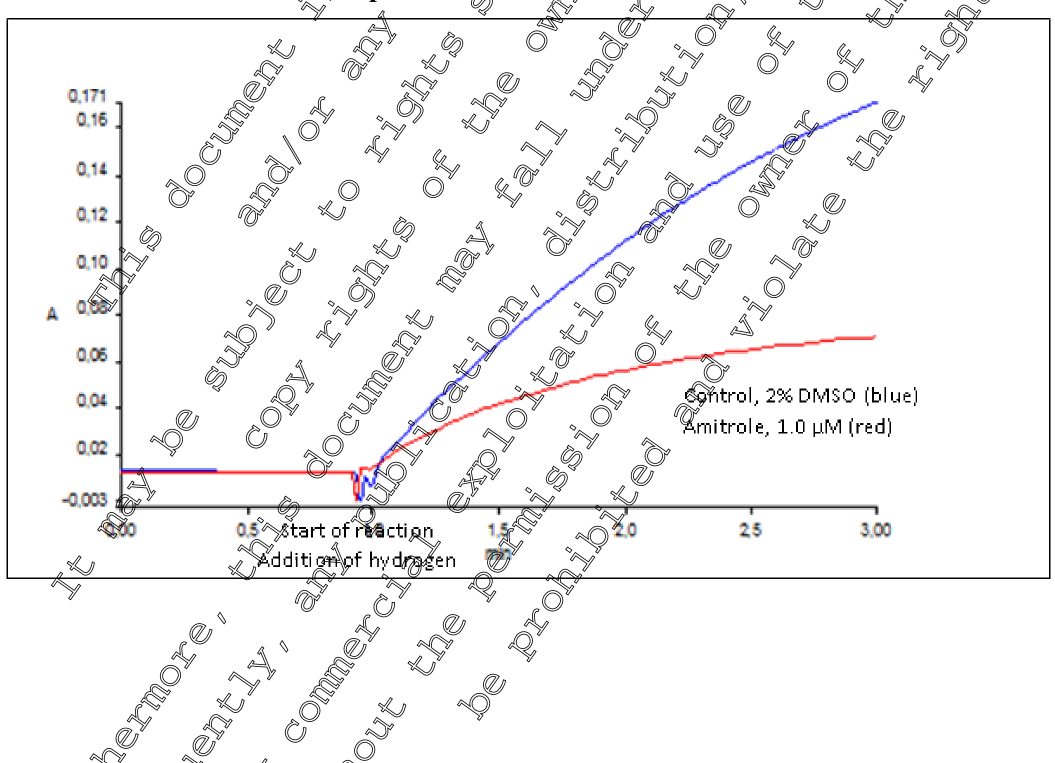

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Figure 5.8.3-2: TPO-catalyzed guaiacol oxidation – Photometer tracings for control and in the presence of aclonifen

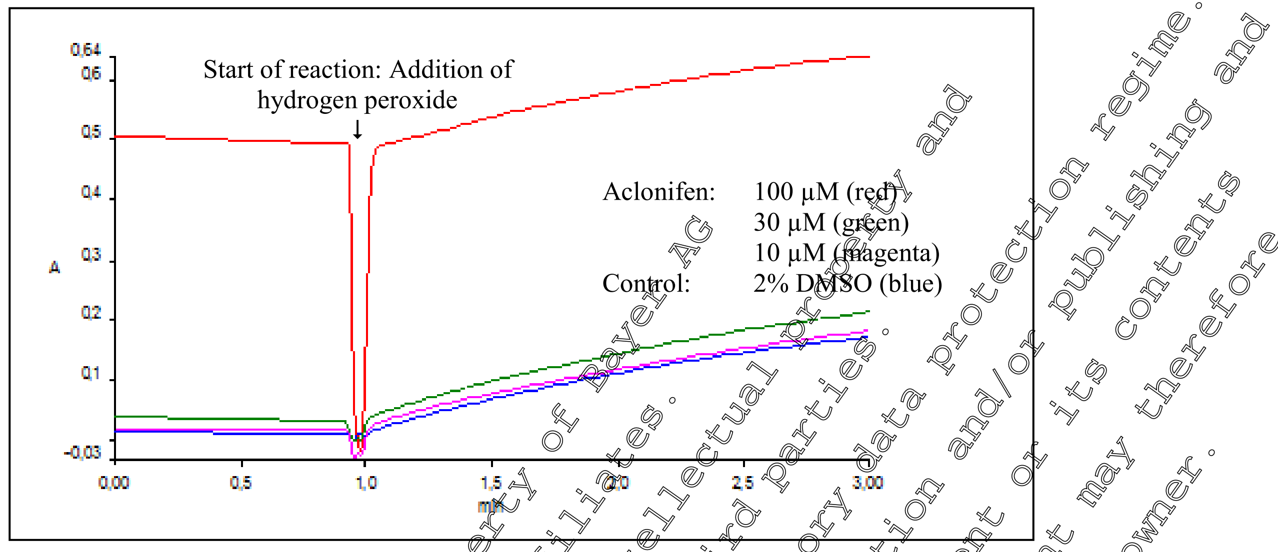

Table 5.8.3-18: Effects of aclonifen and amitrole on TPO-catalyzed guaiacol oxidation

Test Compound	Conc. (µM)	TPO activity slope (ΔE/min)		TPO activity slope (%)	
		Mean	± S.D.	Mean	± S.D.
Vehicle	0	0.123	0.006	100.0	5.0
Amitrole	1.0	0.054	0.003	44.3	2.8
Aclonifen	10	0.107	0.008	87.0	6.4
Aclonifen	30	0.115	0.010	93.5	7.9
Aclonifen	100	0.102	0.015	83.2	12.0

3. Iodine assay

The TPO inhibitor amitrole at 0.1 µM inhibited iodine formation resulting from TPO-catalyzed iodide oxidation by about 62%. ETU at a concentration of 5 µM initially almost fully suppressed iodine formation, but after some time iodine formation reappeared. In the presence of aclonifen the baseline for iodine formation showed a clearly higher extinction before the start of the reaction, during the preincubation period, in comparison to control. The magnitude of this increase depended on the concentration of aclonifen and likely represented UV absorption of aclonifen at the wavelength used to follow iodine formation. Irrespective of the concentration, in the presence of aclonifen the slope of TPO-catalyzed iodine formation was similar to the corresponding control slope, being in the range 83% to 118% of the respective control activity and thus in line with the criterion >75% of control activity as the criterion for no TPO inhibition.. No concentration dependency was observed, and no IC_{50} value could be derived. An overview of the results is given in the following figures and table.

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Figure 5.8.3-3: **TPO-catalyzed guaiacol oxidation – Photometer tracings for control and in the presence of amitrole and ethylenethiourea**

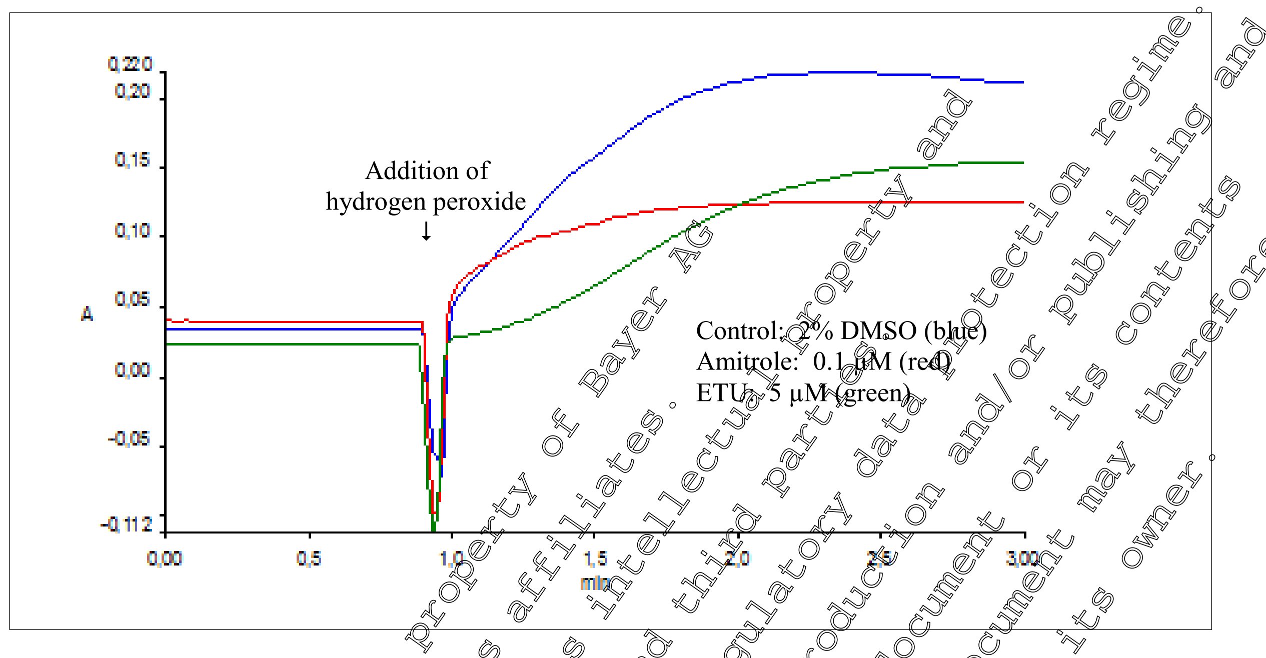

Figure 5.8.3-4: **TPO-catalyzed guaiacol oxidation – Photometer tracings for control and in the presence of aclonifen**

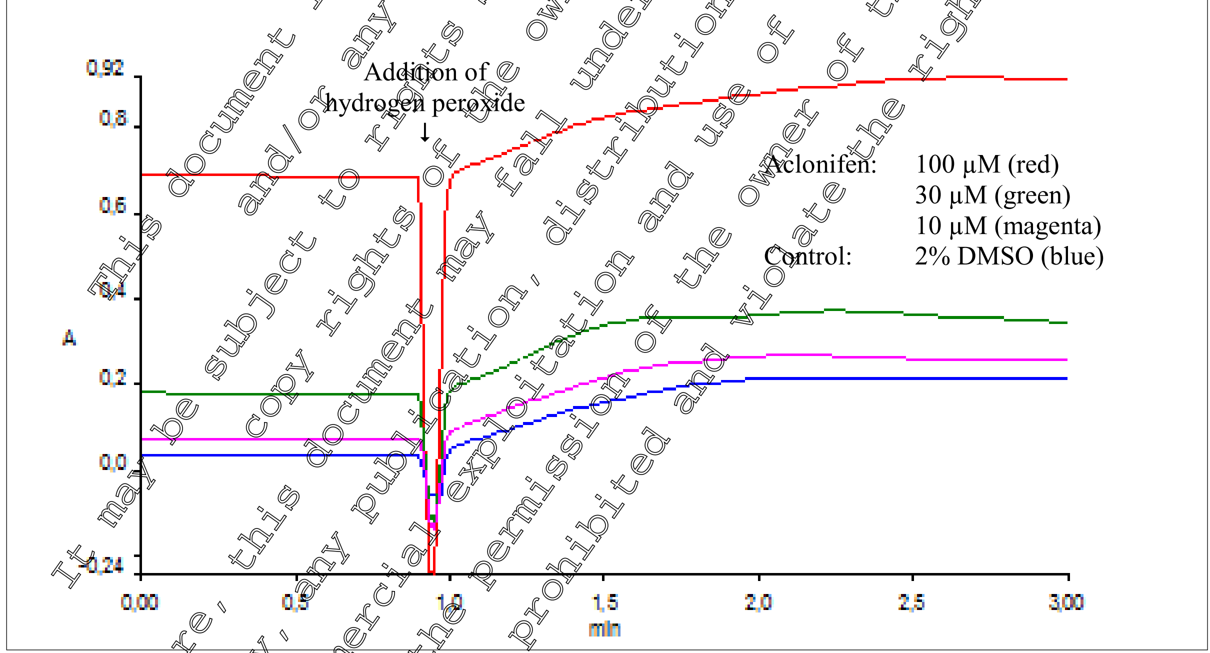

Table 5.8.3-19: **Effects of aclonifen and amitrole on TPO-catalyzed iodine formation**

Test Compound	Conc. (µM)	TPO activity slope (ΔE/min)		TPO activity slope (%)	
		Mean	± S.D.	Mean	± S.D.
Vehicle	0	0.261	0.029	100.0	11.1
Amitrole	1.0	0.100	0.007	38.2	2.6

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Aclonifen	10	0.273	0.015	104.8	5.9
Aclonifen	30	0.309	0.019	118.4	7.2
Aclonifen	100	0.283	0.007	108.4	2.5

III. CONCLUSION

It was indicated that aclonifen did not interfere with TPO-catalyzed reactions *in vitro* in a valid test system and was thus unable to interfere with thyroid hormone synthesis at the level of TPO.

Assessment and conclusion by applicant:

Assessment and conclusion by applicant:

A valid *in vitro* study with an adequately supported conclusion of no interference with thyroid hormone synthesis.

Assessment and conclusion by RMS:

Data Point:	KCA 5.8.3/05
Report Author:	[redacted]
Report Year:	2019
Report Title:	Aclonifen: Study on potential interactions with rat and human sodium/iodide symporter (NIS)
Report No:	TXCL0040
Document No:	M-676484-01-1
Guideline(s) followed in study:	There is currently no existing guideline for this assay.
Deviations from current test guideline:	No deviation
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

The objective of this study was to screen aclonifen *in vitro* for its potential for iodide uptake mediated by rNIS and hNIS. The NIS activity was monitored in rat PCCL3 cells and HEK 293 cells (stably transformed, expressing the human NIS) by measuring the intracellular accumulation of radioactive iodide (I^{125}) over periods of 5, 15 or 45 min by liquid scintillation counting.

Three independent experiments were performed on different days. The first two used 50 µM, 160 µM, or 500µM aclonifen, the third being conducted at 5 µM, 16 µM, or 50 µM aclonifen. The vehicle was DMSO.

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For induction of metabolism, a pre-incubation of aclonifen at 10, 32 or 100 µM was performed in the absence or presence of S9 mix, followed by exposure for 45 min at 5, 16 or 50 µM aclonifen. Each concentration was tested in triplicate, and a positive (inhibitory) control was included (sodium perchlorate). Two independent experiments were conducted on different days for each cell line.

Sensitivity of the NIS assay was demonstrated by the DMSO vehicle control showing a significant level of iodide uptake in all experiments. The uptake was abolished in the presence of the positive control sodium perchlorate (at 100 µM).

In the absence of S9 mix, aclonifen induced a dose-dependent inhibition of NIS activity, up to levels comparable to the sodium perchlorate control, when cells were exposed to 16 µM or ~50 µM of aclonifen in the rat and human cells, respectively. In contrast, pre-incubation with S9 fractions almost totally abolished the NIS inhibition observed in the absence of S9 in both cell lines, suggesting that once metabolized, aclonifen loses its ability to interfere with NIS activity.

It was concluded that in the absence of metabolic transformation, aclonifen interfered with NIS activity *in vitro* in a dose dependent manner, while in the presence of S9 mix-mediated metabolism the effect on NIS activity was almost totally abolished. This suggests that, once metabolized, aclonifen loses its ability to reduce NIS-mediated iodide uptake.

I. MATERIALS AND METHODS

Study dates: Study start: 15 February 2019
Completion: 17 November 2019

A. MATERIALS

1. Test Item — Aclonifen (AE F068300)
Batch No.: AE F068300-01-28
Purity: 99.9%
Appearance: Yellow powder
Expiry: 13 November 2020

2. Reference compound — Sodium perchlorate (Sigma-Aldrich)
Batch No.: MKBJ3906V / MKCL1340
Purity: ≥98%
Expiry: 31 December 2020

3. Control (solvent) — Dimethylsulfoxide (DMSO), Sigma-Aldrich
Batch No.: RNBH2447
Purity: 99.7%
Expiry: 31 December 2020

4. Cells
Rat: PCCL3 cells (thyroid follicular cell line), an established model for mechanistic investigation of NIS activity
Human: HEK 293 cells (human embryonic kidney), stably expressing human NIS, an established model for mechanistic investigation of NIS activity

5. Radioactive iodide — Isobio ARI-102, 2 mCi/vial (ARC)
Batch Nos.: 190208, 90405, 90709, 191018
Half-life: 59 days

6. Sodium iodide: 30 mM solution (Sigma-Aldrich)
Batch No.: MKCJ5428

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	Expiry:	28 February 2022
7.	**HBSS:**	Stock solution (Gibco)
	Batch Nos.:	1854779, 2027174
	Expiry:	31 May 2019, 28 February 2021
8.	**HEPES:**	Stock solution 10 mM (Gibco)
	Batch Nos.:	2010326, 2091561
	Expiry:	31 March 2020, 31 December 2020

B. METHODS

Rat PCCL3 or human hNIS-HEK 293 cells were exposed for 5, 15, or 45 minutes to at least three concentrations of aclonifen. Each concentration was tested in triplicate.

NIS activity was monitored by measuring the intracellular accumulation of radioactive iodide by liquid scintillation counting over time.

Three independent experiments were performed on different days.

Test item and reference compound were dissolved in dimethylsulfoxide (DMSO). Concentrations to be achieved in the final biochemical incubations and in the corresponding stock solution in DMSO are compiled in the following table. The highest testable concentration of aclonifen ("X") was tested in a dissolution test. For preparation of the formulations the content of test item/reference compound was calculated based on an assumed purity of 100% and was performed based on volume, not taking into account the density of the vehicle.

Compound	Final concentration for assay
Aclonifen, experiments 1 and 2	50, 166, 500 µM
Aclonifen, experiment 3	5, 16, 50 µM
Sodium perchlorate	100 µM

The maximum testable concentration of aclonifen was selected based on its cytotoxicity and maximum solubility in DMSO, up to 1mM. The maximum concentration of 500µM was initially selected based on its solubility. The third independent experiment was conducted at lower concentrations, to detect any possible dose related effect on NIS activity.

An additional independent experiment was conducted in both cell lines in the presence or absence of mammalian microsomal liver fraction (S9) obtained from Aroclor 1254-treated rats, to assess the effect of metabolism of aclonifen on NIS activity.

Assay summary

− Incubate 50 ml of HBSS, HEPES 10 mM at 37°C.

− Add 50 µL of 30 mM NaI solution (final concentration 30 µM).

− Add 1 µL of radioactive iodide (3.7 MBq) in 50 mL HBSS.

− Collect 10 mL in a fresh tube and add 10 µL of 100 mM sodium perchlorate solution (final concentration 100 µM) for negative control.

− For 3 tested concentrations of the compound, 3x10 mL of the radioactive solution were collected and place in a new tube.

− Wash each well 2 times with 1 mL of HBSS, HEPES 10 mM at room temperature.

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− To start the uptake incubation, remove the HBSS and add 1 mL of source solution or negative control in each well. Add the same volume of radioactive solution to all wells.

− Incubation times: 5min, 15min, 45min.

− After incubation, wash three times with HBSS, HEPES 10 mM at 4°C.

− Add 0.5 ml/absolute ethanol to each well to permeabilizing the membranes.

− Incubate 20 minutes.

− Transfer to vials containing 2ml of liquid scintillator.

− Prepare also 3 vials with 0.5 ml of radioactive solution alone.

− Accumulated radioactive iodide is measured by liquid scintillation counting.

In terms of results, only descriptive statistics were calculated, mean activity in the absence of test item (vehicle control or vehicle with S9 mix) being set at 100%, while activity in the presence of test item or reference compound was expressed as percent activity relative to vehicle control.

II. RESULTS AND DISCUSSION

1. Solubility of aclonifen

Aclonifen was readily dissolved in DMSO at up to 50 mM. Under the conditions of the assay, it could be dissolved at up to 500 µM.

2. Cytotoxicity

Aclonifen was not cytotoxic to either cell line in the MTT cytotoxicity assay (Sigma-Aldrich, batch MKBZ4723V) at up to 500 µM. *(These data were not shown in the study report).*

3. NIS activity

Sensitivity of the NIS assay was demonstrated by the DMSO control vehicle condition (positive uptake control) showing in all experiments a significant level of iodide uptake. The uptake was efficiently blocked in the presence of the reference compound sodium perchlorate (at 100µM).

Two independent experiments were conducted on rat PCCL3 and human hNIS-HEK 293 cells. A marked inhibition of the NIS activity, compared to the reference compound sodium perchlorate, was observed at all tested concentrations of aclonifen (50 µM, 166 µM, and 500 µM).

To detect a dose-response inhibition effect, a third experiment was conducted using both cell lines at lower doses of 0.5 µM, 16 µM and 50 µM.

Table 5.8.3-20: Effects of aclonifen on NIS activity, Experiment 1

Cell line	Compound	Iodide uptake incubation time (min)	Mean uptake (picomol/well)	Mean uptake normalized to control (%)
hNIS-HEK 293	Control	5	653.1 ± 55.5	100 ± 8.5
		15	1060.8 ± 69.9	100 ± 6.6
		45	1587.6 ± 44.9	100 ± 2.8
	Perchlorate 100 µM	5	30.2 ± 3.2	5 ± 0.5
		15	46.6 ± 4.0	4 ± 0.4
		45	58.2 ± 3.5	4 ± 0.2
	Aclonifen 0.05 mM	5	328.9 ± 9.7	50 ± 1.5
		15	351.7 ± 25.6	33 ± 2.4

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Cell line	Compound	Iodide uptake incubation time (min)	Mean uptake (picomol/well)	Mean uptake normalized to control (%)
		45	208.7 ± 9.2	13 ± 0.6
	Aclonifen 0.166 mM	5	229.3 ± 16.1	35 ± 2.5
		15	184.5 ± 12.8	17 ± 1.2
		45	70.9 ± 8.7	4 ± 0.6
	Aclonifen 0.5 mM	5	147.1 ± 4.8	23 ± 0.7
		15	119.7 ± 6.0	11 ± 0.6
		45	57.7 ± 2.2	4 ± 0.1
Rat PCCL3	Control	5	118.5 ± 7.6	100 ± 6.4
		15	177.5 ± 9.9	100 ± 5.6
		45	174.0 ± 26.7	100 ± 15.3
	Perchlorate 100 µM	5	17.2 ± 3.0	15 ± 2.6
		15	26.8 ± 3.8	15 ± 2.1
		45	16.7 ± 2.4	10 ± 1.4
	Aclonifen 0.05 mM	5	29.5 ± 2.5	25 ± 2.1
		15	36.0 ± 1.7	20 ± 1.0
		45	25.1 ± 4.8	14 ± 2.7
	Aclonifen 0.166 mM	5	25.9 ± 6.2	22 ± 5.3
		15	29.3 ± 0.7	17 ± 0.4
		45	28.4 ± 4.8	16 ± 2.7
	Aclonifen 0.5 mM	5	29.6 ± 16.5	25 ± 13.9
		15	31.9 ± 2.1	18 ± 1.2
		45	22.9 ± 4.4	13 ± 2.5

Table 5.8.3-21: Effects of aclonifen on NIS activity, Experiment 2

Cell line	Compound	Iodide uptake incubation time (min)	Mean uptake (picomol/well)	Mean uptake normalized to control (%)
hNIS-HEK 293	Control	5	538.7 ± 68.2	100 ± 12.7
		15	856.2 ± 95.9	100 ± 11.2
		45	1258.5 ± 78.0	100 ± 6.2
	Perchlorate 100 µM	5	29.3 ± 2.1	5 ± 0.4
		15	42.5 ± 3.3	5 ± 0.4
		45	54.8 ± 4.6	4 ± 0.4
	Aclonifen 0.05 mM	5	239.0 ± 11.8	44 ± 2.2
		15	264.2 ± 22.9	31 ± 2.7
		45	137.6 ± 8.7	11 ± 0.7
	Aclonifen 0.166 mM	5	168.2 ± 13.0	31 ± 2.4
		15	125.1 ± 10.8	15 ± 1.3
		45	64.5 ± 5.3	5 ± 0.4
	Aclonifen 0.5 mM	5	117.2 ± 1.8	22 ± 0.3
		15	92.0 ± 7.4	11 ± 0.9
		45	49.3 ± 5.2	4 ± 0.4
Rat PCCL3	Control	5	101.1 ± 5.4	100 ± 5.4
		15	160.6 ± 7.5	100 ± 4.7
		45	169.2 ± 14.3	100 ± 8.4

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Cell line	Compound	Iodide uptake incubation time (min)	Mean uptake (picomol/well)	Mean uptake normalized to control (%)
	Perchlorate 100 µM	5	17.0 ± 1.6	17 ± 1.6
		15	23.0 ± 2.2	14 ± 1.4
		45	21.0 ± 5.4	12 ± 3.2
	Aclonifen 0.05 mM	5	32.6 ± 3.7	32 ± 3.7
		15	35.5 ± 9.3	22 ± 5.8
		45	25.1 ± 6.0	15 ± 3.6
	Aclonifen 0.166 mM	5	25.3 ± 2.7	25 ± 2.7
		15	22.2 ± 6.6	14 ± 4.1
		45	20.7 ± 5.5	12 ± 3.3
	Aclonifen 0.5 mM	5	24.0 ± 4.6	24 ± 4.6
		15	31.2 ± 6.0	19 ± 3.8
		45	22.1 ± 3.3	13 ± 1.9

Table 5.8.3-22: Effects of aclonifen on NIS activity, Experiment 3

Cell line	Compound	Iodide uptake incubation time (min)	Mean uptake (picomol/well)	Mean uptake normalized to control (%)
hNIS-HEK 293	Control	5	415 ± 40	100 ± 9.6
		15	858 ± 30	100 ± 3.6
		45	998 ± 27	100 ± 2.7
	Perchlorate 100 µM	5	40 ± 7	10 ± 1.7
		15	54 ± 2	6 ± 0.2
		45	44 ± 33	4 ± 3.3
	Aclonifen 5 µM	5	428 ± 21	103 ± 5.1
		15	712 ± 24	83 ± 2.8
		45	784 ± 16	79 ± 1.6
	Aclonifen 16 µM	5	341 ± 21	82 ± 5.1
		15	445 ± 24	52 ± 2.8
		45	404 ± 28	41 ± 2.8
	Aclonifen 50 µM	5	NC	NC
		15	234 ± 21	27 ± 2.4
		45	127 ± 14	13 ± 1.4
Rat PCCl3	Control	5	71 ± 7	100 ± 9.6
		15	103 ± 19	110 ± 18.0
		45	126 ± 21	91 ± 16.6
	Perchlorate 100 µM	5	22 ± 2	31 ± 2.8
		15	20 ± 18	20 ± 17.5
		45	20 ± 6	16 ± 5.1
	Aclonifen 5 µM	5	46 ± 7	65 ± 9.8
		15	55 ± 7	54 ± 6.6
		45	39 ± 3	31 ± 2.2
	Aclonifen 16 µM	5	29 ± 3	41 ± 4.7
		15	35 ± 3	33 ± 3.1
		45	34 ± 4	27 ± 3.5
	Aclonifen	5	32 ± 7	44 ± 10.5

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Cell line	Compound	Iodide uptake incubation time (min)	Mean uptake (picomol/well)	Mean uptake normalized to control (%)
	50 µM	15	35 ± 4	34 ± 3.8
		45	30 ± 3	24 ± 2.1
NC = Not calculated: values considered aberrant and excluded				

Figure 5.8.3-5: **Mean I^{125} uptake in rat PCCL3 and hNIS-HEK 293 cells**

To evaluate the effect of metabolism on the inhibitory effect of aclonifen, the test item was pre-incubated for 60 min at 37° in the presence of mammalian microsomal liver fraction (S9 fraction) obtained from Aroclor 1254-treated rats supplemented with co-factors. A pre-incubation control with only the S9 co-factors was included.

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The NIS assay was then conducted in both cell lines at the single timepoint of 45 min of incubation, each concentration being tested in triplicate, in two independent experiments. A sodium perchlorate positive (inhibitory) control was included.

Sensitivity of the assay was again demonstrated by the DMSO vehicle condition showing a significant level of iodide uptake, which was blocked by the sodium perchlorate.

NIS inhibition by aclonifen was confirmed in both controls (no pre-incubation and control incubation). Pre-incubation in the presence of S9 fraction almost abolished the inhibition by aclonifen. Activity remained at approximately 100% at 5 and 16 µM in both cell lines, compared with reduction to approximately 60% or 40% in the corresponding controls. Minimal NIS activity occurred at the high dose of 50 µM aclonifen (reduction to approximately 70%), compared with complete inhibition (similar to sodium perchlorate) in the corresponding controls.

These results indicate that, once metabolized, aclonifen loses its ability to reduce NIS-mediated iodide uptake.

Table 5.8.3-23: Effects of aclonifen on NIS activity in the presence or absence of S9 mix, two experiments (mean uptake normalized to control as %)

Cell line	Pre-incubation	Compound	First experiment	Second experiment	Overall
hNIS-HEK 293	None	Control	100 ± 1.4	100 ± 6.0	100 ± 9.7
		Perchlorate 100 µM	9 ± 0.2	9 ± 0.8	15 ± 6.6
		Aclonifen 5 µM	86 ± 1.7	89 ± 1.3	69 ± 21.9
		Aclonifen 16 µM	50 ± 3.1	58 ± 1.9	44 ± 16.5
		Aclonifen 50 µM	18 ± 10.2	27 ± 1.9	26 ± 4.4
	Yes (control mix)	Control	100 ± 2.7	100 ± 6.3	100 ± 7.5
		Perchlorate 100 µM	9 ± 0.4	12 ± 1.6	14 ± 3.0
		Aclonifen 5 µM	82 ± 4.1	95 ± 6.2	70 ± 28.6
		Aclonifen 16 µM	52 ± 3.3	77 ± 3.7	48 ± 31.2
		Aclonifen 50 µM	19 ± 1.0	32 ±1.0	27 ± 6.3
	Yes (S9 mix)	Control	100 ± 1.4	100 ± 4.3	100 ± 4.1
		Perchlorate 100 µM	5 ± 0.8	7 ± 1.1	12 ± 5.7
		Aclonifen 5 µM	95 ± 3.8	96 ± 0.3	99 ± 4.9
		Aclonifen 16 µM	93 ± 4.0	95 ± 3.6	98 ± 7.8
		Aclonifen 50 µM	83 ± 4.2	88 ± 3.6	73 ± 17.1
Rat PCCL3	None	Control	100 ± 11.5	100 ± 14.1	100 ± 7.3
		Perchlorate 100 µM	33 ± 3.5	20 ± 3.2	21 ± 13.3
		Aclonifen 5 µM	55 ± 5.2	49 ± 6.9	71 ± 17.2
		Aclonifen 16 µM	31 ± 4.7	29 ± 8.0	40 ± 11.0
		Aclonifen 50 µM	32 ± 3.8	25 ± 6.5	22 ± 12.9
	Yes (control mix)	Control	100 ± 8.0	100 ± 10.1	100 ± 5.4
		Perchlorate 100 µM	23 ± 5.5	15 ± 3.7	16 ± 8.1
		Aclonifen 5 µM	39 ± 6.4	44 ± 2.6	60 ± 24.6
		Aclonifen 16 µM	26 ± 6.1	20 ± 1.6	39 ± 14.9
		Aclonifen 50 µM	34 ± 4.7	22 ± 4.7	27 ± 8.9
	Yes (S9 mix)	Control	100 ± 5.9	100 ± 4.8	100 ±3.8
		Perchlorate 100 µM	21 ± 2.1	17 ± 1.2	13 ± 8.9
		Aclonifen 5 µM	97 ± 5.0	102 ± 5.6	96 ± 4.1
		Aclonifen 16 µM	99 ± 10.0	101 ± 10.5	96 ± 7.5
		Aclonifen 50 µM	68 ± 14.9	58 ± 5.8	75 ± 13.0

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Figure 5.8.3-6: **Relative I^{125} uptake in rat PCCL3 and hNIS-HEK 293 cells, from mean values in two experiments**

Rat PCCL3 cells +/- S9 mix
Relative I^{125} uptake (%)
0
20
40
60
80
100
120
Control uptake
Perchlorate positive ct
Aclonifen 5 µM
Aclonifen 16 µM
Aclonifen 50 µM
No pre-incubation
Control pre-incubation
S9 mix pre-incubation

hNIS-HEK 293 cells +/- S9 mix
Relative I^{125} uptake (%)
0
20
40
60
80
100
120
Control uptake
Perchlorate positive ct
Aclonifen 5 µM
Aclonifen 16 µM
Aclonifen 50 µM
No pre-incubation
Control pre-incubation
S9 mix pre-incubation

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III. CONCLUSIONS

The results indicated that in the absence of metabolic transformation, aclonifen interfered with NIS activity *in vitro* in a dose dependent manner, while in the presence of S9 mix-mediated metabolism the

effect on NIS activity was almost totally abolished. This suggests that, once metabolized, aclonifen loses its ability to reduce NIS-mediated iodide uptake.

Assessment and conclusion by applicant:

A valid *in vitro* study with adequately supported conclusions that, while aclonifen does interfere with iodide uptake, metabolism abolishes this activity.

Assessment and conclusion by RMS:

Data Point:	KCA 5.8.3/04
Report Author:	[redacted]
Report Year:	2019
Report Title:	Aclonifen: Assessment of gene expression profiles of phase I and phase II liver enzymes in primary cultures of rat hepatocytes
Report No:	S20A19
Document No:	M-675870-01-1
Guideline(s) followed in study:	There is no guideline for this type of study
Deviations from current test guideline:	No deviation
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

The objective of this study was to determine potential effects of aclonifen on gene expression profiles of phase I and phase II liver enzymes by qPCR in primary rat hepatocytes cultures following exposure of the hepatocytes to the test compound for 48h. Aclonifen was tested at three concentrations with the highest concentration based on prior cytotoxicity evaluations, alongside the reference compounds betanaphthoflavone (AhR inducer), Clofibrate (PPARα inducer), and Fluopyram, (CAR/PXR inducer).

Reference compounds gave the expected gene expression profiles within the historical ranges indicated by existing validation data, confirming the responsiveness of the cells to stimulation with liver nuclear receptor inducers.

Aclonifen showed a gene expression profile similar to that of Fluopyram, with induction of up to ~75-fold for Cyp3a23, up to ~2.8 for Ugt1a6, and up to ~5.4 for Ugt2b1. In addition, similarly to the reference compound Fluopyram, the fold induction of Ugt2b1 was stronger than for Ugt1a6, confirming the CAR/PXR like profile of aclonifen. The inductions corresponded to ≥20% of the respective levels induced by the positive control. Despite the absence of a dose-related effect for aclonifen, the observed liver enzyme transcript inductions were considered biologically relevant.

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It was concluded that aclonifen showed a CAR/PXR-like gene expression profile, similar to the reference CAR/PXR inducer.

I. MATERIALS AND METHODS

Study dates:	Study start:	20 May 2019
	Completion:	19 December 2019

A. MATERIALS

1.	**Test Item**	Aclonifen (AE F068300)
	Batch No.:	PTDF001324
	Purity:	99.9%
	Appearance	Yellow powder
	Expiry:	13 November 2020
2.	**Reference compound**	Fluopyram ( )
	CAS No.:	658066-35-4
3.	**Reference compound**	β-naphthoflavone
	CAS No.:	6051-87-2
4.	**Reference compound**	Clofibrate
	CAS No.:	637-07-0
5.	**Vehicle**	Dimethylsulfoxide

B. METHODS

Rat primary hepatocytes were first exposed for 48 hrs to four concentrations of Aclonifen at 3, 10, 30, or 100 µM for cytotoxicity determination. Cytotoxicity levels were assessed using the ATPlite kit from . Selection of the highest testable concentration of aclonifen was based on the outcome, with an acceptability threshold of <20-25% reduction in viability. Starting from the highest testable concentration, two further lower concentrations, representing ~3/10 and 1/10 of the top concentration, were derived.

β-naphthoflavone, clofibrate and fluopyram were included as a reference compounds to demonstrate the responsiveness of the cells to liver nuclear receptor inducers. Concentrations of these reference compounds were selected based on proprietary validation data, taking into account cytotoxicity levels and gene expression induction levels of selected Cyps in primary rat hepatocytes. DMSO at 0.1% was used as the vehicle control.

1. Isolation of rat primary hepatocytes

Male Wistar rats aged 7 weeks (approx. 250 g) were perfused using HEPES based buffers with collagenase substituted by liberase (a synthetic enzyme). Once the perfusion was completed, the liver was transferred to HepatoZYME (Life technologies) cell media containing 10% Nu-Serum, 1% ITS premix and 1% penicillin-streptomycin (HepatoZYME + Supplements) at approximately 4°C and the cells were gently scraped from the liver. After filtration and centrifugation, the collected pellet was resuspended in cell media (HepatoZYME + Supplements).

The cells were seeded in HepatoZYME + supplements and kept for 24 h with a change of media 4 h after seeding. The next day, the cell media was replaced by Williams'E (Life technologies) containing

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1% ITS premix, 1% Penicillin-Streptomycin and 0.005% hydrocortisone (Williams'E + Supplements). The cells were maintained at 37°C ± 1°C /5% CO_2.

2. Test conditions

The cells were seeded at 40x10^3 cells/well into 96 well plates and incubated at 37°C ± 1°C /5% CO2 for 24h prior to treatment.

Twenty-four hours after seeding, the medium in each well was removed and replaced with 100 µl of fresh medium (Williams'E + Supplements). Then, the test item or reference substances were added to the wells to reach the final concentrations. DMSO (0.1%) was used as the solvent control. The cells were exposed in triplicate to the test substance, the positive control, or DMSO for 48h at 37°C ± 1°C /5% CO2.

After cell treatment, the cells were lysed in RA1 lysis buffer (NucleoSpin kit from) and stored at approximately -20°C ± 5°C until analyzed for gene expression.

A real-time quantitative reverse-transcriptase-polymerase chain reaction (RT-qPCR) approach was used to measure the expression levels of selected Cyps transcripts.

- Total RNA was isolated from the primary rat hepatocyte samples using the NucleoSpin kit from in accordance with the manufacturer's instructions.
- cDNA was synthesized from total RNA for each sample by reverse transcription using random hexamer as primers.
- qPCR reactions were performed using TaqMan probes for Cyp1a1, Cyp2b1, Cyp3a23, Cyp4a1, Ugt1a1, Ugt1a6 and Ugt2b1 from Applied Biosystems on a Biomark machine from Fluidigm. β-2 microglobulin (B2m) was selected as an internal reference gene for the normalization of gene expression.

3. Data evaluation

The data were presented as fold change versus control, calculated from the mean values. The gene signature of each test compound at each concentration was compared with the gene signature of the reference compounds and DMSO.

Any value that was ≥20% of the positive control was considered an indication of nuclear receptor activation and liver and/or thyroid tumor formation. Values in the 10-20% range of the positive control cannot be clearly interpreted. Values <10% of the positive control were not considered an alert, as this magnitude of induction is not correlated with known *in-vivo* adverse effects. However, other factors such as the general trend of the data, were also taken into consideration when analyzing the data.

No statistical analysis was performed on the data, owing to the low sample size (n = 3/concentration).

II. RESULTS

1. Cytotoxicity

The concentration of 100 µM induced ~50% cytotoxicity in the rat primary hepatocytes, so 30 µM was defined as the highest testable concentration, 10 µM and 3 µM being retained as mid- and low-doses.

Table 5.8.3-24: Cytotoxicity evaluation of aclonifen

Test item	Concentration	Mean Relative Luminescence Units ± SD (n = 3)	% of control

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DMSO	0.1%	805550 ± 33078	-
Aclonifen	100 µM	421447 ± 22263	52%
	30 µM	679383 ± 16037	84%
	10 µM	675487 ± 29762	84%
	3 µM	601863 ± 5093	75%

2. Gene expression for Phase I and Phase II liver enzymes

Mean relative gene expression values were used to calculate the fold change relative to the mean DMSO control values, as well as the percentage change relative to the selected reference compound induction, shown in the tables below, including an overall interpretation of the induction profile.

Table 5.8.3-25: Gene expression profiles for aclonifen – induction relative to negative control

Compound	**DMSO**	**Fluopyram**	**β-NF**	**Clofibrate**	**Aclonifen**		
Concentration	**0**	**30 µM**	**5 µM**	**1000 µM**	**3 µM**	**10 µM**	**30 µM**
Fold change							
Cyp 1a1	1.0	26.5	156.9	10.1	9.3	12.4	23.6
Cyp 2b1	1.0	1.1	0.1	75.8	23.9	17.8	4.1
Cyp 3a23	1.0	79.6	2.0	10.7	74.7	74.7	60.9
Cyp 4a1	1.0	0.1	0.2	149.3	15.5	14.9	7.3
Ugt 1a1	1.0	1.5	0.3	1.4	1.3	1.5	1.4
Ugt 1a6	1.0	2.7	5.0	1.1	1.9	2.3	2.8
Ugt 2b1	1.0	7.2	0.6	1.6	4.5	5.4	4.8
Induction profile	Negative control	CAR/PXR	AhR	PPARα	CAR/PXR-like	CAR/PXR-like	CAR/PXR-like

Reference compounds gave the expected gene expression profiles within the historical ranges (proprietary validation data), confirming responsiveness of the cells to stimulation with liver nuclear receptor inducers. Specifically, the inductions in gene expression for the reference compounds were:

- Fluopyram induced ~80 fold increase in Cyp3a23, a 2.7 fold increase in Ugt1a6, and a 7.2 fold increase in Ugt2b1. Despite the absence of induction of Cyp2b1, the observed Phase I and phase II transcript level inductions were in line with the known profile of fluopyram (CAR/PXR activation being the molecular initiating event, subsequent activation of Phase I and phase II enzymes involved in T4 metabolism).
- Betanaphthoflavone induced a ~157 fold increase in Cyp1a1 and 5.0 fold increase in Ugt1a6.
- Clofibrate induced a ~150 fold increase of Cyp4a1.

Aclonifen showed a gene expression profile similar to that of fluopyram with an induction of up to ~75-fold for Cyp3a23, an induction of up to ~2.8 for Ugt1a6, and an induction of up to ~5.4 for Ugt2b1. The inductions corresponded to ≥20% of the respective levels induced by the positive control. In addition, similarly to the reference compound fluopyram, the fold induction of Ugt2b1 is stronger than the fold inductions of Ugt1a6, confirming the CAR/PXR like profile of aclonifen.

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III. CONCLUSION

Aclonifen showed a CAR/PXR-like gene expression profile, similar to the reference CAR/PXR inducer, fluopyram.

Assessment and conclusion by applicant:

A valid *in vitro* study with an adequately supported conclusion that aclonifen exhibits a gene expression profile similar to another known CAR/PXR inducer.

Assessment and conclusion by RMS:

Data Point:	KCA 5.8.3/07
Report Author:	[redacted]; [redacted]
Report Year:	2020
Report Title:	Appendix I - Assessment of the endocrine disrupting properties of the active substance aclonifen in accordance with Commission Regulation (EU) 2018/605
Report No:	M-676736-01-1
Document No:	M-676736-01-1
Guideline(s) followed in study:	in Accordance with Commission Regulation (EU) 2018/605
Deviations from current test guideline:	--
Previous evaluation:	
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	

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Executive Summary

The potential of aclonifen to interact with endocrine systems in mammals has been reviewed, to facilitate an assessment of whether aclonifen may be judged to be an endocrine disrupter (ED) within the framework of European legislation.
Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 has been published (EFSA/ECHA, JRC, 2018). This guidance document describes how to gather, evaluate and consider all relevant information for the assessment, conduct a MoA analysis, and apply a WoE approach, in order to establish whether the ED criteria are fulfilled. The guidance states that a substance shall be considered as having endocrine disruption properties if it meets all of the following criteria:

i. It shows an adverse effect in an intact organism or its progeny, which is a change in the morphology, physiology, growth, development, reproduction, or, life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment

of the capacity to compensate for additional stress, or an increase in susceptibility to other influences.

ii. It has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system.

iii. The adverse effect is a consequence of the endocrine mode of action.

Standard toxicology and ecotoxicology studies conducted to meet to the data requirements under Regulation (EU) 283/2013 have been submitted in this renewal dossier. A literature search was conducted to find relevant studies in the open literature conducted in the last 10 years. Further *in vitro* studies have been conducted to investigate EATS-mediated endocrine activity.

A summary of all relevant studies is provided in the excel spreadsheet Appendix E.

Conclusion on endocrine disruption

EAS and T modalities in mammals have been sufficiently investigated.

Aclonifen caused adversity and changes in thyroid hormones via the T-modality. The MoA analysis provided sufficient evidence to demonstrate the most plausible MoA was via enhanced hepatic clearance of thyroid hormones. It is a well-documented MoA, and species differences in thyroid hormone metabolism mean this MoA is of limited relevance to humans.

Aclonifen is not an ED via the EAS-modality in mammals as there was no evidence of EAS adversity in *in vivo* studies. *in vitro* findings of endocrine activity via the A and S-modalities were not replicated *in vivo*.

Assessment and conclusion by applicant:

The review of the available data is considered to be acceptable and hence the conclusions drawn are considered to be valid.

EAS and T modalities in mammals have been sufficiently investigated.

Aclonifen caused adversity and changes in thyroid hormones via the T-modality. The MoA analysis provided sufficient evidence to demonstrate the most plausible MoA was via enhanced hepatic clearance of thyroid hormones. It is a well-documented MoA, and species differences in thyroid hormone metabolism mean this MoA is of limited relevance to humans.

Aclonifen is not an ED via the EAS-modality in mammals as there was no evidence of EAS adversity in in vivo studies. in vitro findings of endocrine activity via the A and S-modalities were not replicated in vivo.

Assessment and conclusion by RMS:

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CA 5.9 Medical data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Data Point:	KCA 5.9.1/01
Report Author:	
Report Year:	2003
Report Title:	Surveillance medicale pour le personnel travaillant a la production aclonifen
Report No:	C033463
Document No:	M-233211-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

Aclonifen technical material manufacture started at the at the end of 1988 with the first production completed in 1989. This report of 2003 therefore covers the health surveillance of the workers at this plant over a 14 year period. Employees were examined by the work medical officer for clinical signs, body weight, blood pressure, heart rate, skin, eye and mouth lesions every six month, urinalysis was performed every year and haematology, clinical chemistry analyses and lung function tests were performed every two years. No substance-related changes were indicated in the workers from these clinical and laboratory examinations and no adverse health problems were reported amongst the workers. No dermal allergenic reactions were detected or reported.

I. MATERIALS AND METHODS

Manufacturing site:

Product: Aclonifen technical material

Manufacturing dates: 1989 – 2003

Employees: 49 (1 engineer, 1 supervisor, 1 co-ordinator, 7 specialised technicians, 10 technicians and 29 workers)

Health monitoring: Every 6 months: Clinical examination (body weight, blood pressure, heart rate, skin, eye and mouth lesions), biometrics

Every year: Urinalysis (glucose, ketone, specific gravity, blood, pH, protein, nitrogen, white blood cell count), clinical examination, neurological test,

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medical history and Ishihara test. Additional examinations if triggered by the clinical observations included colonoscopy, ultrasound and scans.

Every two years: Lung function test, chest x-ray, clinical biochemistry (liver function, haematology, kidney function, prostatic antigen and lipid balance) and hemoccult test.

The liver function test comprised of:

Serum glutamic oxaloacetic transaminase	Gamma-glutamyl transferase
serum glutamate-pyruvate transaminase	alkaline phosphatase

The haematology test comprised of:

Red blood cell count	White blood cell count
Haemoglobin	Haematocrit
Mean corpuscular volume	Neutrophils, basophils, eosinophils
Mean corpuscular haemoglobin concentration	Mean corpuscular haemoglobin
Platelet count	Thyroid-stimulating hormone

The kidney function test comprised of:

Urea	Creatinine

The lipid balance test comprised of:

Total cholesterol	HDL cholesterol
LDL cholesterol	Triglycerides

Any abnormal results or work-related ill health problems are referred to the Company Medical Advisor.

II. RESULTS AND DISCUSSION

49 workers at the [redacted] which manufactures aclonifen technical material were monitored for 14 years over the period 1989 – 2003. Monitoring included clinical examination, urinalysis, neurological tests, Ishihara tests, lung function tests, chest x-ray, clinical biochemistry (liver function, haematology, kidney function, prostatic antigen and lipid balance) and hemoccult test. No substance-related changes were indicated in the workers from these clinical and laboratory examinations and no adverse health problems were reported amongst the workers. No dermal allergenic reactions were detected or reported.

III. CONCLUSIONS

There were no indications of any health abnormalities attributed to contact with aclonifen amongst the employees at the manufacturing plant [redacted] following health monitoring over the period 1989 – 2003. No CMR intermediates are used in the production of aclonifen.

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Assessment and conclusion by applicant:

Assessment and conclusion by applicant:

GLP is not relevant for this study, there is no relevant OECD test guideline. This study was deemed to be acceptable in the DAR (2006).

There were no indications of any health abnormalities attributed to contact with aclonifen amongst the employees at the manufacturing plant following health monitoring over the period 1989 – 2003. No CMR intermediates are used in the production of aclonifen.

Assessment and conclusion by RMS:

Data Point:	KCA 5.9.1/02
Report Author:	
Report Year:	2019
Report Title:	Summary of medical data known for aclonifen provided to
Report No:	M-670215-01-1
Document No:	M-670215-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	Not applicable (non-guideline study)
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

Aclonifen technical material has been manufactures at the since 2008. This report of 2019 therefore covers the health surveillance of the workers at this plant over a 11 year period. Employees were examined annually for clinical signs, complete blood count, urinalysis, chest and whole abdomen x-ray, ultrasound, audiometry and spirometry. No substance-related changes were indicated in the workers from these clinical and laboratory examinations and no adverse health problems were reported amongst the workers.

I. MATERIALS AND METHODS

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Manufacturing site:	████████
Product:	Aclonifen technical material
Manufacturing dates:	2008 – 2019
Employees:	59 (23 production, 22 quality, 14 logistics)
Health monitoring:	Annually comprising complete blood count, urinalysis (routine & microscopic), ECG, chest and whole abdomen x-ray, ultrasound, audiometry, and spirometry.

The complete blood count test comprised of:

Reticulocytes count	Erythrocyte sedimentation rate
Serum bilirubin	Serum alkaline phosphatase
Serum glutamic-oxaloacetic transaminase	Cholesterol
Total protein	Serum albumin
Blood urea	Serum creatinine
Random blood sugar	HbA1c
Blood group	

II. RESULTS AND DISCUSSION

59 workers at the ████████ which manufactures aclonifen technical material were monitored annually for 11 years over the period 2008 – 2019. Monitoring included clinical examination, complete blood count, urinalysis (routine & microscopic), ECG, chest and whole abdomen x-ray, ultrasound, audiometry, and spirometry. No substance-related changes were indicated in the workers from these clinical and laboratory examinations and no adverse health problems were reported amongst the workers. No accidental exposures to aclonifen occurred during this period and no referrals to medical professionals were required.

III. CONCLUSIONS

There were no indications of any health abnormalities attributed to contact with aclonifen amongst the employees at the ████████ following health monitoring over the period 2008 – 2019.

Assessment and conclusion by applicant:

GLP is not relevant for this study, there is no relevant OECD test guideline.

There were no indications of any health abnormalities attributed to contact with aclonifen amongst the employees at the ████████ following health monitoring over the period 2008 – 2019.

Assessment and conclusion by RMS:

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CA 5.9.2 Data collected on humans

No studies/information available.

CA 5.9.3 Direct observations

No studies/information available.

CA 5.9.4 Epidemiological studies

No studies/information available.

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

There has not been accidental poisoning resulting from production, formulation and agricultural use of aclonifen. It is anticipated that poisoning could result in non-specific signs similar to those observed in animal experiments. However, the risk of poisoning must be considered as low.

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

First aid measures in the case of suicidal intake of aclonifen should consist of standard measures, i.e. decontamination and symptomatic treatment of non-specific symptoms. In addition, based on the toxicological data available it is advisable to monitor liver and kidney function.

CA 5.9.7 Expected effects of poisoning

No accidental poisoning resulting from production, formulation and agricultural use of aclonifen is known to [redacted]. It is assumed that exposure to high doses of the active substance may possibly result in non-specific signs similar to those observed in the animal studies.

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