Document Title

Summary of the toxicological and metabolism studies for fluopicolide

Part 2

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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Version history

Date [yyyy-mm-dd]	Data points containing amendments or additions[1] and brief description	Document identifier and version number

[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 5.8 Other toxicological studies

Toxicity studies were performed for the metabolites M-01 (AE C653711, BAM), M-02 (AE C657188), M-04 (AE C657378), M-05 (AE 1344122), M-09 (AE B102859), M-10 (AE 1344123), M-14 (AE 1388273) and M-15 (AE 1413903). The toxicology data package mainly consists of genotoxicity studies and for some metabolites also acute and subacute studies. The study results indicate that none of these metabolites are genotoxic and that there is no toxicological concern.

Furthermore, supplementary studies on fluopicolide on the mode of action of the observed liver tumours are available. These studies overall confirmed that the mode of action (MoA) for the liver tumours was related to liver enzyme induction, especially of CAR/PXR. This MoA is similar to the MoA for liver tumours after phenobarbital which is known to be non-relevant to humans.

CA 5.8.1 Toxicity studies of metabolites

Toxicological studies have been conducted on the metabolites M-01 (AE C653711, BAM), M-02 (AE C657188, PCA), M-04 (AE C657378), M-05 (AE 1344122), M-09 (AE B102859), M-10 (AE 1344123), M-14 (AE 1388273) and M-15 (AE 1413903). The metabolites M-04 and M-09 are dietary metabolites, M-10, M-14, and M-15 are groundwater metabolites and M-01, M-02 and M-05 are both groundwater and dietary metabolites.

For the metabolite M-01, an EFSA-set ADI of 0.05 mg/kg bw/d (based on NOAEL of the rat chronic study of 5.7 mg/kg bw/d and the 2-year dog study of 4.5 mg/kg bw/d, with a safety factor of 100) and an ARfD of 0.3 mg/kg bw/d are available and are considered to be still valid for this renewal. For the metabolites M-02, M-04, M-05 and M-10, 28-day repeated dose toxicity studies are available. In accordance with the EFSA guidance (EFSA Scientific Committee; Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. EFSA Journal 2012;10(3):2579. [32 pp.] doi:10.2903/j.efsa.2012.2579), a safety factor of 1000 is proposed for these metabolites, comprising 10 for intra-species differences and 10 for inter-species differences. An additional default value for extrapolation from sub-acute to chronic studies is not explicitly proposed in the guidance; however, it is recommended that such an extrapolation is considered on a case by case basis. An additional uncertainty factor of 10 for the duration of exposure, in order to extrapolate from sub-acute to chronic accounts for missing data such as an additional species and long-term studies (including reproductive and developmental toxicity testing). Therefore, an overall safety factor of 1000 is considered appropriate.

Owing to the recent classification of fluopicolide as toxic to reproduction, category 2 (H361d) at the 53rd meeting of the Committee for Risk Assessment (RAC-53), further studies will be conducted (after alignment with the RMS) to demonstrate that the leaching metabolites do not share the properties of the parent.

M-01

An extensive data package exists for M-01 comprising ADME studies, acute oral toxicity studies in rats, short-term toxicity studies in rats (8- and 90-days) and dogs (90-days and 2-years), a combined long-term and carcinogenicity study in rats and reproductive toxicity studies in rats (3-generation) and rabbits (developmental toxicity). Genotoxicity was investigated in a battery of *in vitro* studies and one *in vivo* study. It was concluded that M-01 (BAM) is not genotoxic or carcinogenic and there was no indication of any concern relating to reproductive performance or embryofoetal development. M-01 was slightly acutely toxic with an LD_{50} of 2000/500 mg/kg bw in M/F and a NOAEL of 180 ppm (14 mg/kg bw/d) was derived from the 90-day day study in rats. Based on the assessment of M-01, the EFSA-set ADI of 0.05 mg/kg bw/d (based on NOAEL of the rat chronic study of 5.5 mg/kg bw/d and the 2-year dog study of 4.5 mg/kg bw/d, with a safety factor of 100) and the ARfD of 0.3 mg/kg bw/d are still valid.

M-02

A robust data set is available for M-02 (PCA), comprising an acute oral toxicity study and a short-term (28-day) study in rats, as well as a battery of *in vitro* genotoxicity studies in bacterial cells (Ames test) and mammalian cells (V79/HPRT test and a chromosome aberration assay and MNT, both in human lymphocytes). M-02 has an LD_{50} of >2000 mg/kg bw and a short-term NOAEL of 1880 mg/kg bw/d was established in rats. An ADI of 1.6 mg/kg bw/d can be derived based on this NOAEL with safety factor of 1000. M-02 was not mutagenic in bacterial cells and was not mutagenic, clastogenic nor aneugenic in mammalian cells *in vitro*. Overall, it can be concluded that there is no toxicological concern for this metabolite based on the available data.

M-04

A robust data set is also available for the metabolite M-04, comprising an acute oral toxicity study and a 28-day oral toxicity study in rats, a battery of *in vitro* and *in vivo* genotoxicity tests is also available. M-04 is not acutely toxic (LD_{50} >2000 mg/kg bw) and a short-term NOAEL of 2000 ppm (equivalent to 159/231 mg/kg bw/d) was established in rats. An ADI of 0.16 mg/kg bw/d can be derived from this NOAEL with a safety factor of 1000. M-04 was not mutagenic *in vitro* in bacterial or mammalian cells (Ames and HPRT/V79 tests respectively). A weakly positive result for the induction of chromosome aberrations *in vitro* in human lymphocytes was followed up with an *in vivo* mouse micronucleus test (with demonstrable target tissue exposure), which confirmed that was no concern for clastogenicity or aneugenicity *in vivo*. Furthermore, M-04 did not induce DNA repair an *in vivo* rat liver UDS test. M-04 is therefore not genotoxic *in vivo*. It is concluded that there is no toxicological concern for this metabolite based on the available data.

M-05

A robust data set is available for the metabolite M-05 which comprises an acute oral toxicity study and a short-term (28-day) oral toxicity study in rats. Several *in vitro* genotoxicity studies are also available. The LD_{50} of M-05 was found to be >2000 mg/kg bw and a NOAEL of 152 mg/kg bw/d was established from the 28-day rat study, an ADI of 0.15 mg/kg bw/d can be derived from this NOAEL with a safety factor of 1000. There was no evidence that M-05 was mutagenic in bacteria in an Ames test or in mammalian cells in a mutation test at the HPRT locus of V79 cells of the Chinese hamster. Furthermore, there was no evidence that M-05 could induce chromosome aberrations in cultured human peripheral blood lymphocytes. A recently conducted *in vitro* micronucleus test confirmed that M-05 was not clastogenic nor aneugenic. Overall, it is concluded that there is no toxicological concern for this metabolite based on the available data.

M-09

The metabolite M-09 has been tested in a battery of *in vitro* genotoxicity tests. M-09 did not induce chromosome aberrations *in vitro* in rat lymphocytes, whilst a recently conducted *in vitro* micronucleus test (MNT) confirmed that M-09 was not clastogenic or aneugenic. There was no evidence that M-09 was mutagenic *in vitro* in mammalian cells when tested at the HPRT locus in chines hamster ovary cells; however, a slight but reproducible increase in the number of revertant colonies plate was observed in an Ames test with tester strain TA 1535 in the presence of metabolic activation (+S9) and in the absence of metabolic activation in tester strain WP2*wvr*A. This weakly positive *in vitro* result for mutagenicity in bacterial cells is being followed up *in vivo* with a mammalian alkaline comet assay, which is currently ongoing and will be submitted in November 2020. With the proviso of the weakly positive *in vitro* result in bacterial cells not being reflected in the follow up *in vivo* comet assay, then the metabolite M-09 is not genotoxic.

M-10

A robust data set is available for the metabolite M-10 which comprises an acute oral toxicity study and a 28-day oral toxicity study in rats. Several *in vitro* and *in vivo* genotoxicity tests are also available. M-10 was not acutely toxic via the oral route (LD_{50}>2000 mg/kg bw) and a NOAEL of 20000 ppm (the highest tested dose, equivalent to 1748/2299 mg/kg bw/d in M/F) was proposed from the 28-day rat study. An ADI of 1.7 mg/kg bw/d can be derived from this NOAEL with a safety factor of 1000. M-10 was not mutagenic *in vitro* in bacterial cells (Ames test) but was weakly mutagenic *in vitro* in mammalian cells. This weakly positive *in vitro* result is followed up *in vivo* in a mammalian alkaline comet assay, which is currently ongoing and will be submitted in November 2020. M-10 also induced a weak increase in chromosome aberrations in human lymphocytes at pH altering doses. This *in vitro* result was followed up with an appropriate *in vivo* test (MNT) in which no evidence of clastogenicity or aneugenicity was seen. Although exposure of the target tissue was not explicitly shown (there was no alteration in the NCE/PCE ratio and no clinical signs of toxicity), exposure of the bone-marrow is likely, owing to the route of exposure (i.p injection) and the highest dose tested being the limit dose (2000 mg/kg bw/d). The lack of clastogenic potential for M-10 was confirmed in a recently conducted *in vitro* MNT, in which no evidence of clastogenicity or aneugenicity was seen. On the proviso that *in vivo* comet assay (currently ongoing) confirms the lack of mutagenic potential, there is no evidence that M-10 is genotoxic. Overall, it is concluded that there is no toxicological concern for this metabolite based on the available data.

M-14

The metabolite M-14 has been tested in a battery of *in vitro* and *in vivo* genotoxicity tests. M-14 was not mutagenic *in vitro* in bacterial cells as evidenced in a negative Ames test when tested up to the highest required concentrations, in the presence and absence of metabolic activation (provided by S9 mix), or in mammalian cells when tested at the HPRT locus of the Chinese hamster V79 cells. However, M-14 induced chromosome aberrations *in vitro* in cultured human peripheral blood lymphocytes, following both a 20-hour continuous exposure in the absence of metabolic activation (S9) and a 3-hour pulse exposure in the presence of S9. This *in vitro* result was followed up with an appropriate *in vivo* test (mouse micronucleus test), in which no evidence of clastogenicity or aneugenicity was seen and exposure of the target tissue was demonstrated by a depression of the ratio between the polychromatic and normochromatic erythrocytes. Furthermore, the route of administration (i.p) is highly likely to have resulted in systemic exposure of the test substance (and thus exposure of the highly perfuse bone marrow tissue). Overall, there is no evidence that M-14 is clastogenic or aneugenic *in vivo*. A negative *in vivo* UDS assay showed that M-14 did not induce DNA repair (damage) in isolated rat hepatocytes. Considering the negative results for mutagenicity *in vitro* and the negative *in vivo* MMT and UDS assay, it is concluded that the metabolite M-14 is not genotoxic *in vivo*.

M-15

The metabolite M-15 has been investigated for mutagenicity *in vitro* in bacterial cells and for mutagenicity and clastogenicity/aneugenicity in mammalian cells. M-15 was not mutagenic *in vitro* in bacteria, as evidenced by a negative Ames test, when tested in appropriate strains of *S typhimurium* in the presence and absence of metabolic activation. Neither was there any mutagenic potential seen in mammalian cells *in vitro* when M-15 was tested at the HPRT locus of Chinese hamster V79 cells, with and without metabolic activation. In an *in vitro* micronucleus test in cultured human peripheral blood lymphocytes M-15 did not induce any biologically relevant increases in the numbers of micronucleated cells either in the presence or absence of metabolic activation up to the highest required concentration, thus it can be concluded that M-15 is not clastogenic or aneugenic. Overall, there is no concern that the metabolite M-15 is genotoxic.

Detailed evaluations of the available studies for the metabolites M-01, M-02, M-04, M-05, M-09, M-10, M-14 and M-15 are presented below.

M-01 (BAM, AE C653711)

An extensive data package is available for M-01. The European Food Safety Authority (EFSA) stated in its "*Conclusion on pesticide peer review regarding the risk assessment of the active substance fluopicolide*" (EFSA Scientific Report (2009) 299, 1-158) that:

"*The M01 metabolite may be considered of comparable toxicity as the parent, although slightly more acutely toxic. In principle, the ADI of the parent could have been applied, however, as an acceptable set of studies were conducted with M01 and considering that M01 is also a metabolite of another active substance, an acceptable daily intake (ADI) of 0.05 mg/kg bw/day was set for this metabolite as well as an acute reference dose (ARfD) of 0.3 mg/kg bw.*"

It was additionally stated that M-01 and other fluopicolide metabolites "*…were not considered relevant according to the guidance document on groundwater metabolites even if they appear at levels above 0.1 µg/L in groundwater*".

Bayer has newly evaluated and assessed all existing toxicity and metabolism studies on M-01 (BAM) according to newest guidance and guidelines. No relevant information was found following a time unlimited search of the literature with regard to M-01 (BAM). Bayer's assessment is that the EFSA conclusions above regarding the toxicological non-relevance of M-01 (BAM) for groundwater as well as the EFSA-set ADI of 0.05 mg/kg bw/day and an ARfD of 0.3 mg/kg bw are still valid. The overall conclusions for the individual sections are provided below, followed by the summaries of the available studies.

Absorption distribution, metabolism and excretion (ADME)

Several *in vivo* single dose and an *in vivo* repeated dose study are available which are summarised in section MCA 5.1.1 of this document, in the context of the parent, fluopicolide.

Acute toxicity

An acute oral toxicity study is available (no acute studies *via* the dermal or inhalation route have been conducted and skin or eye irritation and sensitisation studies have not been conducted). The metabolite M-01 is of low acute toxicity in male rats (LD_{50} 2000 mg/kg bw/d) but is slightly acutely toxic in females (LD_{50} 500 mg/kg bw/d), M-01 is therefore slightly more acutely toxic than the parent.

Short-term toxicity

With regard to short-term toxicity, however, M-01 is considered to be less toxic than the parent, fluopicolide. Short-term toxicity studies are available in rats (90-days and 8-days) and in dogs (90-days and 2-years). In rats, A NOAEL of 180 ppm (equivalent to 14 mg/kg bw/day) was derived when BAM was administered to rats *via* the diet for 90 days. At the LOAEL of 600 ppm (49 mg/kg bw/day) reductions in body weight gain and food consumption were noted in females. This is supported by a NOAEL of 200 mg/kg bw/d derived from an eight-day preliminary study, in which reduced body weight, body weight gain and food consumption were seen at the LOAEL of 400 mg/kg bw/d, in addition to mortalities and clinical signs.

Genotoxicity

The genotoxic potential of M-01 (BAM) has been investigated in a series of *in vitro* studies and one *in vivo* investigation (mouse micronucleus test). M-01 (BAM) is not genotoxic *in vitro* in bacterial cells, as demonstrated in two available Ames tests, or in mammalian cells in the V79 cell line derived from male Chinese hamster lung. A micronucleus test in human peripheral blood lymphocytes has demonstrated that BAM is not clastogenic nor aneugenic *in vitro*. In addition, a negative UDS assay is available as supportive information. M-01 (BAM) was negative *in vivo* in a mouse micronucleus test, in which it is likely that the target tissue was exposed, as demonstrated by signs of systemic exposure, and hence exposure of the highly perfuse bone marrow tissue. Overall, based on the evidence currently available, M-01 (BAM) is not mutagenic or clastogenic.

Carcinogenicity

M-01 (BAM) was administered to rats *via* the diet in a 2-year combined chronic toxicity/carcinogenicity study, which broadly conforms to OECD guidelines 451 and 453. A NOAEL of 180 ppm (equivalent to 6.5 and 8.5 mg/kg bw/day in males and females respectively) was proposed from this study. At the LOAEL of 500-ppm (equivalent to 18.7 and 23.8 mg/kg bw/day in males and females respectively) body weight and bodyweight gain were reduced in both sexes whilst food consumption was reduced in females only. Liver weights were increased in females and non-neoplastic findings (vacuolation, degeneration and fat deposition) were confined to this organ in females.

With regard to neoplastic findings, no treatment-related increase in carcinomas was observed, and the overall tumour burden was not increased; however, there was a slightly higher incidence of hepatomas observed in high-dose females only (4/20 compared with 0/8 in the controls animals). This slight, non-statistically significant increase was considered not to demonstrate a specific carcinogenic potential because the increase was not statistically significant, it only occurred in females, was at the upper end of the historical control range, the increase was observed above the MTD and there was no increase in carcinomas or overall tumour burden. Furthermore M-01 is not genotoxic and the potential for cellular proliferation has been excluded in several mechanistic studies.

Overall, there is no evidence that M-01 (BAM) is carcinogenic.

Reproductive toxicity

A three-generation reproduction study in rats, with production of two litters in each generation, has been conducted, which despite predating both GLP and relevant OECD guideline, is informative on general, functional reproductive performance and is considered acceptable for this purpose. An overall NOAEL of 180 ppm (equivalent to 7.5 mg/kg bw/day) was set from this study based on possible slight reductions in bodyweight of dams and weanlings at the highest dose level of 180 ppm.

Developmental toxicity is covered by a prenatal developmental study in rabbits. This predates the current version of the relevant OECD guideline, but was conducted to GLP and is considered acceptable and reliable. From this study, a NOAEL for maternal toxicity can be set at 30 mg/kg bw/day of BAM, based on maternal toxicity at the high dose level of 90 mg/kg bw/day and a developmental NOAEL can be set at ≥90 mg/kg bw/day of BAM, the highest dose applied.

An expert assessment (Anon.; 2019; M-672423-01-1) of the available data relating to the reproductive and developmental toxicity potential of M-01 (BAM) is available.

Overall, there is no indication in the available data of any concern relating to reproductive performance or to embryofoetal development.

Mechanistic studies

Several mechanistic studies are available which investigate the potential for M-01 (BAM) to induce cell proliferation in the liver and to induce specific metabolizing enzymes. In a proliferation study, liver weight and histomorphology were unaffected (no BrdU measurements were available owing to an error in histochemical staining). In an *ex vivo/in vitro* study using rat hepatocytes, M-01 (BAM) showed no evidence of peroxisome proliferation, and there was no indication that M-01 (BAM) had an effect on replicative DNA synthesis (determined by BrdU incorporation). An *in vitro* study using male rat Liverbeads™ investigated the potential for M-01 (BAM) to induce cytochrome P-450 (Cyp 1a1, 1a2, 2b1, 3a3, 4a22) and UDPGT (Ugt 1a6) enzymes. Only a slight upregulation of Cyp 2b1 was evident (approximately 2-fold over control), at the highest concentration of 300 µM, a result considered to be of doubtful/equivocal biological significance.

Acute toxicity

Data Point:	KCA 5.8.1/01
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C653711 (metabolite of AE C638206) - Acute toxicity in the rat after oral administration
Report No:	C038678
Document No:	M-225484-01-1
Guideline(s) followed in study:	EU (=EEC): 67/548/EEC; OECD: 423; USEPA (=EPA): OPPTS 870.1100
Deviations from current test guideline:	Six males and three females were initially dosed with 1000 mg/kg bw, rather than three animals/dose as required by the guidance.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an acute oral toxicity study (2001) conducted according to the acute toxic class method, fasted Wistar rats (3/sex) were administered M-01 (referred to as 2,6-dichlorobenzamide (BAM) in the report) *via* gavage, at an initial dose of 2000 mg/kg bw (an additional group of 3 males were also administered this dose). All females, as well as 2 out of 3 males from the initial 2000 mg/kg bw group died (all males of the additional group survived). Therefore, a further 3 animals/sex were dosed with the next lowest dose of 300 mg/kg bw; all animals survived and so the result was confirmed with an additional 3 animals/sex. A 14-day observation period followed each treatment, in which body weights, mortality and clinical signs were noted. A necropsy was performed on all animals at sacrifice on day 15.

At 2000 mg/kg bw, deaths occurred from six hours until six days following dosing. Clinical signs of toxicity were observed, which comprised abdominal/lateral position, decreased motility, poor reflexes, reduced reactivity, spasmodic state, uncoordinated gait, laboured breathing, tachypnoea, chromodacryorrhea, increased lacrimation, closed eyelids, narrowed palpebral fissure and piloerection.

No mortality was observed at 300 mg/kg bw; however, clinical signs were noted at this dose which comprised decreased motility, uncoordinated gait and narrowed palpebral fissure

Amongst the surviving males at 2000 mg/kg bw, body-weight development was affected, with reductions in body-weight gain being noted in these animals (-57% in comparison with the 300 mg/kg bw dose group). Body-weight development was not affected at 300 mg/kg bw.

In animals that died or were killed in a moribund state, macroscopic examination revealed pale and discoloured liver and spleen.

The acute oral LD_{50} of M-01 (BAM) was 2000 mg/kg bw in males and 500 mg/kg bw in females.

I. Materials and methods

A. Materials

1. Test material:

Test substance:	M-01 (referred to as AEC653711 (BAM) in the report)
Purity:	97%
Batch no.:	8808018

2. Vehicle:

Vehicle:	2% Cremophor EL

3. Test animals:

Species:	Rat
Strain:	HsdCpb:Wu
Age:	8-13 weeks
Weight at dosing:	194-224g (males) and 165-186g (females)
Source:	[redacted]
Acclimation period:	At least five days
Identification:	Cage labels and individual skin markings
Diet:	Provimi Kliba 3883.0.15 Maus/Ratte Haltung, Kaiseraugst Switzerland, withheld for 16-24h before and 2-4h following dosing
Water:	Tap water *ad libitum*
Housing:	Polycarbonate cages with low dust wood granulate bedding
Environmental conditions	
Temperature:	22 ± 2°C
Humidity:	55 ± 5%
Ventilation:	10 changes/hour
Photoperiod:	12 hours light/12 hours dark

B. Study design

1. In-life dates: October 15th, 2003 to November 26th, 2003

2. Animal assignment and treatment

The animals received a single oral gavage dose, prepared to a volume of 10 mL/kg bw. The stability and homogeneity of the formulated test substance were confirmed analytically. The animals were dosed according to the stepwise procedure described in OECD TG 423. An initial 3 animals/sex were dosed with 2000 mg/kg bw with the resulting mortality and/or clinical signs determining the next step (no further testing, dosing three additional animals at the same dose or dosing three additional animals at the next highest/lowest dose). As mortality occurred at this dose, a further 3 animals/sex received a lower dose of 300 mg/kg bw/d; there were no deaths at this dose and so the results were confirmed with a further 3 animals/sex (a second group of 3 males received 2000 mg/kg bw in a deviation from the guideline; however, this does not affect the validity of the study). The day of dosing was designated day one.

C. Methods

1. Observations

Mortality:

The cages were examined for mortalities several times a day on day one, and then at least once daily thereafter, for an observation period of 14 days.

Clinical signs:

Animals were individually examined for clinical signs several times on day one, and then at least once daily throughout the 14-day observation period; the time, nature, duration and severity of the observed clinical signs were noted.

Bodyweights were recorded weekly until the termination of the study and the results presented as mean values.

2. Necropsy

Animals which died or were killed in a moribund state were weighed and examined macroscopically; animals which survived to the end of the study were sacrificed and were also examined macroscopically.

II. Results and Discussion

A. Results

1. Dose-response table (LD_{50})

Table 5.8.1- 1: Acute toxicity in male and female rats following a single oral gavage dose of M-01 (BAM)

Dose (mg/kg bw)	Toxicological Result*	Duration of Sign	Time of Death	Mortality (%)
Male				
(1st) 300	0/3/3	15 min - 2 d	-	0
(2nd) 300	0/3/3	10 min - 3 d	-	0
(1th) 2000	2/3/3	30 min - 7 d	6 d	67
2nd) 2000	0/3/3	15 min - 8 d	--	0
Female				
(1th) 300	0/3/3	30 min - 2 d	-	0
(2nd) 300	0/3/3	30 min - 5 h	--	0
2000	3/3/3	10 min - 3 d	6 h – 3 d	100

* Number of animals which died and/or were sacrificed moribundly / number of animals with clinical signs / total number of animals.

The LD_{50} was calculated to be 2000 mg/kg bw in males (males in both group receiving 2000 mg/kg bw were used for the assessment in reverse order) and 500 mg/kg bw in females.

2. Mortality

Three animals/sex received an initial dose of 2000 mg/kg bw/d. All females and 2/3 males died or were killed *in extremis* by day 3 (females) or day 6 (males) of the study. Of the further three males receiving 1000 mg/kg bw, all survived and the results from both groups of males receiving this dose were used in the calculation of the LD_{50}. A further 3 animals/sex were administered a lower dose of 300 mg/kg bw/d; there were no deaths and so the result was confirmed with a final 3 animals/sex dosed at 300 mg/kg bw/d.

3. Clinical signs

Decreased motility, uncoordinated gait and narrowed palpebral fissure were noted at 300 and 2000 mg/kg bw. Further clinical signs comprising lateral abdominal position, poor reflexes, decreased reactivity, spasmodic state, laboured breathing, tachypnoea, chromodacryorrhea, increased lacrimation, closed eyelids and piloerection were observed only at 2000 mg/kg bw.

4. Body weights

Body weights and body-weight gain were reduced in the surviving animals treated with 2000 mg/kg bw; body-weight gains of males at this dose were 57% lower than those of the 300 mg/kg bw dose group. There was no effect on body weight development at 300 mg/kg bw.

5. Necropsy findings

In the animals that died or were killed in a moribund state, gross necropsy revealed findings in the liver (pale, discoloured and spotted) and the spleen (pale and discoloured). There were no gross necropsy findings amongst the surviving animals.

III. Conclusion

An LD_{50} value of 2000 mg/kg bw in males and 500 mg/kg bw in females was calculated from this acute oral toxicity study conducted according to OECD TG 423 (acute toxic class method).

Assessment and conclusion by applicant:

Study was conducted in accordance with OECD 423 and is valid and acceptable to investigate the acute oral toxicity of M-01. An LD_{50} of 2000 mg/kg bw in males and 500 mg/kg bw in females was determined from this study.

Data Point:	KCA 5.8.1/02
Report Author:	[redacted]
Report Year:	1967
Report Title:	Preliminary toxicity studies with 2,6 dichlorobenzamide a) Acute oral toxicity to rats b) Range finding study in rats - daily oral application for eight days
Report No:	C040448
Document No:	M-228905-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Doses up to 10,000 mg/kg bw were administered, the limit dose in the guidance is 1000 mg/kg bw; no necropsy examinations were performed, or body weights recorded for the acute study.
Previous evaluation:	yes, evaluated and accepted in DAR (2005)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary:

The study was conducted as a combined subacute 8-day preliminary range finding and acute oral toxicity study in rats. The acute and subacute components of the study are presented individually where appropriate.

Acute

Groups of male and female fasted Wistar rats (5/sex/dose) received a single oral gavage dose (volume, 10 ml/kg bw) of M-01 (referred to as 2,6-dichlorobenzamide (BAM) in the report) suspended in 1% w/v aqueous tragacanth. Administered doses were either 1000, 2150, 4640 or 10000 mg/kg bw. The animals were examined daily for mortality and clinical signs over an observation period of 14-days.

Mortalities were noted at all dose levels; 1/5, 4/5, 5/5 and 5/5 males and 0/5, 3/5, 4/5 and 5/5 females died at 1000, 2150, 4640 and 10000 mg/kg bw, respectively. Clinical signs of toxicity in the decedents were indicative of progressive narcosis and it was considered that the resulting deaths were a consequence of medullary depression. Signs of toxicity in surviving animals comprised prostration, relaxed limbs, absent righting reflex, miosis and rapid, shallow respiration. The LD_{50} of M-01 (BAM) after a single oral dose was calculated to be 1470 and 2330 mg/kg bw in males and females respectively, with a confidence interval of 951 – 2270 mg/kg bw in males and 1430 – 3780 mg/kg bw in females.

Subacute

Male and female rats (5/sex/dose) received M-01 (suspended in 1% w/v aqueous tragacanth) daily for eight days at doses of 0, 6.25, 12.5, 25.0, 100, 200, 400, 800 or 1600 mg/kg bw/day.

The animals were examined daily over the eight-day period, for mortality, clinical signs and bodyweight changes; food consumption was measured, and muscle tone was also examined. On sacrifice, gross necropsy and/or microscopic examinations were performed on a selection of organs from designated dose-groups.

Mortalities were noted in 3/5 males and 3/5 females at both 800 and 2500 mg/kg bw/d and also in 1/5 males and 2/5 females at 400 mg/kg bw/d; all other animals survived. Body weight development was impaired at 400 mg/kg bw/d (no measurements were taken at the higher doses). Clinical signs appeared within 2-hours of each dosing, peaked at 2-hours, and had partially regressed by 24-hours. Clinical signs comprised skeletal hypotonia (from 25 and 100 mg/kg bw/d in males and females respectively) and impaired righting reflex, miosis, hypothermia, moderate analgesia and rapid, shallow breathing from 200 mg/kg bw/d in both sexes. Liver and adrenal weights were increased in this study; however, there were no necropsy findings that could be attributed to treatment with M-01 (BAM).

A NOAEL of 200 mg/kg bw/d can be determined from this study. At the LOAEL of 400 mg/kg bw/d final body weight was reduced in comparison with controls by 15% and 9%, bodyweight gain by 200% and 53% and food consumption by 24% and 23% in males and females respectively.

I. Materials and methods

A. Materials

1. Test material:

Test substance:	M-01 (referred to as 2,6-dichlorobenzamide (BAM) in the report), technical quality
Purity:	97.0%
Batch no.:	133/2/4/104

2. Vehicle:

Vehicle:	1% w/v aqueous tragacanth

3. Test animals:

Species:	Rat
Strain:	Wistar
Age:	Not provided
Weight at dosing:	>190g (males) and >150g (females)
Source:	[redacted]
Water	Tap water *ad libitum*

No information was provided with regard to animal housing, environmental conditions, or diet. It was not stated how the animals were identified or whether the animals underwent an acclimatisation period prior to dosing.

B. Study design

1. In-life dates: 15th October 2003 to 26th November 2003

2. Animal assignment and treatment

Animals received the test substance suspended in 1% w/v aqueous tragacanth; the test substance was administered *via* gavage at a volume of 10 mL/kg bw.

Acute

Food was withheld for 18 hours (overnight) and the rats then randomly distributed into 4 dose-groups of 5 rats/sex. Each animal was administered a single gavage dose at either 1000, 2150, 4640 or 10000 mg/kg bw.

Subacute

Animals received the test substance suspended in 1% w/v aqueous tragacanth; the test substance was administered *via* gavage at a volume of 10 ml/kg bw.

The test substance was administered to 5/sex/dose rats, daily for eight days. Rats were randomly selected into groups which received doses of 0 (vehicle control), 6.25, 12.5, 25.0, 100, 200, 400, 800 or 1600 mg/kg bw/day. Food and water were available throughout the experiment.

C. Methods

1. Observations

Acute

Animals were examined daily for mortality and clinical signs over the 14-day observation period.

Subacute

Animals were examined daily over a period of 8-days for mortality, clinical signs and bodyweight; food consumption was measured twice daily on days 6 and 7 only. The muscle tone of the sub-acute animals was independently scored by two examiners on day 4.

2. Necropsy

Acute

No necropsy observations were provided for the acute phase of the study.

Subacute

The animals of the control, 25, 50, 100, 200 and 400 mg/kg bw/d dose-groups were sacrificed 24-hours following the last dose and the weights of the brain, spleen and liver, as well as the left kidney, adrenal, and gonad were recorded.

No necropsy examinations were carried out on animals of the 6.25 and 12.5 mg/kg bw/d dose-groups and only a gross necropsy was performed on surviving animals of the 800 mg/kg bw/d group. All other animals underwent a macroscopic examination and microscopic examination of the above organs in addition to the thyroid, stomach, duodenum, jejunum and colon.

II. Results and Discussion

A. Results

1. Dose-response table (LD_{50}) and mortality

Acute

Table 5.8.1- 2: Mortality and clinical signs observed in male and female rats following a single oral (gavage) dose of M-01 (BAM).

M-01 (mg/kg bw)	Males	Females	Clinical signs
1000	1/5	0/5	Prostrate, limbs relaxed, righting reflex absent but corneal reflex present; miosis and rapid but shallow respiration (refers to surviving animals only)
2150	4/5	3/5	Progressive narcosis to the plane of surgical anaesthesia. Deaths probably due to medullary depression (refers also to death at 1000 mg/kg bw).
4640	5/5	4/5	
10000	5/5	5/5	

Number died/number with clinical signs

Mortalities were observed from 1000 and 2150 mg/kg bw in males and females respectively, with all observed deaths occurring between 3- and 72-hours post-dosing (see dose-response table above).

Subacute

Table 5.8.1- 3: Mortality and clinical signs observed in the sub-acute phase

M-01 (BAM) (mg/kg bw)	Males	Females
6.25	0/5	0/5
12.5	0/5	0/5
25	0/5	0/5
50	0/5	0/5
100	0/5	0/5
200	0/5	0/5
400	1/5	2/5
800	3/5	3/5
1600	3/5	3/5

Number died/number with clinical signs

All deaths occurred between days 2 and 9, and in each case was associated with loss of righting, corneal and pain reflexes, mydriasis, shallow respiration, brachycardia and hypothermia.

2. Clinical signs

Acute

Clinical signs were observed from 10-minutes following dosing. Clinical signs in surviving animals had fully reversed by 24- or 48-hours following onset and comprised (at 1000 mg/kg bw) prostration, relaxed limbs, absent righting reflex (corneal reflex present), miosis and rapid, shallow respiration. Signs of toxicity in animals dosed at 2150 mg/kg bw (including the one death at 1000 mg/kg bw) were indicative of progressive narcosis, and the subsequent deaths were considered to be the result of medullary depression.

Subacute

Skeletal hypotonia was observed from 25 and 100 mg/kg bw/d in male and female rats respectively, accompanied above 200 mg/kg bw/d in both sexes by impaired righting reflex, miosis, hypothermia, moderate analgesia, and rapid, shallow breathing. Clinical signs appeared within 2-hours of each dosing, peaked at 2-hours and had partially regressed by 24-hours.

3. Body weights

Acute

No information regarding body-weight development or food-consumption was reported for the acute phase of the study.

Subacute

In the subacute phase of the study, body weights and bodyweight gain of male rats was impaired at 400 mg/kg bw/d, and bodyweight gain, but not the final body weight was reduced in females at this dose; in both sexes at this dose a reduction in food consumption was observed (see table below).

Table 5.8.1- 4: Selected bodyweight changes during the sub-acute phase

	Males			Females		
	0 (control)	400 mg/kg bw	% difference from control	0 (control)	400 mg/kg bw	% difference from control
Final body weight (g)	219	186	-15%	156	142	-9%
Bodyweight gain (g)	17	-17	-200%	15	7	-53%
Food consumption (g/animal/day)	54	41	-24%	42	32	-23%

There was no effect on bodyweight development or food consumption observed at the lower-dose levels. The 800 and 1000 mg/kg bw/d were not included in the evaluations.

4. Organ weights (subacute phase only)

Liver weights were increased in male rats at 100, 200 and 400 mg/kg bw/d. Adrenal weights were increased in males at 200 and in males and females at 400 mg/kg bw/d. All other changes in organ weights were isolated incidences that were slight, not statistically significant and/or showed no clear dose-response.

5. Necropsy findings (subacute phase only)

There were no gross necropsy or histopathological findings observed in the subacute phase of the study that could be attributed to treatment with M-01 (BAM); none of the findings showed a clear dose-response and they occurred at similar or greater incidences in the control animals.

III. Conclusion

Acute

The LD_{50} of M-01 (BAM) after a single dose to male rats was calculated to be 1470 mg/kg bw with a confidence interval of 951 – 2270 mg/kg bw.

The LD_{50} of M-01 (BAM) after a single dose to female rats was calculated to be 2330 mg/kg bw with a confidence interval of 1430 – 3780 mg/kg bw.

Subacute

The LD_{50} values of M-01 (BAM) in the subacute toxicity study were 677 and 574 mg/kg bw/day in males and females respectively, with corresponding confidence intervals of 410-1117 and 363-907 mg/kg bw/d. A NOAEL of 200 mg/kg bw/d can be determined from this study. At the LOAEL of 400 mg/kg bw/d final body weight was reduced in comparison with controls by 15% and 9%, bodyweight gain by 200% and 53% and food consumption by 24% and 23% in males and females, respectively.

Assessment and conclusion by applicant:

Study was performed pre-guideline and was a combined acute oral toxicity and preliminary study. Owing to the deviations from the current guidelines as described above, the study is acceptable as supportive information.

Short-term toxicity

Data Point:	KCA 5.8.1/04
Report Author:	[redacted]
Report Year:	1967
Report Title:	Dietary administration of 2,6-dichlorobenzamide to male and female rats for 13 weeks
Report No:	C034142
Document No:	M-234461-01-1
Guideline(s) followed in study:	not specified
Deviations from current test guideline:	Endocrine sensitive endpoints were not measured; ophthalmological investigations were not included; only 5/sex/dose animals were investigated (the guidance recommends 10/sex); functional observations were not conducted.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Groups of 10 male and 10 female Wistar rats were administered M-01 (referred to as 2,6-dichlorobenzamide (BAM) in the report) at dietary concentrations of 0, 50, 180, 600 and 2300 ppm for 13 weeks, equating to mean estimated intakes of 0, 4, 14, 49 and 172 mg/kg bw/day (both sexes combined). The animals were regularly observed for mortality and clinical signs, whilst bodyweights and food intake were determined weekly. The effect on skeletal muscle tone was measured at four intervals during the experiment. Clinical chemistry analyses (blood and urine) were performed throughout the study. At the end of the treatment period, the clearance of bromsulphphthalein by the liver and the blood clotting time were evaluated. At sacrifice, organ weights were determined, and tissues subjected to gross and histopathological investigations. Liver glycogen content was also recorded.

There were no treatment-related deaths and clinical signs were confined to females at 600 and 2300 ppm (hair loss during the latter treatment period). Food consumption and bodyweight gain were reduced in females from 600 ppm and in males at 2300 ppm. At 2300 ppm, mean body weights were reduced from week 2 up to termination and bodyweight gain was reduced throughout the study in all animals at 2300 ppm and in females at 600 ppm. No treatment-related body weight or food consumption changes were observed in males at 600 ppm, or in either sex at the lower doses.

A reduction in skeletal muscle tone was observed in both sexes at 600 and 2300 ppm. Blood coagulation times revealed a significant decrease of the thrombotest-time in both sexes at 2300 ppm compared with controls.

There were no clear treatment-related changes in relative organ weights at termination and gross and microscopic examination at necropsy did not reveal any treatment-related changes in the examined organs and tissues.

The NOAEL for M-01 (BAM) in this 90-day dietary study was therefore 180 ppm (equivalent to 14 mg/kg bw/day). At the LOAEL of 600 ppm (49 mg/kg bw/day) reductions in food consumption and bodyweight gain and reduced skeletal muscle tone were observed.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-01 (BAM, referred to as 2,6-dichlorobenzamide in the report)
Purity:	97%
Batch no.:	133/2/4/104

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rats
Strain:	Wistar
Source:	[redacted]
Diet:	Standard rat diet (Hope farms (Holland) N.V.)
Water:	Tap water *ad libitum*
Housing:	Individually housed at random air-conditioned locations
Temperature:	Approx. 22 °C
Humidity:	55 - 65%
Air changes:	Not stated
Photoperiod:	Not stated

B. Study design

1. In-life dates: Not stated

2. Animal assignment and treatment

Rats were randomly selected (3/sex/dose) and were administered the test substance at dietary concentrations of 0, 50, 180, 600 and 2300 ppm, which equated to mean estimated intakes of 0, 4, 14, 49 and 72 mg/kg bw/d (for both sexes combined).

3. Diet preparation and analysis

The test material (technical quality M-01, BAM) was thoroughly mixed with a standard rat diet (Hope farms (Holland) N.V.) in a Hobart mixer. Diets were freshly prepared and provided to the rats once weekly. Mean intakes (mg/kg bw/d) were calculated on the basis of the previous week's food consumption.

4. Statistics

Unless otherwise stated the subsequent records of quantitative estimations have been analysed for a significant ($p \leq 0.05$) difference between the control and each test group by the method of Wilcoxon, F. (1945) Biometrics Bull. 1:80.

C. Methods

1. Observations (mortality and clinical signs)

The animals were examined daily for mortality and clinical signs of toxicity. Skeletal muscle tone was recorded blind by two independent examiners on four separate occasions from day four until the conclusion of the study.

2. Body weight and food consumption

Body-weights and food consumption were recorded weekly; food utilisation efficiency was measured for weeks two to eleven (considered a period of rapid growth).

3. Laboratory investigations (Haematology, clinical chemistry, urinalysis)

Five rats/group were assigned for blood sampling (heparinised blood). Samples were taken from fasted rats via puncture of the venous plexus of the eye orbit (under anaesthesia) one week prior to the study commencing (pre-test) and then during weeks six and twelve.

Clinical chemistry measurements were taken at all of three sampling points, whilst haematology parameters (coagulation, haemoglobin, packed cell volume, total red and white cell counts and white cell differential count) were measured at the 12-week time-point only.

Urine was collected for analyses from the animals and placed in urine collection units, for two hours (8:30am – 10:30 am) at pre-test and during weeks six and twelve. Liver function at the end of the study was investigated in the control and high-dose animals by measuring the clearance of bromosulphthalein.

4. Pathology

The animals were sacrificed by ether inhalation and sectioned. A full macroscopic examination was performed on each animal and the liver, gonads, adrenals, kidneys, thymus, heart, spleen and brain were weighed and subject to histopathological investigations. Histopathology was also performed on the bladder, prostate, seminal vesicles, uterus, stomach, duodenum, jejunum, ileum, colon, rectum, lungs, thyroid, sub-maxillary salivary glands, cervical lymph-nodes, sciatic nerve-gastrocnemius muscle and skin; however, these organs were not weighed. Slides were stained with haematoxylin and eosin, with the liver also being stained with 'oil red 0' to detect fat. Animals of the high-dose and controls groups were initially examined microscopically, with the animals of the other dose-groups only being investigated in the event of unusual findings. Glycogen content was estimated in the remaining liver of the animals selected for urine collection.

II. Results and discussion

D. Results

1. Clinical results

There were no treatment-related deaths, the three deaths that were noted in the control, 600 and 2300 ppm dose-groups, were attributed to an overdose of ether during blood collection and not to treatment with M-01-(BAM). Clinical signs of toxicity (hair loss during the latter half of the study) were confined to females of the mid- and high-dose groups.

There was a statistically significant reduction in skeletal muscle tone amongst both sexes of the 2300 ppm dose-group at all measurements. Additionally, in females at 600 ppm this effect was apparent on days 91 and 92, but in males of the same dose-group the effect was only noted on day four.

Table 5.8.1- 5: Mean muscle relaxation scores throughout the treatment period

Days	Dose level (ppm)									
	Males					Females				
	0	50	180	600	2300	0	50	180	600	2300
Day 4	4	7	6	17*	27*	10	7	19	17	30*
Day 21	5	6	13	16	34*	10	7	8	13	13*
Day 91	6	11	11	10	34*	7	2	9	21*	25*
Day 92	7	11	11	16	36*	6	6	10	23*	27*

** $p < 0.05$; significantly different to controls using the Wilcoxon test

2. Body weight and food and water intake

The terminal body-weights of males and females in the high-dose group were statistically significantly reduced when compared with the concurrent controls, beginning at week two of the study and persisting until termination. Overall body-weight gains were statistically significantly reduced in females from 600 ppm and in males at 2300 ppm throughout the study (see table 5.8.1-6). Food consumption was reduced in females at 600 and 2300 ppm, which correlated with a reduction in food utilisation efficiency at 2300 ppm during weeks 2-11; in males at 2300 ppm food consumption was also reduced but there was no corresponding effect on food efficiency. During the rapid growth period in the first part of the study (weeks 1 to 2), food utilisation efficiency was reduced in females from 600 ppm. A slight increase in food utilisation efficiency in males at 50 ppm during this period is not of toxicological significance.

There was no effect on body-weight development or food consumption in the lower dose-groups in either sex.

Table 5.8.1- 6: Overall body-weight gain and food consumption in male and female rats

Weeks 2 - 11	Dose level (ppm)									
	Males					Females				
	0	50	180	600	2300	0	50	180	600	2300
Mean body weights gain (g) (% control)	159	165	152	146	111** (70)	66	69	67	54** (82)	46** (70)
Mean food consumption (g) (% control)	1311	1344	1297	1255	1103** (84)	980	1010	1010	907** (93)	814** (83)
Food efficiency (g consumed/g growth)	8.84	8.14	8.81	8.60	10.10	15.05	15.06	15.23	18.23	18.95**

** $p < 0.01$; significantly different from control using the Wilcoxon test

3. Laboratory investigations

Haematology:

There was no effect on haematological parameters in males or females at any dose; haemaglobin, packed cell volume and cell counts were similar across all dose-groups including controls (see table 5.8.1-7); however, the mean blood coagulation times measurements revealed a statistically significant reduction in the thrombotest time in males and females of the high-dose group when compared with controls. Accordingly, the thrombotest was repeated for males and females of all dose-groups; after this second measurement the decrease occurred again in males at 2300 ppm and in females at 600 ppm. There was no effect on the thrombotest time at 180 ppm (see table 5.8.1-8).

Table 5.8.1- 7: Haematological parameters

Dose level (ppm)	Hb (%)	Haematocrit (vol.%)	RBC (mill/mm³)	WBC (/mm³)	Eos.	Bas.	Myel.	Young	Stabs.	Mono.
	Male									
Control	15.8 ± 1.8	52 ± 0.9	11.1 ± 0.8	14300 ± 1500	0.8	0.2	0.0	0.0	0.4	2.8
50	15.8 ± 0.8	52 ± 1.6	10.7 ± 1.7	13000 ± 2200	0.6	0.0	0.0	0.0	0.4	1.8
180	16.0 ± 0.2	53 ± 1.6	10.5 ± 1.8	11950 ± 1750	1.0	0.0	0.0	0.0	0.2	1.2
600	14.9 ± 0.8	50 ± 3.6	10.0 ± 0.5	13200 ± 650	1.2	0.2	0.0	0.0	0.4	2.4
2300	15.2 ± 0.8	50 ± 4.0	9.6 ± 0.7	11050 ± 2600	0.4	0.0	0.0	0.0	0.0	0.8
	Female									
Control	14.8 ± 0.4	47 ± 2.4	8.9 ± 1.2	6900 ± 900	1.0	0.0	0.0	0.0	0.0	0.4
50	14.7 ± 0.5	47 ± 1.2	9.0 ± 0.7	7750 ± 2600	0.2	0.0	0.0	0.2	0.2	1.0
180	14.9 ± 0.6	48 ± 2.4	9.2 ± 0.8	7700 ± 800	1.3	0.0	0.0	0.0	0.0	0.2
600	14.2 ± 0.4	47 ± 2.4	9.1 ± 1.1	8100 ± 1650	0.8	0.0	0.0	0.4	0.0	1.4
2300	14.6 ± 0.8	48 ± 2.2	8.8 ± 0.4	7300 ± 750	1.2	0.0	0.0	0.0	0.0	0.2

Table 5.8.1- 8: Mean blood coagulation times at termination

	Dose level (ppm)									
	Males					Females				
	0	50	180	600	2300	0	50	180	600	2300
Thrombotest-time (seconds)	66	-	-	-	52**	56	-	-	-	49*

* p < 0.05; ** p < 0.01 - significantly different from control using the Wilcoxon test

Clinical chemistry:

Table 5.8.1-9 outlines the main clinical chemistry findings. In high-dose males increases in total protein (TP), cholesterol and urea were observed at weeks 6 and 12. In females of the high-dose group, there was no effect on TP, but cholesterol (weeks 6 and 12) and urea (week 6) were increased. An increase in urea was also noted at 600 ppm in females at week 6 and in males at week 12; SGPT was elevated in males and females during the week 6 measurement but was similar to controls by week 12. There was no effect on glucose, creatinine, SGOT, alkaline phosphatase or bilirubin. The liver function test revealed no difference between the bromosulphthalein retention time between the control and high-dose groups and analysis of the glycogen content in the liver showed that there was no statistically significant difference between the control and high-dose groups.

Table 5.8.1- 9: Mean blood chemistry parameters at termination

Parameter	Dose level (ppm)									
	Males					Females				
	0	50	180	600	2300	0	50	180	600	2300
Total protein (g/100 ml)	6.1	6.1	6.0	6.2	6.8**	6.2	6.2	6.1	6.2	6.6
Cholesterol (mg/100 ml)	97	87	108	116	131**	87	100	92	95	122**
Urea (mg/100 ml)	29	30	32	37	45**	36	37	40	39	47**

**p < 0.01; significantly different from control using the Wilcoxon test

Urinalysis:

There was no effect on urinalysis parameters at any dose-level.

4. Pathology

At 2300 ppm, the absolute weights of the thymus and heart (both sexes) and the brain (males only) were statistically significantly increased in comparison with controls, whilst the weight of the spleen in the females at this dose was lower than controls. However, there was no corresponding effect on the relative weight of these organs and the effects are therefore considered to be secondary to the large reduction in final body weight observed at this dose. Other changes in organ weight were either not statistically significant and/or did not show a clear dose-response. There was no clear treatment-related effect on the relative weight of any organ.

Table 5.8.1- 10: Absolute organ weights after administration of M-01 (BAM) to male and female rats for 13 weeks

Dose level (ppm)	Final Body weight	Total Testicle (g)	Total Ovary (mg)	Spleen (g)	Total Adrenal (mg)	Total Kidney (g)	Thymus (g)	Heart (g)	Brain (g)	Liver (g)
	Male									
Control	341	3.24	-	0.46	44.0	2.48	0.29	1.05	1.66	13.32
50	363	3.46***	-	0.51	48.0	2.65	0.32	1.07	1.66	14.10
180	342	3.26	-	0.49	48.5	2.63	0.29	1.05	1.61	13.91
600	322	3.20	-	0.49	50.1	2.86**	0.30	0.99	1.61	14.65
2300	280	3.20	-	0.42	42.0	2.48	0.21***	0.83***	1.54**	13.66
	Female									
Control	201	-	70.0	0.32	50.6	1.48	0.22	0.68	1.57	7.44
50	204	-	65.5	0.31	52.6	1.55	0.22	0.72	1.59	7.75
180	201	-	69.4	0.31	55.4	1.56	0.24	0.73	1.64	8.05
600	191	-	63.3	0.29	49.5	1.57	0.22	0.69	1.52	7.70
2300	178	-	65.1	0.28*	47.2	1.39	0.17***	0.62***	1.50	7.93

* = p<0.05; ** = p<0.02; *** = p<0.01

Table 5.8.1- 11: Relative organ weights after administration of M-01 (BAM) to male and female rats for 13 weeks

Dose level (ppm)	Final Body weight	Total Testicle (g)	Total Ovary (mg)	Spleen (g)	Total Adrenal (mg)	Total Kidney (g)	Thymus (g)	Heart (g)	Brain (g)	Liver (g)
	Male									
Control	341	0.95	-	0.14	13.0	0.73	0.08	0.31	0.49	3.90
50	363	0.96	-	0.14	13.2	0.73	0.09	0.30	0.46	3.89
180	342	0.96	-	0.14	14.2	0.77	0.09	0.31	0.47	4.07
600	322	1.00	-	0.15	15.5	0.89	0.09	0.30	0.50	4.58
2300	280	1.15	-	0.15	15.1	0.88	0.07	0.30	0.56	4.92
	Female									
Control	201	-	33.3	0.16	25.3	0.74	0.11	0.34	0.78	3.71
50	204	-	31.8	0.16	25.6	0.76	0.11	0.35	0.79	3.81
180	201	-	34.0	0.15	27.7	0.78	0.12	0.36	0.82	4.00
600	191	-	33.9	0.15	25.3	0.82	0.11	0.36	0.81	4.05
2300	178	-	36.4	0.16	26.6	0.77	0.09	0.35	0.84	4.44

There were no treatment-related gross necropsy or histopathology findings. Histopathological findings in the respiratory tract, liver and spleen occurred at similar incidences in the control and high-dose groups.

III. Conclusions

Dietary administration of M-01 (BAM) to male and female rats for 13-weeks had no effect on mortality. The only clinical sign of toxicity (hair loss) was confined to females, but a reduction in muscle tone was observed in both sexes from 600 ppm. Body-weight development was affected in females from 600 and in males at 2300 ppm. In females, overall body-weight gain was 18% and 38% lower than controls at 600 and 2300 ppm respectively, and food consumption was 7% and 17% lower than controls at the same respective doses. In males, a 30% reduction in body-weight gain at 2300 ppm was accompanied by lower food consumption in these animals (-16%) compared with controls. For the females at 2300 ppm, the food utilisation efficiency was reduced, indicating that in this instance the reduction in bodyweight may not be secondary to the reduced food consumption.

Haematology, clinical chemistry and urinalysis parameters remained largely unaffected by treatment with M-01 (BAM); at 12 weeks, a decrease in thrombotest-time was noted at 2300 ppm in males and from 600 ppm in females. With regard to clinical chemistry parameters, an increase in cholesterol (both sexes) and an increase in total protein (males) were observed at 6 and 12 weeks, however, the latter increase is reported to be within the normal ranges for the laboratory. A higher level of blood urea was noted from 6 weeks in females from 600 ppm and in males at 2300 ppm, although by 12 weeks the effect was only apparent in males (from 600 ppm). Additionally, there was a transient effect on SGPT levels in both sexes at the high dose. None of these effects are considered to be indicative of an adverse effect on renal or liver function (tissue damage), but rather an effect on the metabolic processes. This is supported by the lack of relevant histopathological findings, urinalysis findings and also the negative data in the majority of the clinical chemistry parameters measured (creatinine, BSP, SGOT, SGPT).

Therefore, a NOAEL of 180 ppm (equivalent to 14 mg/kg bw/d) is proposed from this study. At the LOAEL of 600 ppm (49 mg/kg bw/d) reductions in body-weight gain, food consumption and skeletal muscle tone were observed.

Assessment and conclusion by applicant:

Study was not conducted according to any particular guideline but broadly conforms OECD TG 408 (with the caveat of the deviations described above). The study is valid and acceptable to assess the short-term toxicity of M-01 in rats. A NOAEL of 180 ppm (equivalent to 14 mg/kg bw/d) was determined from this study.

Data Point:	KCA 5.8.1/46
Report Author:	[redacted]
Report Year:	1967
Report Title:	The study of the oral toxicity of the prefix residue 2,6-dichlorobenzamide: 13-week exposure to dogs
Report No:	M-311805-01-1
Document No:	M-311805-01-1
Guideline(s) followed in study:	not specified
Deviations from current test guideline:	Homogeneity/stability of the test substance in the diet not reported, no ophthalmoscopy conducted, individual bodyweights not reported, mean bodyweight not tabulated and evidence of parasitic infection (ascariasis) in examined tissues.
Previous evaluation:	yes, evaluated and accepted in the Addendum 2 to the DAR (2008)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary:

M-01 (BAM) was administered in the diet of 4/sex Beagle dogs at doses of 100, 300 and 2000 ppm (6/sex control animals received untreated diet). The test substance was administered via the diet on consecutive days for 1 weeks. Doses were based on a range finding study in which 2 animals received 100 mg/kg bw/d and 1 animal received 500 mg/kg bw/d of the test substance (administered orally via capsule).

Clinical signs comprising loss of condition, hair-loss and thin, dull and lifeless coat become apparent in high-dose males from week 4. Individual bodyweights were not reported and no tabulated findings from the group measurements were available; however, the study author reports that bodyweights were reduced at 2000 ppm in males from week 3 and in females from week 5.

Alkaline phosphatase was increased in females at 2000 ppm by 66% when compared with controls, but no other clinical chemistry markers that would indicate a dysfunction of the liver were affected.

Liver weights were increased in females at 200 ppm (35% absolute and 47% relative) and 300 ppm (27% absolute and 17% relative).

Histopathology revealed no treatment-related findings but did find evidence of a parasitic infection in the examined tissues, thus limiting the validity of the study.

The NOAEL therefore was 100 ppm, based on increased liver weights in females at the LOAEL of 300 ppm.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-01 (BAM) referred to as 2,6-dichlorobenzamide in the report
Purity:	97%
Batch no.:	133/2/4/104

2. Vehicle and/or positive control

Vehicle:	Acetone/Diet

3. Test animals

Species:	Dogs
Strain:	Beagle
Age:	3 to 4 months old (12-months for range-finding study)
Weight at start:	Not stated
Source:	[redacted]
Acclimation period:	Yes
Diet:	Ful-O-Pep dog food supplied by Quaker Oats Ltd, Southall, Middlesex
Water:	Water *ad libitum*
Housing:	Housed individually
Temperature:	18 °C
Humidity:	Not provided
Air changes:	Not provided
Photoperiod:	Not provided

B. Study design

1. In-life dates: Not provided

2. Animal assignment and treatment

The animals were randomized and assigned to the following test groups:

Table 5.8.1- 12: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	6	6
2	100	4	4
3	300	4	4
4	2000	4	4

Animals were dosed on consecutive days of 13-weeks.

Doses were based on the results of a range-finding study in which two groups of male dogs were administered either 500 mg/kg bw/d (2 dogs) or 100 mg/kg bw/d (1 dog) orally via capsule. The dogs were then observed for 72-hours following dosing. Both high-dose dogs showed a lack of co-ordination in gait within one hour of dosing, progressing to an inability to rise within 2-hours. Both animals had recovered by 24-hours post-dose. No effects were seen at the low-dose and no unusual findings were revealed upon necropsy.

3. Dose preparation and analysis

The homogeneity and stability of the test substance in the diet was not reported; the actual concentration of the test-substance in the diet (ppm) was checked weekly and no loss of test substance was detected.

Diets for the treated groups were prepared from concentrates after pre-dissolving the test-substance in acetone. The concentrates were mixed to apparent homogeneity in a mixer and the solvent allowed to evaporate. Control diet was prepared in the same manner with an equal quantity of acetone.

Diets were freshly prepared every four weeks.

4. Statistics

Statistical analyses of terminal body weight and organ weights were performed using initial bodyweight as a covariate in covariance analysis, whilst haematology and clinical chemistry measurements were examined using analysis of variance.

C. Methods

1. Observations

All animals were observed for clinical signs of toxicity and unusual behaviour.

2. Body weight and food intake

Food consumption was measured daily throughout the exposure period and bodyweights were recorded for each dog.

3. Ophthalmoscopy

Not conducted

4. Laboratory investigations (haematology, clinical chemistry and urinalysis)

Blood samples were taken prior to dosing and then again during weeks 6 and 13. Haematological examinations included, haemaglobin, packed cell volume, erythrocyte, leucocyte and differential leucocyte counts. Clinical chemistry tests comprised bromosulphthalein clearance, serum protein, urea, glutamic pyruvic transaminase activity and plasma alkaline phosphatase activity (liver function tests were performed on control and high-dose animals only).

Overnight urine was collected during week 12 of exposure using special metabolism cages.

5. Sacrifice and pathology

All surviving animals were sacrificed at the end of the 13-week treatment period (over a 5-day period) by sodium phenobarbitone injection, and subject to a gross necropsy. The brain, heart, liver, kidneys and testes were weighed and the following tissues subject microscopic examination: Brain, spinal cord, thyroid, parathyroid, heart, lungs, spleen, liver, kidneys, stomach, small and large intestines, lymph nodes, bone marrow, pancreas, eyes, testes, prostate, ovaries, fallopian tubes, uterus, urinary bladder and skeletal muscle.

II. Results and Discussion

A. Results

1. Clinical results

There were no deaths and clinical signs were confined to high-dose males with loss of condition, hair-loss and thin, dull and lifeless coat becoming apparent from week 4.

2. Body weight and food intake

Body weight

Bodyweight development was reportedly affected at the high-dose of 2000 ppm in both sexes, becoming apparent from week 3 in males and week 5 in females. Body weight was not affected at the lower doses and food consumption was not affected at any dose. Individual bodyweights were not reported, and mean bodyweights were not tabulated in the report (only a graph was included to show the bodyweight changes).

Food consumption

Food consumption was measured but not reported.

3. Laboratory investigations

Haematology:

Packed cell volume was decreased in high-dose females throughout the 13-week treatment period. There were no treatment related effects in females at the lower doses or in males at any dose. An increase in haemaglobin in high-dose males, observed at week 13 of treatment was due to an erroneous result from one animal and therefore not related to treatment with M-01 (BAM).

Clinical chemistry:

There were no treatment-related effects on clinical chemistry parameters in males at any dose. In females at 2000 ppm alkaline phosphatase was increased in comparison with controls by 65%, whilst liver function tests measuring bromosulphthalein did not show any treatment-related changes (see tables below).

Table 5.8.1-13: Selected clinical chemistry findings from the 13-week dog study

Parameter	Dose (ppm)			
	0	100	300	2000
		Males		
GPTA (I/U)	8	9	7	7
ALP (I/U)	91	112	96	99
		Females		
GPTA (I/U)	8	8	7	6
ALP (I/U)	82	102	111	136** (+66%)

Table 5.8.1- 14: Mean results of the Bromsulphphthalein (BSP) liver clearance test

Dose (ppm)	No. animals	Time for BSP plasma concentration to be halved (mins)
Males		
0 (Control)	6	2.3 ± 0.13
2000	4	2.2 ± 0.16
Females		
0 (Control)	6	2.1 ± 0.14
2000	4	2.0 ± 0.18

Urinalysis

Urinalysis revealed no unusual findings.

4. Sacrifice and pathology

Necropsy:

No treatment-related macroscopic abnormalities were seen.

Organ weights:

In females at 2000 ppm absolute and relative liver weights were increased in comparison to controls by 35% and 47% respectively, liver weights in males at this dose were also increased by 12% (absolute) and 18% (relative), although the change was not statistically significant.

In females of the 300-ppm dose group absolute and relative liver weights were increased by 27% and 17% respectively. Left kidney weights of females at 300 ppm were also increased (+24%); however, the there was no evidence of a dose-response. There were no statistically significant effects on organ weights in males at any dose (see table 5.8.1-15 below).

Table 5.8.1- 15: Selected organ weights from the 90-day dog study *(% difference to control)*

Parameter	Dose level (ppm)					
	0	100		300	2,000	
Males						
Terminal BW (kg)	10.5	10.6	-	10.5	9.9*	*-6%*
Liver weight (g)	416	375	*-10%*	434	465	*+12%*
Relative liver weight (% of body weight)	4.0	3.5	*-13%*	4.1	4.7	*+18%*
Right/left kidney weight	28.5/28	24.7/25.5	*-13%/-9%*	27.6/26.5	23.2/23.2[1]	*-19%/-17%*
Females						
Terminal BW (kg)	9.7	10.6	*+9%*	10.5	8.9*	*-8%*
Liver weight (g)	328	326	-	*417**	442**	*+35%*
Relative liver weight (% of body weight)	3.4	3.0	*-12%*	4.0	5.0	*+47%*
Right/left kidney weight	21.8/22.2	21.4/21.6	-	26.3/27.5*	*+22%/+24%*	24/24.7	*+12%/+11%*

* p< 0.05, ** p<0301, significantly different from control, [1]mean of 3 animals

BW: Body weight

5. Histopathology

No treatment-related effects were observed on microscopic examination. Autopsy revealed the presence of an *ascarid* infection. Findings thought to be evidential of this parasitic infection comprised granulomas of the lungs, liver and kidneys (not dose-related), interstitial nephritis and pneumonitis. Because of these findings the acceptability of the study to inform on the risk assessment is reduced.

III. Conclusion

There were no deaths during the study and clinical signs (thin appearance, dull coat and hair loss) were confined to males and females of the high dose. Bodyweight gain at this dose was lower than controls in both sexes, whilst liver weights and alkaline phosphatase were increased in females. Liver weights in females were also increased at 300 ppm.

Therefore, the NOAEL in the 90-day dietary study in dogs was 100 ppm (equivalent to 7.5 mg/kg bw/d). At the LOAEL of 300 ppm, absolute and relative liver weights were increased in comparison to controls by 27% and 17% respectively.

Assessment and conclusion by applicant:

The study was not conducted according to a particular guideline but broadly conforms to OECD TG 409 (with the deviations described above). The study is acceptable as supportive information for the repeated-dose toxicity of M-01 in dogs, owing to the presence of a parasitic infection in most tissues. A NOAEL of 100 ppm (equivalent to 7.5 mg/kg bw/d) was determined.

Data Point:	KCA 5.8.1/47
Report Author:	[redacted]
Report Year:	1971
Report Title:	Toxicity studies on the Prefix residue 2,6-dichlorobenzamide: two year oral experiment with dogs
Report No:	TLGR.0028.71
Document No:	M-301179-01-1
Guideline(s) followed in study:	US-EPA-FIFRA Guideline 83-1
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted Addendum 2 to the DAR (2008)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The test substance, M-01 (BAM, referred to as 2,6-dichlorobenzamide in the report) was administered in the diet of Beagle dogs for 2 years at levels of 0, 60, 100, 180, and 500 ppm (equivalent to 0, 1.5, 2.5, 4.5 and 12.5 mg/kg bw/d). Animals were regularly examined for clinical signs of toxicity and effects on bodyweight development. Blood samples were taken at 3 monthly intervals and haematological and clinical chemistry parameters measured. At sacrifice a range of organs were weighed and the tissues examined microscopically.

There were no deaths or treatment-related clinical signs of toxicity. Bodyweight development in females at the top dose (500 ppm) was affected, with bodyweight gain being lower than controls throughout the study; however, two control females and one female at 60 ppm were exceptionally large animals, which would have contributed to this difference. Males at 500 ppm also presented with lower body weight gains and this trend, although not statistically significant, was present throughout the study.

Relative liver weights were increased in males at 500 ppm, whilst treated females had reductions in absolute and relative liver and kidney weights. However, these reductions were not dose-related and the organ weights of the two larger control females were greater than the normal range. Therefore, taking this into consideration, along with the small number of animals in the control group, the apparent organ weight reductions were considered to be secondary to the larger control group mean weights and not to treatment with M-01 (BAM).

Gross and microscopic examination of the tissues showed no treatment-related changes and the haematology and clinical chemistry measurements taken throughout the study, showed only slight, occasional changes, none of which was considered to be related to treatment with M-01.

A NOAEL of 180 ppm (equivalent to 4.5 mg/kg bw/d in M/F) was therefore derived from this study based on reductions in bodyweight gain in females and increased liver weights in males at the LOAEL of 500 ppm (equivalent to 12.5 mg/kg bw/d in M/F), which was the highest dose tested.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-01 (BAM, referred to as 2,6-dichlorobenzamide in the report)
Purity:	97%
Batch no.:	Batch 133-2-4-104

2. Vehicle and/or positive control

Vehicle:	Solution of compound in a minimum quantity of acetone mixed into the diet

3. Test animals

Species:	Dogs
Strain:	Beagle
Age:	5 months old at receipt
Weight at start:	Not provided
Source:	[redacted]
Acclimation period:	Not provided
Diet:	Powdered laboratory diet ('Ful-O-Pep' after 22 weeks changed to ground 'Laboratory diet A' (Cooper Nutrition Products, Witham, Essex)
Water:	Tap water *ad libitum*
Housing:	Housed individually
Temperature:	21±2 °C
Humidity:	50±5%
Air changes:	Not provided
Photoperiod:	Not provided

B. Study design

1. In-life dates: Not provided

2. Animal assignment and treatment

The dogs were randomized and assigned to the following test groups.

Table 5.8.1- 16: Study design

Group	Dose (ppm)	Number of males	Number of females
1	0	4	4
2	60	4	4
3	100	4	4
4	180	4	4
5	500	4	4

Each animal received 400 g/day diet moistened with an equal weight of water, increasing to 600 g/day after 12 months. The dry diet contained either 0, 60, 100, 180 or 500 ppm M-01 (BAM) according to the animals assigned treatment group (see table 5.5.1/06 above). The administered doses equated to 0, 1.5, 2.5, 4.5 and 12.5 mg/kg bw/d. Water was provided *ad libitum*.

3. Diet preparation and analysis

A 5000-ppm concentrate was prepared by mixing a solution of the compound (in a minimal quantity of acetone) with the appropriate amount of powered dog food. The other dietary concentrations were prepared directly from this concentrate. The control diet was mixed with a quantity of acetone which equated to the amount used in the 6000-ppm preparation.

4. Statistics

Statistical analyses of body and organ weights were made using the initial bodyweight as a covariate in covariance analysis. Haematology and clinical chemistry values were examined using analysis of variance. The significance of any difference between the treatment and control groups of animals was measured using the Student's T test.

C. Methods

1. Observations

The animals were examined daily for clinical signs of toxicity and body-weights were recorded weekly. Blood samples were taken for analysis prior to the commencement of the study and then at three monthly intervals thereafter. At the end of the study the animals were sacrificed, the organs weighed, and the tissues examined microscopically.

2. Laboratory investigations (Haematology, clinical chemistry)

The following haematological and clinical chemistry parameters were measured.

Haematology

Haemoglobin content, heamatocrit, erythrocyte, leucocyte and differential leucocyte counts, prothrombin time and kaolin-cephalin clotting time.

Clinical chemistry

Bromosulphthalein clearance, serum protein, urea, glutamic pyruvic transaminase activity and plasma alkaline phosphatase activity. Serum protein fractions were determined by paper electrophoresis using LKB electrophoresis equipment (LKB buffer pH 8.6 μ = 0.1).

No urinalysis was conducted in this study.

3. Pathology

All dogs were sacrificed and examined macroscopically; the visceral organs were weighed, and a wide selection of tissues were examined microscopically (see table 5.8.1-17).

Table 5.8.1- 17: Organs weighed, and tissues examined microscopically in the 2-year dog study

Organs weighed	Tissues examined microscopically	
Brain	CNS	Thyroid
Heart	Heart	Parathyroid
Liver	Liver	Lungs
Spleen	Spleen	Spleen
Kidneys	Kidneys	Adrenals
Testes	Gonads	Small intestine
	Stomach	Large intestine
	Pancreas	Salivary glands
	Lymphocytes	Bladder
	Prostate	Uterus

II. Results and discussion

1. Clinical results

There were no deaths or treatment-related clinical signs of toxicity; the general health and behaviour of the dogs was similar across the treated and control groups.

2. Body weight and food and water intake

Body weights in high-dose females (500 ppm) were decreased throughout the study in comparison with controls; however, two control and one 60 ppm female were exceptionally large animals which could, at least in part, account for this difference. The body weights of males in the high-dose-group were also lower than controls throughout the study, albeit without statistical significance (see table 5.5.1-11 below).

Table 5.8.1-18: Selected body weight changes in the two-year dog study

Week	Males					Females				
	Dose (ppm)									
	0	60	100	180	500	0	60	100	180	500
	Weight (kg) and % difference from control									
0	10.7	10.5	10.7	10.9	10.8	9.8	10.4	9.9	9.9	10.0
28	13.5 -	13.1 -3%	12.3 -9%	12.9 -4%	12.0 -11%	12.5 -	12.6 -	12.4 -	12.0 -4%	10.6** -15%
54	14.4 -	14.4 -	13.6 -6%	13.3 -8%	12.4 -14%	13.7 -	13.6 -	12.6 -8%	12.4 -9%	10.8** -21%
80	15.2 -	15.0 -	14.4 -5%	14.0 -8%	13.0 14%	14.4 -	14.4 -	13.2 -8%	12.8 11%	11.3** -22%
104	15.5 -	15.5 -	15.1 -	14.3 -6%	13.6 -12%	15.0 -	15.0 -	13.7 -9%	13.4 -11%	11.5** -23%

3. Laboratory investigations

There were no treatment-related effects on haematological or clinical chemistry parameters at any time-point during the study; only sporadic differences were observed, which were slight, not statistically significant and showed no dose-response. An increase in gamma-globulin in females was present throughout the study but can be related to pre-treatment differences and therefore not a consequence of treatment with M-01 (BAM). See table 5.8.1-19 below.

Table 5.8.1- 19: Clinical chemistry findings in the 2-year dog study

Parameter	Males					Females				
	Dose (ppm)									
	0	60	100	180	500	0	60	100	180	500
Protein g/100 mL	6.1	6.1	6.1	6.3	6.3	6.3	6	6.3	6.4	6.1
Urea mg/100 mL	22	29	26	25	32	26	31	30	26	33
ALP i.u	51	25	34	40	49	62	37	46	72	51
GPTA i.u	16.3	17.8	19.3	15.5	17	18	11.3	15.8	13.7	12.8
Cholesterol mg/100 mL	121	116	135	129	126	144	133	151	143	131
Glucose mg/100 mL	75	76	87	78	84	80	85	85	84	83

ALP = alkaline phosphatase, GPT=glutamic pyruvic transaminase activity

4. Pathology

The relative liver weights of high-dose males were increased in comparison with controls (+25%), whilst the absolute and relative liver and kidney weights were decreased in females in all treatment groups (see table 5.8.1-20), but no dose-response was evident and there were no accompanying histopathological findings. The reductions in organ weights seen in the female dogs were attributed to the high organ weights of the two control females with unusually high bodyweights, the liver weights of which were outside the normal range for female beagle dogs. Therefore, owing to this, the lack of histopathology and the small number of animals in the control groups the organ weight changes in females were not considered as related to treatment with M-01 (BAM).

Table 5.8.1- 20: Selected organ weight changes (g) in the 2-year dog study

Organ		Dose level (ppm)									
		Males					Females				
		0	60	100	180	500	0	60	100	180	500
Terminal BW		15.5	15.5	15.1	14.5	13.6	15.0	15.0	13.7	13.4	11.5**
Liver	Abs.	499	531	493	443	544	621	434**	407**	436**	405**
	Rel.	3.27	3.44	3.274	3.04	4.01*	4.14	2.93**	2.99**	3.24**	3.49**
Kidneys	Abs.	69.6	75.3	78.9	70.1	67.0	73.1	62.6	57.4*	58.4*	49.8**
	Rel.	0.460	0.486	0.523	0.477	0.487	0.483	0.427	0.422	0.437	0.433

*p=<0.05, p=<0.01

There were no unusual gross necropsy or microscopic findings that could be related to the dietary administration of M-01 (BAM). In particular, there were no unusual findings that would explain the apparent weight changes in the liver or kidneys.

III. Conclusions

The dietary administration of M-01 (BAM) for 2 years to Beagle dogs resulted in an overall lower bodyweight in high-dose (500-ppm) females of -23% compared with controls and increased relative liver weights in high-dose (500-ppm) males (+25%).

A NOAEL of 180 ppm (equivalent to 4.5 mg/kg bw/d in males and females respectively) is proposed based on body weight reductions in females and increased liver weights in males at the LOAEL of 500 ppm (equivalent to 12.5 mg/kg bw/d in males and females respectively).

Assessment and conclusion by applicant:

The study was not conducted according to any particular guideline but broadly conforms to OECD TG 452 and is valid and acceptable to investigate the short-term repeated exposure of M-01 in the dog. A NOAEL of 180 ppm was determined from this study.

Genotoxicity

Data Point:	KCA 5.8.1/03
Report Author:	[redacted]
Report Year:	1992
Report Title:	Evaluation of possible mutagenic activity of 2,6-dichlorobenzamide in the Ames Salmonella/Microsome Test
Report No:	C040455
Document No:	M-228925-01-1
Guideline(s) followed in study:	OECD 471 (1983); Directive 84/449/EEC. Annex V, B.14. (1984); US-EPA (FIFRA) 84-2
Deviations from current test guideline:	No strain for detecting cross-linking mutagens was included (TA 102 or E. coli WP2 strains)
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The genotoxic potential of M-01 (BAM) was investigated in S. *typhimurium* strains TA 1535, TA 1537, TA 1538, TA 98, and TA 100. Four concentrations of the test substance were tested in triplicate for each strain.

A positive response in the assay system is considered to be a concentration-related, reproducible increase above background (≥ 2-fold) in the mean number of revertant colonies; the response should also be greater than the laboratory's historical data for the solvent control.

Strain TA 1538 showed an increased spontaneous mutant frequency (92-253) in the first mutation assay and so was excluded from the experiments.

No toxicity or precipitation of the test substance was observed during the preliminary study. Therefore, the limit concentration of 5000 µg/plate was selected as the highest concentration to be used in the mutation assays (in the presence and absence of S9-mix).

The following concentrations were therefore used in the main mutation studies: 625, 1,250, 2,500 and 5,000 µg/plate, both in the presence and absence of S9-mix. No increase in revertant colony counts was observed in either experiment in any of the four tester strains, either in the presence or absence of metabolic activation.

The test substance M-01 (BAM) was not mutagenic in the tested strains, (either in the presence or absence of metabolic activation) under the conditions of this *in vitro* gene mutation assay (Ames test) up to the highest concentration tested (5000 µg/plate).

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-01 (BAM, referred to 2,6-dichlorobenzamide in the report)
Purity:	100.0 %
Batch no.:	FUX001000/FUN31G02C
Expiry date:	December 1997

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: Sodium azide: TA 100, TA 1535 4-nitro-o-phenylene diamine : TA 98, TA 1537 +S9 : 2-Amino-anthracene: TA 98, TA 100, TA 1535, TA 1537

3. Activation:

Metabolic activation was provided by S9, obtained from the liver homogenate of six male Wistar rats. The rats had been previously administered Aroclor 1254 in order to stimulate liver enzyme activity. The S9 mix was supplied by CIVO/TNO, Zeist on 12th September 1990 and contained 0.89 µmol cytochrome P450 per gram of protein.

4. Test organisms:

The following test organisms were used in the study (obtained from Berkeley University, California, 1980, 1981 and 1992):

Table 5.8.1- 21: Strains used in the Ames test with M-01 (BAM)

Strain	Gene affected	Mutation type detected	Additional mutations		
			Repair*	LPS**	R Factor***
TA 1535	His G 46	Base-pair substitution	uvr B	rfa	-
TA 1537	His C 3076	Frameshift	uvr B	rfa	-
TA 98	His D 3052	Frameshift	uvr B	rfa	pKM101
TA 100	His G 46	Base-pair substitution	uvr B	rfa	pKM101

* mutation uvr B: Enhances sensitivity to chemically/physically damaged DNA to enhance the strains sensitivity to mutagens.

** mutation rfa: ↑ permeability and leads to defective lipopolysaccharide coat (covers genes involved in biotin synthesis and UV induced DNA damage repair.

*** resistant transfer factor plasmid (R factor): ↑ in error prone DNA repair leading to more mutation/dose of a mutagen and confers antibiotic resistance (ampicillin), marker to detect the presence of plasmid in the cells.

No strains for the detection of cross-linking mutagens (TA 102 or E. coli WP2U strains) were included. Strain TA 1538 caused an unacceptable level of spontaneous mutations and so was excluded from the study.

5. Test compound concentrations used:

A preliminary toxicity test was conducted with the strain TA 100, in the presence and absence of S9-mix. The basis for the selection of the highest concentrations from the toxicity study was either cytotoxicity or precipitation. If no cytotoxicity was observed and solubility was good, then the limit concentration of 5 mg/plate would be selected as the highest concentration.

B. Test Performance

Experimental phase: 5th November to 20th November 1992

1. Assay procedure

Two independent experiments were conducted; each experiment tested four concentrations with and without S9 mix. For each experiment, tubes containing 2 ml histidine-deficient agar (top agar) supplemented with a histidine/biotin mixture (to support initial growth) were maintained at approximately 52°C. Subsequently, 0.1 ml of an overnight growth culture of each of the four strains, 0.1 mL of a solution of test material in DMSO and either 0.5 mL of the S9-mix or 0.5 mL cofactor mix were added to the tubes. The components were mixed and immediately poured onto a selective agar plate. After solidification of the agar, the plates were turned upside down and incubated in the dark at 37°C for 72 hours.

All solutions were regularly checked for sterility and each culture was examined for the number of spontaneous revertants. The viable count of each culture was determined by plating appropriate dilutions of the cultures on agar plates, whilst revertant colonies were counted automatically with an Artek 880 colony counter. Cytotoxicity was determined by reduced background growth and/or a decreased number of colonies.

2. Statistics

No details of the statistical analyses were provided.

3. Evaluation of results, criteria for a positive response

A response was considered positive in this assay if a reproducible, dose-related increase in the mean number of revertant colonies was observed (2-fold or greater above the background spontaneous reversion rate of the solvent control). The response should also be greater than the laboratories historical control data for that particular solvent control.

According the current test guideline (OECD 471, 1997), a positive response is defined by a concentration-related increase and/or a reproducible increase in the number of revertant colonies/plate in at least one strain with or without metabolic activation.

II. Results and discussion

A. Range-finding assay

Based on the results of the range-finding assay (conducted on strain TA 100), concentrations of 625, 1250, 2500 and 5000 µg/plate were selected for the main assays in the presence of absence of S-9 mix.

Table 5.8.1- 22: Number of revertant colonies in the range-finding study

Concentration of M-01 (BAM)	Number of revertants (duplicate plates)	
	Without S9-mix	With S9-mix
0	85, 87	86, 82
100	80, 87	87, 102
312.5	67, 99	116, 103
625	81, 78	96, 100
1250	77, 100	100, 85
2500	88, 101	74, 86
5000	87, 113	87, 92

No toxicity or precipitation was observed at any concentration; therefore, 5000 µg/plate was selected as the highest concentration in the mutation assay (with and without S9-mix).

B. Mutation assays

The mean number of revertant colonies obtained in experiment 1 and 2 are shown in table 5.8.1-23 and table 5.8.1-24. No increases in revertant colony counts were observed in any strain, in either the presence or absence of S9-mix. The positive controls gave the expected increase. Strain TA 1538 showed an increase in spontaneous mutant frequency and so was excluded from the experiments; the remaining strains showed acceptable levels of spontaneous mutant frequency.

Table 5.8.1- 23: Mean revertant colony counts obtained in experiment 1

Concentration (µg/plate)	TA 98	TA 100	TA 1535	TA 1537
Without S9-mix				
0	28±9	89±2	20±1	9±3
625	31±4	88±12	21±6	7±1
1250	26±10	90±7	17±3	9±3
2500	29±2	107±6	17±4	12±6
5000	23±6	106±7	26±7	7±0
Positive control	608±168	544±114	430±78	223±20
With S9-mix				
0	31±5	88±14	14±5	9±1
625	28±6	83±10	15±4	12±4
1250	30±9	106±15	14±8	7±2
2500	29±9	94±13	10±2	11±5
5000	27±8	85±13	12±3	12±2
Positive control	974±39	1159±43	681±1	916±2

Table 5.8.1- 24: Mean revertant colony counts obtained in experiment 2

Concentration (µg/plate)	TA 98	TA 100	TA 1535	TA 1537
Without S9-mix				
0	35±2	168±27	31±5	8±2
625	33±4	142±20	27±9	7±3
1250	21±4	142±10	22±3	8±2
2500	41±11	161±10	35±3	6±3
5000	37±4	146±14	26±6	6±4
Positive control	408±33	518±28	501±159	161±41
With S9-mix				
0	39±15	162±21	26±5	9±2
625	32±3	200±7	26±8	5±2
1250	41±6	217±2	20±1	6±3
2500	35±6	177±16	21±2	6±3
5000	29±5	157±11	23±6	9±4
Positive control	673±52	1072±90	102±34	107±25

III. Conclusions

Under the conditions of this Ames test, the test material M-01 (BAM) showed no evidence of mutagenic potential when tested up to 5000 µg/plate either in the presence or absence of metabolic activation (S9).

Assessment and conclusion by applicant:

Study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of M-01 in *S. typhimurium* strains. M-01 was not mutagenic in bacterial cells when tested up to the limit concentration under the conditions of this study (+/- S9).

Data Point:	KCA 5.8.1/05
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C653711 - Salmonella/microsome test - Plate incorporation and preincubation method
Report No:	AT00853
Document No:	M-225471-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC, B.13/14 (2000); OECD 471 (1997); US-EPA 712-C-98-247, OPPTS 870.5100 (1998)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

M-01 (BAM, referred to as AE C653711 in the report) was initially investigated using the Salmonella/microsome plate incorporation test for point mutagenic effects. Five Salmonella typhimurium LT2 mutants were tested (histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102) at concentrations up to and including 5000 µg per plate. An independent repeat was performed using the preincubation method, with a 20-minute incubation at 37°C. |All other conditions remained unchanged.

Doses up to and including 50 µg per plate did not cause any cytotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed.

At higher doses, the substance induced a weak, strain-specific cytotoxic effect in the plate incorporation assay (no cytotoxicity was noted in the pre-incubation assay). Owing to the weak nature of the effect, all tested concentrations were used for assessment purposes.

No evidence of mutagenicity was seen; no biologically relevant increases in mutant counts were observed when compared with the negative controls.

The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C, cumene hydroperoxide and 2-aminoanthracene induced a marked mutagenic effect, as seen by a biologically relevant increase in mutant colonies when compared with the corresponding negative controls.

Overall, the test substance M-01 (BAM) was not mutagenic either in the presence or absence of metabolic activation, when investigated using plate incorporation and preincubation methods in this Ames test.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-01 (BAM, 2,6-dichlorobenzamide, referred to as AEC653711 in the report)
Purity:	92.2%
Batch no.:	0801SET
Expiry date:	9th October 2004

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive controls:	Without S9-mix Sodium azide (TA 1535) Nitrofurantoin (TA 100) 4-Nitro-1,2-phenylene diamine (TA1537and TA98) Mitomycin C (TA 102 plate incorporation) Cumene hydroperoxide (TA 102 in pre-incubation) With S9-mix 2-Aminoanthracene (all strains)

3. Activation:

Metabolic activation was provided by S9-mix, obtained from the liver homogenates of at least six adult male Sprague Dawley rats, stimulated by a single intraperitoneal injection of Aroclor 1254.

The S9-mix was freshly prepared on the day of use and comprised the S9 fraction with 70 ml of co-factors (composed of $MgCl_2x6H_20$ 162.6mg, KCl 246.0 mg, glucose-6-phosphate, disodium salt 179.1 mg, NADP disodium salt 315.0 mg and phosphate buffer 100.0mM).

4. Test organisms:

S. *typhimurium* strains TA 1535, TA 1537, TA 100, TA 98 and TA 102 were used in the study. Using these particular strains enables the detection of both types of point mutation (base-pair substitutions and frameshift mutations), including cross linking mutagens, which are detected by the strain TA 102. All strains used concur with those recommended in the current test guideline (OECD 471; 1997).

5. Test compound concentrations used:

Concentrations from 0 (solvent control) to 5000 µg/plate were tested in the plate incorporation experiment with and without S9-mix, whilst the same concentrations were tested in the pre-incubation assay without S9-mix.

B. Test Performance

Experimental phase: 21st November to 4th December 2003

1. Assay procedure

Both plate incorporation and pre-incubation methods were carried out. For the initial plate-incorporation test, each concentration, solvent control, and positive control were tested in triplicate. The solvent (DMSO) constituted 0.1 mL/plate of each tested concentration and solvent control. This first experiment was designated a pre-test for toxicity and would determine the concentrations used in the subsequent pre-incubation test (by evaluation of solubility and/or cytotoxicity); however, the plate-incorporation test would be considered as experiment 1 in the event of a negative response.

Experiment 2 was performed as a pre-incubation in a water bath at 37°C, following the pre-incubation period (20 minutes) 2 ml of soft molten agar was added to the tubes and the contents mixed and plated. Each concentration, solvent control and negative control were tested in triplicate.

Cytotoxicity was determined by reduction in background lawn, a marked and dose-dependent reduction in the mutant count/plate and/or determination of the titer. Total bacterial counts were taken from 2 plates/concentration with S9-mix (experiment 1) or without S9-mix (without S9-mix).

2. Statistics

No details of the statistical analyses are provided.

3. Acceptance / assessment criteria:

An assay was deemed acceptable if the negative and positive controls gave the expected results and the density of the bacteria in the suspension was sufficient.

4. Evaluation of results, criteria for a positive response

A reproducible and dose-related increase in mutant counts (in at least one strain) is considered to be a positive result. The increase should be approximately twice that of negative controls for strains TA 1535, TA 100 and TA98 and at least a threefold for strain TA 1537; an increase of approximately 100 mutants is required for strain TA 102.

II. Results and discussion

A. Mutation assays

In the plate-incorporation assay, no cytotoxicity was noted up to and including 50 µg/plate; mean colony counts amongst treated groups were similar to those of controls and no growth inhibition was noted. At the higher doses up to 5000 µg/plate, only a weak, strain-specific cytotoxic effect was noted. No cytotoxicity was noted under pre-incubation conditions up to and including the highest dose tested of 5000 µg/plate

No dose-related or biologically relevant increase in mutant counts, over those observed for the negative controls, were observed in the plate incorporation test, either with or without S9-mix (see table 5.8.1-25). These results were confirmed by the pre-incubation test (see table 5.8.1-26).

Table 5.8.1- 25: Revertant colony counts/plate in the plate incorporation assay

Treatment	Concentration (µg/plate)	Metabolic activation +/- S9	Mean revertant colony counts in strains				
			TA 98	TA 100	TA 1535	TA 1537	TA 102
M-01	5000	-	13	138	21	6	196
	1581	-	14	158	18	7	186
	500	-	14	144	14	6	215
	158	-	19	145	17	6	215
	50	-	14	130	17	6	237
	16	-	15	129	17	7	226
Solvent control	0	-	14	131	17	8	226
M-01	5000	+	31	165	9	11	277
	1581	+	28	163	9	10	268
	500	+	28	153	9	10	261
	158	+	31	149	8	8	277
	50	+	26	153	9	10	241
	16	+	31	159	11	9	262
Solvent control	0	+	24	136	10	9	267
Sodium azide	10	-	NA	NA	645	NA	NA
4-NPDA	0.5-10*	-	143	NA	NA	85	NA
2-nitrofluorene	0.2	-	NA	331	NA	NA	NA
MMC	0.2	-	NA	NA	NA	NA	531
2-aminoanthracene	3	+	1080	1385	210	379	509

NA: not applicable

MMC: Mitomycin C

* 4-NPDA: 4-Nitro-1,2-phenylene diamine - 0.5 µg/plate for TA 98 and 10 µg/plate for TA 1537

Table 5.8.1- 26: Revertant colony counts/plate in the pre-incubation assay

Treatment	Concentration (µg/tube)	Metabolic activation +/- S9	Mean revertant colony counts in strains				
			TA 98	TA 100	TA 1535	TA 1537	TA 102
M-01	5000	-	19	130	14	5	214
	1581	-	24	147	11	9	239
	500	-	25	147	12	4	248
	158	-	22	144	14	9	245
	50	-	21	154	12	6	263
	16	-	26	153	13	6	256
Solvent control	0	-	18	161	11	8	253
M-01	5000	+	42	195	9	13	259
	1581	+	39	210	9	10	278
	500	+	32	197	9	10	299
	158	+	36	179	8	10	275
	50	+	37	184	7	10	291
	16	+	30	182	9	8	248
Solvent control	0	+	34	187	9	12	278
Sodium azide	10	-	NA	NA	674	NA	NA
4-NPDA	0.5-10*	-	147	NA	NA	126	NA
Nitrofurantoin	0.2	-	NA	514	NA	NA	NA
Cumene hydroperoxide	50	-	NA	NA	NA	NA	526
2-aminoanthracene	3	+	1164	1421	165	291	425

P: precipitation
NA: not applicable
4-NPDA: 4-Nitro-1,2-phenylene diamine - 0.5 µg/plate for TA 98 and 10 µg/plate for TA 1537

III. Conclusion

Under the conditions of this Ames test (using pre-incubation and plate incorporation methods), the test material M-01 (BAM) showed no evidence of mutagenic potential, when tested up to 5000 µg/plate, either in the presence or absence of metabolic activation (provided by S9-mix).

Assessment and conclusion by applicant:

Study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of M-01 in S-typhimurium strains. M-01 was not mutagenic in bacterial cells when tested up to the limit concentration under the conditions of this study (+/- S9).

Data Point:	KCA 5.8.1/06
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C653711 (metabolite of AE C638206) - V79/HPRT-test in vitro for the detection of induced forward mutations
Report No:	AT00610
Document No:	M-218535-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC, B.17 (2000); OECD 476 (1997); US-EPA, 712-C-98-221, OPPTS 870.5300 (1998)
Deviations from current test guideline:	No historical control data was available, and no trend test was performed
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

M-01 (BAM, referred to as AE C653711 in the report) was evaluated for point mutagenic effects at the hypoxanthine-guanine phosphoribosyl transferase locus (forward mutation assay) in V79 cell cultures after treatment with concentrations of up to and including 5000 µg/ml, both with and without S9-mix.

The test substance induced no decreases in survival to treatment nor in relative population growth, either in the presence or absence of S9-mix. Precipitation of the test substance in the culture medium was observed from 3000 µg/ml.

No biologically relevant increase in mutant frequency above that of the vehicle controls was observed, in either the presence or absence of metabolic activation.

The positive controls (ethyl methanesulphonate and dimethylbenzanthracene) induced clear mutagenic effects, thus demonstrating the sensitivity of the test system and the activity of the S9-mix.

The test substance M-01 (BAM) is considered to be non-mutagenic under the conditions of this V79/HPRT forward mutation assay, both in the presence and absence of metabolic activation.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-01 (BAM, 2,6-dichlorobenzamide, referred to as AEC653711 in the report)
Purity:	96.2 % (w/w)
Batch no.:	08018 ET
Expiry date:	9th October 2004

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive controls:	-S9 Ethyl methane sulfonate (EMS), 900 µg/ml +S9 9,10-dimethyl-1,2-benzanthracene (DMBA), 20 µg/ml

3. Activation:

Metabolic activation was provided by S9-mix; S9-fraction was isolated from the liver homogenate of Aroclor 1254 induced male Sprague Dawley rats. The S9-mix was prepared the day of use and stored on ice. Two parts of the S-9 fraction was mixed with three parts of a co-factor solution (co-factors dissolved in 150 mM pH 7.4 sodium phosphate buffer), giving rise to an S9-mix comprised of the following concentrations:

8 mM $MgCl_2$ x $6H_2O$
33 mM KCl
5 mM glucose-6-phosphate
5 mM NADP
40 % (v/v) S9 fraction
60 % (v/v) sodium phosphate buffer

4. Cell cultures and media:

Cells

Cells of the V79 cell line, originally derived from male Chinese hamster lung, were used in this study as supplied by Prof. G. Speit (University of Ulm, Germany). These cells have been re-cloned to maintain karyotypic stability; they have a modal chromosome number of 22 and a rapid population doubling time of 10 to 14 hours. Cells stocks were stored in liquid nitrogen and laboratory cultures were maintained at 37˚C and sub-cultured twice weekly. Cultures were checked routinely for mycoplasma contamination and the karyotype confirmed. The cultures were subcloned (plating 1000/culture) at least every two weeks to reduce the number of spontaneous 6-TG resistant mutants, whilst the spontaneous frequency of HPRT-mutants was kept low by the addition of thymidine (9 µg/mL, hypoxanthine (10 µg/mL), glycine (22.5 µg/mL) and methotrexate (0.3 µg/mL). A 6-TG sensitive sub clone could then be used for the HPRT-test.

Media

The cells were maintained in a culture medium comprising hypoxanthine free Eagle's minimal essential medium (MEM), supplemented with nonessential amino acids, L-glutamine (2mM), MEM-vitamins, $NaHCO_3$, penicillin (100 units/mL), streptomycin (100µg/mL) and heat activated foetal calf serum (10% for maintenance or 2% during treatment). For the selection of mutants, a hypoxanthine-free culture medium containing 10 µg/mL of 6-thioguanine (6-TG) was used.

5. Test compound concentrations used:

A preliminary cytotoxicity test, conducted with and without metabolic activation, did not alter the pH or osmolality of the medium, up to and including the highest concentration tested of 5000 µg/mL. Precipitation in the culture medium began at 2500 µg/mL; no cytotoxic effects were observed even at concentrations were precipitation occurred.

Therefore, based on the results of the preliminary toxicity study, concentrations of 125 to 5000 µg/mL were selected for the mutation experiment, both with and without metabolic activation.

B. Test Performance

Experimental phase: 9th May to 16th July 2003

1. Cytotoxicity assay

Exponentially growing V79 cells were plated in 20 ml culture medium in a 250 mL flask (4x10^6 cells per flask). For each concentration one culture was available. After 16-24 hours (attachment), cells were exposed without S9 mix to vehicle alone and to a range of concentrations of the test substance for 5 hours in 20 mL medium containing 2% PCS. In experiments with metabolic activation 1 mL of medium was replaced by 1 mL S9 mix. Thereafter, cell monolayers were washed with PBS, trypsinised and re-plated in 5 mL culture medium at a density of 200 cells into each of 3 Petri dishes (0 60 mm). These dishes were incubated for 6 to 8 days to allow colony development.

Colonies were then fixed with 95% methanol, stained with Giemsa (Merck, stock solution diluted 1:5 with deionized water) and counted. If practicable (i.e. no interference from precipitation/colouration on the plates), colonies were counted automatically using an Artek counter, model 982B.

Cytotoxicity was expressed by comparison of colonies in treated cultures versus vehicle control cultures (relative cloning efficiency).

2. Main assay

For the assay without metabolic activation, exponentially growing V79 cells were plated in culture medium at a final volume of 20 ml in two 250 ml flasks per concentration (4x10^6 per flask), including all control groups. After attachment (16-24 hours), the cells were exposed for 5 hours in 20 mL culture medium with reduced serum content (2%). The corresponding controls were incubated under the same conditions. Cell monolayers were washed with PBS, trypsinised and re-plated in 20 ml culture medium using 1.5 x10^6 cells per 250 mL flask and in 5 mL culture medium using 200 cells per Petri dish (ø 60 mm). One flask and 3 Petri dishes were used per culture. The Petri dishes were incubated (normally 6 days) to allow colony development and to determine the cytotoxicity associated with each test substance directly after treatment ("Survival to Treatment").

Cells in 250 mL flasks were incubated to permit growth and expression of induced mutations. Cells were sub-cultured (= count 1, normally after 3 days) by reseeding 1.5x10^6 cells into 20 mL medium in 250 mL flasks. At the end of the expression period (= count 2, normally a total of 6 days), cultures were reseeded in Petri dishes (0 100 mm) at 3x10^5 cells per dish (8 dishes per culture) in 20 mL culture medium without hypoxanthine but containing 10 µg/mL 6-TG for selection of mutants. In addition, 200 cells per dish (ø 60 mm, 3 dishes per culture) were seeded in 5 mL culture medium in order to determine the absolute cloning efficiency for each concentration. After incubation for 6 to 8 days, the colonies were fixed, stained with Giemsa and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.

At least two experiments were performed with mutant frequencies for at least four concentrations being determined in each.

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

3. Acceptance Criteria

For an assay to be deemed acceptable, an expert analysis based on the following criteria was conducted:

- The average cloning efficiency should be at least 50%
- The average mutant frequency of the vehicle controls should not exceed $25x10^{-6}$ cells
- The mutant frequency of the two vehicle and/or negative control cultures should be similar (difference no greater than $5x10^{-6}$)
- The positive controls should induce a mutant frequency at least 3 times that of the controls
- The highest concentration should be precipitative or induce toxicity of approximately 80-90%. Survival at the lowest concentration should be in the range of the negative control.
- Five plates/culture with relative survival to treatment, relative population growth and absolute cloning efficiency of at least 10% should be available

Any mutant frequencies not meeting these criteria were excluded from the statistical analyses.

4. Assessment criteria

An assay is considered positive if a concentration related and reproducible increase in mutant frequencies is observed of at least two to three that of the highest concurrent negative/vehicle control and no change in osmolality compared with the vehicle control is observed.

An assay is considered equivocal if one or more concentrations induces a reproducible and biologically relevant (but not concentration dependent) increase and an assay is considered negative if no reproducible and relevant increase in mutant frequencies is observed.

5. Statistical analysis

Mutant frequencies, meeting the acceptance criteria, were subjected to a weighted analysis of variance and a weighted recursive regression, followed by pairwise comparisons with the vehicle control (Dunnett's test). The two mutant frequencies obtained per group were considered as independent measurements.

II. Results and discussion

Precipitation was noted in the medium from 3000 µg/mL with and without metabolic activation and no cytotoxicity was observed. Good cloning conditions for the experiments was indicated by a cloning efficiency 75% and 83.9% respectively in the two mutation experiments without S9-mix and 53.9%, 66.8% and 81.3% in the experiments with S9-mix. The results of the experiments with and without metabolic activation are summarised in table 5.8.1-27 and table 5.8.1-28 below.

1. Mutation Assay without Metabolic Activation:

The test substance did not induce an increase in mutant frequencies at any concentration in either experiment performed without metabolic activation. No concentration related decreases were observed in relative survival or relative population growth.

The mutant frequencies of the negative and vehicle controls were within normal ranges and the positive control (EMS) gave the expected results in both trials.

Therefore, M-01 (BAM) was non-mutagenic in the absence of metabolic activation.

Table 5.8.1- 27: Results of the mutation assay without metabolic activation

Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency
M-01	5000	78.3p	2.45
	3000	81.2p	0.65
	1000	101.3	1.75
	500	72.4	0.70
	250	109.6	0.85
	125	94.1	1.95
Negative control	0	87.4	1.70
Solvent control	0	100.0	0.50
EMS	900	17.9	508.95
M-01	5000	74.6p	3.05
	3000	93.2p	1.85
	1000	77.6	2.65
	500	75.6	3.65
	250	100.9	0.75
	125	84.45	0.25
Negative control	0	95.3	2.85
Solvent control	0	100.0	1.70
EMS	900	10.5	412.9

2. Mutation Assay with Metabolic Activation:

The test substance induced no relevant increases in mutant frequencies in any of the experiments performed with metabolic activation (confirmed by overall statistical analysis). No concentration related decreases were observed in either relative survival or relative population growth. The mutant frequencies of the negative/solvent controls were within normal ranges, and the positive control (DMBA) induced a clear mutagenic and statistically significant effect in all trials, as expected.

Therefore, M-01 (BAM) was not mutagenic in the presence of metabolic activation.

Table 5.8.1- 28: Results of the mutation assay with metabolic activation.

Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency
M-01	5000	115.7p	0.55
	3000	110.1p	0.35
	1000	92.1	0.75
	500	115.6	1.10
	250	133.1	0.25
	125	96.6	0.50
Negative control	0	120.5	0.80
Solvent control	0	100.0	0.80
DMBA	20	30.9	32.65
M-01	5000	78.8p	9.30
	3000	77.5p	2.10
	1000	83.7	1.45
	500	110.5	4.90
	250	116.5	4.80
	125	107.7	0.00
Negative control	0	132.8	1.10
Solvent control	0	100.0	2.85
DMBA	20	93.3	65.35

III. Conclusions

The test substance M-01 (BAM) was not mutagenic in mammalian cells both in the presence (three trials) and absence (two trials) of metabolic activation, when tested up to the limit concentration of 5000 µg/mL. The positive controls, EMS and DMBA produced a marked mutagenic effect whilst the negative and vehicle controls gave the expected results, thus demonstrating the validity of the test system to detect mutagens.

Assessment and conclusion by applicant:

The study was conducted in accordance with OECD TG 476 and is valid and acceptable to assess the potential of M-01 to induce point mutations in V79 cells. M-01 was not mutagenic in mammalian cells under the conditions of this study when tested up to the limit concentration (+/- S9).

Data Point:	KCA 5.8.1/07
Report Author:	[redacted]
Report Year:	1993
Report Title:	Evaluation of DNA repair inducing ability of 2,6-dichlorobenzamide (BAM) in a primary culture of rat hepatocytes (with independent repeat)
Report No:	C034068
Document No:	M-234323-01-1
Guideline(s) followed in study:	EEC Directive 67/584/EEC (1988); OECD 482 (1986); US-EPA TSCA, FIFRA, 84-2, §798.5550
Deviations from current test guideline:	Not applicable, guideline now withdrawn as this specific type of study is no longer accepted
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The potential of M-01 (referred to as 2,6-dichlorobenzamide (BAM) in the report) to induce DNA repair or unscheduled DNA synthesis (UDS) in a primary culture of rat hepatocytes was investigated. Concentrations up to and including 1000 µg/mL were tested and two independent experiments were conducted.

The OECD test guideline (OECD 482) for unscheduled DNA repair synthesis (UDS) is no longer available, as this specific test is no longer a data requirement; therefore, this test is included as supportive information.

No significant dose-related increase in the nuclear grain count was observed in either of the two independent experiments.

Therefore, under the conditions of this experiment, M-01 (BAM) did not induce DNA repair in primary cultures of rat hepatocytes.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-01 (referred to as 2,6-dichlorobenzamide (BAM) in the report)
Purity:	100%
Batch no.:	FUX0010000/FUN81G02C
Expiry date:	December 1997

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive controls:	Without metabolic activation (-S9-mix): 4-Nitroquinoline-N-oxide (4-NQO), 0.01mM & 0.02mM in DMSO With metabolic activation (+S9-mix): 7,12-Dimethylbenzanthracene (DMBA), 0.04 mM & 0.05 mM in DMSO (two concentrations of positive control were tested but only one was scored)

3. Cell cultures and medium

Rat hepatocytes isolated from male Wistar rats (age 8-10 weeks) were used. The culture medium comprised Williams E medium supplemented with foetal calf serum (10%), L-glutamine (2 mM) and gentamycin (50 µg/ml). All incubations were carried out in a humid atmosphere (80-95%) containing 5% CO_2, in the dark at 37˚C.

4. Test concentrations

The concentration used in the UDS assay were selected on the basis of a preliminary toxicity test. Cytotoxicity was determined by a reduction in trypan blue dye exclusion after exposure to the test substance. The concentration which produced a 90% decrease in viability in comparison to controls was determined. The highest concentration used in the UDS assay was the EC10, and the viability of the lowest concentration was comparable with the control (with a further 4 doses spaced evenly between). If solubility was a factor, then this determined the highest concentration instead; in any case a dose higher than 5 mg/mL was not tested.

B. Test performance

Experimental phase: 19th October 1992 to 11th March 1993

1. Unscheduled DNA-synthesis assay

Approximately two hours after the seeding, the cells were exposed overnight (18 hours) to the test compound, together with ^{3}HTdR (10µCi/mL; specific activity 18-30 Ci/mmol). Following exposure, the cells were rinsed with HBSS and fixed with methanol-acetic acid 3:1 (v/v). Two independent experiments were carried out with each dose (including controls) being tested in triplicate.

Autoradiographic procedure:

The cells were fixed and mounted on slides, which were then dipped in Ilford K5D at 42˚C and dried for 2 hours at room temperature in the dark. After drying, the slides were placed in light tight boxes in the presence of silica gel. The photographic emulsion was exposed for 7-14 days at 4˚C and developed for 4 minutes and fixed. The slides were then rinsed, and the cells stained with haematoxylin/eosin.

Scoring:

Slides were randomly coded and examined. The number of grains above the nuclei of 50 cells/slide were counted and the mean ± SD calculated. The grain counts over nuclear areas were compared with the grain counts over the adjacent cytoplasm. Where the cytoplasmic grain counts of treated cells did not differ from controls, the corrected nuclear grain count was calculated for each cell by subtracting the cytoplasmic grain counts from nuclear grain counts. The background grain counts per average nuclear area was recorded.

2. Acceptability of assay

An UDS-assay is considered acceptable if it meets the following criteria:

a) The background counts of grains are below 20 per average nuclear area.

b) The positive control substances should produce significant increases in the number of grains.

c) The selected dose range should include a toxic concentration as demonstrated by the preliminary toxicity range-finding test or should extend to 5 mg/mL or should extend to the limit of solubility.

3. Evaluation of assay

A test substance was considered positive in the UDS-assay if:

a) It induced a statistically significant (two-way analysis of variance) dose-related increase in the average number of grains per nucleus. At least two consecutive concentrations should produce grain counts which exceed those of the control by at least two standard deviations of the control value.

b) The results were reproducible in an independently repeated assay.

A test substance was considered negative in the UDS-assay if:

a) No positive response, as defined above, was observed.

b) The results were reproducible in an independently repeated experiment.

The final result depends upon expert evaluation.

II. Results and discussion

1. Cytotoxicity test and dose selection

The range finding experiment was carried with concentrations of 0.1 to 1000 µg/mL. Concentrations higher than 1000 µg/ml could not be tested because of limited solubility of the test substance in the culture medium. The pH and the osmolality of the culture medium containing 500 µg/ml test substance and the highest concentration without precipitate were 7.88 and 447 mOsm/kg respectively (compared with 7.66 and 456 mOsm/kg of culture medium containing 1% DMSO). The results showed a 39% decrease in the number of viable cells after exposure relative to the blank at 1000 µg/ml.

Based on these results the following doses were selected for the DNA-repair test:

Experiment 1 and 2: 1, 3, 10, 33, 100, 333 and 1000 µg/ml.

Based on these observations the following doses were selected for scoring of UDS:

Experiment 1 and 2: 3, 10, 33, 100, 333 and 1000 µg/ml.

Table 5.8.1- 29: Results of the range-finding experiment

Concentration (µg/ml)	No cells/well ($x10^4$)	Viable cells/dead cells (total)	No viable cells ($x10^4$)	Mean percentage of viable cells relative to blank
0	4.50	89/11 (100)	4.0	100
0	3.60	84/16 (100)	3.0	
0.1	4.48	90/10 (100)	4.0	116
0.1	4.88	85/15 (100)	4.1	
0.3	4.25	69/31 (100)	2.9	99
0.3	4.90	81/19 (100)	4.0	
1	4.13	84/14 (98)	3.5	97
1	3.98	83/17 (100)	3.3	
3	4.63	73/27(100)	3.4	93
3	3.68	83/17 (100)	3.1	
10	4.93	69/31(100)	3.4	94
10	4.20	77/23 (100)	3.2	
33	4.35	68/32 (100)	3.0	127
33	8.98	66/34 (100)	5.9	
100	2.38	67/33(100)	1.6	74
100	5.78	62/38(100)	3.6	
333	4.70	57/43(100)	2.7	74
333	4.13	60/40(100)	2.5	
1000*	3.30	66/34(100)	2.2	61
1000*	3.78	56/44(100)	2.1	

* Slight precipitation in the culture medium

2. DNA-repair assay

No increase in the number of grains per nucleus or cytoplasm was detectable. Furthermore, the corrected nuclear grain counts (number of grains over the nucleus minus the number of grains over a nuclear-sized area of the cytoplasm of the same cell) revealed no increase at any test substance concentration.

The positive control substances DMBA and 4-NQO produced significant, 44-to 146-fold, increases in the number of grains per nucleus. In the scored coverslips the mean nuclear background was less than 20 grains per average nuclear area. Therefore, it can be concluded that the test conditions were optimal and that the metabolic activation system functioned properly.

Table 5.8.1- 30: Induction of UDS in rat hepatocytes, experiment 1

Test substance	Concentration (µg/ml)	Mean percentage of viable cells	Mean nuclear grain count corrected for cytoplasm
M-01	0	100	2
	3	89	1
	10	78	2
	33	88	1
	100	91	1
	333	57	1
	1000	61	0 P
4-NQO	10	75	44
DMBA	50	50	47

P: precipitation
4-NQO: 4-Nitroquinoline-N-oxide = positive control without S9 mix
DMBA: 7,12-Dimethylbenzanthracene = positive control with S9 mix

Table 5.8.1- 31: Induction of UDS in rat hepatocytes, experiment 2

Test substance	Concentration (µg/ml)	Mean percentage of viable cells	Mean nuclear grain count corrected for cytoplasm
M-01	0	100	-5
	3	83	-5
	10	93	-7
	33	82	-5
	100	98	-6
	333	87	-6
	1000	80	-6 P
4-NQO	10	97	143
DMBA	50	93	146

P: precipitation
4-NQO: 4-Nitroquinoline-N-oxide = positive control without S9 mix
DMBA: 7,12-Dimethylbenzanthracene = positive control with S9 mix

III. Conclusions

The test substance M-01 (BAM) was negative under the conditions of this DNA-repair assay, conducted using primary cell cultures of rat hepatocytes.

Assessment and conclusion by applicant:
The study was conducted according to OECD TG 482 (guideline now withdrawn) and is valid and acceptable to assess the ability of M-01 to induce DNA damage and unscheduled repair. M-01 did not induce unscheduled DNA synthesis in primary cultures of rat hepatocytes *in vitro*.

Data Point:	KCA 5.8.1/53
Report Author:	[redacted]
Report Year:	2019
Report Title:	AE C653711: Micronucleus test in human lymphocytes in vitro
Report No:	1969601
Document No:	M-673139-01-1
Guideline(s) followed in study:	OECD Test Guideline No. 487 (July 2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

M-01 (BAM, referred to as AE C653711 in the report) was evaluated for its potential to induce micronuclei in human lymphocytes *in vitro* in two independent experiments; Experiment I comprised a four hour exposure period in the presence and absence of metabolic activation (provided by S9) whist experiment II provided a 20-hour exposure period in the absence of S9. Concentrations up to 1975 µg/mL were tested in duplicate and 1000 binucleated cells/culture were examined for cytogenetic damage.

In experiment I, no cytotoxicity was observed (+/- S9) up to the highest evaluated concentration at which precipitation was noted. In experiment II (-S9), moderate cytotoxicity and precipitation was observed at the highest tested concentration.

Treatment with M01 (BAM) did not cause a statistically significant or biologically relevant increase in the number of micronucleated cells in experiment I or II, either in the presence or absence of S9 mix. Appropriate reference substances gave the expected statistically significant increase in the number of micronucleated cells.

The test substance M-01 (BAM) can be considered neither clastogenic nor aneugenic under the conditions of this *in vitro* micronucleus test, when tested up to precipitative concentrations, both in the presence and absence of metabolic activation.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-01 (BAM, 2,6-dichlorobenzamide, referred to as AEC653711 in the report)
Purity:	96.2 % (w/w)
Batch no.:	0801E1
Expiry date:	4th August 2020

2. Vehicle and/or positive control

Vehicle: DMSO

Positive controls: -S9

Mitomycin C (MMC), 0.8 µg/mL (98% purity, dissolved in deionized water)
Demecolcine, 150 ng/mL (purity ≥98%, dissolved in deionized water)
+S9
Cyclophosphamide (CPA), 17.5 µg/ml (purity 97-103%, dissolved in saline)

3. Activation:

Metabolic activation was provided by S9-mix derived from phenobarbital/ß-naphthoflavone induced rat liver homogenate; each batch of prepared S9 has been routinely evaluated for its ability to activate known mutagens.

An appropriate quantity of S9 was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation used for this study was 30.7 mg/mL.

4. Cell cultures and media:

Cells

Blood was drawn from healthy non-smoking donors, not receiving medication. Blood from a male donor (21 years old) and a female donor (31 years old) were used in experiment I and II respectively. The lymphocytes have been shown to respond well to PHA and positive control substances; furthermore, both donors have an established low incidence of micronuclei in peripheral blood lymphocytes.

Media

Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hours following collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1), supplemented with 200 mM GlutaMAX™. The medium was further supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (foetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).

All incubations were carried out at 37 °C with 5 % CO2 in humidified air.

5. Test compound concentrations used:

Test concentrations for the first experiment were 0 (solvent control), 12.8, 22.5, 39.3, 68.8, 120, 211, 369, 645, 1129 & 1975 µg/mL, both with and without S9 mix.

In the second experiment a continuous (20 hour) treatment was used in the absence of S9 mix at test concentrations of 0 (solvent control), 39.9, 68.8, 120, 211, 369, 645, 1129 & 1975 µg/mL, in the absence of S9 mix.

B. Test Performance

Experimental phase: 28th August 2019 to 11th October 2019

1. Preliminary assay

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity (characterized by the percentages of reduction in the CBPI in comparison to the controls and expressed as % cytostasis) was determined from 500 cells/culture. The experimental conditions in this pre-experimental phase were identical to those described for the mutagenicity assay.

The pre-test was performed with 10 concentrations of the test item, separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hours (with and without S9 mix). The preparation interval was 40 hours after start of the exposure.

This preliminary test was designated Experiment I, since the cultures fulfilled the criteria for cytogenetic evaluation and appropriate concentrations could be selected for evaluation.

2. Cytogenetic experiment

Cells were subject to either 4 hours pulse exposure (with and without S9; experiment I) or 20 hours continuous exposure (without S9; experiment II). The succeeding procedure for cell preparation was the same for both exposure periods.

Pulse exposure

Approximately 48 hours following seeding, two blood cultures of 10 mL each were set up in parallel in 25 cm² cell culture flasks (for each test item concentration). The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H2O, 192 mg/L Na2HPO4 • 2 H2O and 150 mg/L KH2PO4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

Continuous exposure

Approximately 48 hours following seeding, two blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells re-suspended in and washed with "saline G". The washing procedure was repeated once as described. Following washing, the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

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

Preparation of cells

Following the procedures above (approximately 40 hours from the start of treatment), the cultures were harvested by centrifugation. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

3. Acceptance Criteria

The micronucleus assay is deemed acceptable if it fulfils the following criteria:

- The concurrent solvent control will normally be within the laboratory historical solvent control data range. The average mutant frequency of the vehicle controls should not exceed 25×10^{-6} cells
- The concurrent positive controls should produce a statistically significant increase in the micronucleus frequency and should be within the laboratory historical positive control data range. The positive controls should induce a mutant frequency at least 3 times that of the controls
- Cell proliferation criteria in the solvent control are considered acceptable.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.

Any mutant frequencies not meeting these criteria were excluded from the statistical analyses.

4. Assessment criteria

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative if, in all of the experimental conditions examined:

- None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase
- The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system. Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- The increase is concentration-related in at least one experimental condition
- The results are outside the range of the laboratory historical solvent control data

When all of the criteria are met, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system.

There is no requirement for verification of a clear positive or negative response.

In case the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. narrow concentration spacing, other metabolic activation conditions, i.e. S9 concentration or S9 origin) could be useful.

5. Statistical analysis

Statistical significance was confirmed by the Chi square test ($p < 0.05$), using a validated test script of "R", a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those values that indicated an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression was performed using a validated test script of "R" to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Both, biological and statistical significance were considered together.

II. Results and discussion

In Experiment I, precipitation of the test item in the culture medium was observed at 1129 µg/mL and above in the absence of S9 mix and at 645 µg/mL and above in the presence of S9 mix at the end of treatment. In addition, precipitation occurred in Experiment II in the absence of S9 mix at 1975 µg/mL at the end of treatment.

No relevant influence on osmolarity or pH was observed.

In Experiment I in the absence and 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, moderate cytotoxicity (44.1% cytostasis) was observed at the highest applied concentration, which showed precipitation.

The results of both experiments, with and without metabolic activation, are summarised in table 5.8.1-32 below:

Table 5.8.1- 32: Summary of results of experiment I and II

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	Historical control data	
						95% Ctrl limit	Min - Max
Exposure period 4 hrs without S9 mix							
I	40 hrs	Solvent control[1]	1.80		0.45	0.01 – 1.20	0.00 – 1.55
		Positive control[2]	1.67	16.8	**5.50**[S]	2.66 – 22.74	3.95 – 28.60
		369	1.73	9.1	0.60		
		645	1.75	6.5	0.10		
		1129[P]	1.64	20.3	0.35		
Exposure period 20 hrs without S9 mix							
II	40 hrs	Solvent control[1]	1.94		0.40	0.00 – 1.14	0.05 – 1.60
		Positive control[3]	1.48	48.8	**3.95**[S]	1.15 – 6.44	1.95 – 8.80
		645	1.80	14.9	0.35		
		1129	1.66	30.4	0.35		
		1975[P]	1.53	44.1	0.85		
Exposure period 4 hrs with S9 mix							
I	40 hrs	Solvent control[1]	1.89		0.50	0.00 – 1.24	0.10 – 1.30
		Positive control[4]	1.84	5.5	**2.95**[S]	1.01 – 7.34	1.80 – 8.85
		211	1.78	12.1	0.50		
		369	1.81	9.6	0.35		
		645[P]	1.85	4.5	0.35		

* For the positive control groups and the test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
P Precipitation occurred at the end of treatment
S The number of micronucleated cells is statistically significantly higher than corresponding control values
1 DMSO 0.5 % (v/v)
2 MMC 0.8 µg/mL
3 Demecolcine 150 ng/mL
4 CPA 17.5 µg/mL

In both independent experiments, neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells was observed after treatment with the test item.

Demecolcine (150 ng/mL), MMC (0.8 µg/mL) and CPA (17.5 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei, thus confirming the validity of the assay to detect clastogens/aneugens.

III. Conclusions

The test substance M-01 (BAM) did not induce micronuclei in this *in vitro* micronucleus test in human lymphocytes, when tested up to precipitative concentrations.

Therefore M-01 (BAM) is considered to be neither clastogenic nor aneugenic under the conditions of this assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 487 and is valid and acceptable to assess the clastogenicity of M-01. M-01 did not induce micronuclei when tested up to precipitative concentrations and is therefore not considered clastogenic or aneugenic under the conditions of this assay (+- S9).

Data Point:	KCA 5.8.1/08
Report Author:	[redacted]
Report Year:	1993
Report Title:	Micronucleus test in bone marrow cells ot the mouse with 2,6-dichlorobenazmide (BAM)
Report No:	C034071
Document No:	M-234329-01-1
Guideline(s) followed in study:	Directive 67/548/EEC, B.12 (1984); OECD 474 (1983); US-EPA TSCA § 798.5395, FIFRA, 84-2 (1989)
Deviations from current test guideline:	1000 polychromatic erythrocytes/animal scored (current guidance recommends at least 4000); exposure of the target tissue could not be clearly demonstrated
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The clastogenic potential of M-01 (referred to as 2,6-dichlorobenzamide (BAM) in the report), 100% purity, was investigated in a guideline compliant *in vivo* mouse micronucleus test. Following dose finding preliminary testing, a single dose containing 250 mg/kg bw test substance or vehicle control was administered *via* gavage to groups of Swiss mice (5/sex/group), the animals were sacrificed at 24-, 48- or 72-hours following dosing for preparation of bone marrow smears. A positive control group of 5/sex received cyclophosphamide and were sacrificed for sampling 48-hours following dosing. The incidence of micronucleated cells was determined from 1000 polychromatic erythrocytes/animal.

There were no deaths or treatment-related signs of toxicity. No statistically significant increase was noted in the number of micronucleated polychromatic or normochromatic erythrocytes at the 24, 48- or 72-hour sacrifices. A large statistically significant increase was observed in the frequency of micronucleated polychromatic erythrocytes in the cyclophosphamide treated group. There was no change in the ratio of polychromatic to normochromatic erythrocytes in treated animals at any sampling period. A reduction in the PCE:NCE ratio was observed in the positive control group.

It was concluded that M-01 (BAM) showed no evidence of mutagenic potential when administered orally in this *in vivo* study. Bone marrow exposure could not be clearly demonstrated by a reduction in PCE:NCE ratio; however, according to the guidance this is only one way of demonstrating exposure of the target tissue to the test substance. Mice dosed with 250 mg/kg bw/d in the accompanying dose-range findings displayed clinical signs following dosing which comprised ataxia and lethargy, indicating that M-01 (BAM) was systemically available after oral administration. Similarly, in rat studies at similar or lower doses, clinical signs of toxicity and reductions in bodyweight occurred (see section 1.3.2 of this document). Furthermore, although plasma concentrations of M-01 (BAM) have not been measured directly, it has been shown in rat ADME studies that after oral administration (150 mg/kg bw/d) BAM is distributed throughout the carcass. This indicates that the test substance was likely to have been systemically available in this mouse micronucleus test, and as the bone marrow is a highly perfuse tissue it is likely to have also been exposed to M-01.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-01 (referred to as 2,6-dichlorobenzamide (BAM) in the report)
Purity:	100%
Batch no.:	FUX0010000/FUN81G02C

2. Vehicle and/or positive control

Vehicle:	Corn oil
Positive control:	Cyclophosphamide (CP), 50 mg/kg bw dissolved in 0.9% NaCl

3. Test animals

Species:	Mouse
Strain:	Swiss mice, OF-1 (SPF-quality)
Age:	Approx. 7 weeks
Weight at start:	34-41 g (males), 23-29 g (females)
Source:	[redacted]
Acclimation period:	Yes
Diet:	Standard laboratory animal diet (Kliba 343 from Klingentalmühle Ag, Kaiseraugst, Switzerland).
Water:	Tap water, ad libitum
Housing:	Air-conditioned rooms
Temperature:	21 ± 3°C
Humidity:	40 – 70 %
Air changes:	15/hour
Photoperiod:	12 hours

4. Test compound doses

A single oral gavage dose of 250 mg/kg bw (maximum tolerated dose) was administered at a dose volume of 10 mL/kg bw. The selected dose was based on the results of a range-finding study, in which 3/sex animals were orally dosed with 100, 250 and 500 mg/kg bw, with an additional 3 males/dose receiving 100, 2000 and 4000 mg/kg bw.

B. Test performance

Experimental phase: 28th September 1992 to 30th November 1992

1. Treatment and sampling times

Five males and five females per sampling time were administered a single dose and sampled according to the following design:

Table 5.8.1- 33: Study design

Treatment	Dose (mg/kg bw)	Sampling times (hours)
Vehicle	-	24, 48, 72
M-01 (BAM)	250	24, 48, 72
Cyclophosphamide	50	48

In the event of unexpected deaths, additional animals could be used.

2. Removal and preparation of the bone marrow

The animals of the treatment and negative control groups were sacrificed by cervical dislocation at 24, 48- and 72-hours following dosing, whilst those of the positive control groups were sacrificed 48-hours post-dose. Following sacrifice both femurs were detached and the blood and muscle removed. The bone was shortened at both ends to reveal the marrow canal, which was then flushed with 2 mL of foetal calf serum. The cell suspensions were then collected and centrifuged 1000 rpm for 5 minutes.

The supernatant was removed, leaving a drop of serum to be aspirated with the cells of the pellet. A drop of the cell suspension was placed then spread on a clean slide and marked with the animal number. The preparations were air dried, fixed for 5 minutes in 100% methanol and further air-dried overnight.

3. Staining of the bone marrow smears

The slides were stained automatically using the "Wright stain procedure" in an Ames HEMA-tek slide stainer. The dry slides were cleared by dipping in xylene before being embedded in DePex and mounted with a coverslip.

4. Analysis of the bone marrow smears for micronuclei

Slides were randomly coded before examination and the identification covered with a coded label. The slides were initially examined under a magnification of 100 x in order to locate regions of cells that were well spread, undamaged and well stained. The selected regions were then subject to examination at 1000 x. The number of micronuclei per 1000 polychromatic erythrocytes were counted, and the ration of polychromatic to normochromatic erythrocytes was determined in parallel by counting and differentiating the first 1000 erythrocytes.

5. Acceptability

A micronucleus test is considered acceptable if it meets the following criteria:

a) The positive control substance induced a statistically significant (Wilcoxon Rank Sum Test, two-sided test at $P < 0.05$) increase in the frequency of micronuclei.

b) The incidence of micronuclei in the control animals should reasonably fall within the laboratory historical control data range.

6. Evaluation criteria/statistical methods

A test substance is considered positive in the micronucleus test if:

It induced a biologically as well as a statistically significant (Wilcoxon Rank Sum Test; two-sided test at $P < 0.05$) increase in the frequency of micronuclei (at any dose or at any sampling time) in the combined data for both sexes or in the data for male or female groups separately.

A test substance is considered negative in the micronucleus test if:

None of the tested concentrations or sampling times showed a statistically significant ($P \leq 0.05$) increase in the incidence of micronuclei in the combined data (both sexes) or the individual data (males and females).

The final outcome of the study should be based on expert evaluation of the data.

II. Results and discussion

1. Pilot study/Dose selection

The highest doses were administered to males only and all animals died shortly following dosing (4000 and 2000 mg/kg bw) or one day following dosing (1000 mg/kg bw). Males and females receiving 500 mg/kg bw/d were comatose after dosing and all females either remained so or died. Animals administered 250 mg/kg showed ataxia (female) and lethargy (both sexes) on the day of dosing but recovered by the following day. Animals receiving the lowest dose of 100 mg/kg bw showed lethargy after dosing, but all recovered.

Therefore, based on these results a dose of 250 mg/kg bw was selected for the micronucleus test.

2. Micronucleus Test

No decrease in the PCE/NCE ratio was observed; therefore, although no increase in the frequency of micronuclei was observed in the polychromatic erythrocytes of the bone marrow of the treated animals, exposure of the target tissue (bone-marrow) could not be satisfactorily demonstrated.

The incidence of micronuclei in the control animals was within the range of the historical control data (0.59 ± 0.89; N=1120). The positive control substance induced a statistically significant increase in the number of micronuclei in both sexes and exposure of the target tissue was demonstrated by a toxic effect of the positive control substance on erythropoiesis (decrease in the ratio of polychromatic to normochromatic erythrocytes).

Table 5.8.1- 34: Mean number of micronuclei/1000 polychromatic cells and PCE/NCE ratio

Treatment	Sampling time (hours)	Sex	Ratio PCE/NCE	MNPCE/1000 PCE
M-01 (BAM)	24	Male	1.01	1.0
	48		0.89	0.2
	72		0.97	0.0
Vehicle	24		1.01	0.4
	48		0.96	0.8
	72		0.97	1.0
CP	48		0.32	16.8*
M-01 (BAM)	24	Female	1.00	0.6
	48		1.04	0.4
	72		0.96	0.2
Vehicle	24		1.01	0.2
	48		1.03	0.4
	72		1.05	0.2
CPA	48		0.60	8.8*

CPA: cyclophosphamide

* $p < 0.05$ using non-parametric Wilcoxon ranking test

III. Conclusions

M-01 (BAM) showed no evidence of mutagenic potential when administered orally in this *in vivo* study. Exposure of the bone marrow was not directly demonstrated; however clinical signs of toxicity at the same dose in the pilot study and similar doses in rat short-term toxicity studies demonstrate systemic exposure and hence exposure of the highly perfuse bone-marrow tissue.

Assessment and conclusion by applicant:

The study was conducted according to OECD 474 and is valid and acceptable to assess the mutagenic potential of M-01 in vivo (with the caveat of the deviations described above). M-01 was not mutagenic in this study in which target tissue exposure was demonstrated by signs of systemic toxicity at similar doses in other rat studies.

Long-term toxicity and carcinogenicity

Data Point:	KCA 5.8.1/09
Report Author:	[redacted]
Report Year:	1971
Report Title:	Effect of BAM in dietary administration to rats for two years
Report No:	C034294
Document No:	M-234669-01-1
Guideline(s) followed in study:	US-EPA FIFRA 83-1, 83-2
Deviations from current test guideline:	Low number of animals in groups, no purity of the test item reported
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Sprague Dawley CD rats (35/sex/dose) were administered M-01 (BAM, referred to as 2,6-dichlorobenzamide in the report) for two years. Nominal dietary concentrations of 0, 60, 100, 180 and 500 ppm equated to mean estimated intakes of 0, 2, 3.5, 5.7 & 17.6 mg/kg bw/d in males and 0, 2.7, 4.1, 8.6 & 21.3 mg/kg bw/d in females. Throughout the study animals were examined for clinical signs, bodyweight, food and water consumption, ophthalmoscopy, haematology, clinical chemistry, and urinalysis. Organs were weighed on sacrifice and a range of tissues were subject to macroscopic and microscopic investigations.

There were no treatment-related deaths. At the high dose of 500 ppm, bodyweights were statistically significantly lower than controls (-16% and -26% in males and females respectively on week 106), in females being associated with a slight reduction in food consumption (-8% compared with controls); there was no effect on bodyweight development at the lower doses. Ophthalmoscopic examination of high dose animals did not reveal any treatment-related changes when compared with controls. Haematology tests showed occasional minor depression of red cell parameters (haemoglobin concentration, erythrocyte counts and haematocrit) at 500 ppm, predominantly in males but also, to a lesser extent in females, although statistical significance was not always attained. There were no treatment-related alterations to haematological parameters at the lower doses and clinical chemistry and urinalysis did not reveal any treatment-related effects at any dose.

Relative liver and adrenal weights were increased in females at 500 ppm by 25% and 48% respectively, but with no corresponding effect on the absolute weights of these organs, therefore, these increases in relative weights may be secondary to the 20% decrease in final body weight of females in this dose-group. No effects were noted on the weights of any other organs and gross examination at necropsy did not reveal any treatment-related abnormalities.

Treatment-related histopathological findings were confined to the livers of high-dose females. Liver changes in high-dose females were characterized by hepatocyte vacuolation, degeneration and fat deposition.

There was no effect on the overall tumour burden in either sex, but a slightly higher incidence of hepatoma was observed in females at 500 ppm (4/20); however, the slight increase was not considered to be indicative of a carcinogenic potential of M-01 (see separate position paper).

The NOAEL in the 2-year dietary study with M-01 (BAM) was 180 ppm (equivalent to 5.7 and 8.6 mg/kg bw/day in males and females, respectively). At the LOAEL of 500 ppm (17.6 and 21.3 mg/kg bw in males and females respectively) histopathological changes in the liver were observed in females and body weight and bodyweight gain were reduced in both sexes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-01 (BAM, referred to as 2,6-dichlorobenzamide in the report)
Purity:	Not provided
Batch no.:	Batch No. 195

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rats
Strain:	CD (SPF)
Age:	Not provided
Weight at start:	Not provided; group weight ranges differed by less than ±2.5g
Source:	[redacted]
Acclimation period:	Eight days
Diet:	Spiller's Laboratory Small Animals Diet (autoclaved)
Water:	Tap water *ad libitum*
Housing:	Suspended cages with wire-mesh floors, housed five/cage
Temperature:	21± 2 °C
Humidity:	50± 5%
Air changes:	Not stated
Photoperiod:	Not stated

B. Study design

1. In-life dates: 7th November 1968 to 4th October 1971

2. Animal assignment and treatment

The rats were randomized and assigned to the following the test-groups:

Table 5.8.1- 35: Study design

Group	Dose (ppm)	Number of males	Number of females
1	0	35	35
2	60	35	35
3	100	35	35
4	180	35	35
5	500	35	35

3. Diet preparation and analysis

The above dietary concentrations were achieved by the direct dietary dilution of a premix containing 3900 ppm M-01 / BAM (prepared weekly). The diets were homogenized by mixing for 10 minutes with double-rotary cone blender and stored in heat-sealed, opaque polythene bags until use. The dietary content of M-01 (BAM) was analysed by gas-liquid chromatography on suitable concentration of the diet or premix; in preparation for the analyses, 20g samples were extracted from the diet with ethyl acetate, made up to 200 ml and mixed thoroughly. Periodic chemical analyses indicated that the achieved dietary concentrations of M-01 (BAM) differed from the nominal concentrations by up to 9%.

4. Statistics

Where the data suggested a treatment-related response, the students T-test was employed to assess the significance of inter-group variability.

C. Methods

1. Observations

Animals were examined for mortality and clinical signs of toxicity (frequency of examinations not stated); animals found dead or killed *in extremis* were subject to a full macroscopic examination and the tissues preserved.

2. Body weight

Individual bodyweights were recorded prior to dosing, and then at weekly intervals thereafter and the group mean bodyweights calculated.

3. Food intake

Food intake was measured, and the mean weekly food intake was calculated per cage. Food utilisation efficiency was assessed by calculating the mean food conversion ratios (FCR values) during the fastest growth period, calculated as food consumed (g) per body-weight gain (g). Water intake was measured by a visual inspection of the water bottles, no further investigations were necessary as an effect was not evident.

4. Ophthalmoscopic examination

During weeks 0, 13, 26, 52 and 104, the eyes of all animals from groups 1 and 5 were examined with a Koeler indirect ophthalmoscope.

5. Laboratory investigations

Haematology

Blood samples were collected for haematological investigations from selected animals of the control and high-dose groups (10/sex). The samples were collected from the orbital sinus during weeks 0, 13, 26, 52 and 103 and the following parameters were measured:

Packed cell volume (PCV), haemaglobin concentration (Hb), red cell count (RBC), white cell count (WBC) and differential white cell count (neutrophils [N], lymphocytes [L], eosinophils [E], basophils [B] and monocytes [M].

From these measurements the mean corpuscular haemaglobin content (MCHC) and the mean cell volume (MCV) were calculated.

During weeks 26, 39 and 103 weeks of treatment, the haematology examinations were extended to the animals of group 4.

Clinical Chemistry

Blood was collected for clinical chemistry analyses form the orbital sinus of 5/sex rats of the control and high-dose group, during weeks 13, 26, 52 and 103. The following parameters were measured: Plasma urea, glucose, serum alkaline phosphatase (SAP), serum glutamate pyruvate transaminase (SAP), sodium (Na^+), potassium (K^+), total protein, albumin and globulin.

Urinalysis

Overnight urine samples were collected from selected animals of the control and high-dose groups (5/sex) and the following quantitative tests performed: pH, specific gravity (SG) and protein; reducing substances, glucose, ketones, bile pigments and urobilin were also measured and graded as either – (negative), tr (trace), 1 (positive), 2 (strong positive) and 3 (maximal positive). The centrifuged deposit was additionally microscopically examined for the presence of epithelial cells (E), polymorphonuclear cells (P), mononuclear cells (M), erythrocytes (R), organisms (O), casts (C) and abnormal constituents (A); graded in severity from 1 (none) to 3 (many).

6. Sacrifice and pathology

The rats were sacrificed by carbon dioxide asphyxiation and the adrenals, pituitary, brain, spleen, heart, testes, kidneys, thyroid, liver, uterus, and ovaries were weighed. Relative weights (percentage BW x 100) were also calculated.

7. Histopathology

The weighed organs (above) as well as the colon, duodenum, eyes, ileum, lungs, lymph nodes, pancreas, salivary glands, spleen, stomach, thymus, and urinary bladder were subject to a full histopathological examination. In addition, any unusual macroscopic findings (including tumours) were also examined microscopically. The eyes were preserved in Davidson's fixative and all other tissues were preserved in buffered 4% formaldehyde.

The aorta, caecum, femur, oesophagus, prostate, sciatic nerve, skin, seminal vesicles, skeletal muscle and tongue were preserved but not examined further.

The microscopic examination was initially confined to rats that died prematurely, all rats at 500 ppm and seven males and eight females of the control group. As microscopic findings were evident in the liver and/or mammary glands of the high-dose group, the livers of selected animals (10/sex) and the mammary tissue of all rats, of the 60, 100 and 180 ppm dose-groups were further examined.

II. Results and Discussion

A. Results

1. Clinical results

There were no treatment-related deaths or clinical signs of toxicity; With regard to mortality, 20/18, 19/18, 22/18, 16/22 and 20/15 males/females at 0, 60, 100, 180 and 500 ppm respectively, were found dead or killed in extremis during the treatment period. The deaths were noted consistently across all dose-groups (including controls) and showed no clear dose response; therefore, the deaths are not considered to be related to treatment with M-01 (BAM).

2. Body weights

Body-weight development was affected at 500 ppm; both males and females at this dose showed a statistically significant reduction in body-weight gain in comparison with controls. The effect became evident at week 26 and the difference was maintained until the end of the study at week 106 (see table 5.5.1-02). By week 106 of the study, the mean body weights were 13.4% and 20.6% lower than controls in males and females respectively and the overall bodyweight gains the end of the study were 16% and 26% lower than the corresponding male and female controls. The body-weight changes noted in the lower dose groups were slight, not statistically significant and/or did not show a dose response; therefore, the body weight changes seen at the lower doses are considered to be unrelated to treatment with M-01 (BAM).

Table 5.8.1- 36: Selected body weight changes in the 2-year rat study with M-01 (BAM)

Week	Males					Females				
	Dose (ppm)									
	0	60	100	180	500	0	60	100	180	500
	Weight (g) and % difference from control									
0	137	137	137	137	137	122	122	122	122	122
26	629 -	609 -3.2	618 -1.7	610^{NS} -3	580** -7.8	346 -	349 -	354 +2.3	336^{NS} -3	311** -10.11
52	720 -	701 -2.6	704 -2.2	698^{NS} -3	664* -7.7	429 -	414 -3.5	425 -	399^{NS} -7	368*** -14
78	808 -	773 -4.3	777 -3.8	759^{NS} -6	732* -9.4	520 -	494 -5	512 -1.5	495^{NS} -4.8	454* -12.7
106	792 -	759 -4	740 -6.6	766^{NS} -3.3	686* -13.4	616 -	580 -5.8	572 -7.1	533^{NS} -13.5	489** -20.6

	Males					Females				
	Dose (ppm)									
	0	60	100	180	500	0	60	100	180	500
Week	Weight (g) and % difference from control									
Week	Body weight gain (g) and % difference from controls									
26	492 -	472 -4%	481 -2%	473 -4%	443 -10%	224 -	227 -1%	232 4%	214 -4%	189 -16%
52	583 -	564 -3%	567 -3%	561 -4%	527 -10%	307 -	292 -5%	303 -1%	277 -10%	246 -20%
78	671 -	636 -5%	640 -5%	622 -7%	595 -11%	398 -	372 -7%	390 -2%	373 -6%	332 -17%
106	655 -	622 -5%	603 -8%	629 -4%	549 -16%	494 -	458 -7%	450 -9%	411 -17%	367 -26%

* P<0.05, **P<0.01, ***P<0.001, NS = not statistically different from controls p<0.01

3. Food intake

Food consumption was affected in females at 500 ppm and was approximately 8% lower than controls, correlating with a minimal decrease in food utilisation efficiency. Food consumption in males at this dose, and in both sexes at the lower doses, remained comparable with controls throughout the duration of the study.

4. Ophthalmoscopic examinations

Upon ophthalmoscopic examination, the incidence of ocular abnormalities and/or variations was similar across the control and high dose groups; therefore, no treatment-related effect was apparent.

5. Laboratory investigations

Haematology

A minor (and only occasionally statistically significant) depression of certain red cell parameters (RBC, Hb and PCV) was observed on several occasions throughout the study.

In high-dose males, no significant differences were noted up to and including 13 weeks; however, from week 26, mean Hb concentrations in high-dose males were significantly lower when compared with controls and remained so throughout the study. Heamatocrit and erythrocyte counts of high-dose males were statistically significantly lower than controls at weeks 39 & 103 and weeks 26 and 39, respectively. In females of this dose group, lower red cell counts at week 26 and heamatocrit at week 52 were the only statistically significant differences from controls. To further investigate the effects noted in the high-dose group, rats from the 180-ppm dose group were sampled at weeks 26 and 103 (both sexes) and week 40 (males only). In the 180-ppm dose group, the same parameters were slightly lower than controls, but were within normal ranges; furthermore, a clear dose response was not evident. Therefore, it is likely that the findings in the high-dose group are incidental and not related to treatment with M-01 (BAM).

Table 5.8.1- 37: Selected haematology findings from the 2-year rat study

Parameter	Dose level (ppm)									
	Males					Females				
	0	60	100	180	500	0	60	100	180	500
Week 13										
Haematocrit (%)	47	-	-	-	44	44	-	-	-	43
Haemoglobin (g%)	15.5	-	-	-	15.1	15.3	-	-	-	15.0
RBC ($x10^6$/cmm)	7.34	-	-	-	7.23	6.79	-	-	-	6.68
Week 26										
Haematocrit (%)	45	-	-	-	44	44	-	-	-	42
Haemoglobin (g%)	14.8	-	-	-	14.3*	14.2	-	-	-	13.9
RBC ($x10^6$/cmm)	8.17	-	-	-	7.47***	7.00	-	-	-	6.65*
Week 39										
Haematocrit (%)	48	-	-	-	46	45	-	-	-	44
Haemoglobin (g%)	15.1	-	-	-	13.9***	14.3	-	-	-	13.7^{NS}
RBC ($x10^6$/cmm)	8.81	-	-	-	8.15***	7.48	-	-	-	7.36^{NS}
Week 40										
Haematocrit (%)	47	-	-	-	46	-	-	-	-	-
Haemoglobin (g%)	14.9	-	-	-	14.6	-	-	-	-	-
RBC ($x10^6$/cmm)	8.38	-	-	-	7.84**	-	-	-	-	-
Week 52										
Haematocrit (%)	47	-	-	-	47	46	-	-	-	43**
Haemoglobin (g%)	14.8	-	-	-	13.8*	13.8	-	-	-	13.6
RBC ($x10^6$/cmm)	8.39	-	-	-	8.08^{NS}	7.70	-	-	-	7.31^{NS}
Week 103										
Haematocrit (%)	46	-	-	45	41*	42	-	-	43	43
Haemoglobin (g%)	14.9	-	-	14.8	13.5*	14.2	-	-	13.9	13.5
RBC ($x10^6$/cmm)	7.80	-	-	7.69	7.34	7.12	-	-	6.78	7.29

*p<0.5, ** p<0.01, *** p<0.001, ^{NS} not significantly different from controls (p<0.05)

Clinical Chemistry

No treatment related changes were noted, all parameters measured were at similar levels to controls.

Urinalysis

No treatment related changes to urinalysis parameters were noted.

6. Pathology

No treatment related macroscopic findings were evident.

There were several isolated incidences of organ weight differences across the control and treated groups, statistically significant in females only. At 500 ppm the relative liver weights and relative adrenal weights were increased in females by 25% and 48% respectively, in comparison with controls, however there was no corresponding effect on the absolute weights, suggesting that the increases in relative weights may have been secondary to the 20% reduction in final bodyweight in females of this dose-group. Particularly with regard to the adrenal weights, for which there were no accompanying histopathological findings. All other changes were slight, not statistically significant and showed no dose response or consistent effect across absolute and relative weights.

Table 5.8.1- 38: Absolute and relative organ weights (g) from the 2-year rat study

Organ		Dose level (ppm) (% difference from controls)									
		Males					Females				
		0	60	100	180	500	0	60	100	180	500
Terminal BW		784	754	747	777	718	602	569	561	526	481**
		-	-	-	-	-8%	-	-	-	-	(-20%)
Heart	Abs.	2.1	2.1	1.9	1.9	2.0	1.6	1.6	1.6	1.4*	1.4**
	Rel.	27	28	25	25	30	28	30	29	27	30
Liver	Abs.	29.3	28.5	28.0	27.8	27.2	23.3	22.1	23.6	22.4	23.6
	Rel.	378	383	375	362	398NS	392	396	425	430NS	489***
		-		-	-	-	-	-	-	-	(+25%)
Spleen	Abs.	1.3	1.2	1.2	1.1	1.2	0.9	0.9	0.9	0.8NS	0.7**
	Rel.	17	16	16	14	17	16	16	16	16	15
Kidneys	Abs.	6.2	6.4	6.0	6.4	6.6	3.8	3.8	4.2	3.6	3.5
	Rel.	81	86	80	84	98NS	65	71	79	69	75NS
Adrenals (x10^{-3})	Abs[1].	99	111	83	92	118	124	118	131	116	152NS
	Rel.	1.2	1.5	1.1	1.2	1.4NS	2.5	2.2	2.5	2.3	3.7*
Gonads	Abs.	5.7	5.6	5.4	5.2	5.7	183[1]	185[1]	252[1]	186[1]	182[1]
	Rel.	75	76	72	69	76	3.3	3.4	5.3	3.6	3.8
Thyroid (x10^{-3})	Abs[1]	45	59	48	47	40	39	42	38	39	35
	Rel.	0.6	0.8	0.6	0.6	0.7	0.7	0.8	0.7	0.8	0.8
Brain	Abs.	2.2	2.2	2.2	2.1	2.2	2.0	2.0	1.9	1.9	1.9
	Rel.	28	30	29	28	32	35	37	37	38	42
Pituitary (x10^{3})	Abs.	30	34	21	29	18	35	58	84	44	28
	Rel.	0.4	0.3	0.3	0.4	0.3	0.6	0.9	1.4	0.9	0.6
Uterus	Abs.	-	-	-	-	-	0.9	0.9	0.9	0.9	0.8
	Rel.	-	-	-	-	-	17	18	17	16	18

*p<0.5, ** p<0.01, [NS] not significantly different from controls (p<0.05), [1] x10^{3}

7. Histopathology

Non-neoplastic findings:

Treatment-related microscopic findings were confined to the liver (see table 5.8.1-39). The liver findings were present only in females at 500 ppm and comprised marked vacuolation, fat deposition and hepatocyte degeneration.

Table 5.8.1- 39: Liver histopathology in males and females sacrificed at week 107

	Dose level (ppm)									
	Males					Females				
	0	60	100	180	500	0	60	100	180	500
N° of rats subjected to liver histology	7	10	10	10	15	8	10	10	10	20
Vacuolation, fat deposition, hepatocyte degeneration	0	1	1	0	1	0	3	0	2	9
Hepatoma	1	0	1	0	1	0	0	0	0	4

Other histopathological findings were isolated, not statistically significant and/or are common findings in laboratory rats of this age and strain. Therefore, they are not considered to be related to treatment.

Neoplastic findings:

No tumours were observed that were considered to be related to treatment with M-01 (BAM). Table 5.8.1-40 below summarises the tumour distribution amongst rats sacrificed at 107 weeks.

Table 5.8.1- 40: Tumours in males and female control and high-dose rats sacrificed at week 107

Tumour type	0 ppm (control)		500 ppm	
	Males	Females	Males	Females
Thyroid adenoma	0	2	1	1
Parathyroid adenoma	1	0	1	0
Adrenal adenoma	1	0	3	2
Pituitary adenoma	1	3	0	1
Pancreatic adenoma	0	0	0	1
Islet cell adenoma	1	0	0	0
Pulmonary adenoma	2	0	0	0
Interstitial cell adenoma	0	0	1	0
Ovarian adenoma (arrhenoblastoma)	0	0	0	2
Uterine carcinoma	0	1	0	0
Mammary tumours (all types)	0	2	0	13
Hepatoma	1	0	1	4
Fibroma	0	0	1	0
Fibro-sarcoma	0	0	1	0
Lipoma	1	0	0	0
Skin tumours (all types)	3	1	0	0
Haemangioma	1	0	0	0
No. animals examined	7	8	15	20

Four females presented with hepatocellular adenomas (two of which were associated with the marked degeneration and fat deposition).The adenomas only appeared in rats sacrificed at the end of the study; in animals that died during the study the incidence of hepatocellular adenoma was 1/0, 0/0, 1/0, 2/1 and 0/1 in M/F at 0, 60, 100, 180 and 500 ppm respectively. The higher incidence of hepatocellular adenomas in females at 500 ppm, was not also statistically significant and no carcinomas were recorded in any female group; furthermore, no increase in hepatocellular tumours was seen in male rats (see table 5.8.1-40 above).

Although contemporaneous HCD on spontaneous incidences of hepatoma in this strain of rats are not available, data published by the animal supplier from 11 studies completed between 1977 and 1985 indicate there was a general background incidence in controls of up to 3.5% for males (average 0.8%) and 1.3% for females (average 0.2%). More recent data published by the supplier, for 20 studies conducted between 2001 and 2009 indicate a range of control group incidence in males of 1% to 6.2% (average 2.0%) and in females of 1% to 10% (average 2.2%), representing a range of incidences in females from 1 to 5 for those 10 studies in which hepatoma occurred in the control group females. Therefore, the incidence of hepatoma is at the upper end of the expected range. The slight increase in late-onset benign hepatocellular adenomas in high-dose females is considered a non-adverse finding associated with lifelong metabolism of M-01 and its metabolites, at high-doses above the MTD (see Anon.; 2019; M-672504-01-1).

The apparent higher number of mammary tumours in the high dose group (13/20) compared with 0/7 in the control), showed no clear dose-response when the rats that died during treatment were included in the calculation. The values were then found to be 16/35, 14/34, 21/34, 16/35 and 20/35 at 0, 60, 180 and 500 ppm, respectively. Therefore, this is an incidental finding and not related to treatment with M-01 (BAM).

III. Conclusion

No carcinogenic activity was detected for M-01 (BAM) in this study, up to and including the high dose of 500 ppm (17.6 and 21.3 mg/kg bw/d in males and females respectively).

Administration of M-01 (BAM) to male and female rats for 2-years resulted in reduced body-weight gain at the high dose of 500 ppm; overall bodyweight gain at week 106, was 16% and 26% lower than controls in males and females, respectively. In females, this was associated with lower food consumption and a slight reduction in food utilisation efficiency.

The liver was identified as a target organ in this study; in high-dose females liver weight was increased by 24.7% and microscopic findings comprising hepatocyte vacuolation and degeneration along with fat depositions were noted. Hepatomas were observed in 4/20 high-dose females (compared with 0/8 in controls); however, there was no similar effect in the males, no hepatocellular carcinomas were observed in any female group and there was no overall increase in tumours in males or females. Furthermore, M-01 (BAM) has been shown to be non-genotoxic in a battery of in vivo and in vitro tests. Therefore, this slight increase in incidence is not considered to show any specific carcinogenic potential. The non-significance of the benign hepatocellular adenomas is presented in Anon.; 2019; M-672504-01-1.

A NOAEL of 180 ppm (equivalent to 5.7 and 8.6 mg/kg bw/d in males and females) is therefore proposed; at the LOAEL of 500 ppm (equivalent to 17.6 and 21.3 mg/kg bw/d), increased relative liver weights and histopathological findings were noted in females and decreased bodyweight gain in both sexes. The overall weight of evidence suggests that M-01 (BAM) is not carcinogenic.

Assessment and conclusion by applicant:

The study was conducted according to US EPA FIFRA 83-1, 83-2 and broadly conforms to OECD TG 453. The study is valid and acceptable to assess the long-term toxicity and carcinogenic potential of M-01 in rats. A NOAEL of 180 ppm (equivalent to 5.7 and 8.6 mg/kg bw/d in males and females) was determined from this study. The weight of evidence suggests that M-01 is not carcinogenic.

Data Point:	KCA 5.8.1/45
Report Author:	[redacted]
Report Year:	2008
Report Title:	Effects of BAM (2,6-dichlorobenzamide) in dietary administration to rats for 2 years (3980/71/138; CA-515-1)
Report No:	M-299662-01-1
Document No:	M-299662-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted in the Addendum 2 to the DAR (2008)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Supportive only

Assessment and conclusion by applicant:

This position paper is a statement from study director discussing the unacceptability of the BAM carcinogenicity study based on a lack of histopathological investigations and statistical analyses. Subsequent histopathological investigations and new statistical analyses have since been conducted on the stored slides: [redacted] 1996; M-234672-01-1. Therefore, this paper is no longer relevant and has not been considered further for this renewal.

In the original 2-year rat study, whilst all animals in the high-dose group were examined histopathologically (35/sex), only a limited number of surviving animals in the intermediate groups (10/sex) and the control group (7 males and 8 females) were examined. Therefore, in 1996, a re-evaluation of the histopathological findings in the slides produced from the liver sections taken in the 2-year rat study was conducted ([redacted] 1996; M-234672-01-1), and an accompanying expert opinion on the carcinogenic potential of M-01 ([redacted] 2007; M-287543-01-1) was prepared. On the basis of this re-evaluation, further statistical evaluation of the liver histopathology was undertaken by Bayer in 2006, and a position paper addressing the carcinogenic potential of M-01 (BAM) was produced. These reports are summarised below.

Data Point:	KCA 5.8.1/10
Report Author:	[redacted]
Report Year:	1996
Report Title:	Re-assessment of liver lesions/tumors from study PDR/49 BAM: Dietary administration to rats for 2 years
Report No:	C034295
Document No:	M-234672-01-1
Guideline(s) followed in study:	US-EPA Guideline 83-2
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Data Point:	KCA 5.8.1/43
Report Author:	[redacted]
Report Year:	2007
Report Title:	Expert opinion on the carcinogenic potential of BAM (2,6-dichlorobenzamide)
Report No:	M-287543-01-1
Document No:	M-287543-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	not applicable
Previous evaluation:	yes, evaluated and accepted Addendum 1 to the DAR (2007)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary:

The pathological findings in slides produced from liver sections in the 2-year rat study (M-234669-01-1) were re-examined. Owing to the age of the study and procedures in use at the time not all archived liver sections were available for re-examination. Additional liver sections from the control rats of three contemporaneous studies were also examined for comparison purposes only and were not referred to in the results.

Non-neoplastic findings (eosinophilic and basophilic hepatocytes) were noted in the 100, 180 and 500 ppm dose-groups, being more pronounced in females. An increased incidence of vacuolation of centrilobular hepatocytes was seen in both sexes at 500 ppm. With regard to neoplastic findings, statistical analysis revealed a slight increase in the incidence in hepatocellular adenomas in female rats treated with 500 ppm (p=0.049 for the pairwise comparison and p=0.003 for the trend test using the time to tumours method). No effect was seen in females at the lower doses or in males at any dose and no hepatocellular carcinomas were seen in females of any dose. An expert statement (M-287543-01-1) later concluded that the observed slight increase in hepatocellular adenomas was not related to treatment with M-01 (referred to as BAM in the report) and that the overall weight of evidence suggests that M-01 (BAM) has no carcinogenic potential.

I. Materials and Methods

1. Study design

In the original study, an apparent increase in benign hepatocellular adenomas was noted in females only; therefore, although the re-evaluation of the liver slides was conducted on both sexes, the statistical analyses was focused on females.

In the original study 35 female rats received 0, 60, 100 or 500 ppm M-01 (BAM). A macroscopic examination was performed on all livers and a microscopic examination was performed on the livers of all decedents (18, 18, 18, 22 and 15 females), as well as a further 8, 10, 10, 10 and 20 terminal females of the 0, 60, 100, 180 and 500 ppm dose-groups respectively.

The archived tissue (embedded in paraffin wax) from the 2-year rat study was re-sectioned as necessary to improve quality. Owing to the age of the study and the procedures in situ at the time of the investigation, only the archived liver sections of the following animals in each group were available and suitable for analysis (see table below).

Table 5.8.1- 41: Liver sections available for re-examination (by animal number)

Group	Dose-level (ppm)	Animal numbers (male)	Animal numbers (female)
1	0 (control)	2-21, 24, 27, 287, 30, 32, 34	176-188, 190-191, 196, 197, 199, 200, 202, 204, 206-208, 210
2	60	36-40, 42-57, 59, 61, 63, 64, 66, 67, 70	211-213, 215-229, 231, 233-237, 240-242, 244
3	100	71-91, 93-95, 97-102, 104, 105	246, 248-263, 268-272, 274-277, 279, 280
4	180	106-119, 122-126, 128, 129, 131, 132, 136, 139	281-300, 302-305, 307-313, 315
5	500	141-164-166-175	316-350

2. Statistical analysis

The data analysed in the statistical analyses included the data obtained from this most recent examination of the slides, in conjunction with information from the original *post-mortem* examination. Slides from animals 205 (control), 214 (60 ppm) and 251 (100 ppm) were not available at re-analysis.

The number of animals with hepatocellular tumours (all benign) were analysed by logrank methods. Tumours were classified as non-incidental (considered to have caused the death of the animal), macroscopic incidental or microscopic incidental and split into time intervals (times at which the deaths occurred) for each category. The expected tumour frequencies were then calculated for each time-interval under a hypothesis of no difference between groups. The observed and expected frequencies were totalled over time intervals and across categories, with the observed and expected tumour frequencies being compared across groups using a χ^2 statistic.

The following statistical analyses were carried out:

One-tailed test for a trend against dose level (repeated excluding the highest dose group until no longer significant)

One-tailed pairwise comparison of each treated group against the control group were carried out.

II. Results and Discussion

A. Results

1. Non-neoplastic findings

Increased incidences of focal and diffuse eosinophilic and basophilic hepatocytes were noted in rats from the 100, 180 and 500 ppm dose groups, with the effect being more pronounced in females. Centrilobular hepatocyte vacuolation was detected in both sexes at 500 ppm, in females associated with areas and foci of eosinophilic hepatocytes; foci are considered an adaptive change and not an adverse effect and therefore do not contribute to the NOAEL of 180 ppm derived for the 2-year rat study.

Table 5.8.1- 42: Non-neoplastic findings

Dose (ppm)	Males					Females				
	0	60	100	180	500	0	60	100	180	500
Total no. examined	**26**	**28**	**32**	**25**	**34**	**25**	**28**	**28**	**32**	**35**
Eosinophilic hepatocytes focal										
Total	5	12	17**	14	21**	5	4	7	16*	23**
Minimal	5	9	16	10	11	5	4	6	13	10
Moderate	0	3	1	1	9	0	0	1	3	12
Marked	0	0	0	0	1	0	0	0	0	1
Eosinophilic hepatocytes diffuse										
Total	1	3	0	2	4	2	2	1	5	18**
Minimal	1	3	0	2	3	2	2	1	5	10
Moderate	0	0	0	0	1	0	0	0	0	7
Marked	0	0	0	0	0	0	0	0	0	1
Basophilic hepatocytes- focal										
Total	7	11	5	6	9	9	10	6	14	23*
Minimal	7	11	5	6	9	8	10	6	14	19
Moderate	0	0	0	0	0	1	0	0	0	4
Basophilic hepatocytes- diffuse										
Total	1	0	1	0	1	3	3	0	2	5
Minimal	1	0	1	0	1	3	3	0	1	3
Moderate	0	0	0	0	0	0	0	0	1	1
Marked	0	0	0	0	0	0	0	0	0	1
Centrilobular hepatocyte vacuolation										
Total (minimal)	5	7	10	5	16*	5	7	5	8	11
Minimal	4	6	9	2	12	4	2	4	8	6
Moderate	0	1	1	2	4	1	5	1	0	4
Marked	1	0	0	1	0	0	0	0	0	1

*p=<0.05, **p=<0.01 with fisher's exact test

2. Neoplastic findings

A slight increased incidence (p=0.049 for the pairwise comparison) in hepatocellular adenomas was observed in female rats at 500 ppm, and statistical analysis using the time to tumour method (trend test p=0.003) demonstrated an increase in hepatocellular tumours in this dose group; no hepatocellular carcinomas were observed in females.

Table 5.8.1- 43: Neoplastic findings

Dose (ppm)	Males					Females				
	0	60	100	180	500	0	60	100	180	500
Total no. examined	26	28	32	25	34	24	28	27	32	35
Hepatocellular adenoma	1	0	1	0	1	0	1	0	0	5
Hepatocellular carcinomas	2	1	2	1	0	0	0	0	0	0

B. Discussion

As in the previous 2-year rat study, this re-evaluation confirmed that the liver was identified as a target organ and that a slightly higher incidence of hepatocellular adenomas was found in females of the high-dose group.

An accompanying expert opinion on the carcinogenic potential of M-01 (BAM), based on the findings of this re-evaluation ([redacted] 2007; M-287543-01-1) and on the overall findings of the original 2-year rat study, concluded that nevertheless, no evidence of carcinogenicity has been observed with M-01 (BAM, 2,6-dichlorobenzamide) for the following reasons:

- The adenomas were only seen at the end of the treatment period
- There were no treatment-related neoplastic findings in male rats in any dose group
- No hepatocellular carcinomas were reported in females of any dose group
- There was no effect on the overall tumour burden in either sex
- M-01 (BAM) had no known structural analogy to carcinogens and is not genotoxic

Assessment and conclusion by applicant:

A re-assessment of the slides from the 2-year rat study and an accompanying expert statement confirmed that M-01 was not carcinogenic.

Data Point:	KCA 5.8.1/42
Report Author:	[redacted]
Report Year:	2006
Report Title:	Position paper - Re-assessment of liver lesions/tumors from study PDR/49 - BAM: Dietary administration to rats for 2 years - Complementary statistical analysis of hepatocellular tumors in female rats
Report No:	M-273467-01-1
Document No:	M-273467-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	not applicable
Previous evaluation:	yes, evaluated and accepted Addendum 1 to the DAR (2007)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

1. Methods

Owing to the discrepancies between the statistical analyses of the original 2-year rat study and the 1996 re-evaluation, the data from the re-analysis of the liver slides from the 2-year rat carcinogenicity study was subject to further statistical analysis. Only the data relating to the females was analysed.

Survival adjusted analyses considering the possible incurrent mortality differences owing to competing toxicity amongst treated groups were performed on lesions.

For non-palpable tumours, each was characterized as either fatal (contributed to the animal's death) or incidental. Only one animal had a fatal tumour (500 ppm); therefore, all tumour data was analysed using incidental tumour procedures.

Logistic regression analysis of tumour prevalence (LOPRAN.EXE) was used, based on the assumption that the diagnosed adenomas were not directly responsible for the animal's death. Treated and control group lesion rates were compared using the corrected score test. The results reflect 1-sided testing.

Statistical significance was evaluated at the 5% and 1% level of significance using SAS programs (version 8.2).

2. Results and discussion

The data obtained in the complementary statistical analysis is summarised in the table below. The liver slides of animals 222 and 235 (both 60 ppm) were excluded from the analysis because no week of death was recorded in the available reports (required for the statistical analyses); however, no adenoma or carcinomas were found in these animals.

Table 5.8.1- 44: Statistical significance of the hepatocellular adenomas in female rats

Dose level (ppm)	0	60	100	180	500
Total no. examined	25	26	27	32	35
Hepatocellular adenoma	0	1	0	0	5
Logistic regression tests[1]	-	NS (p=0.4740)	-	-	NS (p=0.1226)

[1]p-value corresponds to the pairwise comparisons between the treated and control group (all statistical tests were one-sided), NS=not statistically significant

The statistical analyses showed that the treated group females were not different from the control group females with regard to the incidence of liver adenoma findings. The disparity between this statistical analysis and the previous one is addressed in a separate document ([redacted] 2019; M-672006-01-1), which concluded that the logistic regression analysis used here is the most appropriate approach.

Assessment and conclusion by applicant:

A reassessment of the statistical analysis from the 2-year rat study suggested that the incidence of hepatomas in females was no different form the control group.

Data Point:	KCA 5.8.1/41
Report Author:	[redacted]
Report Year:	2006
Report Title:	2.6-dichlorobenzamide (BAM) - Toxicity profile and lack of carcinogeniety potential
Report No:	M-274220-02-1
Document No:	M-274220-02-1
Guideline(s) followed in study:	not specified
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted in the Addendum 1 to the DAR (2007)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Supportive only

Assessment and conclusion by applicant:

This position paper, has been superseded by a new 2019 paper: Anon.; 2019; M-672504-01-1 and therefore has not been considered further for this renewal.

Data Point:	KCA 5.8.1/54
Report Author:	[redacted]
Report Year:	2019
Report Title:	2,6-dichlorobenzamide (BAM): Appropriate statistical analysis of hepatoma incidence among females in a 2-year carcinogenicity study in rats
Report No:	VC/19/037-04
Document No:	M-672006-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive summary:

This review was commissioned as part of the preparations for a renewal submission on behalf of fluopicolide. M-01 (BAM) is a metabolite of fluopicolide. The review considers the various approaches to the statistical analysis of tumour incidence observed in the 2-year carcinogenicity study in rats with M-01 and concludes on the most valid result for statistical significance of the hepatoma incidence in high dose females.

The following statistical approaches were used:

a) Original study report ([redacted] 1971; M-234669-01-1): Statistical analysis comprised Fisher's Exact tests, comparing individual treatment groups to control and concluded no statistical significance (p=0.1219, one-sided).

b) Review of histological sections ([redacted] 1996; M-234672-01-1): Incidences were examined using a survival -adjusted method for non-lethal tumours and concluded a positive trend (p=0.003) for the incidence of hepatomas in the 500ppm female group, and a marginal statistical significance (p=0.049) for the pairwise comparison of this group to controls.

c) Complementary statistical analysis of the same data ([redacted] 2006; M-273467-01-1): A different approach to the survival-adjusted analysis, with logistic regression analysis of tumour prevalence was employed. The logistical regression tests showed that there were no statistically significant differences from control (p=0.1226 for the high dose group).

The review concluded that the logistic regression approach (c) above, is the most robust in terms of efficiency and assumptions being made. Therefore, it is concluded overall that there is no statistical significance for hepatomas among high dose females.

Assessment and conclusion by applicant:

This position paper examined all of the statistical data relating to the apparent increase in hepatomas in female rats in the 2-year combined chronic and carcinogenicity study. The analysis confirmed that the logistic regression was the most robust approach and that the incidence of hepatomas in females was not statistically significant.

Data Point:	KCA 5.8.1/55
Report Author:	Anon.
Report Year:	2019
Report Title:	2,6-dichlorobenzamide (BAM): Review of the significance of benign hepatocellular adenomas in the 2-year carcinogenicity study in rats
Report No:	M-672504-01-1
Document No:	M-672504-01-1
Guideline(s) followed in study:	none
Deviations from current test guideline:	Not applicable
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

This review was commissioned as part of the preparations for a renewal submission on behalf of fluopicolide. M-01 (BAM) is a metabolite of fluopicolide. The review considers the significance of the low incidence of benign hepatocellular adenomas observed in high-dose female rats in the 2-year rat carcinogenicity study with M-01 (BAM).

The review concluded that the observed increase in the incidence of benign hepatomas can be considered as **not demonstrating carcinogenic potential** for the following reasons:

- The incidence was only at the upper end of the historical control range
- The difference from concurrent control was not statistically significant
- The difference occurred at a dosage that exceeded the MTD (as it is normally defined)
- The difference occurred only in in females
- There was no accompanying increase in hepatocellular carcinoma
- There was no increase in overall tumour burden in either sex
- There was no evidence of reduced time-to-tumour (onset at an earlier age)
- A chronic toxicity study in dogs gave no supporting evidence for any early-onset carcinogenicity
- Mechanistic studies did not show potential for inducing cellular proliferation
- BAM is not genotoxic

Assessment and conclusion by applicant:
This review confirms that M-01 has no carcinogenic potential.

Data Point:	KCA 5.8.1/56
Report Author:	Anon.
Report Year:	2018
Report Title:	AE C653711 - Derek Nexus report
Report No:	M-672008-01-1
Document No:	M-672008-01-1
Guideline(s) followed in study:	none
Deviations from current test guideline:	Not applicable
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

M-01 (BAM) was submitted for processing for a Derek Nexus prediction on 9th October 2018. The selected knowledge database was Derek KB 2018 1.1 and the following alerts were reported:

- Alpha-2-mu-Globulin in mammal is EQUIVOCAL
- Carcinogenicity in mammal is PLAUSIBLE
- Hepatotoxicity in mammal is PLAUSIBLE
- Mutagenicity in vitro in bacterium is INACTIVE
- Skin sensitization in mammal is NON-SENSITISER

The plausible alert for carcinogenicity is based on alert 116 (Polyhalogenated aromatic compounds). Examples of these compounds are hexachlorobenzene and para-dichlorobenzene which show some activity in rodents via a non-genotoxic mechanism. However, the activity of para-dichlorobenzene has not been demonstrated in the liver of rats, but in the kidneys by interaction with alpha-2-mu Globulin (see alert above). This mechanism is not relevant in man and can result in kidney tumours in rats; there is no evidence that such activity is occurring in the kidneys of rats after administration of M-01 (BAM). There is also no evidence that hexachlorobenzene can lead to carcinogenicity in man, despite evidence of some activity in rodents.

It is likely that the 'plausible' alert for carcinogenicity is secondary to the alert for alpha-2-mu globulin, which can lead to kidney tumours in male rats. This mechanism is not relevant to man and has not been shown to occur in rats following administration of M-01 (BAM).

Therefore, the Derek Nexus alert for plausible carcinogenicity can be discounted.

Assessment and conclusion by applicant:

This report from Derek Nexus contains some alerts for plausible carcinogenicity; however, the alert is not human relevant.

Data Point:	KCA 5.8.1/44
Report Author:	[redacted]
Report Year:	2008
Report Title:	The non-relevance of the fluopicolide metabolite M01 (AE C653711): 2,6-dichlorobenzamide (also known as BAM)
Report No:	M-300114-01-1
Document No:	M-300114-01-1
Guideline(s) followed in study:	not specified
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted in the Addendum 2 to the DAR (2008)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Supportive only

Assessment and conclusion by applicant:

This position paper discusses the non-relevance of M-01 (BAM). The values discussed in the paper are no longer correct. Therefore, this paper has not been further considered for this renewal. An updated discussion on this topic is available in document M1.

Reproductive toxicity

Data Point:	KCA 5.8.1/49
Report Author:	[redacted]
Report Year:	1993
Report Title:	Results of reproduction study of rats fed diets containing 2,6-dichlorobenzamide (BAM) over three generations
Report No:	CA-512
Document No:	M-301025-01-1
Guideline(s) followed in study:	U.S. EPA-FIFRA Guideline 83-4
Deviations from current test guideline:	The 3-generation, 2 litters/generation design has been superseded by a 2 generation, single litter/generation design, with added requirements for special histopathology for the reproductive system and endpoints relevant for DNT (optional) and ED. These additional requirements were not specifically included in the present study
Previous evaluation:	yes, evaluated and accepted Addendum 2 to the DAR (2008)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

[a] These deficiencies in endpoints/data derive from the fact that they were not specific requirements at the time of the study, although the potential problems they address can often be inferred, or at least signalled, from the nature and pattern of effects seen on other endpoints/data in the study, in context with information from other toxicity studies with the test material.

Executive summary

M-01 (referred to as BAM,2,6-dichlorobenzamide in the report, 99.5% purity) was administered to groups of Long-Evans rat weanlings (10 males and 20 females per group) at 60, 100 or 180 ppm in the diet for 79 days prior to mating, then through a 2-week mating period (males rotated to a second cogroup female during that period) and the subsequent gestation and lactation periods. The F1a litters were discarded at weaning and the F0 parents were re-mated 10 days later. Randomly selected pups (excluding runts) from the F1b litters were maintained on the test diets and mated at 100 days of age. This schedule was repeated for 3 successive generations.

The number of pups in each litter was recorded at birth (day 1) and on day 5, at which time litters larger than 10 were culled to 10. On day 21 the weanlings were counted and weighed, the necessary numbers being retained for the next generation and the remainder sacrificed. The parents were then weighed, sacrificed, and necropsied. Of the F3b weanlings, 10/sex from the control and high dose, and 5/sex from the other groups, were selected for necropsy. Individual body weight and brain, liver and kidney weights were recorded, then brain, heart, lung, liver, spleen, kidney and testes were examined histologically.

There were no clinical signs except for hyper-excitability in 4 litters of the F1b generation only, at 180 ppm. There was no meaningful effect on fertility, litter size or pup survival. Small reductions from control (11-15%), statistically significant, were noted for mean weanling weight for the F1b at 60 and 180 ppm, and for the F3a and F3b at 180 ppm. The lack of significant change at 100 ppm in the F1b made the biological significance of the F1b differences from control doubtful, there was no worsening of the apparent effect through the succeeding generations, and indeed there was no notable effect for either the F2a or the F2b. This inconsistency of outcomes casts doubts overall on the biological significance of any of the apparent weanling weight differences.

Average weight of parents at sacrifice was comparable across all groups, although for F2b females at 180 ppm a 6% reduction from control achieved statistical significance. Other differences from the control mean of a similar magnitude did not achieve significance, and thus the apparent effect in F2b females is considered incidental.

There were no treatment related findings at necropsy for either the parents or the selected F3b offspring. F3b female weanling kidney weight was 12% higher than control for females at 180 ppm (statistically significant). F3b weanling liver weight was 10% higher than control for males at 180 ppm and females at 100 and 180 ppm (statistically significant), and there was an evident trend for a similar increase in liver weight for all groups receiving test material. No histological changes were noted for the F3b tissues examined.

It was concluded in the report that an overall NOAEL of 60 ppm in the diet was demonstrated, while 100 ppm probably had no effect and 180 ppm had a definite effect, based on the parameters measured in the study. This conclusion took account of the pattern of non-significant trends in body weight decreases in the parents, the slightly reduced body weight among weanlings at 180 ppm, and the increased kidney and liver weight in the (F3b) weanlings examined.

The 180 ppm dietary concentration is indicated to be <u>approximately</u> equivalent to 14 mg/kg bw/day of M-01 (BAM), based on a 90-day dietary study (Boschman, 1967), and represents a NOAEL for the adult parents in terms of general functional reproductive performance, under the conditions of the study. The liver and kidney weight increases were likely a consequence of adaptive metabolism. A precautionary overall NOAEL of 100 ppm can be assigned, equivalent to approximately 7.5 mg/kg bw/day parental dose, based on possible slight body weight reduction in dams and offspring at 14 mg/kg bw/day parental dose.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-01 (referred to as BAM, 2,6-dichlorobenzamide in the report)
Purity:	99.5%
Batch no.:	Batch No. 195

2. Vehicle

Vehicle:	Powdered rat diet, from Limonsen Laboratories, CA, USA

3. Test animals

Species:	Rats
Strain:	Long-Evans
Age:	21 days
Weight at start:	Not provided
Source:	[redacted]
Acclimation period:	Not given
Diet:	Powdered rat diet, from Limonsen Laboratories, CA, USA
Water:	Tap water *ad libitum*
Housing:	Wire cages, males housed five/cage, females one/cage
Temperature:	Not stated
Humidity:	Not stated
Air changes:	Not stated
Photoperiod:	Not stated

B. Study design

1. In-life dates: Study completed in October 1970.

2. Animal assignment and treatment

The rats were randomized and assigned to the following the test-groups:

Table 5.8.1- 45: Study design

Group	Dose (ppm)	Number of males	Number of females
1	0	10	20
2	60	10	20
3	100	10	20
4	180	10	20

3. Diet preparation

Stock solutions of 6, 8 and 9% M-01 (BAM) were prepared in acetone and stored refrigerated. Addition of 1.0, 1.25 or 2.0 mL of the respective solution to each kilogram of feed resulted in the desired dietary concentrations of 60, 100 or 180 ppm. The diets were mixed fresh weekly in a twin-shell blender and stored at room temperature. Controls received plain diet. The diets were not analysed.

4. Statistics

Numbers of pups born, percent survival and weanling weight at 21 days, parental weight at sacrifice and organ/bodyweight ratios for F3b weanlings were analysed (presumably by ANOVA) and treated group means compared with control using Dunnett's test. Fertility, gestation, viability and lactation indices were analysed using the Chi^2 test.

C. Methods

1. Exposure and mating plan

The F0 parents were exposed from 21 days of age, for 79 days (to 100 days of age), then paired for mating for a 2-week period (one male to one female, each male rotated to a second cogroup female during that period). The resulting F1a litters were discarded at weaning (day 21 from birth), then the parents were re-mated 10 days later. The parents remained on their test diets throughout, until termination after weaning of their second (F1b) litters (after approximately 28 weeks of exposure overall). From the F1b weanlings, the next generation was randomly selected (excluding runts), retained on the respective test diets and mated at 100 days of age, as for the F0. Their F2a litters were discarded and after a further 10 days they were re-mated to produce F2b litters, from which the parents of the next generation were selected and retained on the test diets, as before. The F3a litters were discarded and the F3b litters then provided weanlings for terminal investigations.

2. Clinical observations

The animals were observed for any clinical signs of toxicity (frequency not stated).

3. Body weight

Individual bodyweights of parents were recorded at sacrifice. Weanlings (day 21) were weighed as an entire litter, individual weights being derived by dividing the litter weight by the total number of pups.

4. Food intake

Food intake was not measured. No evaluation of achieved exposure possible.

5. Fertility, gestation, litter survival

Offspring were counted (total in each litter) on the day of birth (day 1), then on days 5 and 21 (at weaning).

The fertility index (percent of matings resulting in pregnancy), gestation index (percent of pregnancies resulting in live pups), viability index (percent of pups born surviving to day 5) and lactation index (percent pup survival from day 5 to weaning at day 21) were calculated.

Not monitored (deviations from current guidance/practice): Oestrous cycles, pre-coital time, mating index, gestation length, individual pup counts and weights by sex, developmental milestones (including sexual maturation).

6. Necropsy, gross pathology

The parents were necropsied (gross examination only). Of the offspring at termination (at weaning), from the F3b generation 10/sex from the control and high dose groups, and 5/sex from the other groups, were necropsied, and brain, liver and kidney weights were recorded, then the brain, heart, lungs, liver, spleen, kidneys and testes were preserved in formalin.

7. Histopathology

The F3b offspring preserved tissues (above) were examined histopathologically.

Not examined (deviations from current guidance/practice): Pituitary, adrenals, thyroid, ovaries, uterus, epididymides, prostate, seminal vesicles and coagulating gland. Full detailed evaluation of testes (with attention to the spermatogenic cycle) and the post-lactational ovary, not done.

II. Results and Discussion

1. Clinical results

Rats in the treated groups were indistinguishable from the controls and there were no mortalities among the adults. A small number of pups (within four F1b litters) at 180 ppm were recorded as being 'hyper-excitable'.

2. Body weight (parents)

Average weight of parents at sacrifice was generally comparable across all groups. While the reduction from control in F2b females at 180 ppm achieved statistical significance, other differences from control of a similar magnitude did not achieve significance, and in the absence of a consistent dose relationship none of the intergroup differences were considered biologically meaningful.

Table 5.8.1- 46: Body weight of parents at termination in the 3-generation reproduction study with M-01, BAM (group mean (g) and % difference from control)

	Males				Females			
	Dose (ppm in diet)							
	0	60	100	180	0	60	100	180
F0	488	499 +2.3%	488 0	481 -1.4%	339	328 -3.2%	331 -2.4%	319 -5.9%
F1b	488	499 +2.3%	465 -4.7%	461 -5.5%	339	317 -6.5%	323 -4.7%	320 -5.6%
F2b	490	487 -0.6%	456 -6.9%	466 -4.9%	325	322 -0.9%	309 -4.9%	305* -6.2%

Statistical significance of difference from control: * P<0.05 (Dunnett's test)

3. Reproductive performance

There was no effect of M-01 (BAM) on fertility, as indicated by the fertility indices, and the gestation index was optimal (100%) in all groups and generations.

Table 5.8.1- 47: Fertility indices in the 3-generation reproduction study with M-01 (BAM)

Generation		Dose (ppm in diet)			
		0	60	100	180
F1a	Ratio	17/20	18/20	20/20	20/20
	Index (%)	85	90	100	100
F1b	Ratio	20/20	18/20	18/20	19/19
	Index (%)	100	90	90	100
F2a	Ratio	18/20	19/20	20/20	19/20
	Index (%)	90	95	100	95
F2b	Ratio	18/20	20/20	20/20	20/20
	Index (%)	90	100	100	100
F3a	Ratio	19/20	20/20	20/20	20/20
	Index (%)	95	100	100	100
F3b	Ratio	19/20	20/20	19/20	20/20
	Index (%)	95	100	95	100

4. Litter size, survival and weanling weight

There were no meaningful differences from control in terms of litter size or pup survival. Occasional statistically significant departures occurred from control values but were inconsistent in direction and/or without dose relationship and were considered incidental.

Average weights of pups at 21 days were significantly less than the controls for F1b litters in the 60 and 180 ppm groups and for F3a and F3b litters in the 180 ppm group. The biological significance of the reduced weight in the 60 ppm group was considered doubtful, considering the lack of weight reduction in the 100 ppm group, and there was no worsening of the apparent effect through the succeeding generations (there was no notable effect for either the F2a or the F2b). Such an inconsistency of outcomes casts doubts overall on the biological significance of any of the apparent weanling weight differences.

Table 5.8.1- 48: Litter parameters in the 3-generation reproduction study with M-01 (BAM)

Mean per litter ± Standard Error[a]	Dose (ppm in diet)			
	0	60	100	180
F1a generation				
Litters born (day 1)	17	18	20	20
Total pups born/litter	10.5 ± 0.4	9.9 ± 0.73	10.6 ± 0.4	9.3 ± 0.6
Live on day 5/litter	10.1	9.5	10.0	9.0
Viability Index (%)	172/178 (96.6)	171/179 (95.5)	190/212 (89.6)**	180/186 (96.8)
Live pups (litters) d 21	6.9 (17)	6.7 (18)	7.6 (20)	6.1 (20)
Lactation Index (%)	117/159 (73.6)	121/162 (74.7)	145/174 (83.3)*	115/172 (66.9)
Day 21 pup weight (g)	38.7 ± 1.5	38.0 ± 1.2	37.9 ± 1.1	41.0 ± 1.5*
F1b generation				
Litters born (day 1)	20	18	18	19
Total pups born/litter	10.6 ± 0.6	12.2 ± 0.4	11.0 ± 0.5	11.3 ± 0.6
Live on day 5/litter	9.8	11.0	10.1	10.9
Viability Index (%)	197/213 (92.5)	198/219 (90.4)	181/198 (91.4)	207/214 (96.7)
Live pups (litters) d 21	6.0 (20)	6.8 (18)	6.5 (18)	7.3 (19)
Lactation Index (%)	114/180 (63.3)	122/172 (70.9)	104/163 (63.8)	132/179 (73.7)*
Day 21 pup weight (g)	38.9 ± 1.5	33.4 ± 0.9*	35.1 ± 1.5	33.1 ± 1.3*
F2a generation				
Litters born (day 1)	18	19	20	19
Total pups born/litter	8.8 ± 0.5	9.3 ± 0.5	8.7 ± 0.7	9.6 ± 0.3
Live on day 5/litter	8.3	9.3	8.4	9.1
Viability Index (%)	150/158 (94.9)	177/180 (98.3)	167/174 (96.0)	172/182 (94.5)
Live pups (litters) d 21	7.7 (18)	8.4 (19)	7.6 (20)	8.8 (19)
Lactation Index (%)	139/144 (96.5)	160/166 (96.4)	151/156 (96.8)	167/170 (98.2)
Day 21 pup weight (g)	34.4 ± 1.5	32.6 ± 1.4	32.8 ± 1.5	32.7 ± 1.1
F2b generation				
Litters born (day 1)	18	20	20	20
Total pups born/litter	9.4 ± 0.7	10.2 ± 0.5	10.0 ± 0.5	10.4 ± 0.8
Live on day 5/litter	9.3	10.1	9.7	9.8
Viability Index (%)	161/170 (94.7)	202/205 (98.5)*	194/200 (97.0)	186/207 (89.9)
Live pups (litters) d 21	8.6 (18)	8.8 (20)	9.0 (20)	8.2 (20)
Lactation Index (%)	146/151 (96.7)	175/184 (95.1)	180/182 (98.9)	156/163 (95.7)
Day 21 pup weight (g)	38.6 ± 1.0	35.5 ± 1.4	36.7 ± 1.6	36.8 ± 1.1
F3a generation				
Litters born (day 1)	19	20	20	20
Total pups born/litter	9.1 ± 0.7	11.2 ± 0.4**	10.6 ± 0.4	10.8 ± 0.3*
Live on day 5/litter	9.1	11.0	10.2	9.7
Viability Index (%)	164/172 (95.3)	219/224 (97.8)	205/211 (97.2)	194/215 (90.2)
Live pups (litters) d 21	7.3 (19)	8.8 (20)	8.3 (20)	8.0 (20)
Lactation Index (%)	132/152 (86.8)	159/193 (82.4)	157/187 (84.0)	160/178 (89.9)
Day 21 pup weight (g)	35.0 ± 1.3	32.9 ± 0.6	34.4 ± 1.5	30.9 ± 1.0*
F3b generation				
Litters born (day 1)	19	20	19	20
Total pups born/litter	9.8 ± 0.7	11.2 ± 0.4	10.5 ± 0.6	10.2 ± 0.5
Live on day 5/litter	9.9	10.8	10.0	9.2
Viability Index (%)	178/186 (95.7)	217/223 (97.3)	190/199 (95.5)	175/203 (86.2)**
Live pups (litters) d 21	8.5 (19)	7.5 (20)	8.1 (19)	8.3 (20)
Lactation Index (%)	153/164 (93.3)	175/194 (90.2)	145/168 (86.3)*	157/167 (94.0)
Day 21 pup weight (g)	36.1 ± 1.1	33.8 ± 1.0	35.3 ± 1.2	31.1 ± 1.0**

a Where given in the report
Statistical significance of difference from control: * P<0.05, ** P<0.01 (Dunnett's test)

5. Gross pathology and histopathology

No abnormalities were seen at parental necropsies, except for lung pathology in one F2b male in the 100 ppm group that was considered unrelated to treatment.

Examination of the selected F3b weanlings revealed only a distended bladder and fluid-filled kidneys in one male in each of the 100 and 180 ppm groups, and a slightly enlarged spleen and supernumerary liver lobe in one 180 ppm female. These are considered incidental. F3b female weanling kidney weight was 12% higher than control for females at 180 ppm (statistically significant), and F3b weanling liver weight was approximately 10% higher than control for both sexes at all dosages, statistically significant for males at 180 ppm and for females at 100 and 180 ppm, while no histological changes were noted for any of the F3b tissues examined. It is likely that the liver and kidney weight effects were related to early adaptive metabolic changes, considering that the weanlings would have begun eating the diet during the last week of lactation, in addition to any exposure through the milk.

Table 5.8.1- 49: Organ weights, absolute (g) and relative to body weight (%) of F3b weanlings in the 3-generation reproduction study with M-01 (BAM) (group mean ± Standard Error)

Organ		Dose level (ppm)			
		0	60	100	180
Males					
Brain	Abs.	1.22	1.25	1.25	1.23
	Rel.	3.50 ± 0.06	3.57 ± 0.08	3.53 ± 0.09	3.53 ± 0.07
Kidney	Abs.	0.434	0.457	0.475	0.450
	Rel.	1.24 ± 0.03	1.31 ± 0.05	1.34 ± 0.05	1.28 ± 0.05
Liver	Abs.	1.42	1.55	1.53	1.56
	Rel.	4.05 ± 0.09	4.44 ± 0.08	4.33 ± 0.21	4.46 ± 0.10*
	%diff		+9.6%	+6.9%	+10%
Females					
Brain	Abs.	1.20	1.21	1.20	1.18
	Rel.	3.51 ± 0.06	3.46 ± 0.10	3.44 ± 0.07	3.42 ± 0.05
Kidney	Abs.	0.435	0.470	0.467	0.487
	Rel.	1.26 ± 0.03	1.34 ± 0.04	1.33 ± 0.04	1.41 ± 0.02**
	%diff		+6.3%	+5.6%	+12%
Liver	Abs.	1.46	1.61	1.62	1.62
	Rel.	4.24 ± 0.06	4.60 ± 0.13	4.62 ± 0.05*	4.65 ± 0.11**
	%diff		+8.5%	+9.0%	+9.7%

Statistical significance of difference from control: * P<0.05 (Dunnett's test)

III. Conclusions

It was concluded in the report that an overall NOAEL of 60 ppm in the diet was demonstrated, while 100 ppm probably had no effect and 180 ppm had a definite effect, based on the parameters measured in the study. This conclusion took account of the pattern of non-significant trends in body weight decreases in the parents, the slightly reduced body weight among weanlings at 180 ppm, and the increased kidney and liver weights in the (F3b) weanlings examined.

The liver and kidney weight increases were likely a consequence of adaptive metabolism. The 180 ppm dietary concentration is indicated to be approximately equivalent to 14 mg/kg bw/day of M-01 (BAM), based on a 90-day dietary study (Boschman, 1967; M-234461-01-1), and represents a NOAEL for the adult parents in terms of general functional reproductive performance under the conditions of the study. A precautionary overall NOAEL of 100 ppm can be assigned, equivalent to approximately 7.5 mg/kg bw/day parental dose, based on possible slight body weight reduction in dams and offspring at 14 mg/kg bw/day parental dose.

Assessment and conclusion by applicant:

The study was conducted according to a 3-generation design which has now been superseded; however, the study is acceptable for investigating limited endpoints focused on general, functional reproductive performance, considering its various limitations. The was no evidence of reproductive toxicity in this 3-generation study with M-01.

Data Point:	IICA 5.8.1/48
Report Author:	[redacted]
Report Year:	1993
Report Title:	2,6-dichlorobenzamide: Oral (gavage) teratology study in the rabbit
Report No:	CA-2613
Document No:	M-301030-01-1
Guideline(s) followed in study:	US-EPA Guideline 83-3
Deviations from current test guideline:	12 pregnancies/group instead of current requirement for 20/group at termination. Treatment over major organogenesis (GD 7-19), instead of current requirement for at least from implantation until the day before expected parturition.
Previous evaluation:	yes, evaluated and accepted Addendum 2 to the DAR (2008)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

M-01 (BAM, referred to as 2,6-dichlorobenzamide in the report, 99.4% purity) was administered to groups of New Zealand White rabbits (16 mated/group) once daily by oral gavage on gestation days (GD) 7-19 (GD 0 = day of mating), at dosages of 0 (vehicle control), 10, 30 or 90 mg/kg bw/day. The dosing vehicle was 1% aqueous gum tragacanth, at a dose volume of 2 ml/kg bw, and dose volumes throughout were based on individual body weight at GD 7.

The rabbits were checked daily for clinical signs of toxicity, and body weight was recorded on GD 0, 7, 13, 19, 23 and 28. Food consumption was recorded for the intervals GD 0-7, 7-13, 13-19, 19-23 and 23-28. Surviving females were killed on GD 28 and necropsied. The ovaries and uterus were removed and the numbers of corpora lutea and implants were recorded (live foetuses, early and late intra-uterine

deaths). Live foetuses were killed, examined externally, then dissected for examination of the viscera. After initial fixation in alcohol, the heads were sliced through the fronto-parietal suture to permit examination of the brain in that region. Following processing and staining with Alizarin, the foetal skeletons were examined.

Five females at 90 mg/kg/day were killed between GD 19 and 22 following abortion or deteriorated condition. These deaths were considered treatment-related following body weight loss, thin appearance and reduced food intake in the majority of these animals during the dosing period. However, no consistent treatment-related macroscopic changes were observed at necropsy. Two females receiving 30 mg/kg/day were killed on GD 12 or 14 following deterioration in physical condition, while one female receiving 10 mg/kg/day was killed following abortion on GD 23, again with no necropsy findings. Among controls, one female was killed following abortion on GD 24 and a further female was killed on GD 20 following deterioration in physical condition. Review of the information regarding these deaths (see the related review document CA 5.6.3) indicates that the abortions in the 10 and 30 mg/kg/day groups were incidental, while the other losses in those groups were probably attributable to dosing accidents. Meanwhile, the abortions at 90 mg/kg/day appeared related to the marked maternal toxicity at that dosage, and the other losses were again indicated to have probably been dosing errors; there is also the context of the incidence of mortality in the control group.

There was a marked decrease in weight gain during the first week of dosing at 90 mg/kg/day, with 9 of the 11 survivors losing weight over this period. The weight gain of these animals was then less than control for the rest of the dosing period. The low and intermediate dose animals showed no effect on body weight gain. Food consumption levels reflected the changes in body weight during dosing, with some recovery after cessation of dosing.

There was no effect of treatment on numbers of corpora lutea or implantations, or on pre- and post-implantation loss. Live litter size was comparable across all groups, and the foetal sex ratio was unaffected. There was a marginal reduction in mean foetal weight (-6% from control) at 90 mg/kg/day. However, it was not statistically significant, was within the normal background range, and was considered merely secondary to the maternal toxicity. Mean foetal weight at 10 and 30 mg/kg/day was indistinguishable from control.

There was no treatment relationship in the incidences of major foetal abnormalities. There was a slightly higher foetal incidence of minor external and visceral defects in the 30 and 90 mg/kg/day groups, but no adverse effect of treatment was evident from the type and distribution of defects observed. The overall foetal incidence of visceral variants was also slightly higher in the 90 mg/kg/day group, owing to increased prevalence of the common, non-adverse variant abnormal common carotid artery. In the absence of other alterations in prevalence of minor heart or vessel abnormalities/variants, an association with treatment was considered very unlikely.

The foetal incidence of minor skeletal defects in the BAM treated groups was higher than in the control group. There was no indication of a dose response relationship and the group mean values were mostly similar to background control. Despite the intergroup variations, the type and incidence of skeletal variants were considered generally similar in all groups and the various indicators of ossification status were considered together not to indicate an adverse effect of treatment.

It was concluded in the report that 90 mg/kg bw/day BAM in rabbits was associated with maternal toxicity characterized by mortality following deterioration in condition and/or abortion, body weight loss and reduced food consumption. Effects on foetuses from surviving dams at this dosage were confined to a marginal reduction in foetal weight (not statistically significant), considered secondary to the maternal toxicity observed. There was no indication of an effect on foetal development (incidence or type of external, visceral or skeletal alterations). There were no adverse effects of treatment at 30 mg/kg bw/day of BAM, and this dose was concluded as a NOAEL for maternal toxicity, while a developmental NOAEL can be set at ≥90 mg/kg bw/day, the highest dose applied.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-01 (BAM, referred to as 2,6-dichlorobenzamide in the report)
Purity:	99.4%
Batch no.:	FUX 001000

2. Vehicle

Vehicle:	1% aqueous gum tragacanth

3. Test animals

Species:	Rabbits
Strain:	New Zealand White
Age:	Not provided
Weight at start:	3.10 to 4.14 kg
Source:	[redacted]
Acclimation period:	15 days
Diet:	Ranch Rabbit Diet (pelleted) on arrival, then Standard Rabbit Diet SQC from SDS Ltd, UK
Water:	Filtered tap water *ad libitum*
Housing:	Steel grid-floored cages, one/cage
Temperature:	16-22°C
Humidity:	40-70% RH
Air changes:	15/h
Photoperiod:	14 h light

B. Study design

1. In-life dates: 13 March to 05 May 1986

2. Animal assignment and treatment

Each female rabbit was mated naturally with a single male of the same strain and source. The day of mating was considered as gestation day (GD) 0.

The mated females were assigned to the following the test-groups, using a stratified randomization procedure based on body weight. The dose levels were based on information from previous dose range finding studies:

Table 5.8.1- 50: Study design

Group	Dose (mg/kg/day)	Number of females
1	0	16
2	10	16
3	30	16
4	90	16

The test material was formulated in the vehicle and individual doses were administered once daily by oral gavage at a dose volume of 2 mL/kg/day, based on body weight at GD 7. Samples of the dosing formulations (from the first batch prepared during the study) were analysed by the study sponsor for achieved concentration (details of the method not provided in the study report).

3. Clinical observations

The females were checked twice daily for mortality/morbidity. They were examined daily during gestation for any clinical signs of reaction to treatment.

4. Body weight

Individual body weights were recorded on GD 0, 7, 13, 19, 23 and 28.

5. Food consumption

Individual food consumption was recorded for the periods GD 0-7, 7-13, 13-19, 19-23 and 23-28.

6. Necropsy and pathology

Any dead or moribund animals, or any aborting their pregnancy, were necropsied to determine if possible, the cause of each animal's condition, and the pregnancy status was recorded.

Surviving females were killed on GD 28 and necropsied, any abnormalities being recorded. The ovaries and uterus were removed and examined for the numbers of corpora lutea and implants (live foetuses, early and late intra-uterine deaths). Each live foetus was weighed and examined externally, killed and dissected for examination of the viscera (and to determine sex), then eviscerated and fixed in alcohol. The head was sliced through the fronto-parietal suture to permit examination of the brain in that region. Following processing and staining with Alizarin, the foetal skeleton was then examined.

Foetal abnormalities were categorized as major (rare and/or possibly lethal), minor (common deviations from normal), or variants (retarded sternebral or phalangeal ossification, asymmetric pelvic insertion, or supernumerary ribs).

7. Statistical analysis

Appropriate analyses were conducted as necessary: Analysis of variance (parametric or non-parametric), with t-tests or the Wilcoxon rank-sum test for differences between control and treated groups (2-sided tests). Incidence data were analysed with Fisher's two-sum randomization (permutation) test with Monte Carlo simulation for computing significance levels; a square-root transformation was used for weighting the incidences and adjusting for litter size. Significances were quoted for a one-sided risk. The litter was the experimental unit in all cases.

II. Results and Discussion

1. Dosing formulation analysis

Analysis of formulations prepared for the first day of dosing showed that they were acceptably close to the nominal concentrations: 5.2, 14.8 and 43.1 mg/mL for the respective dose groups.

2. Clinical observations, mortality

Three females receiving 90 mg/kg/day were killed following abortion on days 19, 21 or 22 of gestation. A further two females were killed following deterioration in physical condition on days 21 or 22 of gestation. These abortions and deaths were considered treatment-related following body weight loss, thin appearance and reduced food intake in the majority of these animals during the dosing period. However, no consistent treatment-related macroscopic changes were observed at necropsy.

Two females receiving 30 mg/kg/day were killed following deterioration in physical condition, on days 12 and 14 of gestation. One female receiving 10 mg/kg/day was killed following abortion on day 23 of gestation, with no necropsy findings.

Among controls, one female was killed following abortion on day 20 of gestation and a further female was killed following deterioration in physical condition on day 24 of gestation.

Although M-01 (BAM) involvement in the 10 and 30 mg/kg/day group deaths could not be entirely excluded, it was considered unlikely in the context of the similar incidence of mortality in the control group.

The majority of high dose females had a thin appearance and fur staining, which persisted after cessation of dosing on GD 19. The intermediate and low dose females were comparable with control in clinical condition, any findings being of the incidental type commonly found in the strain of animals.

The information regarding these deaths (see the related review document CA 5.6.3) indicates that the abortions in the 10 and 30 mg/kg/day groups were incidental, while the other losses in those groups were probably attributable to dosing accidents. Meanwhile, the abortions at 90 mg/kg/day appeared related to the marked maternal toxicity at that dosage, and the other losses were again indicated to have probably been dosing errors.

3. Body weight

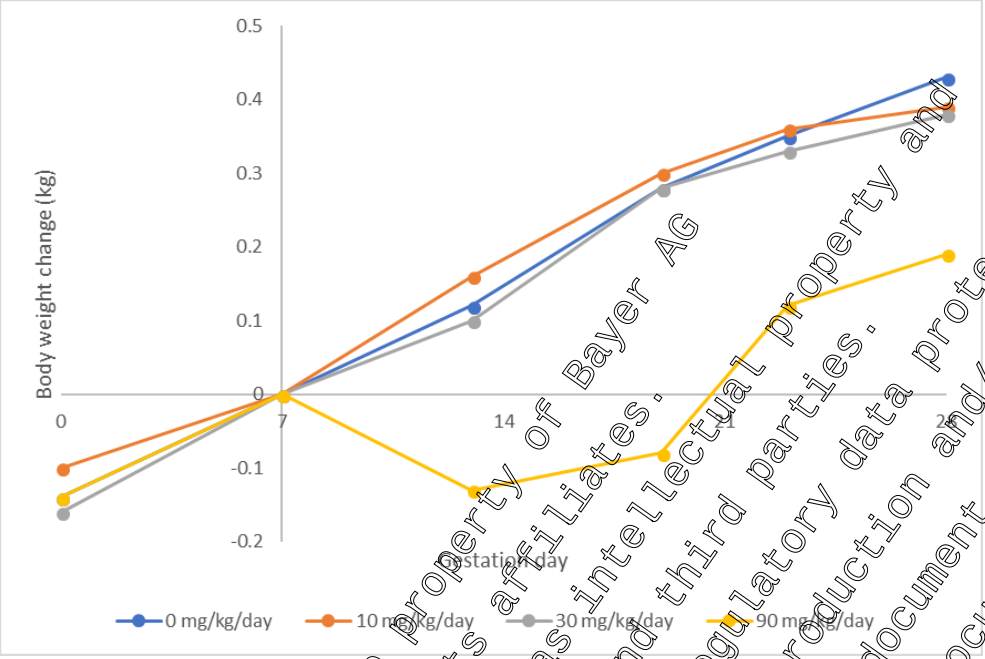
There was a marked decrease in weight gain during the first week of dosing at 90 mg/kg/day, with 9 of the 11 survivors losing weight over this period. The weight gain of these animals was then less than control for the rest of the dosing period. The low and intermediate dose animals showed no effect on body weight gain.

Table 5.8.1- 51: Body weights in the rabbit teratology study with M-01 (BAM) (group mean, kg)

Gestation Day	Dose level (mg/kg bw/day)			
	0	10	30	90
0	3.60	3.55	3.57	3.66
7	3.74	3.65	3.73	3.80
13	3.86	3.81	3.83	3.67
19	4.02	3.95	4.01	3.72
23	4.09	4.01	4.06	3.92
28	4.17	4.04	4.11	3.99
% change 0-28	**16%**	**14%**	**15%**	**9%**
% change 7-19	**7.5%**	**8.2%**	**7.5%**	**-2.1%*****

Statistical significance of difference from control: *** P<0.001(only body weight gains analysed)

Figure 5.8.1- 1: Body weight change in the rabbit teratology study with M-01 (BAM) (group mean, kg)



4. Food consumption

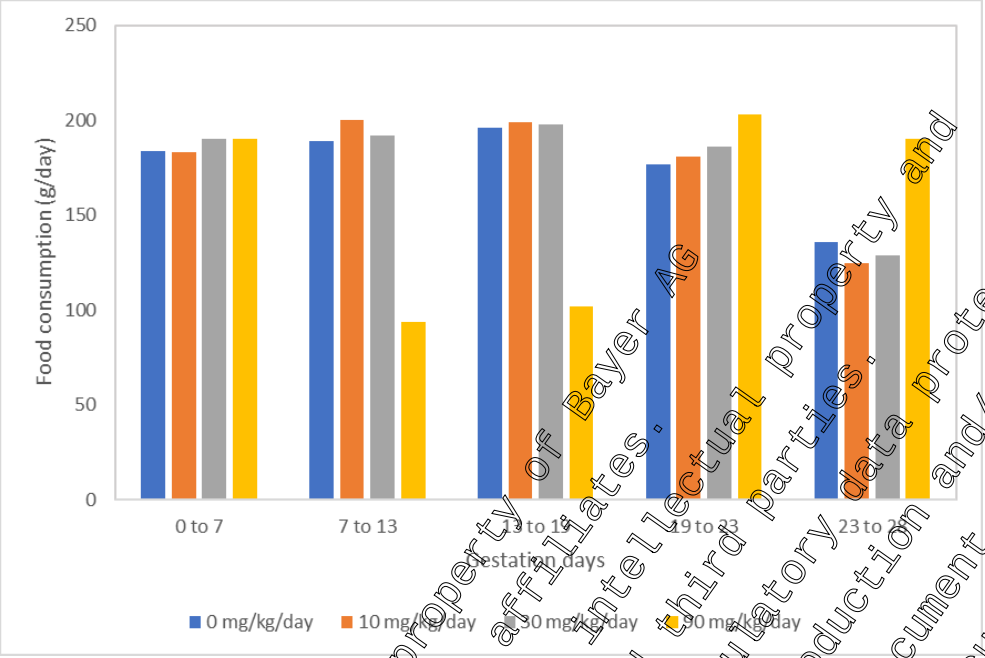
Food consumption levels reflected the changes in body weight during dosing, with some recovery after cessation of dosing.

Table 5.8.1- 52: Food consumption in the rabbit teratology study with M-01 (BAM) (group mean, g)

	Dose level (mg/kg bw/day)			
Gestation Day	**0**	**10**	**30**	**90**
0-7	184	183	190	190
7-13	189	200	192	94***
13-19	196	199	198	102***
19-23	177	181	185	203
23-28	136	125	129	190

Statistical significance of difference from control: *** P<0.001

Figure 5.8.1- 2: Food consumption in the rabbit teratology study with M-01 (BAM) (group mean, g)



5. Pregnancy parameters

There was no effect of treatment on numbers of corpora lutea or implantations or on pre- and post-implantation loss. Live litter size was comparable across all groups, and the foetal sex ratio was unaffected. There was a marginal reduction in mean foetal weight (-6% from control) at 90 mg/kg/day, not statistically significant, and only just outside the normal background range for group means (34.7 to 39.4 g, from six studies). Foetal weight at 10 and 30 mg/kg/day was indistinguishable from control.

Table 5.8.1- 33: Pregnancy parameters in the rabbit teratology study with M-01 (BAM)

	Dose level (mg/kg bw/day)			
	0	**10**	**30**	**90**
Number of pregnant females	16	16	16	16
Surviving to GD 28	14	15	14	11
Mean number of corpora lutea	10.9	9.7	10.1	10.3
Mean implantations	10.4	9.0	8.4	9.5
Pre-implantation loss (%)	4.6	7.5	16.3	8.0
Number (mean) early intrauterine deaths	18 (1.3)	7 (0.5)	2 (0.1)	5 (0.5)
Number (mean) late intrauterine deaths	6 (0.4)	3 (0.2)	6 (0.4)	3 (0.3)
Post-implantation loss (%)	16.6	7.4	6.8	8.7
Mean live foetuses	8.5	8.3	7.9	8.6
Foetal sex ratio m:f	1 : 0.95	1 : 1.16	1 : 0.75	1 : 0.79
Mean litter weight (g)	310.6	294.8	284.9	293.3
Mean foetal weight (g)	36.0	36.4	36.8	33.9
Background range (6 studies)				34.7 – 39.4
Mean foetal weight, males (g)	36.6	36.8	36.8	33.5
Mean foetal weight, females (g)	35.7	36.3	37.0	33.7

Litter means calculated from those dams with live foetuses at scheduled termination

6. Foetal abnormalities and variants

The major abnormalities noted are shown in the following table. Although there were a few more in the low dose group than in control, there was evidently no dosage relationship and all occurrences were considered incidental.

Table 5.8.1- 54: Major foetal abnormalities in the rabbit teratology study with M-01 (BAM) (number of foetuses and litters affected)

Abnormality	Dose Level (mg/kg bw/day)			
	0	10	30	90
Number of foetuses (litters) examined	121 (14)	125 (15)	110 (14)	95 (11)
Multiple vertebral defects including fusion and agenesis	1	1a	1	1b
Unilateral arthrogryposis		1a		
Left 12th rib absent, bifurcated rib				1b
Additional ossification site between 1st and 2nd lumbar vertebral arches	1			
Multiple defects including cebocephaly, unilateral arthrogryposis and vertebral defects		1		
Fused ribs		2 (2)		
Bifurcated ribs		2 (1)		
Right 12th rib absent		1		
Additional floating rib			1	

Showing: number of foetuses (number of litters) affected
a: Abnormality occurred in the same foetus
b: Abnormality occurred in the same litter

The incidences of minor abnormalities and variants are shown in the following table:

Table 5.8.1- 55: Minor foetal abnormalities/variants in the rabbit teratology study with M-01 (BAM) (number and % of foetuses affected)

Minor abnormality/variant	Dose Level (mg/kg bw/day)				Relevant background control data (%)[a]
	0	10	30	90	
Number of foetuses (litters) examined	**121 (14)**	**125 (15)**	**110 (14)**	**95 (11)**	
External and visceral					
Ocular opacity, bilateral				1 (1.1)	
Dark foetus				1 (1.1)	
Atrium/atria reduced	5 (4.1)	4 (3.2)	8 (7.3)	3 (3.2)	
Atrium/atria enlarged	1 (0.8)	7 (5.6)		3 (3.2)	
Post-caval lung lobe agenesis			1 (0.9)	3 (3.2)	1.2
Abnormal common carotid	21 (17.4)	23 (18.4)	11 (10.0)	26 (27.4)	17.9
Red fluid in abdomen				1 (1.1)	
Incomplete closure abdominal muscle layer		3 (2.4)	1 (0.9)		
Gaseous distension of stomach	2 (1.7)	5 (4.0)	4 (3.6)	3 (3.2)	
Gall bladder reduced in size	2 (1.7)			2 (2.1)	
Gall bladder agenesis	1 (0.8)	1 (0.8)		1 (1.1)	
Kidney reduced in size			1 (0.9)		
Ovary reduced in size			2 (1.8)		
Ovaries cystic				1 (1.1)	

Minor abnormality/variant	Dose Level (mg/kg bw/day)				Relevant background control data (%)[a]
	0	10	30	90	
Number of foetuses (litters) examined	121 (14)	125 (15)	110 (14)	95 (11)	
Skeletal:					
Minor fusion of frontals		1 (0.8)	2 (1.8)		
Interparietal incomplete ossification	1 (0.8)	2 (1.6)	9 (8.2)	7 (7.4)	
Interparietal bipartite	1 (0.8)	1 (0.8)	2 (1.8)	4 (4.2)	0.3
Interparietal asymmetrically ossified				1 (1.1)	
Parietals incompletely ossified		1 (0.8)	1 (0.9)	1 (1.1)	
Cleft in parietal		2 (1.6)	1 (0.9)		
Nasals incompletely ossified		1 (0.8)			
Cleft in nasal		1 (0.8)	1 (0.9)		
Sutural bone between frontals	9 (7.4)	6 (4.8)	6 (5.5)	3 (3.2)	
Sutural bone between parietals	1 (0.8)	1 (0.8)	2 (1.8)	2 (2.1)	
Sutural bone between nasals			1 (0.9)		
Sutural bone between parietals and frontals	1 (0.8)	1 (0.8)		1 (1.1)	
Abnormal suture line between parietals		1 (0.8)	1 (0.9)		
Frontals incompletely ossified	33 (27.3)	36 (28.8)	30 (27.3)	38 (40.0)	
Cleft in frontal(s)	43 (35.5)	28 (22.4)	22 (20.0)	26 (27.4)	
Cervical centrum 3 asymmetrically ossified				1 (1.1)	
Thoracic centrum 10 incompletely ossified				1 (1.1)	
Clubbed ribs	3 (2.5)	4 (3.2)	5 (4.5)	3 (3.2)	
Spatulate ribs			1 (0.9)		
Cervical ribs, vertebra 7	1 (0.8)	1 (0.8)	2 (1.8)		
Supernumerary ribs, lumbar vertebra 1	51 (42.1)	35 (28.0)	39 (35.5)	36 (37.9)	37.0
Supernumerary rib, lumbar vertebra 1	9 (7.4)	14 (11.2)	11 (10.0)	18 (18.9)	12.9
Sternebra(e) asymmetrically ossified	8 (6.6)	10 (8.0)	11 (10.0)	9 (9.5)	
Sternebra(e) bipartite	1 (0.8)	6 (4.8)	3 (2.7)	2 (2.1)	
Minor fusion of sternebrae	2 (1.7)	10 (8.0)	5 (4.5)	7 (7.4)	
Additional site between sternebrae 5 and 6	4 (3.3)	2 (1.6)	6 (5.5)		
Additional site fused to sternebra 5	2 (1.7)	3 (2.4)			
Sternebrae 1-4 incompletely ossified	1 (0.8)	1 (0.8)	2 (1.8)		
Sternebra 5 incompletely/unossified	7 (5.8)	18 (14.4)	11 (10.0)	7 (7.4)	
Sternebra 6 incompletely/unossified	12 (9.9)	11 (8.8)	8 (7.3)	1 (1.1)	
Pubis incompletely/unossified		3 (2.4)	11 (10.0)		
Pelvis inserted on sacral vertebra 2	41 (33.9)	33 (26.4)	23 (20.9)	26 (27.4)	
Unilateral insertion on sacral vertebra 2	6 (5.0)	6 (4.8)	2 (1.8)	7 (7.4)	
Bilateral insertion on lumbar vertebra 7		1 (0.8)			
Metacarpals incompletely/unossified	2 (1.7)	10 (8.0)	5 (4.5)	3 (3.2)	
Astragali incompletely/unossified	5 (4.1)	6 (4.8)	10 (9.1)	10 (10.5)	
Forelimb phalanges unossified	40 (33.1)	31 (24.8)	30 (27.3)	20 (21.1)	
Hindlimb phalanges unossified	3 (2.5)	2 (1.6)	3 (2.7)	4 (4.2)	

Showing: number of foetuses (%) affected
a: BCD (cumulative incidences) from 1375 examined foetuses

There was a slightly higher foetal incidence of minor external and visceral defects in the 30 and 90 mg/kg/day groups, but no adverse effect of treatment was evident from the type and distribution of defect observed.

The overall foetal incidence of visceral variants was also slightly higher in the 90 mg/kg/day group owing to increased prevalence of the common, non-adverse variant abnormal common carotid artery. This incidence was also higher than the cumulative background incidence appended to the study. In the absence of other alterations in prevalence of minor heart or vessel abnormalities/variants, an association with treatment for such an isolated occurrence was very unlikely. A similar apparent trend also occurred for incidence of the inconsequential, non-adverse variant agenesis of the post-caval lung lobe, which generally is a relatively common background variant in rabbit strains, as is variation in the lobe's size.

The foetal incidence of minor skeletal defects in the BAM treated groups was higher than in the control group, but there was no indication of a dose response relationship. Despite the intergroup variations, the type and incidence of skeletal variants were considered generally similar in all groups. The marginal increase in unilateral supernumerary (lumbar) rib at the highest dose was inconsistent with the unaffected incidence of bilateral supernumerary rib and with the unaffected incidence of asymmetric insertion of the pelvis and can be disregarded as incidental. In addition, such supernumerary ribs at the thoraco-lumbar border are generally non-adverse, and such small additional ossification sites are commonly absorbed later into the vertebral lateral processes.

The various indicators of ossification status were considered together not to indicate an adverse effect of treatment. The incidence of 'bipartite' interparietal is commonly associated with local variation in the intramembranous ossification status of this bone, which is naturally quite variable (as is ossification status of the other bones of the cranial vault, which can be inconsistent). 'Bipartite' here refers to a manifestation of local retardation of ossification, the appearance arising when the paired centres at which ossification begins have not yet become confluent. In this case, incidence of this variant was indeed accompanied by a parallel slight increase in generally incomplete ossification of the interparietal, which is also interpreted as not being of biological significance overall. In this context, it should be noted that reverting to BCD is not appropriate for interpretation of ossification status, which can only be meaningfully done by assessing multiple indicators in the skeleton in context with foetal size/weight, within the particular study.

III. Conclusions

It was concluded in the report that 90 mg/kg bw/day M-01 (BAM) in rabbits was associated with maternal toxicity characterized by mortality following deterioration in condition and/or abortion, body weight loss and reduced food consumption. Effects on foetuses from surviving dams at this dosage were confined to a marginal reduction in foetal weight (not statistically significant), considered secondary to the maternal toxicity observed at this dose level. There was no indication of an effect on foetal development (incidence or type of external, visceral or skeletal alterations).

There were no adverse effects of treatment at 30 mg/kg bw/day of M-01 (BAM), and this was concluded to be an overall NOAEL for maternal toxicity, while a developmental NOAEL could be set at ≥90 mg/kg bw/day, the highest dose applied.

Assessment and conclusion by applicant:

The study was conducted according to US EPA 83-3 and was broadly compliant to OECD TG 414, with the caveat of the deviations described above. The study is valid and acceptable to assess the developmental toxicity potential of M-01 in rabbits. A NOAEL of 30 mg/kg bw/d for maternal toxicity was determined from this study. There were no developmental effects up to the highest dose tested of 90 mg/kg bw/d.

Data Point:	KCA 5.8.1/57
Report Author:	Anon.
Report Year:	2019
Report Title:	2,6-dichlorobenzamide (BAM, or M-01): Review of the reproductive and developmental studies
Report No:	VC/19/037-1
Document No:	M-672423-01-1
Guideline(s) followed in study:	Regulation (EC) 283/2013
Deviations from current test guideline:	Not applicable (position paper)
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive summary

This review and evaluation were commissioned as part of the preparations for a renewal submission on behalf of fluopicolide. M-01 (BAM) is a metabolite of fluopicolide. The review considers the two available studies addressing potential reproductive and developmental toxicity of M-01 (BAM), including their reliability and acceptability.

The three-generation reproduction study has some deficiencies in terms of design and reporting, which limit its utility to evaluation of general functional reproductive performance. Many of the deficiencies in endpoints/data derive from the fact that they were not specific guideline requirements at the time of the study. The potential problems they address can often be inferred, or at least signalled, from the nature and pattern of effects seen on other endpoints/data in the study, in context with information from other toxicity studies with the test material.

There was no significant general toxicity at the highest dosage used (180 ppm in the diet), but the study provides a NOAEL for parental general toxicity and reproductive performance at this level, equivalent to approximately 14 mg/kg bw/day of M-01 (BAM). This dosage is consistent with the NOAEL derived from a 90-day dietary study in rats (Boschman, 1967) that showed reduced food intake and body weight gain at 49 mg/kg bw/day. An overall NOAEL for the reproduction study can be set at 100 ppm in the diet, equivalent to approximately 7.5 mg/kg bw/day, based on possible slight body weight reduction in dams and offspring at 14 mg/kg bw/day.

The developmental toxicity study in rabbits has only minor differences from current guidance that do not significantly compromise its utility. There were a number of intercurrent decedents in the study, owing to abortion or moribund condition, and the report implied that involvement of M-01 (BAM) in these deaths at the low and intermediate dosages could not be entirely excluded. However, a review of the information surrounding these deaths lead to a different conclusion, that the maternal losses in the control, 10 and 30 mg/kg/day groups were incidental and not due to toxicity: The two abortions appear to have been spontaneous events and the three terminations due to moribund condition were probably attributable to partial mis-dosing (rubber gavage errors, dosing into the trachea/lungs). Meanwhile the three abortions at the high dose of 90 mg/kg/day appeared a consequence of the marked maternal toxicity observed at this dose and the two instances of moribund condition again were indicated to have probably been partial mis-dosing. These intercurrent deaths resulted in rather sub-optimal numbers of litters for evaluation (11-13 per group), but it was still possible to conclude quite safely that there was no effect on foetal development in the study, despite a marginal reduction (-6%) in foetal weight at 90 mg/kg/day maternal dose, not statistically significant and not accompanied by a meaningful shift in the pattern of skeletal indicators of retarded development. Such an effect on foetal weight, marginal and

of doubtful biological significance, can be considered merely a secondary effect of the quite marked maternal toxicity at this dose. A NOAEL for maternal toxicity in the study can be set at 30 mg/kg bw/day of M-01 (BAM), based on maternal toxicity at the high dose. A developmental NOAEL can be set at ≥ 90 mg/kg bw/day of M-01 (BAM), the highest dose applied.

While the reproduction study does not cover all the specific endpoints that are required by current guidance, and embryofoetal development is addressed only by an adequate study in rabbits, the available data does not indicate that there is any particular concern relating either to overall reproductive performance or to embryofoetal development

Assessment and conclusion by applicant:

In this position paper the available data pertaining to the potential reproductive and developmental toxicity of M01 was reviewed. The analysis concluded that there was no concern for reproductive or developmental toxicity.

Mechanistic studies

Data Point:	KCA 5.8.1/58
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE C653711 (metabolite of AE C638206) - Preliminary 7-day toxicity study for proliferation assessment in the Sprague-Dawley female rat
Report No:	C042014
Document No:	M-231818-01-1
Guideline(s) followed in study:	not specified
Deviations from current test guideline:	Not applicable , non-guideline mechanistic 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:

Groups of 15 female Sprague-Dawley rats were given either diet containing 500 ppm M-01 (BAM) which equated to 31 mg/kg bw/d, or untreated control diet for 7 days. Animals were checked for mortality and clinical signs and bodyweights and food and water intake were measured. After 7 days the rats were necropsied and all major organs, tissues and body cavities were examined. Any abnormalities were further examined microscopically. Histopathological examinations were performed on the livers of all animals.

A solution of 5-bromo-2'-deoxyuridine (BrdU) was delivered to animals in water bottles between days 1 and 7. Hepatic cell cycling was assessed by using immunohistochemical staining to visualize the incorporation of BrdU. A section of the duodenum was used as a positive control owing to its high proliferation rate. The labelling index was expressed as the number of BrdU positive hepatocytes per 1000 hepatocytes.

There were no deaths or clinical signs of toxicity; bodyweight losses were observed in the treated group compared with a gain in controls and food and water consumption were also reduced in this group. There were no differences in liver weights between the treated and control group and there were no treatment-related histopathology findings.

The histopathological examination of the livers did not reveal any evidence of hepatocellular proliferation; however, the result could not be confirmed with BrdU staining owing to a technical error.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-01 (BAM) (referred to as AE C653711)
Purity:	96.02%
Batch no.:	08018 ET

2. Vehicle and/or positive control

Vehicle:	Diet
Positive control	None

3. Test animals

Species:	Rat
Strain:	Sprague-Dawley
Age:	Approx. 9 weeks
Weight at start:	231-259g
Source:	[redacted]
Acclimation period:	8 days
Diet:	Certified rodent powdered and irradiated diet A04C-10P1 from S.A.F.E, Epinay-sur-Orge, France
Water:	Tap water provided *ad libitum*
Housing:	Housed individually in suspended steel wire mesh cages.
Temperature:	20˚C - 24˚C
Humidity:	40% - 70%
Air changes:	10 to 15 changes per hour
Photoperiod:	12 hours light, 12 hours dark

B. Study design

1. In-life dates:

October 8, 2003 to October 21, 2003

2. Animal assignment and treatment

A total of 30 female rats were randomly selected for the study. An automatic randomization procedure was used to select animals for the study from the middle of the weight range of the available animals that ensured a similar body weight distribution among groups for each sex. The dose level was set based on the results of the 2-year rat study where hepatocellular adenomas were observed in females at 500 ppm. Groups of 15 female rats were given either diet containing 500 ppm M-01 (BAM) which equated to 31 mg/kg bw/d, or untreated control diet.

Table 5.8.1- 56: Study design

Group no.	Dose	No. females/group
1	0 ppm (control)	15
2	500 ppm	15

3. Diet preparation and analysis

The test substance was incorporated into the diet to provide the required dietary concentration. The test substance was ground into a fine powder before being incorporated into the diet by dry mixing. There was one preparation for the entire study. No analysis of the homogeneity or stability of the test substance in the diet was reported.

4. Statistics

Statistical analyses were carried out using Path/Tox system V4.2.2. (Module Enhanced Statistics).

Variables analysed:

- Bodyweight parameters
- Food consumption
- Water consumption
- Organ weight parameters

Means and standard deviations (STD) were calculated for each group at each time period. Group means were compared at the 5% and 1% level of significance. The F test was performed to compare the homogeneity of group variances.

Bodyweight gain/day parameters and organ weights

If the F test was not significant (α=0.05), the mean of the exposed group was compared to the mean of the control group using the t-test (2-sided). If the F test was significant (α=0.05), the mean of the exposed group was compared to the mean of the control group using the modified t-test (2 sided).

Body weight and average food or water consumption/day parameters

If the F test was not significant (α=0.05), the mean of the exposed group was compared to the mean of the control group using the t-test (2-sided). If the F test was significant (α=0.05) data were transformed using the log transformation. If the F test on log transformed data was not significant (α=0.05) the mean of the exposed group was compared to the mean of the control using the t-test (2-sided) on log transformed data. If the F test was significant (α=0.05) even after log transformation, the mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

If one or more group variance(s) equalled 0, the means were compared using the non-parametric Mann-Whitney test (2-sided).

C. Methods

1. Observations

All animals were checked for mortality twice daily (once daily at weekends). The nature, severity reversibility and duration of clinical signs were recorded at least once daily between days 1-6.

2. Body weight and food intake

Each animal was weighed during the acclimatisation period (days 1 and 7) and fasted animals were weighed prior to necropsy. Food consumption was recorded weekly.

3. Cell proliferation (in-life)

A solution of 5-bromo-2'-deoxyuridine (BrdU) in drinking water was prepared at 0.8 g/L and delivered to animals in water bottles between days 1 and 7. Mean water consumption/day was calculated.

4. Post-mortem examinations

All animals were fasted for 24-hours and then sacrificed on day 8 by pentobarbital injection and exsanguination. The necropsy of all animals included the examination of all major organs, tissues and body cavities. Any abnormalities were further examined microscopically. Histopathological examinations were performed on the livers of all animals.

5. Cell proliferation assessment

Hepatic cell cycling was assessed by using immunohistochemical staining to visualize the incorporation of BrdU. A section of the duodenum was used as a positive control owing to its high proliferation rate.

Two liver samples/animal and one duodenum sample underwent an immunohistochemical reaction that involved an incubation with a monoclonal antibody raised against BrdU, an amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex and a detection of the complex with the chromogen diamino-benzidine (DAB) and a Feulgen nuclear counterstaining.

The labelling index was expressed as the number of BrdU positive hepatocytes per 1000 hepatocytes.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities or clinical signs of toxicity.

2. Body weight and food and water intake

At 500 ppm there was a bodyweight loss of 0.5g/day compared with a gain of 1.5 g/day in the control group (days 1-7), whilst mean overall bodyweight in treated animals (day 7) was 5% lower than controls. In treated animals, food consumption was statistically significantly when compared with controls reduced by 23% for days 1-7 and likewise water consumption for the same period was 42% lower than controls.

Table 5.8.1- 57: Mean body weight and body weight gain

	Dose level (ppm)	
	0	**500**
Body weight (g)		
Day 1	249	247
Day 7	251	244*
Bodyweight gain/day (g)		
Days 1-7	1.5	-0.5*
Food consumption/day (g)		
Days 1-7	19.4	14.9*

3. Post-mortem examinations

There was no difference in mean or absolute liver weights between the treated and control groups and no treatment related macroscopic or microscopic findings were noted.

4. Cell proliferation assessment

Owing to a technical error, the determination of the labelling index was not possible for the control group animals; therefore, the results were not reported.

III. Conclusion

Administration of M-01(BAM) to the diet of Sprague-Dawley rats for 7-days did not lead to mortalities or clinical signs. Bodyweight losses were observed in the treated group compared with a gain in controls and food and water consumption were also reduced in this group. There were no differences in liver weights between the treated and control group and there were no treatment-related histopathology findings.

The histopathological examination of the livers did not reveal any evidence of hepatocellular proliferation, however, the result could not be confirmed with BrdU staining owing to a technical error.

Assessment and conclusion by applicant:
The study is valid and acceptable to assess the potential for M-01 to induce hepatocellular proliferation. No evidence of proliferation was seen, but the result could not be confirmed with BrdU staining.

Data Point:	KCA 5.8.1/59
Report Author:	[redacted]
Report Year:	2007
Report Title:	A study with 2,6-dichlorobenzamide (BAM) in cultured rat hepatocytes
Report No:	CXR0584
Document No:	M-293647-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	Not applicable, non-guideline mechanistic 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:

The aim of this study was to investigate the potential for M-01 to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and to determine the potential for peroxisome proliferation using CN⁻ insensitive palmitoyl CoA oxidation in cultured hepatocytes of Sprague Dawley rats.

The known peroxisome proliferator in rat hepatocytes, WY 14,643 was used as the positive control for peroxisome proliferation (0.1 and 10 µM), whilst Epidermal Growth Factor (EGF, 12.5 and 25 ng/mL) was included as a positive control for hepatocyte proliferation. Hepatocyte viability was measured by trypan blue exclusion. M-01 (BAM) was tested at concentrations ranging from 1 to 1000 µM.

The mean viability of the cultured hepatocytes was 87.2%. BAM induced peroxisome proliferation only from 3000 µM, although not to a marked extent; the maximum increase for BAM was 1.6-fold, compared with 7-fold in the positive control. BAM did not induce replicative DNA synthesis in cultured rat hepatocytes. The positive control (EGF) stimulated S phase by approximately 3-fold at both tested concentrations.

Overall, M-01 (BAM) did not show the properties expected of a peroxisome proliferator in cultured rat hepatocytes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-01 (BAM) (referred to as 2,6-dichlorobenzamide in the report)
Purity:	96.2%
Batch no.:	AE C653711 1B96 0001

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive controls:	Wyeth 14,643 (Wy) and epidermal growth factor (EGF)

3. Test animals

Species:	Rat
Strain:	Sprague-Dawley
Age:	6-8 weeks
Source:	Harlan, UK
Acclimation period:	5 days
Diet:	RM1 pelleted diet, Special Diet Services Ltd., Stepfield, Witham, Essex, UK
Water:	Tap water, *ad libitum*
Housing:	3/cage in sawdust in solid-bottomed polypropylene cages
Temperature:	19-23˚C
Humidity:	40-70%
Air changes:	14-15/hour
Photoperiod:	12-hours light/12-hours dark

B. Study conduct

1. Experimental procedures

Hepatocyte isolation

Mice were terminally anaesthetised using Euthatal™ and hepatocytes isolated by *in situ* perfusion according to Mitchell A.M. et al., (1984)[1]. Viabilities of the hepatocyte preparations, as determined by Trypan Blue exclusion (Laboratory Method Sheet (LMS) Tic-002), were in excess of 80%. Hepatocytes pooled from two independent perfusions were used.

Hepatocyte culture

Primary monolayer cultures of hepatocytes were prepared in 6-well plastic tissue culture plates, using Leibowitz (LMS-TIC-001) as the medium. The hepatocytes were cultured in Leibowitz complete medium for 4 hours. The medium was then changed, and the cells exposed to the test substance at 7 concentrations (1, 3, 10, 30, 100 & 300µM and 1mM). Untreated and vehicle (DMSO) controls, as well as positive controls (2 concentrations of Wyeth 14,643 (Wy) and 2 concentrations of epidermal growth factor (EGF) were cultured concurrently. The media, with appropriate additions, were replenished daily for a further 3 days. There were 5 replicates for each culture and the test substance was formulated in DMSO (the concentration of DMSO in the media did not exceed 1% v/v).

Hepatocyte viability

Viabilities of the isolated hepatocyte preparations were assessed individually, prior to pooling, by trypan blue exclusion.

[1] Mitchell, A.M *et al* (1984), Arch. Toxicol., 55: 239-256

Peroxisome proliferation

Cells were harvested after 96 hours by scraping them into SET buffer (0.25M sucrose, 5mM EDTA and 20 mM Tris-HCL, pH 7.4), sonicating the mixture and storing at -70˚until analysis. Cyanide-insensitive acyl CoA oxidation (which is a peroxisomal enzyme marker) was determined spectrophotometrically using palmitoyl CoA as a substrate, according to LMS Spec-003. Cyanide was used to inhibit the re-oxidation of NADH by the mitochondria. Wy 14,643 (0.1µM and 10 µM, n=5) acted as the positive control.

Replicative DNA synthesis (S-phase)

The number of cells undergoing replicative DNA synthesis (S-phase) in any given cell population can be determined by the incorporation of BrdU followed by immunostaining. S-phase was immunocytochemically following the incorporation of BrdU into hepatocyte nuclei over the last 3 days of culture. Immunostaining was performed after fixation at 96-hours. Data were expressed as a labelling index (% of total cells that had incorporated BrdU). EGF (12.5 ng/L and 25 ng/L, n=5) was included as a positive control.

Statistical analysis

Statistical comparisons between treated and control groups were undertaken using a student's t-test (2-sided) to a significance of $p<0.05$, $p<0.01$ and $p<0.001$.

II. Results and Discussion

A. Results

1. Hepatocyte viability

The mean viability was 87.2% (see table below)

Table 5.8.1- 58: Hepatocyte viability

Rat	Live cells	Total cells	% viability	Concentration	Vol. used
1	320	358	89.4%	4.48 x 10^6/ml	3.4 ml
2	152	179	84.9%	2.13 x 10^6/ml	28.2 ml

2. Peroxisome proliferation

M-01 (BAM) induced palmitoyl proliferation at concentrations above 300µM; however, the effect was not particularly marked, with the maximum increase being 1.6-fold. The positive control (Wy14,643), which is a well-known peroxisome proliferator in rats, induced palmitoyl CoA proliferation at both concentrations to a much more marked extent (maximum 7-fold increase).

Table 5.8.1- 59: Peroxisome proliferation in cultured rat hepatocytes

Palmitoyl CoA oxidation (nmol NAD^+ reduced/min/mg. protein)		
Concentration (µM)	**BAM**	**Wy14,643**
0[1]	0.66 ± 0.26 (61.2 ± 24.1)***	
DMSO	1.08 ± 0.09 (100.0 ± 8.2)	
0.1		4.27 ± 1.51 (395.4 ± 139.7)**
1	1.08 ± 0.04 (100.3 ± 3.8)	
3	0.65 ± 0.12 (60.2 ± 11.1)***	
10	0.71 ± 0.10 (65.9 ± 9.5)***	7.77 ± 0.24 (719.1 ± 21.8)***
30	1.03 ± 0.09 (95.1 ± 8.8)	
100	1.16 ± 0.15 (107.5 ± 13.7)	
300	1.38 ± 0.29 (127.9 ± 26.4)	
1000	1.69 ± 0.12 (156.8 ± 10.8)***	

Values are Mean ± SD (% control), n=5/group. [1]n=10 for untreated controls, *p<0.05, **p<0.01, ***p<0.001

3. Replicative DNA synthesis (S-phase)

BAM had no effect on S-phase; as expected, the positive control (EGF) simulated S-phase by approximately 3-fold at each concentration.

Table 5.8.1- 60: Peroxisome proliferation in cultured rat hepatocytes

Hepatocytes in S-phase (% of total)		
Concentration (µM)	**BAM**	**EGF**
0[1]	40.7 ± 1.25 (51.5 ± 15.4)*	
DMSO	8.10 ± 3.15 (100 ± 38.9)	
1	8.59 ± 1.17 (106 ± 14.5)	
3	9.60 ± 2.11 (118.5 ± 26)	
10	5.91 ± 1.53 (73 ± 18.9)	
30	8.90 ± 0.98 (109.9 ± 12.1)	
100	4.97 ± 1.53 (61.3 ± 18.9)	
300	7.30 ± 1.57 (90.1 ± 19.4)	
1000	4.20 ± 1.73 (51.9 ± 21.4)*	
12.5 ng/ml		20.32 ± 3.81 (250.8 ± 47)***
25 ng/ml		21.31 ± 6.75 (263.1 ± 83.3)**

Values are mean ± SD (% control), n=5/group, [1]n=10 for untreated controls, *p<0.05, **p<0.01, ***p<0.001

III. Conclusion

In this study, M-01 (BAM) did not exhibit the expected properties of a peroxisome proliferator.

There was no marked induction of CN^- insensitive palmitoyl CoA oxidation or increase in replicative DNA synthesis (labelling index, S-phase). The positive controls gave the expected results.

Assessment and conclusion by applicant:

The study is valid and acceptable to assess the potential of M-01 to induce peroxisome proliferation. No evidence of peroxisome proliferation was observed.

Data Point:	KCA 5.8.1/60
Report Author:	[redacted]
Report Year:	2009
Report Title:	AE C653711 (BAM) - In vitro induction of cytochrome P-450 and UDPGT mRNa levels in hepatocytes (liverbeads) from male rat
Report No:	SA 07324
Document No:	M-345184-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	Not applicable, non-guideline mechanistic 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:

M-01 (BAM) was tested at concentrations of 100, 200 and 300 µM in suspensions of male Wistar rat LiverBeads™. Following a 48-hour incubation, the potential of M-01 (BAM) to induce cytochrome P-450 and UDPGT enzymes was analysed using mRNA extraction and subsequent qPCR analysis with different CYP and UGT probes. Phenobarbital, clofibric acid and β-naphthoflavone, which are well-known inducers of CYP2B family, CYP4 family and CYP1A family respectively, were included as positive controls. The parent compound fluopicolide was also included in the evaluation.

M-01 did not demonstrate a clear potential to induce cytochrome P450 and UDPGT isoenzymes; only a slight (2-fold) upregulation of cyp2b1 transcripts was observed at the highest concentration, with no dose response. The positive controls gave the expected results. No analysis of fluopicolide could be carried out owing to a technical error.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-01 (BAM)) referred to as AE C653711 in the report*
Purity:	96.2% (w/w)
Batch no.:	1B96 001

* the parent compound fluopicolide (purity 97.9%, batch PFV052K012 was also included)

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive controls:	Phenobarbital (liver CYP 2B induction), clofibric acid (liver CYP 4A induction) and β-naphthoflavone (Liver CYP 1A induction)
Supplier:	Sigma-Aldrich (saint Quentin-Fallavier, France)

3. Test system

Test system	Liverbeads™
Supplier	Biopredic International, Rennes, France
Source	Wistar male rat
Characteristics	10^6 cells/vial, immobilized hepatocytes entrapped within an alginate matrix
Batch	LVB008001

B. Study conduct

1. Experimental procedures

Preparation and incubation with Liverbeads™

After thawing the liverbeads™ were pooled in Hepatozym medium containing calf serum 910%), insulin (4 µg/ml), glutamine (2mM), streptomycin (50 µg/ml) and penicillin (50 UI/ml) and seeded ($3x10^5$ cells) in 12 well plates. The plates were incubated (with 5% CO_2) under gentle shaking for 3 hours at 37˚C. After incubation, the media was replaced by another Hepatozym media containing the test substance (M-01, fluopicolide or positive control) in DMSO (1%). control incubations without the test substance were also included.

The concentrations of M-01 (BAM) and fluopicolide were 100, 200 and 300 µM, whilst the concentrations for the positive controls were 100, 200 and 50 µM for phenobarbital, clofibric acid and ß-naphthoflavone respectively. Each assay was run in duplicate.

After a further 48-hours incubation, the matrix of the LiverBeads™ was dissolved by the addition of 300 µl EDTA Na_2 to each well for 10 minutes. Following centrifugation, the hepatocytes were washed with PBS and disrupted by incubation with lysis buffer. The resulting cell lysates were used for RNA isolation.

Quantitative (q)PCR analysis

The cell lysates were thawed, and cytoplasmic RNA isolated from each sample using RNeasy minikit (Qiagen). RNA quality controls were performed based on ribosomal RNA electrophoretic profiles.

Approximately 2.5 µg total RNA were used for reverse transcription (RT) using a high capacity cDNA archive kit. The assay was performed in duplicate on an ABI prism 7900 HT machine using taqman probes and 1/50 diluted first strand cDNA and AmpliTaq Gold PCR Master Mix. A negative control was included for each gene transcript in which H_20 MQ was used in place of first strand cDNA.

Beta-2 microglobulin was selected as a reference gene for the quantitative calculations of transcripts.

II. Results and Discussion

A. Results

A slight upregulation of cyp2b1 gene transcripts was measured in the hepatocytes treated with M-01 (BAM) at the highest dose level; however, the results are considered to be of doubtful biological relevance as was no clear dose-response and the increase was small compared with the positive controls (see table below).

Table 5.8.1- 61: Results of qPCR analysis

	Mean relative quantity of gene transcripts (compared to control mean values)						
Gene transcripts	**Control**	**B-NF 50 µM**	**PB 100 µM**	**CLO 200 µM**	**BAM 100 µM**	**BAM 200 µM**	**BAM 300 µM**
Cyp2b1	1.000	0.598 (-40%)	8.239 (+724%)	4.515 (+352%)	1.819 (+82%)	1.454 (+45%)	3.172 (+217%)
Cyp1a1	1.000	27.371 (+2637%)	0.940 (-6%)	0.105 (-90%)	2.110 (+111%)	1.053 (+5%)	2.022 (+102%)
Cyp1a2	1.000	8.830 (+2637%)	1.126 (+13%)	0.939 (-6%)	2.430 (+143%)	1.658 (+66%)	2.064 (+106%)
Cyp3a3	1.000	0.121 (-88%)	1.130 (+13%)	1.224 (+22%)	0.997	0.557 (+44%)	0.900 (-10%)
Cyp4a22	1.000	0.074 (-93%)	1.115 (+12%)	17.855 (+1686%)	1.944 (+94%)	1.585 (+59%)	1.889 (+89%)
Ugt1a6	1.000	3.007 (201%)	0.829 (-17%)	0.551 (-45%)	2.143 (+114%)	1.544 (+54%)	1.699 (+70%)
Ugt2b	NR	NR	NR	NR	NR	NR	NR

NR= no transcripts for Ugt2b could be measured to due to a technical error (invalid Taqman assay), no qPCR analysis were performed on LiverBeads containing fluopicolide due to poor RNA extraction

The positive controls gave the following expected results:

- Upregulation of cyp2b1 in hepatocytes treated with phenobarbital (8-fold) and clofibric acid (4-fold)
- Upregulation of cyp1a1 (27-fold) and cyp1a (9-fold) in hepatocytes treated with ß-naphthoflavone
- Cyp3a3 not affected by any positive control
- Upregulation of cyp4a22 in hepatocytes treated with clofibric acid (18-fold)
- Upregulation of Ugt1a6 in hepatocytes treated with ß-naphthoflavone (3-fold)

III. Conclusion

In this *in vitro* test, M-01 (BAM) did not demonstrate the potential to induce cytochrome P-450 and UDPGT isoenzymes. Only a slight, non-concentration-related upregulation of cyp2b1 gene transcripts was observed at the highest concentration of 300µM. the positive controls gave the expected responses.

Assessment and conclusion by applicant:

The study is valid and acceptable to assess the potential of M-01 to induce cytochrome P450 and UDPGT isoenzymes.M-01 did not demonstrate the potential to induce P-450 of UDPGT enzymes

M-02 (PCA, AE C657188)

M-02 is not acutely toxic *via* the oral route, as evidenced by an acute oral toxicity study in which an LD_{50} of >2000 mg/kg bw was established in rats.

A short-term oral toxicity study in rats is available for M-02 in which no adverse effects were noted up to the highest dose tested of 20000 ppm (approximately 1580 mg/kg bw in both sexes), this dose level was therefore considered to be the NOAEL. An ADI of 1.6 mg/kg bw/d with a safety factor of 1000 can be derived from this study.

A battery of *in vitro* genotoxicity tests is available for M-02. The metabolite M-02 is not mutagenic in bacterial cells in a negative Ames test, nor in mammalian cells *in vitro* as evidenced by a negative V79/HPRT mutation test. M-02 did not induce chromosome aberrations *in vitro* in human peripheral blood lymphocytes, whilst a recently conducted *in vitro* MNT revealed that M-02 was neither clastogenic nor aneugenic.

Overall, the available data confirm that there is no toxicological concern for the metabolite M-02. The available studies for M-02 are summarised below.

Data Point:	KCA 5.8.1.11
Report Author:	[redacted]
Report Year:	2011
Report Title:	AE C657188 00 1B99 0002 (plant metabolite of AE C638206) - Rat acute oral toxicity
Report No:	AES018/003625/AC
Document No:	M-197257-02-1
Guideline(s) followed in study:	Directive 96/54/EEC, Part, Method B.1 tris. (1996); OECD 423 (1996)
Deviations from current test guideline:	Doses of 4000 mg/kg bw were tested in a preliminary study; the guidance recommends a top dose of 2000 mg/kg bw; 6 animals/dose were tested rather than 3/dose as recommended in the guidance
Previous evaluation:	yes, evaluated and accepted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

An acute toxicity study was conducted according to the acute toxic class method, by exposing fasted Sprague-Dawley rats to M-02 (referred to as AE C657188 in the report).

In a preliminary toxicity assessment, two animals dosed at 4000 mg/kg died following treatment. Macroscopic examination revealed congestive changes in the majority of organs and tissues. Dose levels for the main test were based on these findings.

Initially a group of six rats (three males and three females) was treated at 500 mg/kg bw, a dosage selected after review of preliminary study results. On the basis of findings at this dosage a further six rats (three males and three females) were dosed at 2000 mg/kg bw.

In males and females dosed at 500 and males at 2000 mg/kg, clinical signs of reaction to treatment were confined to piloerection on Day 1 following dosing.

No signs of ill health or toxicity were observed for females at 2000 mg/kg.

Recovery of surviving rats, as judged by external appearance and behaviour, was complete in all instances by Day 2.

No macroscopic abnormalities were observed.

All animals were considered to have achieved satisfactory bodyweight gains throughout the study, with the exception of a low bodyweight gain on Day 15 in two females dosed at 500 mg/kg.

In conclusion, the acute lethal oral dose in rats of M-02 was greater than 2000 mg/kg bw and less than 4000 mg/kg bw.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C657188
Code number:	AEC657188001B990002
Chemical name:	3-chloro-5-(trifluoromethyl) pyridine-2-carboxylic acid
Purity:	99.7%
Batch no.:	RAW2440451
Appearance:	Off-white crystalline solid
Expiry:	6 September 2000

2. Vehicle and/or positive control

Vehicle:	1% w/v aqueous methylcellulose

3. Test animals

Species:	Rat
Strain:	Male and female CD rats of Sprague-Dawley origin (Hsd: Sprague-Dawley(CD))
Age:	8-11 weeks
Weight at start:	214 g (204 – 232 g) in males and 210 g (202 – 221 g) in females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Special Diet Services RM1(E) SQC expanded pellet
Water:	Water *ad libitum*
Housing:	In groups of up to five rats of the same sex in metal cages (RS Biotech Sub-Dividable Rodent Cages - polished stainless steel).
Temperature:	22 ± 3 °C
Humidity:	30 – 70%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: May 3 to June 1, 2000

2. Animal assignment and treatment

Groups of six rats (three males and three females) were treated at 500 and 2000 mg/kg bw, the dosage being selected after review of preliminary study results with administration of a dose of 4000 mg/kg bw. The appropriate dose volume of the test substance was administered to each rat by oral gavage using a syringe and catheter of the appropriate gauge. The day of dosing was designated Day 1.

C. Methods

1. Observations

Mortality:

Cages of rats were checked at least twice daily for mortalities.

Clinical signs:

Animals were observed soon after dosing and at frequent intervals for the remainder of Day 1. On subsequent days animals were observed on two occasions during the day (once in the morning and again at the end of the experimental day, with the exception of the day of study termination – morning only). The nature and severity of clinical signs and the time these were noted were recorded at each observation. Animals in the preliminary and main study were observed for 3 or 14 days respectively following dosing.

The body weights of each rat in the preliminary study were recorded on Day 1 (prior to dosing) and 3 (after all animals were dead) and in the main study on Days 1 (prior to dosing), 8 and 15.

2. Necropsy

All surviving animals were killed by carbon dioxide asphyxiation at study termination.

Animals were subjected to a macroscopic examination, which consisted of opening the cranial, thoracic and abdominal cavities. The macroscopic appearance of all examined organs was recorded.

II. Results and Discussion

A. Results

1. Dose-response table (LD_{50})

The results of the study for acute oral toxicity in the fasted rat, including the LD_{50}, are summarized in Table 5.8.1-62.

Table 5.8.1- 62: Dose response

Dose (mg/kg bw)	Toxicological result*	Occurrence of signs	Time of death	Mortality (%)
Males				
Prelim. 4,000	1/1/1	1/1	Day 3	100
500	0/3/3	3/3	-	0
2,000	0/3/3	3/3	-	0

Dose (mg/kg bw)	Toxicological result*	Occurrence of signs	Time of death	Mortality (%)
Females				
Prelim. 4,000	1/1/1	1/1	Day 3	100
500	0/3/3	3/3	-	0
2,000	0/3/3	3/3	-	0

* Number of animals which died and/or were sacrificed moribundly / number of animals with signs / total number of animals.

The LD_{50} was therefore **greater than 2000 mg/kg bw and less than 4000 mg/kg bw**

2. Clinical signs

In the preliminary study dosed at 4000 mg/kg bw, clinical signs comprised piloerection, hunched posture, lethargy, abnormal gait, shallow respiration, reduced body temperature, dull, partially closed eyes, body tremors, pallor of the extremities and lacrimation.

In the main study, in males and females dosed at 500 and males at 2000 mg/kg, clinical signs of reaction to treatment were confined to piloerection on Day 1 following dosing.

No signs of ill health or toxicity were observed for females at 2000 mg/kg.

Recovery of surviving rats, as judged by external appearance and behaviour, was complete in all instances by Day 2.

3. Body weights

All animals were considered to have achieved satisfactory bodyweight gains throughout the study, with the exception of a low bodyweight gain on Day 15 in two females dosed at 500 mg/kg.

4. Necropsy findings

In the preliminary study, a post-mortem macroscopic examination revealed congestion in the subcutaneous tissue, brain, heart, liver, spleen and kidneys and congestion with fluid contents in the gastrointestinal tract.

The necropsies performed at the end of the main study did not reveal any unusual findings.

III. Conclusion

The acute oral LD_{50} of M-02 in rats was greater than 2000 mg/kg bw and less than 4000 mg/kg bw.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 423 and is valid and acceptable to investigate the acute oral toxicity of M-02 in rats. M-02 was not acutely toxic via the oral route under the conditions of this study. An LD_{50} of >2000 mg/kg bw was established.

Short-term toxicity

Data Point:	KCA 5.8.1/15
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C657188 (PCA) Preliminary 28-day toxicity study in the rat by dietary administration
Report No:	SA 01176
Document No:	M-204953-03-1
Guideline(s) followed in study:	OECD 407 (1995)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

AE C657188 (PCA), was administered continuously *via* the diet to groups of Sprague-Dawley rats (5/sex/group) for 28 days at concentrations of 0, 20, 200, 2000 and 20 000 ppm (equivalent to 1.50, 15.0, 149 and 1574 mg/kg bw/day in males and to 1.63, 15.9, 162 and 1581 mg/kg bw/day in females).

A similarly constituted group received untreated diet and acted as a control. Animals were observed daily for mortality and clinical signs. Body weight and food consumption were recorded once weekly. During the acclimatization phase all animals were subjected to an ophthalmic examination.

All animals at 0 and 20 000 ppm were re-examined at the end of Week 3. Haematology, plasma chemistry and urine parameters were determined at the end of the study. All animals were necropsied; selected organs were weighed, and a range of tissues were fixed and examined microscopically.

The dietary administration of M-02 (PCA) to Sprague-Dawley rats at 20, 200, 2000 and 20000 ppm for 28 days did not induce treatment-related mortalities or clinical signs. Food consumption and mean body weights were unaffected by treatment with the exception of a slight reduction for females at 20000 ppm. No ocular abnormalities were observed at the ophthalmological examination. With regard to haematology, no toxicologically significant changes were noted. However, clinical chemistry analyses revealed a tendency towards lower values in inorganic phosphorus concentration in males at 20000 ppm. Urinalysis revealed no toxicologically significant changes. There was no effect on mean terminal body weights, mean absolute and relative organ weights, and gross pathology in all treated groups. No treatment-related findings were seen at histopathological examination in any dose group.

A NOAEL of 20000 ppm (approximately 1580 mg/kg bw in both sexes), the highest dose tested, is therefore proposed from this study.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-02 (PCA, referred to as AE C657188 in the report)
Purity:	99.1 %
Batch no.:	D 0526

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rat
Strain:	Crl: CD(SD)1GS Br
Age:	6-7 weeks old
Weight at start:	220 to 243 g for the males and 161 to 198 g for the females
Source:	[redacted]
Diet:	Certified rodent and irradiated powder diet AO4C-10P1 from U.A.R. (Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge, France)
Housing:	housed individually in suspended stainless-steel wire mesh cages.
Temperature:	20 – 24 °C
Humidity:	40 - 70 %
Air changes:	10-15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates:

June 12 – July 12,13, 2001

2. Dose preparation

The test substance was incorporated into the diet to provide the required dietary concentrations. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. There was one formulation for the study for each concentration. When not in use, the diet formulations were stored at approximately -18° C. The stability of the dietary formulations was checked for the lowest and highest concentrations over a duration which covers the period of storage and usage for the study. The homogeneity of AE C657188 (PCA) in diet was verified for the lowest and highest concentrations to demonstrate adequate formulation procedures. Dietary levels of the test substance were verified for each concentration.

Results for homogeneity of diets with AE C657188 at 20 and 20 000 ppm were within the target ranges (86 to 108% of the nominal value). Concentration checks for all doses were in a range of 90 to 98% of the nominal values. Diet mixtures were found stable over a duration period (41 days) that covered the conditions of storage and usage for this study.

2. Animal assignment and treatment

Groups of 5 male and 5 female rats were given control diet or the appropriate diet mixture according to the following study design.

Table 5.8.1- 63: Study design

Group	Diet concentration (ppm)	No. of males	No. of females
1	0	5	5
2	20	5	5
3	200	5	5
4	2000	5	5
5	20000	5	5

C. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals during the study. The nature, onset, severity, reversibility, and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose faeces.

2. Body weights and food consumption

Each animal was weighed at least once during the acclimatization period, on the first day of test substance administration, then at weekly intervals throughout the treatment period and before necropsy.

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. The mean achieved dosage intake in mg/kg bw/day for each week and for weeks 1 to 4 was calculated.

3. Ophthalmological examinations

During the acclimatization period all animals were subjected to an ophthalmic examination. After instillation of an atropinic agent (Mydriaticum, Merck Sharp and Dohme) each eye was examined by means of an indirect ophthalmoscope. At the end of Week 3. animals from control and top dose groups were re-examined.

4. Clinical pathology

Blood sampling:

On study Days 22 or 23 blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus. An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were fasted overnight prior to bleeding and anesthetized by inhalation of isoflurane. Blood was collected on EDTA for haematology (0.5 mL), on lithium heparin for plasma chemistry (2.5 mL) and on sodium citrate for coagulation parameters (0.9 mL).

Haematology:

Red blood cell count, haemoglobin, heamatocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, white blood cell count and differential count evaluation and platelet count were assayed using a Technicon HI (Bayer Diagnostics, Puteaux, France).

A blood smear was prepared and stained with Wright stain. It was examined when the results of Technicon HI determinations were abnormal. Reticulocytes were stained with brilliant cresyl blue. A smear was prepared but not examined since no significant red blood cell changes were observed. Prothrombin time was assayed on an ACL 3000 (Instrumentation Laboratory, Paris, France).

Clinical chemistry:

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

Urine collection:

On study Days 29 or 30, in the morning, prior to sacrifice, overnight urine samples were collected from all animals in all groups. An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Feed and water were not accessible during urine collection.

Urinalysis:

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

Quantitative parameters:

Urinary volume was measured. pH was assayed using a Clinitek 200+ and Ames Multistix dipsticks (Bayer Diagnostics. Division Ames, Puteaux, France). Urinary refractive index was measured using an Atago clinical refractometer (Bioblock Scientific, Ulkirch, France).

b/Semi-quantitative parameters

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

Microscopic examination of the sediment:

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

5. Post-mortem examinations

On study Days 29 or 30, all animals from all groups were sacrificed by exsanguination under deep anaesthesia (pentobarbital, intraperitoneal injection of 50 or 60 mg/kg body weight). An approximately equal number of animals randomly distributed amongst all groups were sacrificed on each day. Animals were fasted overnight prior to sacrifice.

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

Adrenals, brain, epididymides. heart, kidneys, liver, ovaries, pituitary gland, prostate, spleen, testes, thymus, thyroid (with parathyroid) and uterus (including cervix) were weighed fresh. Paired organs were weighed together.

The following organs or tissues were sampled:

Adrenal gland
Aorta
Articular surface (femoro-tibial)
Bone (sternum)
Bone marrow (sternum)
Brain
Epididymis
Esophagus
Eye and optic nerve
Harderian (lachrymal) gland
Heart
Intestine (duodenum, jejunum, ileum, cecum, colon, rectum)
Kidney
Larynx
Liver
Lung
Lymph nodes (submaxillary, mesenteric)
Mammary gland
Ovary
Pancreas
Pituitary gland
Prostate
Sciatic nerve
Seminal vesicle
Skeletal muscle
Skin
Spinal cord (cervical, thoracic, lumbar)
Spleen
Stomach
Submaxillary (salivary) gland
Testis
Thymus
Thyroid (with parathyroid)
Tongue
Trachea
Urinary bladder
Uterus (including cervix)
Vagina

A bone marrow smear was prepared from one femur, stained with May-Grünwald Giemsa, but not examined. Samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative.

Histology was performed at Propath UK Ltd, Willow Court, Netherwood Road, Hereford HR2 6.1U England.

Embedding and preparation of histological sections (stained with haematoxylin and eosin) were performed for all tissues selected for microscopic examination. Adrenal gland, brain, epididymis, heart, kidney, liver, lung, ovary, pituitary gland, prostate, spleen, seminal vehicle, testis, thymus, thyroid gland, parathyroid gland, uterus and vagina were examined histologically on all the animals in the control and high dose groups. Macroscopic findings were examined in all animals.

6. Statistics

Variables analysed:
- body weight parameters
- food consumption
- haematology parameters (except eosinophils, basophils, monocytes and large unstained cells, absolute counts and percentages)
- clinical chemistry parameters
- urinary parameters (only pH, volume and refractive index)
- organ weight parameters

Statistical analysis:

Means and standard deviations (STD) were calculated for each sex separately for each group at each time period. The following procedures were performed when considered useful.

For body weight, food intake, plasma chemistry, haematology and organ weight parameters, results were inter-compared for each treated and control group by use of

Bartlett's test for homogeneity of variances between groups. When Bartlett's test indicated heterogeneous variances, any significant differences were identified by using the combination of Analysis of variance (ANOVA) and Dunnett's test.

When Bartlett's test indicated heterogeneous variances, any significant differences were identified by using a modified t-test. All tests were performed at 5% and 1% levels.

II. Results and Discussion

1. Clinical signs

There were no treatment-related mortalities during the study.

There were no treatment-related clinical signs. The few clinical signs recorded in control and 20000 ppm groups were recorded once only and were considered not to be related to AE C657188 administration.

2. Body weight and food consumption

There were no modification of the mean body weights and mean body weight changes in the treated groups compared to control animals, with the exception of a slight (non-significant) reduction on Weeks 3 and 4 for the females at 20000 ppm.

In male animals, food consumption was unaffected by treatment. In female animals, a slight reduction of food consumption was observed at 20 000 ppm. The differences versus control group were marginal (9% lower on Weeks 2 and 4) but statistically significant on Week 3 (-15%). No differences were observed at 20, 200 and 2 000 ppm when compared to control group.

3. Ophthalmoscopic examinations

No abnormal findings were observed.

4. Clinical pathology

Haematology:

No toxicologically significant or treatment-related changes were noted.

Plasma chemistry:

A tendency towards lower values was noted in inorganic phosphorus concentration, in males at 20000 ppm when compared to the control group (-12%, α=0.01). The other statistically significant changes seen were few and considered to be incidental and/or of no toxicological relevance.

Urinalysis:

No toxicologically relevant change was noted. The statistically significant difference noted in urinary volume, in males at 2 000 ppm, was judged to be incidental.

5. Post mortem examinations

Mean terminal body weights and mean absolute and relative organ weights from treated groups did not show any significant difference from the control group.

Gross pathology:

The gross changes seen were few and considered to be incidental and unrelated to the treatment.

Microscopic pathology:

Histopathological findings were few and considered to be incidental in origin and within the range of expected changes for animals of this age kept under laboratory conditions.

III. Conclusion

A dietary administration of AE C657188 (PCA) to Sprague-Dawley rats at 20, 200, 2000 and 20000 ppm for 28 days induced no treatment-related deaths or clinical signs. Food consumption and mean body weights were unaffected by treatment, with the exception of a slight reduction for females at 20 000 ppm. At the ophthalmological examination, no ocular abnormalities were observed. No toxicologically significant haematological changes were noted. With regard to clinical chemistry, a tendency towards lower inorganic phosphorus concentrations were noted in males at 20000 ppm. Urinalysis revealed no toxicologically significant changes. No change was seen in mean terminal body weights, mean absolute and relative organ weights, and gross pathology in all treated groups. No treatment-related findings were seen at histopathological examination in animals treated at 20000 ppm.

The No Observed Adverse Effect Level (NOAEL) was therefore 20000 ppm (approximately 1580 mg/kg in both sexes).

Assessment and conclusion by applicant:

The study was conducted according to OECD 407 and is valid and acceptable to investigate the short-term toxicity of M-02. A NOAEL of 20000 ppm (1580 mg/kg bw/d) was derived from this study.

Genotoxicity

Data Point:	KCA 5.8.1/12
Report Author:	[redacted]
Report Year:	2000
Report Title:	AE C657188 (plant metabolite of AE C638206) - Bacterial mutation assay
Report No:	AES017/002836
Document No:	M-197258-01-1
Guideline(s) followed in study:	Directive 92/69/EEC B.13, B.14 (1992); JMAFF NohSan No. 4200 (1985); OECD 471 (1997); US-EPA 712-C-98-247, OPPTS 870.5100 (1998); Joint Directives of JEPA, JMHW, JMJTI (1997); Directive of JMOL No. 67 (1997)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

This *in vitro* mutagenicity test assessed the potential of AE C657188 to induce reverse gene mutation in bacteria according to prevailing OECD, US-EPA and JMAFF testing guidelines.

Histidine dependent auxotrophic mutants of Salmonella typhimurium (strains TA1535, TA1537, TA98 and TA100) and a tryptophan dependent mutant of Escherichia coli (strain WP2MvrA/pKM101 (CM891)) were exposed to the test substance diluted in dimethyl sulphoxide, which was also used as a negative control.

Two independent mutation tests were performed in the presence and absence of liver preparations from Aroclor 1254-induced rats (S9 mix). The first was a standard plate incorporation assay, the second involved a pre-incubation stage.

Dose levels of up to 5000 µg/plate were tested in the mutation tests. This is the standard limit dose recommended in the regulatory guidelines this assay follows. Other dose levels used were a series of approx. half-log10 dilutions of the highest concentration.

No signs of toxicity were observed towards the tester strains in either mutation test. No precipitation was observed.

No evidence of mutagenic activity was seen at any dose level of AE C657188 in either mutation test.

The concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the liver preparations.

It is concluded that, when tested in dimethyl sulphoxide, AE C657188 showed no evidence of mutagenic activity in this *in vitro* bacterial system.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-02 (referred to as AE C657188 in the report)
Purity:	99.7 % w/w
Batch no.:	R001739

2. Vehicle and/or positive control

Vehicle:	DMSO (concentration not given)
Positive control:	Without S9 mix: Na-azide: TA 100, TA 1535 9-Aminoacridine : TA 1537 2-Nitrofluorene : TA 98 2-(2-Furfuryl) -3-(5-nitro-2-furyl) acrylamide (AF-2) : E.coli WP2uvrA/pKM101 (CM891) With S9 mix : 2-Aminoanthracene : TA 1535, E.coli WP2uvrA/pKM101 (CM891) Benzo[a]pyrene: TA 1537, TA 98, TA 100

3. Test organisms :

The following strains were used:

S. typhimurium TA1535, S. typhimurium TA1537, S. typhimurium TA98, S. typhimurium TA100 (is the same as TA1535 but contains the pKM101 plasmid, in addition to base-pair substitutions, it is also able to detect certain frameshift mutagens), E. coli WP2uvrA/pKM101 (CM891).

The strains of S. typhimurium were obtained from Professor Bal. Ames, University of California, Berkeley, California, USA. The strain of E. coli was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Batches of the strains were obtained from master stocks held in liquid nitrogen. The test batches were aliquots of nutrient broth cultures and were stored at -80°C. Dimethyl sulphoxide (DMSO) was added to the cultures at 8% v/v as a cryopreservative. Each batch of frozen strain was tested, where applicable, for cell membrane permeability (rfa mutation), sensitivity to UV light and the pKM101 plasmid, which confers resistance to ampicillin. The responses of the strains to a series of diagnostic mutagens were also assessed.

For use in tests an aliquot of frozen culture was added to 25 mL of nutrient broth (Merck No.2) and incubated with shaking, at 37°C for 10 hours. These cultures provided at least 2 x 10^9 cells per mL which were measured photometrically.

4. Activation:

S9 fraction was prepared from a group of 8 male Sprague-Dawley rats. Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intraperitoneal injection in corn oil at a dosage of 500 mg/kg bw. On the fifth day after injection, following overnight fasting, the rats were sacrificed, and their livers aseptically removed.

The following steps were carried out at 0-4°C under aseptic conditions. The livers were placed in 0.15 M KCl (3 mL KCl : 1 g liver) before being transferred to an Ultra-Turrax homogeniser. Following preparation, the homogenate was centrifuged at 9000 g for 10 minutes. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80°C until required. Each batch of S9 fraction was tested for sterility and efficacy.

S9 mix contained: S9 fraction (10% v/v), MgCl2 (8 mM), KC1 (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADPH (4 mM) and NADH (4 mM). All cofactors were filter-sterilised before use.

5. Test compound concentrations used:

Experiment 1: 0, 5, 15, 50, 150, 500, 5000 µg/plate

Experiment 2: 0, 50, 150, 500, 1500, 5000 µg/plate

B. Test Performance

Experimental phase: 28 April to 8 May 2000

Experiment 1:

The test substance was added to cultures of the five tester strains at seven concentrations separated by approx. a half-log10 interval. The highest concentration of AE C657188 tested was 50 mg/mL in the chosen solvent, which provided a final concentration of 5000 µg/plate. This is the standard limit concentration recommended in the regulatory guidelines. The negative control was the chosen solvent, dimethyl sulphoxide. The appropriate positive controls were also included.

An aliquot of 0.1 mL of a 10-hour bacterial culture and 0.5 mL S9 mix or 0.5 mL 0.1 M sodium phosphate buffer (pH 7.4) were placed in glass bottles. An aliquot of 100 µL of the test solution was added, followed immediately by 2 mL of molten agar containing 0.05 mM histidine/biotin/tryptophan. The mixture was thoroughly shaken and overlaid onto previously prepared petri dishes containing 25 mL minimal agar. Each petri dish was individually labelled with a unique code corresponding to a sheet, identifying the dish's contents. Three petri dishes were used for each dose level. Plates were also prepared without the addition of bacteria in order to assess the sterility of the test substance, S9 mix and sodium phosphate buffer. All plates were incubated at 37°C for ca 72 hours. After this period the appearance of the background bacterial lawn was examined and revertant colonies counted using a Seescan automated colony counter.

Any toxic effects of the test substance would be detected by a substantial reduction in revertant colony counts or by the absence of a complete background bacterial lawn. In the absence of any toxic effects the top concentration normally used in the second test would be the same as that used in the first. If toxic effects were observed a lower concentration may be chosen. It should be ensured that if a lower concentration was chosen, signs of bacterial inhibition are present at the top concentration. Ideally a minimum of three non-toxic concentrations should be obtained.

Experiment 2:

As a clear negative response was obtained in the first test, a variation to the test procedure was used for the second. The variation used was the pre-incubation assay in which the bottles, which contained mixtures of bacteria, buffer or S9 mix and test solution, were incubated at 37°C for 30 minutes with shaking before the addition of the agar overlay. 5000 µg/plate was again chosen as the top concentration, but only five dose levels were used as it was known from the first test that the test substance was non-toxic.

2. Statistics

Statistical analysis is not required

3. Assessment of results

For a test to be considered valid the mean of the solvent control revertant colony numbers for each strain should lie within the 99% confidence limits of the current historical control range of the laboratory. The historical range will be maintained as a rolling record over five years. Also, the positive control compounds must cause at least a doubling of mean revertant colony numbers over the negative control. The mean number of revertant colonies for all treatment groups is compared with those obtained for the solvent control groups. The mutagenic activity of a test substance is assessed by applying the following criteria:

- If treatment with a test substance produces an increase in revertant colony numbers of at least twice the concurrent solvent controls, with some evidence of a positive dose-relationship, in two separate experiments, with any bacterial strain either in the presence or absence of S9 mix, it is considered to show evidence of mutagenic activity in this test system. No statistical analysis is performed.
- If treatment with a test substance does not produce reproducible increases of at least 1.5 times the concurrent solvent controls in either mutation test, it is considered to show no evidence of mutagenic activity in this test system. No statistical analysis is performed.
- If the results obtained fail to satisfy the criteria for a clear "positive" or "negative" response given in paragraphs (a) and (b), additional testing may be performed in order to resolve the issue of the test substance's mutagenic activity in this test system. Should an increase in revertant colony numbers then be observed which satisfies paragraph (a) the substance is considered to show evidence of mutagenic activity in this test system. No statistical analysis is performed.

If no clear "positive" response can be obtained, the test data may be subjected to analysis to determine the statistical significance of any observed increases in revertant colony numbers. The statistical procedures used will be those described by Mahon et al (1989) and will usually be analysis of variance followed by Dunnett's test. Biological significance should always be considered along with statistical significance. It should be noted that it is acceptable to conclude an equivocal response if no clear results can be obtained.

II. Results and discussion

Experiment 1:

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to AE C657188 at any concentration in either the presence or absence of S9mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to AE C657188, indicating that the test substance was non-toxic. No precipitation was observed.

The following table gives an overview.

Table 5.8.1- 64: Revertant colony counts – experiment 1

Treatment	Concentration (µg/plate)	+/- S9	Mean revertant colony counts in strains				
			TA 98	TA 100	TA 1535	TA 1537	WP2*uvr*A/pKM101
M-02	5000	-	23	107	15	7	200
	1500	-	25	121	17	9	184
	500	-	25	97	13	11	197
	150	-	22	98	16	11	184
	50	-	27	95	13	11	200
	15	-	26	113	13	10	198
	5	-	27	114	13	11	187
Solvent control	0	-	21	103	11	11	197
M-02	5000	+	26	120	13	8	182
	1500	+	26	111	14	12	192
	500	+	25	113	16	12	179
	150	+	24	123	16	11	200
	50	+	27	108	14	10	185
	15	+	23	105	13	11	203
	5	+	27	112	16	9	202
Solvent control	0	+	26	107	15	13	192
Sodium azide	0.5	-	NA	558	197	NA	NA
AF-2	0.05	-	NA	NA	NA	NA	2134
9-Aminoacridine	30	-	NA	NA	NA	117	NA
2-nitrofluorene	1	-	400	NA	NA	NA	NA
Benzo[a]pyrene	5	+	257	804	NA	72	NA
2-aminoanthracene	2 - 10 *	+	NA	NA	133	NA	1866

AF-2 : 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide
NA : not applicable
*: 2 µg/plate for TA 1535, and 10 µg/plate for WP2*uvr*A

Experiment 2:

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to AE C657188 at any concentration in either the presence or absence ofS9mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to AE C657188. No precipitation was observed.

The mean revertant colony counts for the solvent controls were within the 99% confidence limits of the current historical range of the laboratory. Appropriate positive control compounds (with S9 mix where required) induced substantial increases in revertant colony numbers with all strains, confirming sensitivity of the cultures and activity of the S9 mix.

The following table gives an overview.

Table 5.8.1- 65: Revertant colony counts – experiment 2

Treatment	Conc. (µg/plate)	+/- S9	Mean revertant colony counts in strains				
			TA 98	TA 100	TA 1535	TA 1537	WP2*uvr*A pK M101
M-02	5000	-	22	105	14	8	183
	1500	-	23	98	10	8	225
	500	-	23	113	14	11	213
	150	-	21	98	12	12	201
	50	-	24	111	8	8	197
Solvent control	0	-	24	111	14	9	204
M-02	5000	+	23	94	13	7	198
	1500	+	28	104	11	12	213
	500	+	24	97	15	13	218
	150	+	23	94	16	13	218
	50	+	26	115	12	10	203
Solvent control	0	+	28	98	15	12	196
Sodium azide	0.5	-	NA	502	168	NA	NA
AF-2	0.05	-	NA	NA	NA	NA	2295
9-Aminoacridine	30	-	NA	NA	NA	110	NA
2-nitrofluorene	1	-	166	NA	NA	NA	NA
Benzo[a]pyrene	5	+	250	630	NA	85	NA
2-aminoanthracene	2 - 10 *	+	NA	NA	140	NA	1939

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide
NA : not applicable
*: 2 µg/plate for TA 1535 and 10 µg/plate for WP2*uvr*A

III. Conclusions

It is concluded that M-02 (AE C657188) showed no evidence of mutagenic activity in this *in vitro* bacterial system.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 471 and is valid and acceptable to assess the potential of M-02 to induce gene mutations *in vitro* in bacterial cells. M-02 was not mutagenic under the conditions of this assay.

Data Point:	KCA 5.8.1/14
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C657188 - V79/HPRT-test in vitro for the detection of induced forward mutations
Report No:	AT00551
Document No:	M-235459-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC (2000), OECD 476 (1997), US-EPA 712-C-98-221, OPPTS 870.5300 (1998)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

M-02 (AE C657188) was evaluated for point mutagenic effects at the hypoxanthine-guanine phosphoribosyl transferase locus (forward mutation assay) in V79 cell cultures after treatment with concentrations of up to and including 5000 µg/mL, both with and without S9 mix.

Without and with S9 mix AE C657188 induced no decreases in survival to treatment or in relative population growth. However, AE C657188 was tested up to its limit of solubility under culture conditions. Precipitation of AEC657188 in the culture medium was observed at 4000 µg/mL and above, so that at 5000 µg/mL no further evaluation was possible.

Without and with S9 mix there was no biologically relevant increase in mutant frequency above that of the vehicle controls.

The positive controls ethyl methanesulphonate and dimethylbenzanthracene induced clear mutagenic effects, thus demonstrating the sensitivity of the test system and the activity of the S9 mix.

Based on these results, M-02 is considered to be non-mutagenic in the V79/HPRT Forward Mutation Assay, both with and without metabolic activation.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-02 (referred to as AE C657188 in the report)
Purity:	99.1 % (analytical result dated May 4, 2001) 97,7 % (analytical result dated February 18, 2003)
Batch no.:	D0526 = OP21 50091

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: Ethyl methane sulfonate (EMS) +S9: 9,10-dimethyl-1,2-benzanthracene (DMBA)

3. Cell cultures and medium:

The V79 cell line was originally derived from the lung of a male Chinese hamster. The V79 cells used in this study (designated V79 in this report) were a kind gift from Prof. G. Speit, University of Ulm, Germany. These cells have since been re-cloned to maintain karyotypic stability. They have a modal chromosome number of 22 and a rapid population doubling time (10 to 14 hours).

V79 cell stocks are stored in liquid nitrogen. Laboratory cultures were maintained in plastic tissue culture vessels at 37°C in a humidified atmosphere containing approximately 5% CO2. Exponential growth of cell cultures was maintained by sub-culturing at least twice a week. For cell detachment in order to subculture, a solution consisting of 0.1% trypsin and 0.04% EDTA (ethylenediamine-N.N.N'.N'-tetra acetic acid) in phosphate buffered saline (PBS) has been employed.

The V79 cells were checked on January 16, 2003 for karyotype stability utilising a modified protocol of the method of Moorhead et al. (Exp. Cell Res. 20, 613-616,) and the karyotype (modal chromosomes number: 22) was confirmed. A routine check for mycoplasma was performed on February 17, 2003 using a DNA-Staining Kit (Biochrom) according to the method provided by the supplier. There was no evidence of mycoplasma contamination.

To keep the number of spontaneous 6-TG resistant mutants at a low level, cell cultures were subcloned by plating about 1,000 cells per culture vessel at least every two weeks. If necessary, the spontaneous frequency of HPRT-mutants was additionally reduced by supplementing the culture medium with thymidine (9 µg/mL), hypoxanthine (10 µg/mL), glycine (22.5 µg/mL) and methotrexate (0.3 µg/mL). A 6-TG sensitive subclone was then used for the HPRT-test.

In all parts of this study incubation was performed at 37°C in a humidified atmosphere with about 5% CO2.

Cells were maintained in hypoxanthine-free Eagle's Minimal Essential Medium (MEM, Gibco). The hypoxanthine-free MEM has been proven suitable for the growth of V79 cells (Abbondandolo et al., 1977). The MEM was supplemented with nonessential amino acids, L-glutamine (2 mM), MEM-vitamins, NaHCO3, penicillin (100 units/ml), streptomycin (100 ug/ml) and heat-inactivated foetal calf serum (final concentration: 10%) (Seromed). This medium is referred to as culture medium. During treatment with AE C657188, the serum content was reduced to 2%.

For selection of mutants, a hypoxanthine-free culture medium was used, containing 10 µg/ml of 6-thioguanine (6-TG).

4. Activation:

S9 mix was used for the simulation of the mammalian metabolism. The S9 fraction was isolated in house from the livers of Aroclor 1254 induced male Sprague Dawley rats.

The S9 fraction was kept frozen at -80°C. Prior to its first use in a V79/HPRT-test, a sample of this batch was tested for contamination and cytotoxicity. For use, the S9 fraction was thawed at room temperature. Cofactors were freshly dissolved in sodium phosphate buffer (150 mM, pH 7.4). Three parts of the cofactor solution were mixed with two parts of the S9 fraction giving rise to the following final concentrations in the S9 mix:

8 mM MgCl2 x 6H2O
33 mM KCl
5 mM glucose-6-phosphate
5 mM NADP
40 % (v/v) S9 fraction
60 % (v/v) sodium phosphate buffer

The S9 mix was kept on ice until use the same day.

5. Test compound concentrations used:

A preliminary cytotoxicity test was conducted without and with metabolic activation using concentrations of AE C657188 ranging from 1 µg/mL to 5000 µg/mL. Concentrations of up to 5000 µg/mL AE C657188 did not change the pH in the medium of the pre-test. The osmolality in the medium of the pre-test was not changed by concentrations of up to 5000 µg/mL AE C657188.

Precipitation of the test item in the culture medium was observed from 5000 µg/mL. No relevant cytotoxic effect could be seen.

Therefore, M-02 was tested in the respective first mutation experiment in concentrations ranging from 16 µg/mL to 5000 µg/mL without and with metabolic activation.

The concentrations of further trials were selected on the basis of the results of the first mutation experiment. Owing to the substance's precipitation, concentrations ranging from 16 to 4000 µg/mL were chosen for the repeat test(s) without and with metabolic activation.

B. Test Performance

Experimental phase: May 20 to June 17, 2003

1. Cytotoxicity assay

Exponentially growing V79 cells were plated in 20 mL culture medium in a 250 mL flask (4x106 cells per flask). For each concentration one culture was available. After attachment (16-24 hours later), cells were exposed without S9 mix to vehicle alone and to a range of concentrations of the test substance for 5 hours in 20 mL medium containing 2% FCS. In experiments with metabolic activation 1 mL of medium was replaced by 1 mL S9 mix. Thereafter, cell monolayers were washed with PBS, trypsinised and re-plated in 5 mL culture medium at a density of 200 cells into each of 3 Petri dishes (Ø 60 mm). These dishes were incubated for 6 to 8 days to allow colony development. Thereafter, colonies were fixed with 95% methanol, stained with Giemsa (Merck; stock solution diluted 1:5 with deionized water) and counted. If not interfered e.g. by precipitation on the plates or coloration of the plates, colonies were counted automatically using an Artek counter, model 982B. Data were transferred to a PC and processed with the released and DOS 6.0 based software HPRT-Test (V1.08) of Bayer AG, which includes also counting of mutant colonies.

2. Main assay

Treatment Protocol without Metabolic Activation:

The method is based on the publication of Myhr and DiPaolo (1978, Cancer Res. 38, 2539-2543). Exponentially growing V79 cells were plated in culture medium at a final volume of 20 mL in two 250 mL flasks per concentration (4x106 per flask) including all control groups. After attachment (16-24 hours later), the cells were exposed for 5 hours in 20 mL culture medium with reduced serum content (2%). The corresponding controls were incubated under the same conditions. Thereafter, cell monolayers were washed with PBS, trypsinised and replated in 20 ml culture medium using 1.5 x 10⁶ cells per 250 mL flask and in 5 mL culture medium using 200 cells per Petri dish (Ø 60 mm). Per culture one flask and 3 Petri dishes were used. The Petri dishes were incubated (normally 6 days) to allow colony development and to determine the cytotoxicity associated with each test substance directly after treatment ("Survival to Treatment").

Cells in 250 mL flasks were incubated to permit growth and expression of induced mutations. Cells were sub cultured (= count 1, normally after 2 days) by reseeding 1.5x106 cells into 20 mL medium in 250 mL flasks. At the end of the expression period (= count 2, normally a total of 6 days), cultures were reseeded in Petri dishes (Ø 100 mm) at 3x10⁵ cells per dish (8 dishes per culture) in 20 m culture medium without hypoxanthine but containing 10 µg/mL 6-TG for selection of mutants. In addition, 200 cells per dish (Ø 60 mm, 3 dishes per culture) were seeded in 5 ml culture medium to determine the absolute cloning efficiency for each concentration. After incubation for 6 to 8 days, the colonies were fixed, stained with Giemsa, and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.

At least two trials were performed. Mutant frequencies for at least four concentrations should be determined in each trial.

Treatment Protocol with Metabolic Activation:

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

3. Acceptance Criteria

The following criteria have to be met:

- The average cloning efficiency of the negative and vehicle controls should be at least 50%.
- The average of mutant frequency of the vehicle controls should not exceed 25x10-6 cells.
- The mutant frequency of the two cultures of the vehicle and/or the negative control should differ only to an acceptable extent. As a rule of thumb, the difference of mutant frequencies should not be greater than 5 x 10^{-6}.
- The positive control should induce an average mutant frequency of at least three times that of the vehicle control.
- If not limited by the solubility of the test substance in the vehicle the highest concentration should induce cytotoxicity of about 80 to 90% or should be a concentration where precipitation occurs in the medium. The survival at the lowest concentration should be in the range of the negative control.
- For the calculation of an acceptable mutant frequency at least 5 dishes per culture should be available and relative survival to treatment, relative population growth and absolute cloning efficiency should be 10% or greater.

However, these criteria may be overruled by good scientific judgement.

4. Assessment criteria

- Mutant frequencies will only be used for assessment, if at least 5 dishes per culture were available and relative survival to treatment, relative population growth and absolute cloning efficiency were 10% or greater.
- A trial will be considered positive if a concentration-related and in parallel cultures reproducible increase in mutant frequencies is observed. To be relevant, the increase in mutant frequencies should be at least two to three times that of the highest negative or vehicle control value observed in the respective trial. If this result can be reproduced in a second trial, the test substance is considered to be mutagenic.
- Despite these criteria, a positive result will only be considered relevant, if no significant change in osmolality compared to the vehicle control can be observed. Otherwise, unphysiological culture conditions may be the reason for the positive result.
- A test substance will be judged as equivocal, if there are not strictly concentration related increase in mutation frequencies but if one or more concentrations induce a reproducible and biologically relevant increase in mutant frequencies in all trials.
- An assay will be considered negative if no reproducible and relevant increases of mutant frequencies were observed.

5. Statistical analysis

The statistical analysis relies on the mutant frequencies which are submitted to a weighted analysis of variance as well as to a weighted recursive regression, both with Poisson derived weights (Hsie, A.W. et al., (1981). Mutation Res.86, 193-214. 1981; Arlett et al., (1989), in: Kirkland, D.J. (ed.). Statistical evaluation of mutagenicity test data. UKEMS sub-committee on guidelines for mutagenicity testing. Report Part III, Cambridge University Press, Cambridge, 66-101). According to the acceptance criteria given in point 3, mutant frequencies based on less than 5 plates and/or on a relative survival to treatment and/or a relative population growth and/or an absolute cloning efficiency below 10% are not included in the statistical analysis.

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

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

II. Results and discussion

1. Mutation Assay without Metabolic Activation:

Under nonactivation conditions two trials were performed (Table 5.8.1-66 and Table 5.8.1-67). The mutant frequencies of the negative controls and of the vehicle controls were all within the normal range. The positive control EMS induced clear mutagenic and statistically significant effects in all trials.

For AE C657188 treated cultures no relevant concentration-related decreases were observed both in relative survival to treatment and relative population growth. However, AE 657188 was tested up to the limit concentration of 5000 µg/mL. Relevant AE C657188 induced increases in mutant frequencies could not be found. In addition, the overall statistical analysis did not reveal a statistically significant increase of biological relevance.

Therefore, M-02 (AE C657188) was evaluated as non-mutagenic in the non-activation trial.

Table 5.8.1- 66: Relative survival and mean mutation frequency (mutant colonies per 1 million cells) – Experiment 1-without S9 mix

Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency
M-02	5000	ND	ND
	1600	164.9	1.70
	500	158.5	2.25
	160	139.8	0.60
	50	95.1	1.75
	16	137.2	1.10
Negative control	0	97.9	0.85
Solvent control	0	100.0	0.65
EMS	900	43.9	601.25

ND : not determined
EMS : ethylmethanesulfonate

Table 5.8.1- 67: Relative survival and mean mutation frequency (mutant colonies per 1 million cells) – Experiment 2-without S9 mix

Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency
M-02	4000	122.5	1.00
	2000	130.4	2.50
	1000	74.6	2.40
	500	61.4	1.80
	160	88.3	2.35
	50	85.8	1.35
	16	86.2	1.30
Negative control	0	96.2	2.05
Solvent control	0	100.0	1.15
EMS	900	14.8	329.2

ND : not determined
EMS : ethylmethanesulfonate

2. Mutation Assay with Metabolic Activation:

Two trials were performed with S9 mix (Tables 5.8.1-68 and 5.8.1-69). In all experiments, cytotoxic effects were induced. The mutant frequencies of the negative controls and of the vehicle controls were all within the normal range. The positive control DMBA induced a clear mutagenic and statistically significant effect in all trials.

The AEC657188 treated cultures showed no relevant concentration-related decreases in relative survival to treatment and in relative population growth. However, AE 657188 was tested up to the limit concentration of 5000 µg/mL.

AEC657188 induced no relevant increases in mutant frequencies. In addition, the overall statistical analysis reveals no statistically significant increase.

With metabolic activation AE G657188 was therefore evaluated as non-mutagenic.

Table 5.8.1- 68: Relative survival and mean mutation frequency (mutant colonies per 1 million cells) – Experiment 1-with S9 mix

Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency
M-02	5000	1.0	ND
	1600	107.2	0.60
	500	103.1	0.70
	160	99.0	1.80
	50	84.65	1.85
	16	83.5	1.75
Negative control	0	103.4	1.40
Solvent control	0	100.0	0.90
DMBA	20	63.2	80.75

ND : not determined
DMBA : dimethylbenzanthracene

Table 5.8.1- 69: Relative survival and mean mutation frequency (mutant colonies per 1 million cells) – Experiment 2-with S9 mix

Treatment	Concentration (µg/mL)	Relative survival (%)	Mutation frequency
M-02	4000	128.85	1.15
	2000	129.65	1.65
	1000	95.1	1.65
	500	90.3	2.65
	160	93.5	2.15
	50	112.3	2.75
	16	96.35	3.35
Negative control	0	115.6	0.70
Solvent control	0	100.0	3.40
DMBA	20	47.6	71.00

ND : not determined
DMBA : dimethylbenzanthracene

III. Conclusions

M-02 (AE C657188) was tested in the V79/HPRT-test in concentrations ranging from 16 µg/mL to 5000 µg/mL without and with S9 mix. Under both activation conditions, no cytotoxic effects were induced. However, AEC657188 was tested up to the limit concentration.

AE C657188 induced no biologically relevant increases in mutant frequencies.

The positive controls EMS and DMBA had a marked mutagenic effect, as was seen by a biologically relevant increase in mutant frequencies as compared to the corresponding negative controls and thus demonstrated the sensitivity of the test system and the activity of the used S9 mix.

M-02 (AEC657188) is considered to be non-mutagenic in the V79/HPRT forward mutation assay, both with and without metabolic activation.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 476 and is valid and acceptable to assess the potential of M-02 to induce gene mutations in mammalian cells (V79/HPRT). M-02 was not mutagenic when tested up to the limit concentration under the conditions of this assay.

Data Point:	KCA 5.8.1/13
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C657188 (metabolite of AE C638206): Induction of chromosome aberrations in cultured human peripheral blood lymphocytes
Report No:	2014768-D6172
Document No:	M-234744-01-1
Guideline(s) followed in study:	ICH Tripartite Harmonised Guideline on Genotoxicity, S2A; OECD 473 (1997)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

AE C657188 was tested in an in vitro cytogenetics assay using duplicate human lymphocyte cultures prepared from the pooled blood of three female donors in two independent experiments. Treatments covering a broad range of doses, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9). The test article was dissolved in sterile anhydrous analytical grade Dimethylsulphoxide (DMSO) and the highest dose level used, 2256 mg/mL, was equivalent to 10 mM.

In experiment 1, treatment in the absence and presence of S-9 was for 3 hours followed by a 17-hour recovery period prior to harvest (3+17). The S-9 used was prepared from a rat liver post-mitochondrial fraction (S-9) from Aroclor 1254 induced animals. The test article dose levels for chromosome analysis were selected by evaluating the effect of AEC657188 on mitotic index.

Chromosome aberrations were analysed at the dose levels of 0, 739.2, 1444, 2256 µg/mL (without S-) and of 0, 378.5, 924.1, 2256 µg/mL (with S-9). The highest concentration chosen for analysis, 2256 mg/mL, induced approximately 34% and 37% mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9 respectively.

In experiment 2, treatment in the absence of S-9 was continuous for 20 hours. Treatment in the presence of S-9 was for 3 hours only followed by a 17-hour recovery period prior to harvest (3+17). Chromosome aberrations were analysed at the dose levels of 0, 739.2, 1444, 2256 µg/mL (without S-9) and of 0, 378.5, 924.1, 2256 µg/mL (with S-9). The highest concentrations chosen for analysis, 739.2 ng/mL and 2256 mg/mL, induced approximately 47% and 20% mitotic inhibition in the absence and presence of S-9, respectively.

Appropriate negative (solvent) control cultures were included in the test system in both experiments under each treatment condition. The proportion of cells with structural aberrations in these cultures fell within historical solvent control ranges. 4-Nitroquinoline 1-oxide (NQO) and cyclophosphamide (CPA) were employed as positive control chemicals in the absence and presence of liver S-9 respectively. Cells receiving these were sampled in each experiment, 20 hours after the start of treatment; both compounds induced statistically significant increases in the proportion of cells with structural aberrations.

Treatment of cultures with AE C657188 (metabolite of AE C638206) in the absence and the presence of S-9 resulted in frequencies of cells with structural aberrations which were similar to those in concurrent negative controls. Numbers of aberrant cells (excluding gaps) in all treated cultures fell within historical negative control ranges. No increases in the frequency of cells with numerical aberrations, which exceeded the historical negative control range, were observed in cultures treated with AE C657188 in the absence and presence of S-9.

It is concluded that AEC657188 (metabolite of AEC638206) did not induce chromosome aberrations in cultured human peripheral blood lymphocytes, when tested to a maximum concentration of 10 mM (following 3+17 hour treatments in the absence and presence of S-9) or to its limit of cytotoxicity (following 20+0 hour treatment in the absence of S-9).

I. Materials and methods

A. Materials

1. Test material

Test substance:	AE C657188
Purity:	99.1 %
Batch no.:	OP2150091

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: 4-Nitroquinoline 1-oxide (NQO) (1.25-5.00 µg/mL) +S9: Cyclophosphamide (CPA) (3.125-12.5 µg/mL)

2. Cell cultures:

Blood from three healthy, three non-smoking female volunteers was used for each experiment of this study. No donor was suspected of any virus infection nor had been exposed to high levels of radiation or hazardous chemicals; an appropriate volume of whole blood was drawn from the peripheral circulation on the day prior to culture initiation.

3. Culture Medium:

Blood was stored refrigerated and pooled prior to use. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL heparinised blood into 9.0 mL Hepes-buffered RPMI medium containing 20% (v/v) foetal calf serum and 50 µg/mL gentamycin. Phytohaemagglutinin (PHA reagent grade) was included at a concentration of approximately 2% of culture volume to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37°C and rocked continuously.

4. Activation:

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. The batches of MolTox™ S-9 were stored frozen in aliquots at -80°C and thawed just prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufm-0-dealkylase activities).

Preparation of S-9 mix:

Glucose-6-phosphate (180 mg/mL), NADP (25 mg/mL), 150 mM KCl and rat liver S-9 were mixed in the ratio 1:1:1:2. An aliquot of the resulting S-9 mix was added to each cell culture designated for treatment in the presence of S-9 to achieve the required final concentration in a total of 10 mL. The final concentration of liver homogenate in the test system was 2%. Cultures treated in the absence of S-9 received an equal volume of 150 mM KCl.

5. Test compound concentrations used:

In experiment 1, the dose levels were 0, 739.2, 1444, 2256 µg/mL (without S-9) and of 0, 378.5, 924.1, 2256 µg/mL (with S-9).

In experiment 2, dose levels were 0, 739.2, 1444, 2256 µg/mL (without S-9) and 0, 378.5, 924.1, 2256 µg/mL (with S-9).

B. Test Performance

Experimental phase: March 10 to April 29, 2003

1. Treatment

S-9 mix or KCl (0.5 mL) was added appropriately. One set of quadruplicate cultures (A, B, C and D) for each treatment regime was then treated with the solvent and one set of duplicate cultures with the test article (0.1 mL per culture). Additional duplicate cultures for treatments in the absence of S-9 and in its presence were treated with 0.1 mL of the positive control chemicals. All cultures were then incubated at 37°C. This scheme is illustrated below.

Table 5.8.1- 70: Study scheme

Treatment	S-9	Number of cultures	
		3+17*	20+0*
Experiment 1			
Negative control	-	4	
	+	4	
Test article (all doses)	-	2	
	+	2	
Positive control (all doses)	-	2	
	+	2	
Experiment 2			
Negative control	-		4
	+	4	
Test article (doses as appropriate)	-		2
	+	2	
Positive control (all doses)	-		2
	+	2	

* Hours treatment + hours recovery

Treatment media remained on cultures receiving the continuous treatment until sampling, that is, 20 hours after the beginning of treatment. Cultures received pulse treatments (both in the absence and presence of S-9) for 3 hours only. They were then pelleted (approximately 300 x 'g', 10 minutes), washed twice with sterile saline (pre-warmed at 37°C) and resuspended in fresh medium containing foetal calf serum and gentamycin. Cultures were incubated for a further 17 hours before harvesting.

Table 5.8.1- 71: Treatment conditions

Treatment		S-9	Duration of treatment	Harvest time (hours after start of treatment)
Continuous	20+0		20	20
Pulse	3+17	+	3	20
	3+17	-	3	20

Harvesting:

Approximately 2 hours prior to harvest, colchicine was added to give a final concentration of approximately 1 µg/mL to arrest dividing cells in metaphase. At the defined sampling time cultures were centrifuged at approximately 300 x 'g' for 10 minutes; the supernatant was carefully removed and cells were re-suspended in 4 mL pre-warmed hypotonic (0.075 M) KC1 and incubated at 37°C for 15 minutes to allow cell swelling to occur. Cells were then fixed by dropping the KC1 suspension into an equal volume of fresh, ice-cold methanol/glacial acetic acid (3:1, v/v). The fixative was changed by centrifugation (approximately 300 x 'g', 10 minutes) and resuspension. This procedure was repeated several times (centrifuging at approximately 1250 x 'g', 2-3 minutes) until the cell pellets were clean.

Preparation of metaphase spreads:

Lymphocytes were kept in fixative in the refrigerator before slides were prepared but slides were not made on the day of harvest to ensure cells were adequately fixed. Cells were pelleted and re-suspended in a minimal amount of fresh fixative (if required) so as to give a milky suspension. Several drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance chromosome spreading, and several drops of suspension were transferred to clean microscope slides labelled with the appropriate study details. Slides were flamed, as necessary, to improve metaphase spreading.

After the slides had dried the cells were stained for 5 minutes in 4% (v/v) filtered Giemsa stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried and mounted with coverslips.

Selection of doses for cytogenetic analysis:

Slides were examined, uncoded, for mitotic index (MI) or percentage of cells in mitosis. Slides from enough dose levels from each treatment regime were scored to determine if chemically induced mitotic inhibition had occurred. This is defined as a clear decrease in mitotic index compared with negative controls (based on at least 1000 cells counted), preferably dose-related.

Rationale for dose selection:

The highest dose for chromosome analysis from cultures sampled at 20 hours should be one at which at least 50% mitotic inhibition (approximately) has occurred or should be the highest dose tested. Analysis of slides from highly cytotoxic concentrations is avoided, if possible. Slides from the highest selected dose and two lower doses, such that a range of cytotoxicity from maximum to little or none is covered, were taken for microscope analysis.

For each treatment regime, two solvent control cultures were initially to be analysed for chromosome aberrations. Slides from the remaining solvent control cultures were only to be analysed if considered necessary, for example, to help resolve an equivocal result. A single positive control dose level, which gives satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage, was analysed.

Scoring of aberrations:

Slides from NQO and CPA positive control treatments were checked to ensure that the system was operating satisfactorily. Slides from the selected treatments and from solvent and positive controls were coded using randomly generated letters by a person not connected with the scoring of the slides. Labels bearing only the study reference number, experiment number, the sex of the donor and the code were used to cover treatment details on the slides.

Where possible, one hundred metaphases from each code were analysed for chromosome aberrations. Only cells with 44-46 chromosomes were considered acceptable for analysis of structural aberrations. Any cell with more than 46 chromosomes, that is polyploid, endoreduplicated and hyperdiploid cells, observed during this search was noted and recorded separately. Classification of structural aberrations was based on the scheme described by ISCN ((1995) An International System for Human Cytogenetic Nomenclature; Editor Felix Mitelman; S Karger, Switzerland). Under this scheme, a gap is defined as a discontinuity less than the width of the chromatid and no evidence of displacement of the fragment and a deletion is defined as a discontinuity greater than the width of the chromatid and/or evidence of displacement of the fragment.

Slide analysis was performed off-site by analysts trained in accordance with Covance Laboratories Limited Standard Operating Procedures.

2. Analysis of results

After completion of microscopic analysis, data were decoded. The aberrant cells in each culture were categorised as follows:

1. cells with structural aberrations including gaps
2. cells with structural aberrations excluding gaps
3. polyploid, eridoreduplicated or hyperdiploid cells.

The totals for category 2 in negative control cultures were compared with the current laboratory negative control (normal) ranges to determine whether the assay was acceptable or not. The proportion of cells in category 2 in test article treated cultures were also compared with normal ranges. The statistical significance of any data set was only to be taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentration exceeded the normal range. Under this condition, the statistical method used would be Fisher's exact test. Probability values of $p < 0.05$ were to be accepted as significant. The proportions of cells in categories 1 and 3 were also examined in relation to historical negative control (normal) ranges and statistical analysis by Fisher's exact test may be used.

The proportions of aberrant cells in each replicate were also used to establish acceptable heterogeneity between replicates by means of a binomial dispersion test. Probability values of $p < 0.05$ were to be accepted as significant.

3. Acceptance criteria

The human lymphocyte assay is considered valid if the following criteria are met:

1. the binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures, and
2. the proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range, and
3. at least 160 cells out of an intended 200 are analysable at each dose level, and
4. the positive control chemicals induce statistically significant increases in the proportion of cells with structural aberrations.

4. Criteria for a positive response

A test article is considered as positive in this assay if:

1. the proportions of cells with structural aberrations at one or more concentration exceeds the normal range in both replicate cultures, and
2. a statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurs at these doses.

Increased incidence of cells with gaps or increased proportions of cells with structural aberrations not exceeding the normal range or occurring only at very high or very toxic concentrations are likely to be concluded as "equivocal". Full assessment of the biological importance of such increases is likely only to be possible with reference to data from other test systems. Evidence of a dose-related effect is considered useful but not essential in the evaluation of a positive result. Cells with exchange aberrations or cells with greater than one structural aberration occur very infrequently in negative control cultures. Their appearance is therefore considered to be of particular biological significance.

5. Statistics

Mentioned under point 2.

II. Results and discussion

A. Selection of doses for cytogenetic analysis

The results of mitotic index determinations for the treatments in Experiment 1 were as follows.

Table 5.8.1- 72: Results of mitotic index determinations in Experiment 1

Duration	S-9	Concentrations (µg/mL)	Mitotic inhibition*
3+17 hours	-S-9	739.2, 1444, 2256	34%
3+17 hours	+S-9	378.5, 924.1, 2256	37 %

* At highest concentration analysed

The results of mitotic index determinations for the treatments in Experiment 2 were as follows.

Table 5.8.1- 73: Results of mitotic index determinations in Experiment 2

Duration	S-9	Concentrations (µg/mL)	Mitotic inhibition*
20+0 hours	-S-9	739.2, 1444, 2256	47 %
3+17 hours	+S-9	1001,1385, 2256	20 %

* At highest concentration analysed

B. Chromosome aberration analysis

Validity of study:

The data confirm that:

1. no evidence of significant heterogeneity between replicate cultures was obtained in the binomial dispersion test and
2. the proportion of cells, with structural aberrations (excluding gaps) in negative control cultures fell within the normal range and
3. at least 160 cells out of an intended 200 were analysed at each dose level and
4. the positive control chemicals NQO and CPA induced statistically significant increases in the number of cells with structural aberrations.

Structural aberrations:

Treatment of cultures with AEC657188 in the absence and presence of S-9 resulted in frequencies of cells with structural aberrations which were similar to those in concurrent negative controls Numbers of aberrant cells (excluding gaps) in all treated cultures fell within historical negative control ranges.

Numerical aberrations:

No increases in the frequency of cells with numerical aberrations, which exceeded the historical negative control range, were observed in cultures treated with AEC657188 in the absence and presence of S-9.

The following tables give an overview of the study results.

Table 5.8.1- 74: Mean mitotic indices and number of aberrant human lymphocytes, including and excluding gaps – Experiment 1

Treatment	Concentration (µg/ml)	Metabolic activation +/- S9	Treatment time (h)	Mitotic index	Aberrant cells Including gaps	Aberrant cells Excluding gaps
AE C657188	739.2	-	3	9.9	0	0
	1444	-	3	8.5	3	1
	2256	-	3	7.5	2	1
Solvent control	0	-	3	11.4	2	2
AE C657188	378.5	+	3	12.4	1	1
	924.1	+	3	11.1	0	0
	2256	+	3	9.3	3	2
Solvent control	0	+	3	14.8	2	1
NQO	5	-	3	-	27	19***
CPA	6.25	+	3	-	61	45***

*** p< 0.001 statistically significantly different from controls Fisher's test
NQO : 4-Nitroquinoline 1-oxide
CPA : Cyclophosphamide

Table 5.8.1- 75: Mean mitotic indices and number of aberrant human lymphocytes, including and excluding gaps – Experiment 2

Treatment	Concentration (µg/ml)	Metabolic activation +/- S9	Treatment time (h)	Mitotic index	Aberrant cells Including gaps	Aberrant cells Excluding gaps
AE C657188	320.9	-	20	7.6	0	0
	377.5	-	20	4.8	2	1
	723.2	-	20	4.0	2	1
Solvent control	0	-	20	7.5	1	1
AE C657188	1001	+	3	12.4	2	0
	1385	+	3	9.4	3	3
	2256	+	3	8.3	3	2
Solvent control	0	+	3	10.4	0	0
NQO	2.5	-	20		36	34***
CPA	3.125	+	3	-	41	36***

*** p< 0.001 statistically significantly different from controls Fisher's test
NQO : 4-Nitroquinoline 1-oxide
CPA : Cyclophosphamide

III. Conclusions

It is concluded that M-02 (AE C657188) did not induce chromosome aberrations in cultured human peripheral blood lymphocytes, when tested to a maximum concentration of 10 mM following 3+17 hour treatments in the absence and presence of S-9, or to its limit of cytotoxicity following 20+0 hour treatment in the absence of S-9.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 473 and is valid and acceptable to investigate the potential of M-02 to induce chromosome aberrations in cultured human peripheral blood lymphocytes. M-02 did not induce chromosome aberrations under the conditions of this assay, either in the presence or absence of metabolic activation.

Data Point:	KCA 5.8.1/61
Report Author:	[redacted]
Report Year:	2019
Report Title:	AE C657188: Micronucleus test in human lymphocytes In vitro
Report No:	1969602
Document No:	M-673693-01-1
Guideline(s) followed in study:	OECD Test Guideline No. 487 (July 2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

M-02 (PCA, referred to as AE C657188 in the report) was evaluated for its potential to induce micronuclei in human lymphocytes *in vitro* in two independent experiments. Experiment I comprised a four hour exposure period in the presence and absence of metabolic activation (provided by S9) whilst experiment II provided a 20-hour exposure period in the absence of S9. Concentrations up to 2000 µg/mL were tested in duplicate and 1000 binucleated cells/culture were examined for cytogenetic damage.

In experiment I, no cytotoxicity or precipitation was observed (+/- S9) up to the highest evaluated concentration. Similarly, in experiment II (-S9), no cytotoxicity or precipitation was observed at the highest tested concentration. Therefore, concentrations up to the limit concentration of 2000 µg/mL were evaluated.

No relevant increases in the numbers of micronucleated cells were observed following treatment with the test item either in the presence or absence of S9 mix. However, in experiment I in the absence of S9, a dose dependency, tested via trend test, was observed (p=0.039). Since none of the values exceeded the historical control data or were statistically significantly increased, this finding is considered to be biologically irrelevant.

Appropriate reference substances gave the expected statistically significant increase in the number of micronucleated cells.

The test substance M-02 (PCA) can be considered neither clastogenic nor aneugenic under the conditions of this *in vitro* micronucleus test, when tested up to the highest possible concentration, both in the presence and absence of metabolic activation.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-02 (PCA, referred to as AEC657188 in the report)
Purity:	99.9 % (w/w)
Batch no.:	BCOO 6709-1-1
Expiry date:	16th October 2020

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive controls:	-S9
	Mitomycin C (MMC), 0.8 µg/mL (98% purity, dissolved in deionized water)
	Demecolcine, 100 ng/mL (purity ≥98%, dissolved in deionized water)
	+S9
	Cyclophosphamide (CPA), 15 µg/ml (purity 97-103%, dissolved in saline)

3. Activation:

Metabolic activation was provided by S9-mix derived from phenobarbital-β-naphthoflavone induced rat liver homogenate; each batch of prepared S9 has been routinely evaluated for its ability to activate known mutagens.

An appropriate quantity of S9 was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation used for this study was 30.4 mg/mL.

4. Cell cultures and media:

Cells

Blood was drawn from healthy non-smoking donors, not receiving medication. Blood from a female donor (27 years old) and a male donor (22 years old) were used in experiments I and II, respectively. The lymphocytes have been shown to respond well to PHA and positive control substances; furthermore, both donors have an established low incidence of micronuclei in peripheral blood lymphocytes.

Media

Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hours following collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1), supplemented with 200 mM GlutaMAX™. The medium was further supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (foetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).

All incubations were carried out at 37 °C with 5.5 % CO2 in humidified air.

5. Test compound concentrations used:

Test concentrations for the first experiment were 0 (solvent control), 15.2, 26.5, 46.6, 81.2, 142, 249, 435, 762, 1333 & 2000 µg/mL, both with and without S9 mix.

In the second experiment a continuous (20 hour) treatment was used in the absence of S9 mix at test concentrations of 0 (solvent control), 142, 249, 435, 762, 1333 & 2000 µg/mL, in the absence of S9 mix.

B. Test Performance

Experimental phase: 14th August 2019 to 17th September 2019

1. Preliminary assay

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity (characterized by the percentages of reduction in the CBPI in comparison to the controls and expressed as % cytostasis) was determined from 500 cells/culture. The experimental conditions in this pre-experimental phase were identical to those described for the mutagenicity assay.

The pre-test was performed with 10 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hours (with and without S9 mix). The preparation interval was 40 hours after start of the exposure.

This preliminary test was designated Experiment I since the cultures fulfilled the criteria for cytogenetic evaluation and appropriate concentrations could be selected for evaluation.

2. Cytogenetic experiment

Cells were subject to either 4 hours pulse exposure (with and without S9; experiment I) or 20 hours continuous exposure (without S9; experiment II). The succeeding procedure for cell preparation was the same for both exposure periods.

Pulse exposure

Approximately 48 hours following seeding, two blood cultures of 10 mL each, were set up in parallel in 25 cm² cell culture flasks (for each test item concentration). The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H2O, 192 mg/L Na2HPO4 • 2 H2O and 150 mg/L KH2PO4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

Continuous exposure

Approximately 48 hours following seeding, two blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells re-suspended in and washed with "saline G". The washing procedure was repeated once as described. Following washing, the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

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

Preparation of cells

Following the procedures above (approximately 40 hours from the start of treatment), the cultures were harvested by centrifugation. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

3. Acceptance Criteria

The micronucleus assay is deemed acceptable if it fulfils the following criteria:

- The concurrent solvent control will normally be within the laboratory historical solvent control data range. The average mutant frequency of the vehicle controls should not exceed $25x10^{-6}$ cells
- The concurrent positive controls should produce a statistically significant increase in the micronucleus frequency and should be within the laboratory historical positive control data range. The positive controls should induce a mutant frequency at least 3 times that of the controls
- Cell proliferation criteria in the solvent control are considered acceptable.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- The quality of the slides should allow the evaluation of an adequate number of cells and concentrations
- The criteria for the selection of top concentrations should be met

Any mutant frequencies not meeting these criteria were excluded from the statistical analyses.

4. Assessment criteria

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative if, in all of the experimental conditions examined:

- None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase
- The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- The increase is concentration-related in at least one experimental condition
- The results are outside the range of the laboratory historical solvent control data (95% control limit realized as 95% confidence interval

When all of the criteria are met, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system.

There is no requirement for verification of a clear positive or negative response.

In case the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. narrow concentration spacing, other metabolic activation conditions, i.e. S9 concentration or S9 origin) could be useful.

5. Statistical analysis

Statistical significance was confirmed by the Chi square test ($p < 0.05$), using a validated test script of "R", a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those values that indicated an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression was performed using a validated test script of "R", to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Both, biological and statistical significance were considered together.

II. Results and discussion

In experiment I neither precipitation nor cytotoxicity was observed up to the highest applied concentration, either in the presence or absence of S9, and no relevant influence on osmolarity or pH was observed. Similarly, in experiment II, no precipitation or cytotoxicity was observed up to the highest applied concentration. The highest concentration applied (2000µg/ml) was therefore the limit concentration.

The results of both experiments, with and without metabolic activation, are summarised in the table below:

Table 5.8.1- 76: Summary of results of experiment I and II

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	Historical control data	
						95% Ctrl limit	Min - Max
Exposure period 4 hrs without S9 mix							
I	40 hrs	Solvent control[1]	1.80		0.20	0.01 – 1.20	0.00 – 1.55
		Positive control[2]	1.69	14.1	**11.30[s]**	2.66 – 22.74	3.95 – 28.60
		762	1.79	0.9	0.35		
		1333	1.78	2.6	0.35		
		2000	1.95	n.c	0.50		
Exposure period 20 hrs without S9 mix							
II	40 hrs	Solvent control[1]	1.83		0.30	0.00 – 1.14	0.05 – 1.60
		Positive control[3]	1.43	48.5	**4.20[s]**	1.15 – 6.44	1.95 – 8.80
		762	1.82	0.4	0.25		
		1333	1.75	9.7	0.20		
		2000	1.62	25.6	0.20		
Exposure period 4 hrs with S9 mix							
I	40 hrs	Solvent control[1]	1.72		0.30	0.00 – 1.24	0.10 – 1.30
		Positive control[4]	1.35	50.8	**3.75[s]**	1.01 – 7.34	1.80 – 8.85
		762	1.65	8.7	0.50		
		1333	1.57	21.0	0.40		
		2000	1.62	14.0	0.50		

* For the positive control groups and the test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
s The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c Not calculated as the CBPI is equal to or higher than the solvent control value
1 DMSO 0.5 % (v/v)
2 MMC 0.8 µg/mL
3 Demecolcine 100 ng/mL
4 CPA 15 µg/mL

In both independent experiments, no relevant increases in the numbers of micronucleated cells were observed following treatment with the test item either in the presence or absence of S9 mix. However, in experiment 1 in the absence of S9, a dose dependency tested via trend test was observed (p=0.039). Since none of the values exceeded the historical control data or were statistically significantly increased, this finding is considered to be biologically irrelevant.

Demecolcine (100 ng/mL), MMC (0.8 µg/mL) and CPA (15 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei, thus confirming the validity of the assay to detect clastogens/aneugens.

III. Conclusions

The test substance M-02 (PCA) did not induce micronuclei in this *in vitro* micronucleus test in human lymphocytes, when tested up to precipitative concentrations.

Therefore M-02 (PCA) is considered to be neither clastogenic nor aneugenic under the conditions of this assay.

Assessment and conclusion by applicant:
The study was conducted according to OECD TG 487 and is valid and acceptable to assess the clastogenicity of M-02 in vitro. M-02 is neither clastogenic nor aneugenic under the conditions of this study.

M-04 (AE C657378)

M-04 is not acutely toxic *via* the oral route; in an acute oral toxicity study in rats an LD_{50} of >2000 mg/kg bw was derived.

A 28-day oral toxicity study in the rat is available. A NOAEL of 2000 ppm (equivalent to 159.2 & 230.6 mg/kg bw/d in males and males respectively) was established from this study. At the LOAEL of 20000 ppm (equivalent to 1775 & 1930.8 mg/kg bw/d in males and females respectively), the highest dose tested, reductions in body weight and body weight gain were observed in males in addition to increased kidney weights, urinalysis alterations and histopathology findings. In females at this dose, haematological and clinical chemistry parameters were altered, and increased liver weights and histopathological findings were observed. An ADI of 0.16 mg/kg bw/d can be derived from this study with a safety factor of 1000.

M-04 was tested in a battery of *in vitro* and *in vivo* genotoxicity tests and was not mutagenic *in vitro* in bacterial cells (negative Ames test) or in mammalian cells (negative mutation assay at the HPRT locus in V79 cells of the Chinese hamster lung). However, M-04 induced chromosome aberrations *in vitro* in culture human peripheral blood lymphocytes, when subjected to a 20 hours continuous exposure in the absence of metabolic activation, provided by S9 mix; no aberrations were seen with a 3-hour pulse exposure either in the presence of absence of S9 mix and there was no evidence of numerical aberrations (polyploidy). This result was followed up with an appropriate *in vivo* study. In an *in vivo* mouse micronucleus test (MMT), no evidence of a clastogenic effect of M-04 was seen following two/dose intraperitoneal injections of 1200 mg/kg bw/d, administered 24 hours apart. Exposure of the target tissue was demonstrated by an altered PCE/NCE ratio and was further evidenced by clinical signs and mortality at the high dose; in additional, the mode of administration (i.p) is highly likely to lead to exposure of the target tissue. Therefore, M-04 is not clastogenic or aneugenic *in vivo*. In addition, M-04 did not induce unscheduled DNA synthesis (DNA repair) *in vivo* in a rat liver UDS test.

Overall, there is no toxicological concern for the metabolite M-04.

The available studies for M-04 are summarised below.

Data Point:	KCA 5.8.1/16
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C638206-AE C657378 - Acute toxicity in the rat after oral administration
Report No:	AT00665
Document No:	M-221558-01-1
Guideline(s) followed in study:	Directive 67/548/EEC, Annex IV B, Part B, B. 1 tris (1967); OECD 423 (2001); US-EPA 712-C-98-190, OPPTS 870.1100 (1998)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In an acute toxicity study conducted according to the acute toxic class method, six female fasted Sprague-Dawley rats (3/group) were each administered a single oral gavage dose of 2,000 mg/kg bw of M-04 (referred to as AE C638206 - AE C657378 in the report).

A dose of 2000 mg/kg bw was tolerated by rats without mortalities, effects on weight development or gross pathological findings. Clinical signs comprising decreased motility, laboured breathing and increased salivation were observed, but had fully resolved by day two.

In conclusion, the LD_{50} of M-04 is therefore > 2000 mg/kg body weight.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 - AE C657378 also known as M-04
Chemical name:	2,6-dichloro-3-hydroxy benzamide
Purity:	98.3%
Batch no.:	1119-DC/3
Appearance:	White powder
Expiry:	March 31, 2005

2. Vehicle and/or positive control

Vehicle:	2% aqueous Cremophor EL

3. Test animals

Species:	Rat
Strain:	Female rats of Wistar origin (HsdCpb:Wu)
Age:	9-13 weeks
Weight at start:	176 g (160 - 192 g)
Source:	[redacted]
Acclimation period:	Yes
Diet:	Provimi Kliba 3883.0.15 Maus/Ratte Haltung, Kaiseraugst Switzerland
Water:	Provided *ad libitum*
Housing:	In groups of three rats of the same sex in polycarbonate cages on low dust wood granulate bedding (J. Rettenmaier & Sohne, 73479 Ellwangen-olzmuhle, Germany).
Temperature:	22 ± 2 °C
Humidity:	55 ±5%
Air changes:	Approximately 10 changes per hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: July 24 to August 8, 2003

2. Animal assignment and treatment

Two groups of three female rats were treated at 2000 mg/kg bw. The appropriate dose volume of the test substance was administered to each rat by oral gavage. The day of dosing was designated Day 1.

C. Methods

1. Observations

Mortality:

Cages of rats were checked at least once daily for mortalities.

Clinical signs:

Animals were observed soon after dosing and at frequent intervals for the remainder of Day 1. On subsequent days animals were observed at least once daily. The nature and severity of clinical signs and the time these were noted were recorded at each observation. Animals were observed for 14 days following dosing.

The body weights of each rat were recorded on days 1 (prior to dosing), 8 and 15.

2. Necropsy

The surviving animals were sacrificed by carbon dioxide at the end of the study, dissected and examined macroscopically.

II. Results and Discussion

A. Results

1. Dose-response table (LD_{50})

The results of the study for acute oral toxicity in the fasted rat, including the LD_{50}, are summarized in the table below.

Table 5.8.1- 77: Dose response

Dose (mg/kg bw)	Toxicological result*	Occurrence of signs	Time of death	Mortality (%)
Females				
1st 2,000	0/3/3	3/3	-	0
2nd 2,000	0/3/3	3/3	-	0

* Number of animals which died and/or were sacrificed moribund / number of animals with signs / total number of animals

The LD_{50} is therefore **≥ 2000 mg/kg bw**

2. Clinical signs

The following clinical signs were observed at 2000 mg/kg body weight: decreased motility, laboured breathing, and increased salivation. Recovery of rats was complete by Day 2.

3. Body weights

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

4. Necropsy findings

The necropsies performed at the end of the study did not reveal any unusual findings.

III. Conclusion

M-04 is not acutely toxic via the oral route under the conditions of this study. The LD_{50} was determined to be ≥ 2000 mg/kg bw.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 423 and is valid and acceptable to determine the acute oral toxicity of M-04. M-04 was not acutely toxic under the conditions of this study and an LD_{50} of > 2000 mg/kg bw was established.

Data Point:	KCA 5.8.1/17
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C657378 - Salmonella/microsome test - Plate incorporation and preincubation method AE C657378
Report No:	AT00630
Document No:	M-219454-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC, B.13/14. (2000); OECD 471 (1997); US-EPA 712-C-98-247, OPPTS 870.5100 (1998)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In this study the potential of M-04, to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102 was investigated.

The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test substance was tested at the following concentrations:

Experiment I: 16, 50, 158, 500, 1581 & 5000 µg/plate
Experiment II: 16, 50, 158, 500, 1581 & 5000 µg/tube

Precipitation of the test substance in the overlay agar was observed from 1581 to 5000 µg/plate on the incubated plates and from 1581 to 5000 µg/tube in the test tubes. The plates incubated with the test substance showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

There was no indication of toxicity up to and including 5000 µg/plate; no inhibition of growth was noted.

No dose-related or biologically relevant increase in mutant count was observed in any of the five strains in either experiment, in the presence or absence of S9.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test substance did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, M-04 is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M04 (referred to as AE C657378 in the report)
Purity:	98.3%
Batch no.:	1119-DC/3

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	Without S9 mix:
	Na-azide: TA 1535
	Nitrofurantoin (NF) : TA 100
	4-Nitro-1,2-phenylene diamine (4-NPDA): TA 1537, TA 98
	Mitomycin C (MMC): TA 102
	Cumene hydroperoxide (Cumene): TA 102
	With S9 mix:
	2-Aminoanthracene (2-AA) : TA 1535, TA 100, TA 1537, TA 98, TA 102

3. Activation:

Owing to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

Aroclor 1254 induced rat liver S9 were used as the metabolic activation system. Enzyme induction was induced in at least 6 male SD rats by the administration of a single intraperitoneal injection of 500 mg/kg bw Aroclor 1254. The animals were sacrificed after 5 days and the livers removed under sterile conditions. The liver homogenate was prepared under sterile conditions at 4˚C; the homogenate was centrifuged and the S9 fraction was stored at -80˚C.

S9 mix:

An appropriate quantity of S9 supernatant was thawed before use and the S9 mix prepared with 70 mL of S9 cofactor solution containing:

162.6 mg $MgCl_2 \times 6H2O$
246 mg KCl
179.1 mg glucose-6-phosphate, disodium salt
315 mg NADP, disodium salt
100 mM phosphate buffer

Each batch of S9 was routinely tested for its metabolising capacity by using known reference mutagens. Furthermore, for each S9 batch a sterility test was performed and no contamination of the S9 mix was found.

4. Test organisms:

Histidine-deficient mutants of Salmonella typhimurium LT2 serve as indicators of point mutagenic effects. Point mutations comprise of both base-pair substitutions and frameshift mutations; therefore, strains which cover both types were used. The strains used and the type of mutations that they detect are outlined in the table below.

Table 5.8.1- 78: Salmonella typhimurium strains used

Salmonella typhimurium		
Strains	**Genotype**	**Type of mutations indicated**
TA 1537	his C 3076	Frame shift mutations
TA 98	his D 3052; pKM 101 R-factor	Frame shift mutations
TA 1535	his G 46	Base-pair substitutions
TA 100	his G 46; pKM 101 R-factor	Base-pair substitutions
TA 102	his G 428; pKM 101 R-factor	Base-pair substitutions

In addition, the strains are 'deep rough' i.e. they are partly deficient in lipopolysaccharide side chains in the cell walls which increases their sensitivity to producing mutations. When necessary, the genotype of the stock cultures was regularly checked with regard to histidine requirement, ampicillin resistance, tetracycline resistance, crystal violet sensitivity and UV sensitivity.

The bacterial strains were obtained directly from prof. Bruce Ames.

5. Test substance concentrations used:

Concentrations of 0, 16, 50, 158, 500, 1581 and 5000 µg/plate were tested both in the presence and in the absence of S9 mix. Each concentration was tested in triplicate.

B. Test performance

Experimental phase: July 31 to August 21, 2003

1. Assay procedure

Experiment I (plate-incorporation test)

Three plates were used, both with and without S9, for each strain and concentration. An equal number of plates filled with solvent (minus the test substance) served as the negative control. Each positive control also contained 3 plates per strain. 0.1 mL/plate of solvent was used for the test substances and controls. If not limited by solubility, 5000 µg (5µmL)/plate comprised the highest dose and at least 5 additional doses were used. The results of this first experiment are also considered a pre-test for toxicity.

Experiment II (preincubation test)

The preincubation test was performed in a water bath at 37˚C for 20 minutes. At the end of the preincubation period, 2 mL of molten agar was added to the tubes and the contents mixed and plated. Three plates were used, both in the presence and absence of S9, for each strain and concentration. An equal number of plates filled with solvent (minus the test substance) served as the negative control. Each positive control also contained 3 plates per strain. The concentrations were based on the results of experiment I and are presented as µg/tube.

Assessment of toxicity

Toxicity was assessed in three ways. Firstly, a gross appraisal of background growth reduction was performed. Secondly, a toxic effect was assumed when there was a marked (dose-dependent) reduction in mutant count/plate compared to the negative control and lastly, the titer was determined.

Data recording

The count was made after the plates had been incubated for 48 hours at 37 °C. Colonies were counted automatically using an Artek counter, model 982B. Data were transferred to a PC and processed with the released and DOS 6.0 BioSys software Ames Test III (rev. 3.10/x. if required by the presence of precipitation, the colonies were counted manually.

2. Statistics

A statistical analysis of the data is not required.

3. Acceptance / assessment criteria:

The *Salmonella typhimurium* reverse mutation assay is considered acceptable if it meets the following criteria:

- Negative controls are within the expected range, as defined by published data or the laboratories historical control data
- The positive control substances show sufficient effects as defined by the laboratories experience
- Titer determinations demonstrate sufficient bacterial density in the suspension.

Only trials which met the above criteria were accepted for assessment.

4. Evaluation of results, criteria for a positive response

A reproducible and dose-related increase in mutant counts of at least one strain is considered to be a positive result. For TA 1535, TA 100, and TA 98 the increase should be approximately twice that of the negative controls whilst for TA 1537 at least a three-fold increase should be reached. For strain TA 102 and increase of approximately 100 mutants should be observed. If these criteria are not met the result is deemed negative.

II. Results and Discussion

A. Mutation assays

Precipitation of the test item was observed from 1581 µg/plate; however, no inhibition of growth was noted and there was no indication of a toxic effect up to and including 5000 µg/plate. The total bacterial counts were similar to the negative controls.

In the plate incorporation test, no dose related or biologically relevant increase in mutant counts (over and above those in the negative controls) were observed in any of the five strains, either in the presence or absence of S9 mix. The negative results were confirmed by the preincubation assay.

The positive controls all increased mutant counts to levels well above the negative controls, thus confirming the sensitivity of the assay.

The results are summarised in the following tables.

Table 5.8.1- 79: Summary of experiment I

Test Group	Dose Level (µg/plate)	Mean Revertant Colony Counts				
		TA 1535	TA 100	TA 1537	TA 98	TA 102
Without metabolic activation (-S9)						
M-04	0	20	127	7	35	298
	16	15	126	5	36	283
	50	19	117	9	30	289
	158	22	126	8	30	277
	500	20	121	8	40	267
	1581	17	123	7	33	264
	5000	25	127	6	28	275
Na-azide		441				
NF			314			
4-NPDA				93	164	
MMC						546
With metabolic activation (+S9)						
M-04	0	13	114	9	36	306
	16	11	136	11	37	286
	50	13	117	9	36	260
	158	12	124	8	38	344
	500	13	107	10	37	320
	1581	10	122	9	48	346
	5000	10	103	11	41	281
2-AA		164	1740	305	1095	844

Table 5.8.1- 80: Summary of experiment II

Test Group	Dose Level (µg/tube)	Mean Revertant Colony Counts				
		TA 1535	TA 100	TA 1537	TA 98	TA 102
Without metabolic activation (-S9)						
M-04	0	21	156	6	18	210
	16	21	154	5	14	180
	50	22	144	5	12	210
	158	25	186	5	10	191
	500	30	[149]	5	14	200
	1581	27	151	4	19	202
	5000	25	139	5	12	183
Na-azide		519				
NF			488			
4-NPDA				97	136	
Cumene						464
With metabolic activation (+S9)						
M-04	0	10	106	7	26	194
	16	11	106	4	22	212
	50	8	99	7	23	189
	158	9	109	6	17	201
	500	11	107	6	26	206
	1581	10	109	7	26	185
	5000	7	101	5	23	217
2-AA		77	1349	180	1040	466

III. Conclusion

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test substance did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, M-04 is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of M-04 in *S-typhimurium* strains. M-04 is not mutagenic in bacterial cells under the conditions of this study when tested up to precipitating concentrations (+/- S9), either by base pair changes or frameshift mutations.

Data Point:	KCA 5.8.1/18
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C657378: Induction of chromosome aberrations in cultured human peripheral blood lymphocytes
Report No:	C038440
Document No:	M-224994-01-1
Guideline(s) followed in study:	OECD 473 (1997); ICH Tripartite Harmonised Guideline (1995)
Deviations from current test guideline:	Cytotoxicity was not evaluated by the parameters of relative population doubling (RPD) or relative increase in cell count (RICC). Only 100 metaphases were analysed, rather than a minimum of 300 as recommended in the current guidance.
Previous evaluation:	yes, evaluated and accepted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

This study was performed to assess the ability of M-04 to induce chromosomal aberrations in human lymphocytes cultured *in vitro*.

Human lymphocytes, in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to the test substance both in the presence and absence of S9 mix derived from rat livers. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using Colcemid®, the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

In order to assess the toxicity of M-04 to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. On the basis of these data, the following concentrations were selected for metaphase analysis:

Experiment 1:

- Without S9 mix (20 hours treatment, 0 hours recovery): 1159, 1545 & 2060 µg/mL.
- With S9 mix (3 hours treatment, 17 hours recovery): 1159, 1545 & 2060 µg/mL.

Experiment 2:

- Without S9 mix (3 hours treatment, 17 hours recovery): 843.8, 1318 & 2060 µg/mL.

In the absence of S9 mix (20+0-hour continuous treatment), significantly elevated frequencies of cells with structural aberrations compared to those in concurrent vehicle controls were seen for all analysed concentrations. The numbers of aberrant cells (excluding gaps) in all treated cultures exceeded the normal range and a dose-related increase in the frequency of structural aberrations was evident. Similar findings were not evident with a 3+17-hour pulse treatment in the presence or absence of S9 mix, where the frequencies of cells with structural aberrations were similar to those seen in concurrent vehicle controls; furthermore, the number of aberrant cells (excluding gaps) in all treated cultures for this treatment regime fell within the normal historical control ranges.

The quantitative analysis for polyploidy (numerical aberrations) revealed that normal frequencies (within negative historical control ranges) were observed for the majority of cultures in the presence and absence of S-9 mix.

All positive control compounds caused statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

It is concluded that M-04 has shown evidence of clastogenic activity following a 20-hour continuous exposure in the absence of S9 mix in this *in vitro* cytogenetic test system, under the experimental conditions described.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-04 (referred to as AE C657378 in the report)
Purity:	98.3%
Batch no.:	1119-DC/3

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	+S9: 4-Nitroquinoline 1-oxide (NQO); 2.50 µg/mL -S9: Cyclophosphamide (CPA); 6.25 µg/mL

3. Activation:

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male SD rats inducted with Aroclor 1254 (obtained from Molecular Toxicology Inc., USA). The batches of S9 were stored frozen at -80˚C and thawed prior to use. Each batch was checked for sterility, protein content, ability to convert known pro-mutagens to bacterial mutagens and cytochrome P450-catalysed enzymes activities.

Preparation of S9-mix:

Glucose-6-phosphate (180 mg/mL), NAPD (25 mg/mL), KCl (150 nM) and rat liver S-9 were mixed in the ratio of 1:1:1:2. An aliquot of the resulting S9 mix was added to each required cell culture to achieve the final concentration in a total of 10 mL. the final concentration of liver homogenate in the test system was 2%. Cultures treated in the absence of S9 received an equal volume of 150 mM KCl.

4. Cell cultures and medium:

Blood was collected from 3 healthy non-smoking, female volunteers within one day of culture initiation. Whole blood cultures were established in sterile tubes by placing 0.4 mL heparinised blood into 8.9 mL Hepes-buffered RPMI medium containing 20% (v/v) foetal calf serum and 50 µg gentamycin. Phytohaemagglutinin (PHA) was included at concentration of 2% and 4% of culture volumes (experiments 1 and 2 respectively) to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37˚C and rocked continuously.

5. Test substance concentrations used:

In experiment 1 the tested concentrations were 0 (solvent control), 154.7, 206.2, 275, 366.6, 488.8, 651.8, 869.1, 1159, 1545 & 2060 µg/mL with and without metabolic activation.

In experiment 2 the tested concentrations were 276.5, 345.6, 432, 540, 675.1, 843.8, 1055, 1318, 1648 & 2060 without metabolic activation.

The highest dose selected for chromosome analysis should be one at which at least 50% mitotic inhibition has occurred, or the highest dose tested; slides from highly toxic concentrations should be avoided.

Therefore, based on these criteria the following doses were selected for analysis (the highest selected dose and two lower doses):

Experiment 1 without S9 (20 hours treatment): 1159, 1545 & 2060 µg/mL

Experiment 1 with S9 (3 hours treatment, 17 hours recovery): 1159, 1545 & 2060 µg/mL

Experiment 2 without S9 (3 hours treatment, 17 hours recovery): 843.8, 1318 & 2060 µg/mL

6. Controls

Sterile DMSO was added to negative control cultures. The positive control chemicals were dissolved in DMSO immediately prior to use at the following concentrations:

+S9: 4-Notroquinoline 1-oxide (NQO); 1.25, 2.50 & 5 µg/mL

-S9: Cyclophosphamide (CPA); 3.125, 6.25 & 12.5 µg/mL

Cells treated with 2.50 µg NQO/mL and 6.25 µg CPA/mL gave satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage. Therefore, these concentrations were selected for analysis. Immediately prior to treatment all positive control cultures had 0.1 mL culture medium added to result in a final pre-treatment volume of 9.4 mL.

B. Test performance

Experimental phase: April 24 to September 15, 2003

1. Preliminary Assay and second test

S-9 mix or KCl (0.5 mL) was added appropriately. One set of quadruple cultures (A, B, C and D) for each treatment regime was then treated with the solvent, one set of duplicate cultures treated with RPMI 1640 (untreated controls) and one set of duplicate cultures with the test article (0.2 mL/culture). Additional duplicate cultures for treatment for treatment in the absence of S9 and its presence were treated with 0.1 mL of the positive control chemicals. All cultures were then incubated at 37˚C.

Treatment media remained on cultures receiving the continuous treatment until sampling (20h after start of treatment). Cultures received pulse treatments (+/- S9) for 3 hours only and were then pelleted, washed twice with sterile saline, and resuspended in fresh treatment (with gentamycin and foetal calf serum). These cultures were incubated for a further 17 hours before harvesting.

Harvesting and fixation

Approximately 2 hours prior to harvest, colchicine was added to give a final concentration of 1µg/mL to arrest dividing cells in metaphase. At the defined sampling time cultures were centrifuged, the supernatant carefully removed, and the cells resuspended in 4 mL pre warmed hypotonic (0.075 M) KCl and incubated at 37˚C for 15 minutes to allow cell swelling to occur. Cells were then fixed by dropping the KCl suspension into an equal volume of fresh methanol/glacial acetic acid (3:1, v/v), the fixative was changed by centrifugation and resuspension. The procedure was repeated many times until the pellets were clean. Lymphocytes were kept in fixative in the refrigerator before slides were prepared, slides were not prepared on the day of harvest to ensure cells were adequately fixed.

Slide preparation

The cells were pelleted and resuspended in a minimal amount of fresh fixative to give a milky suspension. Several drops of aqueous acetic acid were added to each suspension to enhance chromosome spreading, and several drops of suspension were transferred to clean slides. Slides were flamed if necessary, to enhance metaphase spreading. After the slides had dried the cells were stained for 5 minutes in 4% (v/v) filtered Giesma stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried and mounted with coverslips.

Microscopic examination

Slides were examined, uncoded for mitotic index (MI) or percentage of cells in mitosis. Slides from enough dose levels from each treatment regime were scored to determine if chemically induced mitotic inhibition had occurred. This is defined as a clear decrease in mitotic index compared with negative controls (based on at least 1000 cells counted), preferably dose related.

For each treatment regime, two solvent control cultures were initially to be analysed for chromosome aberrations. Slides form the remaining solvent control cultures and untreated controls were only analysed if necessary. A single positive control dose, which gave a satisfactory response was analysed.

100 metaphases from each slide were analysed. Only cells with 44-46 chromosomes were acceptable for analysis of structural aberrations.

3. Assessment of results

An assay is considered to be acceptable if the following criteria are met:

- The binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures, and
- The proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range, and
- At least 160 cells out of an intended 200 are analysable at each dose level, and
- The positive control chemicals induce statistically significant increases in the proportion of cells with structural aberrations.

The test substance is considered to cause a positive response if the following conditions are met:

- The proportion of cells with structural aberrations at one or more concentrations exceeds the normal range in both replicate cultures, and
- A statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurs at the concentrations.

A negative response is claimed if no statistically significant increases in the number of aberrant cells above concurrent control frequencies are observed, at any dose level.

A further evaluation may be carried out if the above criteria for a positive or a negative response are not met.

6. Statistics

Aberrant cells in each culture were categorised as:

1) cells with structural aberrations including gaps

2) cells with structural aberrations excluding gaps

3) polyploid, endoreduplicated or hyperdiploid cells

Cells with structural aberrations excluding gaps in the negative control cultures were compared with historical control data to determine the acceptability of the assay and those in the treated cultures were compared with the normal ranges.

The statistical significance of any data set was only taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentrations exceeded the normal range. Fishers exact test would be used in this case. Probability values of $p < 0.05$ would be accepted as significant. The proportions of aberrant cells in each replicate were also used to establish heterogeneity between replicates by means of a binomial dispersion test. Probability values < 0.05 would be significant.

II. Results and Discussion

1. Experiment 1

An initial experiment was performed using pulse (3-hour) treatments in the absence and presence of S-9. However, owing to a technical error the data from this experiment was rejected and not further reported. The experiment was repeated, and the data is presented here as experiment 1.

Experiment 1 was performed using a continuous (20+0-hour treatment) in the absence of S9 and a pulse exposure (3+17-hour treatment) in the presence of S9.

In the presence of S9 mix (3+17-hour pulse treatment) the reduction in mitotic index was 12% at the highest concentration tested of 2060 µg/mL. Concentrations of 1159, 1545 & 2060 µg/mL were selected for the metaphase analysis.

In the absence of S9 mix (20+0-hour continuous treatment) the reduction in mitotic index was 0% at the highest concentration tested of 2060 µg/mL. Concentrations of 1159, 1545 & 2060 µg/mL were selected for the metaphase analysis.

In the presence of S9 mix (3+17-hour pulse treatment) frequencies of cells with structural aberrations were similar to those seen in concurrent vehicle controls. Furthermore, the number of aberrant cells (excluding gaps) in all treated cultures fell within the normal historical control ranges.

In the absence of S9 mix (20+0-hour continuous treatment), significantly elevated frequencies of cells with structural aberrations compared to those in concurrent vehicle controls were seen for all analysed concentrations. The numbers of aberrant cells (excluding gaps) in all treated cultures exceeded the normal range and a dose-related increase in the frequency of structural aberrations was evident.

The quantitative analysis for polyploidy (numerical aberrations) revealed that normal frequencies (within negative historical control ranges) were observed for the majority of cultures in the presence and absence of S-9 mix in experiment 1.

2. Experiment 2

Experiment 2 comprised a pulse exposure (3+17-hour treatment) in the absence of S9. The reduction in mitotic index in the absence of S9 mix was 23% at 2060 µg/mL (the highest concentration tested). Concentrations of 843.8, 1318 & 2060 µg/mL were selected for the metaphase analysis.

In the absence of S9 mix (3+17-hour pulse treatment) frequencies of cells with structural aberrations were similar to those seen in concurrent vehicle controls. Furthermore, the number of aberrant cells (excluding gaps) in all treated cultures fell within the normal historical control ranges.

The quantitative analysis for polyploidy (numerical aberrations) revealed that normal frequencies (within negative historical control ranges) were observed for the majority of cultures in the absence of S-9 mix in experiment 2.

The positive control chemicals NQO and CPA induced statistically significant increases in the number of cells with structural aberrations, thus confirming the sensitivity of the assay for both experiments. The amount of cells with aberrations (excluding gaps) exceeded the range of the HCD for one solvent control replicate from experiment 1 (-S9); however, the remaining 3 fell within the normal range and the remaining two cultures were also analysed and fell within the normal range. The group mean was also with the normal range; therefore, this did not affect the acceptability of the study.

An overview of the results for experiments 1 and 2 is provided in the tables below.

Table 5.8.1- 81: Relative mitotic indices and mean percentage of aberrant human lymphocytes in experiment 1

Treatment	Concentration (µg/ml)	Treatment time (h)	Mitotic index (mean)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
Without metabolic activation (-S9)					
M-04	1159	20	3.7	19	17*
	1545	20	5.2	21.5	8.5*
	2060	20	3.7	23.5	22*
Solvent control	-	20	3.1	11	10
Untreated control	-	20	5.1	4.5	3.5
Positive control NQO	2.5	20	-	19	18.5*

Treatment	Concentration (µg/ml)	Treatment time (h)	Mitotic index (mean)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
With metabolic activation (+S9)					
M-04	1159	3	6.3	1.5	0
	1545	3	5.6	3	2.5
	2060	3	4.2	2	2
Solvent control	-	3	4.7	2.5	2
Untreated control	-	3	6.5	1.5	0.5
Positive control CPA	6.25	3	-	27.5	25*

*Statistically significant p<0.001, exceeding HCD range

Table 5.8.1- 82: Relative mitotic indices and mean percentage of aberrant human lymphocytes in experiment 2

Treatment	Concentration (µg/ml)	Treatment time (h)	MIII* (%)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
Without metabolic activation (-S9)					
M-04	843.8	3	9.8	3	2
	1318	3	8.8	4.5	2
	2060	3	7.9	1.5	0.5
Solvent control	-	3	10.2	3	2.5
Untreated control	-	3	11.6	0.5	0.5
Positive control NQO	2.5	3	-	22	17.5*

*Statistically significant p<0.001

III. Conclusion

M-04 induced structural chromosome aberrations in cultured human peripheral blood lymphocytes following continuous 20-hour treatment in the absence of metabolic activation. A similar effect was not apparent following 3+17-hour treatment either in the presence or absence of metabolic activation, provided by S9 mix, when tested at concentrations up to 10 mM. There was no evidence of numerical aberrations (polyploidy).

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 473 and is valid and acceptable to assess the potential of M-04 to induce chromosome aberrations in human lymphocytes *in vitro*. M-04 was clastogenic in human lymphocytes under the conditions of this study following a 20-hour continuous exposure in the absence of S9. There was no evidence of clastogenicity following a 3-hour pulse exposure in the presence or absence of S9 and there was no evidence of polyploidy.

Data Point:	KCA 5.8.1/19
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C657378 - V79/HPRT-test in vitro for the detection of induced forward mutations
Report No:	AT00628
Document No:	M-219388-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC, B.17. (2000); OECD 476 (1997); US-EPA 712-C-98-221, OPPTS870.5300 (1998)
Deviations from current test guideline:	Concentrations equivalent to 20 mM were tested, the limit concentration in the guidance is 10 mM
Previous evaluation:	yes, evaluated and accepted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The study was performed to investigate the potential of M-04 to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster *in vitro*.

The assay was performed in two experiments with and two without rat liver microsomal activation (S9-mix). The test article was dissolved in DMSO and tested at the following concentrations:

550, 900, 1100, 1800, 2200, 3600 & 4400 µg/mL (+/- S9)

The concentration ranges were based on the results of preliminary testing for solubility and toxicity. The highest concentration was toxic both with and without metabolic activation.

No relevant increase in mutant colony numbers was obtained in any experiment.

The positive controls showed distinct statistically significant increases in induced mutant colonies, thus indicating the sensitivity of the assay whilst the negative controls gave the expected results.

It was concluded that M-04 was not mutagenic in this HPRT test with V79 Chinese hamster cells.

A. Materials

1. Test material

Test substance:	M-04 (referred to as AE C657378 in the report)
Purity:	98.3%
Batch no.:	1119-DC/3

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: Ethyl methanesulphonate (EMS), 900 µg/mL +S9: Dimethylbenzanthracene (DMBA), 20µg/mL

3. Activation:

Metabolic activation was provided by S9 mix. The S9 fraction was isolated in house from the livers of Aroclor 1254 induced male SD rats and was kept frozen at -80˚C. Samples of the batch were tested for contamination and cytotoxicity prior to use. To prepare the S9 mix, two parts of the thawed S9 fraction were mixed three parts of freshly dissolved co-factors (in sodium phosphate buffer) to give the following final concentrations:

$MgCl_2$ x 6H2O (8mM)

KCl (33mM)

Glucose-6-phosphate (5mM)

NADP (1mM)

S9 fraction (40% v/v)

Sodium phosphate buffer (60% v/v)

The S9 mix was stored on ice until use the same day.

4. Cell cultures and media:

Cell cultures

V79 cell stocks (derived from Chinese hamster lung cells) are stored in liquid nitrogen. Laboratory cultures are maintained in plastic tissue culture vessels at 37˚C in a humid atmosphere containing 5% CO_2. Exponential growth was maintained by twice weekly sub-culturing. The cells were checked for mycoplasma contamination of which there was no evidence. To reduce the number of spontaneous 6-TG resistant mutants cell cultures were sub-cloned by plating 1000 cells/culture vessel at least every two weeks. If necessary, the spontaneous frequency of HPRT-mutants was further reduced by the addition of thymidine (9µg/mL), hypoxanthine (10µg/mL), glycine (22.5µg/mL and methotrexate (0.3µg/mL). A 6-TG sensitive sub-clone was then used for the HPRT test.

In all parts of the study incubation was performed at 37˚C humidified air with 5% CO_2.

Media

Cells were maintained in hypoxanthine free Eagle's Minimal Essential medium (MEM, Gibco) which has been proven suitable for growth of V79 cells. The MEM was supplemented nonessential amino acids, L-glutamine (2mM), MEM-vitamins, $NaHCO_3$, penicillin (100 units/mL, streptomycin (100 units/mL) and heat activated foetal calf serum (10%; reduced to 2% in treated cultures).

For the selection of mutants, a hypoxanthine-free culture medium was used containing 10µg/mL of 6-thioguanine (6-TG)

5. Test substance concentrations used:

The following test concentrations were used for the mutation assays (with and without metabolic activation provided by S9 mix):

550, 900 1100, 1800, 2200, 3600 & 4400 µg/mL (+/- S9)

B. Test performance

Experimental phase: June 25 to August 12, 2003

1. Preliminary assay

A preliminary cytotoxicity assay was conducted with and without metabolic activation at the following concentrations in order to select the dose levels for the mutation assays:

1.23, 3.7, 12.3, 37, 123, 370, 1110 & 3330 µg/mL

Cell cultures for the preliminary toxicity assay received the same treatment as in the mutation assays. For each concentration one culture was used.

2. Main assay

Without S9

Exponentially growing V79 cells were plated in culture medium at a final volume of 20 mL in two 250 mL flasks/concentration (4x10^6 per flask) including controls. Following attachment (16-24 hours), the cells were exposed for 5 hours in 20 ml culture medium with reduced serum (2%). Corresponding controls received the same treatment. Thereafter, cell monolayers were washed with PBS, trypsinised and re-plated in 20 mL culture medium using 200 cells per petri dish (one flask and three petri dishes were used per culture). Petri dishes were incubated for 6 days to allow colony development and determine the 'survival to treatment).

Cells in 250 mL flasks were incubated to permit growth and expression of induced mutation. Cells were sub-cultured (=count 1, 3 days) by re-seeding 1.5 x 10^6 cells into 30 ml medium in 250 mL flasks. Following the expression period (=count 2, total 6 days) cultures were re-seeded in petri dishes at 3x10^5 cells per dish (8 dishes per culture) in 20 mL culture medium without hypoxanthine but containing 10 µg/mL 6-Tg for mutant selection. In addition, 200 cells per dish (3 per culture) were seeded in 5 mL culture medium to determine absolute cloning efficiency for each concentration.

After 6-8 days incubation, the colonies were fixed, stained and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.

With S9

The activation assay was performed independently; the procedure was identical to the non-activation assay described above except for the addition of S9 mix. In the activation assay 19mL of culture medium with 1 mL of S9 was used (rather than 20 mL of culture medium) during the treatment period, resulting in a concentration of 5% S9 mix in the cultures.

3. Acceptance Criteria

The assay was considered valid if the following criteria were met:

- The average cloning efficiency of the negative and vehicle controls should be at least 50%.
- The average of mutant frequency of the vehicle control should not exceed $25x10^{-5}$ cells
- The mutant frequency of the two cultures of the vehicle and negative controls should only differ to an acceptable extent (not greater than $5x10^{-6}$)
- The positive control should induce an average mutant frequency of at least 3 times the vehicle control
- If not limited by solubility, the highest concentration should induce cytotoxicity of about 80-90% or should be precipitative concentration. The survival at the lowest concentration should be in the range of the negative control
- For the calculation of an acceptable mutant frequency, at least 5 dishes per culture should be available and relative survival to treatment, relative population growth and absolute cloning efficiency should be 10% or greater

4. Criteria for a positive response

The test substance is classified as mutagenic if:

- A concentration related increase in mutant frequencies is observed in parallel cultures.
- The increase in mutant frequencies should be 2-3 times higher than the highest negative or vehicle control in the respective trial
- The result should be reproduced in the second trial
- the increase should occur in the absence of a change in osmolality compared to the vehicle control

A test substance is considered negative if:

- there is no reproducible or relevant increase in mutant frequencies

A test substance will be considered equivocal if:

- There is no strict concentration related increase in mutant frequencies, but one or more concentrations induce a reproducible and biologically relevant increase in mutant frequencies

Sound scientific judgement should be used in implementing the above criteria.

5. Statistical analysis

The statistical analysis relies on the mutant frequencies which are submitted to a weighted analysis of variance and a weighted recursive regression, both with Poisson derived wights.

II. Results and Discussion

A. Preliminary cytotoxicity assay

There was no effect on the pH or the osmolality of the medium. No precipitation was observed up to the highest tested concentration; however, cytotoxicity was observed from 1100 µg/mL with S9 mix and from 3330 µg/mL without S9 mix. Therefore, concentrations of 550 to 4400 µg/mL were selected for the main mutation assays.

B. Main mutation assays

Without metabolic activation

Two trials were performed in the absence of S9 mix. Concentration related decreases in relative survival to treatment and relative survival to growth in all treated cultures.

No M-04 induced increases in mutant frequencies were observed in the absence of S9; furthermore, statistical analysis revealed no statistically significant increases.

The mutation frequencies of the negative and vehicle controls were all within the normal range and the positive control EMS induced a clear mutagenic and statistically significant effect in both trials, thus demonstrating the validity of the assay.

With metabolic activation

Two trials were performed in the presence of S9 mix. Cytotoxic effects were seen in both trials; concentration related decreases in relative survival to treatment and relative survival to growth in all treated cultures were observed.

No M-04 induced increases in mutant frequencies were observed in the presence of S9; furthermore, statistical analysis revealed no statistically significant increases.

The mutation frequencies of the negative and vehicle controls were all within the normal range and the positive control EMS induced a clear mutagenic and statistically significant effect in both trials, this demonstrating the validity of the assay.

The results from both experiments with and S9 and both experiments without S9 are summarised in the tables below:

Table 5.8.1- 83: Relative survival and mutant frequencies in experiment 1 without metabolic activation

Treatment	Concentration (µg/ml)	Population growth (%)	Mutation frequency
M-04	550	59.3	1.4
	550	91.3	1.8
	900	77	2.1
	900	107.3	2.4
	1100	81.7	0.2
	1100	76.8	2.1
	1800	54.9	0.5
	1800	91.2	0.8
	2200	120.8	0.3
	2200	99.9	0.5
	3600	96.8	0.3
	3600	58.2	0.4
	4400	41.2	0.8
	4400	32.7	0.6
Negative control	-	78	0.3
	-	144.3	0.5
Solvent control	-	100	1.1
	-	100	0.5
Positive control EMS	900	50.5	299.6
	900	70.3	304.1

Table 5.8.1- 84: Relative survival and mutant frequencies in experiment 2 without metabolic activation

Treatment	Concentration (µg/ml)	Population growth (%)	Mutation frequency
M-04	550	99.2	4.2
	550	136.1	0.9
	900	48.7	1.4
	900	69.0	0.4
	1100	56.4	0.7
	1100	67	0.9
	1800	49.1	0.9
	1800	93.2	1.2
	2200	52.0	1.1
	2200	94.0	0.7
	3600	49.4	4.0
	3600	55.0	0.9
	4400	40.2	0.9
	4400	69.4	0.7
Negative control	-	106.2	1.6
	-	97.8	1.6
Solvent control	-	100	2.3
	-	100	1.6
Positive control EMS	900	45.9	252
	900	38.2	219.6

Table 5.8.1- 85: Relative survival and mutant frequencies in experiment 1 with metabolic activation

Treatment	Concentration (µg/ml)	Population growth (%)	Mutation frequency
M-04	550	150	0.6
	550	57.4	0.3
	900	189.4	0.7
	900	63.7	0.4
	1100	185.0	1.2
	1100	94.3	0.3
	1800	161.1	0.4
	1800	57.4	1.6
	2200	63.9	2.1
	2200	35.2	2.3
	3600	53.3	1.8
	3600	19.3	3.8
	4400	41.7	0.3
	4400	11.4	0.3
Negative control	-	105.6	0.7
	-	84.7	0.5
Solvent control	-	100	0.9
	-	100	0.5
Positive control EMS	900	59.8	59.3
	900	24.6	50.9

Table 5.8.1- 86: Relative survival and mutant frequencies in experiment 2 with metabolic activation

Treatment	Concentration (µg/ml)	Population growth (%)	Mutation frequency
M-04	550	121.3	1.0
	550	137.9	0.5
	900	174.8	1.2
	900	117.3	0.5
	1100	-	-
	1100	-	-
	1800	193.8	3.2
	1800	197.7	2.9
	2200	320.6	0.7
	2200	161.4	0.6
	3600	216.6	1.4
	3600	82.4	0.6
	4400	72.3	0.8
	4400	57.1	0.4
Negative control	-	115.9	1.2
	-	107.1	1.3
Solvent control	-	100	0.4
	-	100	0.5
Positive control EMS	900	38.1	76.2
	900	49.7	65.3

III. Conclusion

M-04 did not induce gene mutations in mammalian cells (Chinese hamster lung V79 cells) either in the presence or absence of metabolic activation provided by S9 mix. Appropriate responses exhibited by the concurrent negative, vehicle and positive controls confirmed the validity of the assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD 476 and is valid and acceptable to assess the potential of M-04 to induce gene mutations at the HPRT locus in Chines Hamster Lung V79 cells. M-04 is not mutagenic in mammalian cells under the conditions of this study.

Data Point:	KCA 5.8.1/20
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C657378 - Micronucleus-test on the male mouse
Report No:	C038286
Document No:	M-224671-01-1
Guideline(s) followed in study:	EU (=EEC): 2000/32/EC; OECD 474
Deviations from current test guideline:	2000 rather than 4000 immature erythrocytes were analysed
Previous evaluation:	yes, evaluated and accepted in the DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The micronucleus test was employed to investigate M-04 in male NMRI mice for a possible clastogenic effect on the chromosomes of bone marrow erythroblasts.

Cyclophosphamide (a known clastogen and cytostatic agent) served as the positive control.

Male mice treated with M-04 received two intraperitoneal administrations of 300, 600 or 1200 mg/kg bw, separated by 24 hours. Males of the positive control received a single intraperitoneal treatment with 20 mg/kg bw cyclophosphamide.

The femoral marrow of all groups was prepared 24 hours after the last administration.

Males treated twice with M-04 in doses up to and including 1200 mg/kg bw showed signs of toxicity after administration starting at 300 mg/kg (in addition one male died at 1200 mg/kg bw), thus demonstrating systemic exposure of males to M-04.

There was an altered ratio between polychromatic and normochromatic erythrocytes. Further demonstrating the systemic exposure of the animals to the test substance.

Following two intraperitoneal treatments of males with doses up to and including 1200 mg/kg bw no indications of a clastogenic effect of M-04 were found; a slight statistically significant increase at 300 mg/kg bw was not relevant owing to the lack of a dose response and the fact that the increase was within the range of the negative historical control data.

The positive control produced a clear clastogenic effect, as demonstrated by a biologically relevant increase in polychromatic erythrocytes with micronuclei. The ratio of polychromatic to normochromatic erythrocytes was not altered.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-04 (referred to as AE C657378 in the report)
Purity:	98.3%
Batch no.:	1119-DC/3

2. Vehicle and/or positive control

Vehicle:	0.5% aqueous Cremophor
Positive control:	Cyclophosphamide (CP)

3. Test animals

Species:	Mice
Strain:	Hsd/Win:NMRI
Age:	6-12 weeks
Weight at start:	36-42 g
Source:	[redacted]
Acclimation period:	At least five days
Diet:	Standard diet
Water:	Provided *ad libitum*
Housing:	Housed individually in type 1 cages
Identification	Cage markings
Temperature:	22.5-23 °C
Humidity:	40-70%
Air changes:	10/hour
Photoperiod:	12 hours light/12 hours dark

4. Test substance doses

The selection of doses was based on a pilot test using animals of the same source, strain, and age. In the pilot study 3/mice/sex received the test substance *via* intraperitoneal injections at doses of 1000 and 2000 mg/kg bw, 24 hours later a second injection at the same dose was administered. In males from 1000 mg/kg bw clinical signs comprising apathy, semi-anaesthetised state, weight-loss, staggering gait, lateral recumbency, spasm, difficulty breathing and slitted eyes were recorded for at least 4-hours following the second application. In addition, 3/3 males at 2000 mg/kg bw died. In females from 1000 mg/kg bw clinical signs comprising apathy, semi-anaesthetised state, weight-loss, staggering gait, lateral recumbency, spasm, difficulty breathing, and wide-legged gait were observed for at least 4 hours following the second application and all females (3/3) died at 2000 mg/kg bw.

Based on these findings, 1200 mg/kg bw was selected as the MTD for males. There were no substantial sex differences observed, therefore, females were not included.

B. Test performance

Experimental phase: October 7 to November 5, 2003

1. Treatment and sampling times

The study design of the main study was as follows:

Table 5.8.1- 87: Main study

Experimental group	Dose in mg/kg bw	No. of animals	Route and number of applications	
Negative control	0	5	i.p.	2
M04	300	5	i.p.	2
	600	5	i.p.	2
	1200	5	i.p.	2
	1200*	5	i.p.	2
Positive control (Cyclophosphamide)	20	5	i.p.	1

*Replacement group; only to be used as substitution of animals at 1200 mg/kg bw (for which ratio of PCE/NCE was strongly altered)

Male mice (five/group) received two intraperitoneal administrations of 300, 600 or 1200 mg/kg bw, separated by 24 hours. Mice of the positive control received a single intraperitoneal treatment with 20 mg/kg bw cyclophosphamide, whilst two injections of the negative control (cyclophosphamide dissolved in deionized water) were administered the same way as the treated groups. In all groups the administered volume was 10 mL/kg.

2. Tissues and cells examined

At least one intact femur was prepared from each sacrificed animal (not pre-treated with a spindle inhibitor). A suitable instrument was used to sever the pelvic bones and lower leg.

The femur was separated from muscular tissue. The lower-leg stump, including the knee and all attached soft parts, was separated in the distal epiphyseal cartilage by a gentle pull at the distal end.

The proximal end of the femur was opened at its extreme end with a suitable instrument, e.g. fine scissors, making visible a small opening in the bone-marrow channel.

A suitable tube was filled with sufficient foetal calf serum. A small amount of serum was drawn from the tube into a suitable syringe with a thin cannula. The cannula was pushed into the open end of the marrow cavity. The femur was then completely immersed in the calf serum and pressed against the wall of the tube, to prevent it slipping off. The contents were then flushed several times and the bone marrow was passed into the serum as a fine suspension. Finally, the flushing might be repeated from the other end, after it had been opened. The tube containing the serum and bone marrow was centrifuged in a suitable centrifuge at approximately 1000 rpm for five minutes. The supernatant was removed with a suitable pipette (e.g. Pasteur pipette), leaving only a small remainder. The sediment was mixed to produce a homogeneous suspension.

One drop of the viscous suspension was placed on a well-cleaned slide and spread with a suitable object, to allow proper evaluation of the smear. The labelled slides were dried overnight. If fresh smears needed to be stained, they needed to be dried with heat for a short period.

The smears were stained automatically with an Ames Hema-Tek Slide Stainer from the Miles Company. The slides were then "destained" with methanol, rinsed with deionized water, and left to dry.

Following this treatment, the smears were transferred to a holder. A cuvette was filled with xylene, into which the holder was immersed for approximately ten minutes. The slides were removed singly (e.g. with tweezers) to be covered. A small amount of covering agent was taken from a bottle with a suitable object (e.g. glass rod) and applied to the coated side of the slide. A cover glass was then placed in position without trapping bubbles. The slides were not evaluated until the covering agent had dried.

3. Evaluation

Coded slides were evaluated using a light microscope at a magnification of about 1000. Micronuclei appear as stained chromatin particles in the anucleated erythrocytes. They can be distinguished from artifacts by varying the focus. Normally, 2000 polychromatic erythrocytes were counted per animal. The incidence of cells with micronuclei was established by scanning the slides in a meandering pattern.

It is expedient to establish the ratio of polychromatic to normochromatic erythrocytes for two reasons:

1. Individual animals with pathological bone marrow depressions may be identified and excluded from the evaluation.
2. An alteration of this ratio may show that the test compound actually reaches the target.

Therefore, the number of normochromatic erythrocytes per 2000 polychromatic ones was noted. If the ratio for a single animal amounts to distinctly more than 6000 normochromatic erythrocytes per 2000 polychromatic ones, or if such a ratio seems likely without other animals in the group showing similar effects, then the case may be regarded as pathological and unrelated to treatment, and the animal may be omitted from the evaluation. A relevant, treatment-related alteration of the ratio polychromatic to normochromatic erythrocytes can only be concluded if it is clearly lower for a majority of the animals in the treated group than in the negative control.

In addition to the number of normochromatic erythrocytes per 2000 polychromatic ones, the number of normochromatic erythrocytes showing micronuclei was also established. This information is useful in two ways. Firstly, it permits the detection of individuals already subject to damage before the start of the test. Secondly, combined with the number of micronucleated polychromatic erythrocytes, it permits a representation of the time-effect curve for positive substances.

An increase in the number of micronucleated normochromatic erythrocytes, without a preceding increase in micronucleated polychromatic erythrocytes, is irrelevant to the assessment of a clastogenic effect, since normochromatic erythrocytes originate from polychromatic ones. Before an effect can be observed in normochromatic erythrocytes, there must be a much greater increase in micronucleated polychromatic erythrocytes, due to the "dilution effect" of the "old" cells, i.e. normochromatic erythrocytes already present at the start of the test, and this effect would have been observed previously

4. Evaluation criteria

An assay was considered acceptable if the figures of negative and positive controls were within the expected range, in accordance with the laboratory's experience and/or the available literature data.

5. Criteria for a positive response

A test was considered positive if there was a relevant and significant increase in the number of polychromatic erythrocytes showing micronuclei in comparison to the negative control.

A test was considered negative if there was no relevant or significant increase in the rate of micronucleated polychromatic erythrocytes. A test was also considered negative if there was a significant increase in that rate which, according to the laboratory's experience was within the range of historical negative controls.

In addition, a test was considered equivocal if there was an increase of micronucleated polychromatic erythrocytes above the range of attached historical negative controls, provided the increase was not significant and the result of the negative control was not closely related to the data of the respective treatment group. In this case, normally a second test will be performed.

6. Statistical methods

Treated group(s) with the highest mean (provided this superseded the negative control mean) and the positive control were checked by Wilcoxon's nonparametric rank sum test with respect to the number of polychromatic erythrocytes having micronuclei and the number of normochromatic erythrocytes. A variation was considered statistically significant if its error probability was below 5 % and the treatment group figure was higher than that of the negative control.

The rate of normochromatic erythrocytes containing micronuclei was examined if the micronuclear rate for polychromatic erythrocytes was already relevantly increased. In this case, the group with the highest mean was compared with the negative control using the one-sided chi²-test. A variation was considered statistically significant if the error probability was below 5 % and the treatment group figure was higher than that of the negative control. In addition, standard deviations (1s ranges) were calculated for all the means.

II. Results and Discussion

A. Micronucleus assay

Following two intraperitoneal administrations of 300, 600 and 1200 mg/kg bw M04, the treated males presented with apathy, semi-anaesthetised state, roughened fur, loss of weight, lateral recumbency, spasm and difficulty breathing; thus demonstrating the systemic exposure, and therefore the target tissue exposure, of the animals to the test substance. One male at 1200 mg/kg bw died during the test period as a consequence of these clinical signs. There were no deaths or apparent signs of toxicity in the control groups.

An overview of the genotoxicity evaluation is provided below.

Table 5.8.1- 88: Group mean PCE/NCE ratios and incidences of micronucleated PCE and NCE

Treatment group	Dose (mg/kg bw)	No. of animals	Total no. PCE scored	No. NCE/ 2000 PCE ± SD	No. micronucleated cells/2000 ± SD	
					NCE	PCE
M-04	1200	5	10000	2985±494**	2.9±1.5	3.0±1.9
	600	5	10000	1632±518	2.7±1.8	5±2.4
	300	5	10000	2108±482	1.9±1.6	5.4±1.3*
	0	5	10000	1446±262	0.9±1.3	2.4±1.8
Positive control (Cyclophosphamide)	20	5	10000	1823±609	2.8±0.7	22.4±5.6*

*p<0.05, **p<0.01 in non-parametric Wilcoxon ranking

It can be seen from the above table that the ratio of polychromatic to normochromatic erythrocytes in males was altered by treatment with M-04 and was 2000:1446 (1s=262) in the negative control, 2000:2108 (1s=482) in the 300 mg/kg bw group, 2000:1632 (1s=518) in the 600 mg/kg bw group and 2000:2985 (1s=494) in the 1200 mg/kg bw group. Therefore, the systemic exposure of the test substance has been demonstrated.

There were no biologically relevant variations between control and treated groups with respect to the incidence of micronucleated polychromatic erythrocytes. The incidence of micronucleated cells was 2.4/2000 (1s=1.8) in the negative control and 5.4/2000 (1s=1.3), 5.0/2000 (1s=2.4) and 3.0/2000 (1s=1.9) at 300, 600 and 1200 mg/kg bw, respectively. Similarly, no biologically significant variation between the negative control and treated groups in the number of micronucleated normochromatic erythrocytes was observed, since normochromatic erythrocytes originated from polychromatic ones.

A slight statistically significant increase was noted at 300 mg/kg bw; however, this was not considered relevant as there was no dose response and was within the range of the historical control data.

The positive control cyclophosphamide caused a clear, statistically significant increase in the number of polychromatic erythrocytes with micronuclei. The incidence of micronucleated PCE was 22.4/2000 (1s = 5.6), which represents biologically relevant increases in comparison to the negative control and thus confirms the sensitivity of this study.

III. Conclusion

There was no indication of a clastogenic effects in male mice following two intraperitoneal treatments with doses of up to and including 1200 mg/kg bw.

The ratio of polychromatic to normochromatic erythrocytes was altered by treatment and thus confirmed relevant systemic bone marrow exposure.

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered M-04 in the micronucleus test in male mice, i.e. in a somatic test system *in vivo*.

Assessment and conclusion by applicant:

This study was conducted according to OECD TG 474 and is valid and acceptable to determine the clastogenic potential of M-04 *in vivo*. M-04 was not clastogenic under the conditions of this study.

Data Point:	KCA 5.8.1/21
Report Author:	[redacted]
Report Year:	2003
Report Title:	Subacute toxicity in rats (Administration in the diet for 4 weeks) - Project AE C638206
Report No:	AT00699
Document No:	M-221960-01-2
Guideline(s) followed in study:	MAFF 12-Nousan No 8147 (2001); OECD 407; US-EPA OPPTS 870.3100
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

M-04 (reported as AE C657378) was administered *via* the food to 10 male and 10 female Wistar rats per dose group, at dietary concentrations of 0, 20, 200, 2000 and 20000 ppm for a period of at least 4 weeks. This corresponds to estimated achieved doses of 1.6, 16.2, 159.2 and 1575.0 mg/kg bw/d in males and 2.1, 20.4, 230.6 and 1930.8 mg/kg bw/d in females.

The animals were regularly observed and weighed, and food intake were determined. In addition, ophthalmological investigations, observations in the functional observation battery (FOB) as well as clinical laboratory analyses of blood and urine samples were performed. Organ weights were determined, and organs and tissues were subjected to gross and histopathological investigations.

There were no deaths or clinical signs of toxicity and FOB investigations revealed no indications of neurotoxicity.

At 20000 ppm lower body weights and body weight gains were noted in males, whilst there was no effect on the body weights of females or on the food consumption of both sexes up to the highest dose tested. Ophthalmoscopy revealed no unusual findings.

At 20000 ppm significantly reduced haemoglobin concentration (females) and significantly higher platelet counts (both sexes) were noted. At the same dose, a significant increase in plasma cholesterol level and liver weights was noted in both sexes and in females a minimal cytoplasmic change of periportal hepatocytes was evident, which was accompanied by a concurrent reduction of periportally stored fat.

In 20000 ppm males a reduced mean for urine volume followed by an increase in urine density as well as a higher severity in the degree and increased incidence of basophilic cortical tubules were found. These findings were correlated with an increase in relative kidney weights.

At 20000 ppm thyroid glands exhibited granular or clumpy alterations of the follicular colloid in both genders and flattened follicular epithelium in two often high dose males.

Under the conditions described the dietary administration of M-04 to male and female rats was tolerated without adverse effects up to 2000 ppm.

In conclusion, a NOAEL of 2000 ppm (equivalent to 159.2 and 230.6 mg/kg/day, in males and females respectively) was proposed for M-04.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M04 (referred to as AE C638206)
Chemical name:	2,6-dichloro-3 -hydroxybenzamide
Purity:	98.3%
Batch no.:	1119-DC/3
Appearance:	White powder
Expiry:	March 31, 2005

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rats
Strain:	Wistar (Hsd Cpb:WU)
Age:	4 – 5 weeks of age (calculated from body weights)
Weight at start:	140g (120 g to 154 g) for males and 107 g (95 g to 119 g) for females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Fixed-formula standard diet (supplied by Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland)
Water:	Water ad lib
Housing:	Singly housed
Temperature:	22 ± 2 °C
Humidity:	55% ± 5%
Air changes:	≥ 10 passages per hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: May 16 to June 23, 2003

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 5.8.1- 89: Study design

Group no.	Dose (ppm)	Sex	Number of animals	Animal number
1	0	Males	10 (control)	1 – 10
2	20	Males	10	11 – 20
3	200	Males	10	21 – 30
4	2,000	Males	10	31 – 40
5	20,000	Males	10	41 – 50
6	0	Females	10 (control)	51 – 60
7	20	Females	10	61 – 70
8	200	Females	10	71 – 80
9	2,000	Females	10	81 – 90
10	20,000	Females	10	91 – 100

3. Diet preparation and analysis

Prior to the start of treatment, a procedure was developed to prepare stable mixtures of the test material in the laboratory rodent diet at the required nominal concentrations.

The test item was mixed into the diet intended for the following week. A mixing granulator manufactured by the company Lodige, Paderborn was used. The mixtures were used for not longer than one week.

Analyses to determine homogeneity and stability of the test item in the diet were started prior to the study. Stability was satisfactory over the time of use of the diet, i.e. % nominal levels declined by a maximum of 5% over 8 days storage at room temperature.

During the study the correct concentration in the formulations were checked analytically twice. The results for the test-diet samples analysed and prepared for dosing, were within the range 95% to 108% of nominal (acceptable range +10% to -15% of nominal).

4. Statistics

The statistical evaluation of data related to laboratory investigations, body and organ weights as well as feed intake was performed using SAS® routines.

C. Methods

1. Observations

On working days, the experimental animals were inspected twice a day for morbidity and mortality (once on weekends and public holidays). General clinical examinations (in the home cage) were made daily, detailed clinical examinations were performed weekly. Once before the start of treatment and once weekly thereafter animals were placed into a standard arena (open field) for behavioural observations. Any clinical signs (findings) and abnormalities were recorded. Body surfaces and orifices, posture, general behaviour, breathing, and excretory products were assessed. Findings and abnormalities were recorded either using a coding system or uncoded.

2. Body weight and food intake

The weight of the animals was recorded at receipt. Each animal was weighed at randomisation, at the start of treatment, weekly thereafter and at necropsy. For each sex, the amount of food consumed for each cage of animals was measured at weekly intervals throughout the treatment period.

3. Ophthalmoscopic examination

Ophthalmological examinations were conducted on each animal prior to the start of treatment and on all animals of Groups 1 and 5 just prior to termination.

The pupillary reflex of both eyes was first tested in a darkened room and the anterior regions of the eye were inspected. After dilating the pupils with Mydriaticum Roche® or Mydriaticum Stulln® drops, the refractive elements of the eye as well as iris and fundus were examined using, an indirect ophthalmoscope. In addition, the optical media were examined with a ZEISS photo-slit lamp.

4. Neurotoxicity assessments

Near terminal sacrifice, the appearance, behaviour and functional integrity of each animal was assessed using a Functional Observation Battery (FOB). The FOB was comprised of a combination of examinations that assess the reaction of animals to handling on removal from the cage and observations in an open field standard arena.

The following observations/examinations were performed:

Home cage observation: posture, piloerection, gait abnormalities, involuntary motor movements, vocalization, others

Observations during handling: ease of removing, reaction to being handled, muscle tone, palpebral closure, pupil size, pupil response (only done in those rats investigated ophthalmologically and at that examination), lacrimation, nasal discharge, salivation, stains.

Open field observations: piloerection, respiratory abnormalities, posture, involuntary motor movements, stereotypy, bizarre behaviour, gait abnormalities, vocalization, arousal, rearing, defecation, urination, others.

Manipulative tests: The following were additionally determined in week 4; approach response, touch response, auditory response, tail pinch response, righting reflex, grip strength, landing foot splay, body temperature.

5. Laboratory investigations (haematology, clinical chemistry, urinalysis)

Samples for clinical laboratory investigations were taken near terminal sacrifice (Days 29 or 32).

The blood samples for determination of glucose concentrations were directly taken after the urine sampling period from the caudal vein of fasted (for about 16 hrs), non-anesthetized animals.

The blood samples used for determining the other parameters in peripheral blood were collected in the morning from the retro-orbital venous plexus of fasted (for about 16 hrs) animals anesthetized with diethyl ether. The blood samples for the partial thromboplastin time (PTT) and HQUICK were collected during ether narcosis directly prior to necropsy on fasted rats, which had only received water ad libitum overnight.

Urine samples were collected at room temperature during a period of 16 hours. Prior to the urine collection rats received 10 mL per kg body weight by gavage. Feed was not supplied.

The following parameters were determined:

Table 5.8.1- 90: Haematology

Leukocytes	Mean corpuscular volume (MCV)
Erythrocytes	Mean corpuscular haemoglobin (MCH)
Haemoglobin	Mean corpuscular haemoglobin concentration (MCHC)
Haematocrit	
Reticulocytes	HQUICK (thromboplastin time)
Differential blood count	Platelets
	PTT (partial thromboplastin time)

Table 5.8.1- 91: Clinical Chemistry

Alkaline phosphatase	Creatinine
Aspartate aminotransferase	Total protein
Alanine aminotransferase	Albumin/globulin ratio
Glutamate dehydrogenase	Albumin
Gamma glutamyl transpeptidase	Sodium
	Potassium
Cholesterol	Chloride
Triglyceride	Calcium
Urea	Glucose
Total bilirubin	

Table 5.8.1- 92: Urinalyses

Semi quantitatively:	Quantitatively:
pH	Volume
Glucose	Specific gravity
Ketones	Protein x Vol
Protein	Protein
Bilirubin	
Urobilinogen	
Blood	
Microscopy of sediment	

4. Sacrifice and pathology

Animals were killed by exsanguination under deep ether anaesthesia. All animals were examined thoroughly, and macroscopic abnormalities were recorded.

The following organs from all animals were weighed at necropsy:

Adrenals	Liver	Testes
Brain	Ovaries/oviducts	Thymus
Epididymides	Pituitary (fixed)	Thyroid/parathyroid (one fixed organ only)
Heart	Prostate	
Kidneys	Spleen	Uterus

The following organs and tissues from all animals were evaluated histopathologically:

Adrenal glands*	Liver (Oil-Red-O)*	Stomach (forestomach and glandular stomach)
Brain (cerebrum, cerebellum, brain stem)	Lungs	Testes
Epididymides	Lymph nodes, mandibular	Thymus
Eyes	Lymph nodes, mesenteric	Thyroid glands (with parathyroids)
Femur with Bone Marrow	Ovaries*	Trachea*
Heart	Oviducts	Urinary bladder*
- Duodenum	Prostate	Uterus (with cervix)*
- Jejunum	Sciatic nerve	Organs and tissues with macroscopic findings*
- Ileum	Seminal vesicles (incl. coagulating glands)	
- Cecum	Skeletal muscle (thigh)	
- Colon	Spinal cord (cervical, thoracic, lumbar)	
- Rectum	Spleen	
- Peyer 's Patches	Sternum with Bone marrow	
Kidneys (with Adip. Tissue)		
Liver		

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities and no treatment-related clinical signs.

2. Body weight and food and water intake

Body weight

Compared with controls, body weight was reduced in males at 20000 ppm, being statistically significant on day 22 (-7.7%) and day 29 (-9.7%); body weight gain was also reduced in males at this dose being statistically significant on days 22-29 (-75%). There were no statistically significant effects on body weight or body weight gain in females up to the highest dose tested of 20000 ppm.

Table 5.8.1- 93: Mean body weights and body weight gains per day

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Body weight [g] (% difference to control)					
Day 1	140	140 (±0.0)	143 (+2.1)	139 (-0.7)	137 (-2.1)
Day 8	182	185 (+1.6)	189 (+3.8)	184 (+1.1)	175 (-3.8)
Day 15	228	224 (-1.8)	233 (+2.2)	223 (-2.2)	212 (-7.0)
Day 22	261	252 (-3.4)	267 (+2.3)	254 (-2.7)	241* (-7.7)
Day 29	269	252 (-6.3)	271 (+0.7)	256 (-4.8)	243** (-9.7)
Body weight gain per day [g] (% difference to control)					
Day 1-8	6.0	6.4 (+7.1)	6.6 (+9.5)	6.4 (+7.1)	5.4 (-9.5)
Day 8-15	6.6	5.6 (-15.2)	6.3 (-4.3)	5.6 (-15.2)	5.3 (-19.6)
Day 15-22	4.7	4.0 (-15.2)	4.9 (+3.0)	4.4 (-6.1)	4.1 (-12.1)
Day 22-29	1.1	0.0** (-100.0)	0.6 (-50.0)	0.3* (-75.0)	0.3* (-75.0)
Day 1-29#	4.6	4.0 (-13.2)	4.6 (-0.8)	4.2 (-9.3)	3.8 (-17.8)
Females					
Body weight [g] (% difference to control)					
Day 1	104	109 (+4.8)	106 (+1.9)	107 (+2.9)	108(+3.8)
Day 8	125	129 (+3.2)	125 (±0.0)	128 (+2.4)	128 (+2.4)
Day 15	146	149 (+2.1)	145 (-0.7)	145 (-0.7)	146 (±0.0)
Day 22	159	162 (+1.9)	157 (-1.3)	160 (+0.6)	163 (+2.5)
Day 29	159	162 (+1.9)	158 (-0.6)	160 (+0.6)	161 (+1.3)

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Body weight gain per day [g] (% difference to control)					
Day 1-8	3.0	2.9 (-4.8)	2.7 (-9.5)	3.0 (±0.0)	2.9 (-4.8)
Day 8-15	3.0	2.9 (-4.8)	2.9 (-4.8)	2.4 (-19.0)	2.6 (-14.3)
Day 15-22	1.9	1.9 (±0.0)	1.7 (-7.7)	2.1 (+15.4)	2.4 (+30.8)
Day 22-29	0.0	0.0 (±N/A)	0.1 (±N/A)	0.0 (±N/A)	-0.3 (±N/A)
Day 1-29#	2.0	1.9 (-3.6)	1.9 (-5.5)	1.9 (-3.6)	1.9 (-3.6)

* $p < 0.05$; statistically different to controls using Dunnett's test
** $p < 0.01$; statistically different to controls using Dunnett's test

Food intake

In 20000 ppm females there was a lower food intake per animal as well as per g/kg body weight at 200 and 20000 ppm, which reached a statistical significance only in the second week (intake per animal only).

Because a clear dose correlation is lacking, the differences to controls are small and a significance is stated for one time point for 20000 ppm females only these findings are not considered as toxicologically relevant, but incidental.

Table 5.8.1- 94: Mean food consumption (g/animal/day) (% difference to control)

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Week 1	17.4	16.9 (-2.9)	17.7 (+1.7)	16.9 (-2.9)	18.2 (+4.6)
Week 2	19.8	18.9 (-4.5)	19.8 (±0.0)	18.4 (-7.1)	19.1 (-3.5)
Week 3	20.5	18.6 (-9.3)	20.4 (-0.5)	19.7 (-3.9)	20.4 (-0.5)
Week 4	18.3	16.9 (-7.7)	18.9 (+3.3)	17.1 (-6.6)	18.2 (-0.5)
Females					
Week 1	16.8	15.3 (-8.9)	14.5 (-13.7)	17.2 (+2.4)	14.2 (-15.5)
Week 2	19.4	16.6 (-14.4)	16.5 (-14.9)	17.5 (-9.8)	14.6* (-24.7)
Week 3	17.1	15.6 (-8.8)	15.0 (-12.3)	15.8 (-7.6)	14.6 (-14.6)
Week 4	14.8	15.1 (+2.0)	13.1 (-11.5)	15.8 (+6.8)	13.6 (-8.1)

* $p < 0.05$; statistically different to controls using Kruskal-Wallis test

Table 5.8.1- 95: Mean daily food intake (g/kg/day) (% difference to control)

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Week 1	96	91 (-5.2)	93 (-3.1)	92 (-4.2)	104 (+8.3)
Week 2	87	84 (-3.4)	85 (-2.3)	82 (-5.7)	90 (+3.4)
Week 3	79	74 (-6.3)	76 (-3.8)	77 (-2.5)	85 (+7.6)
Week 4	68	67 (-1.5)	69 (+1.5)	67 (-1.5)	75 (+10.3)
Females					
Week 1	135	118 (-12.6)	116 (-14.1)	137 (+1.5)	111 (-17.8)
Week 2	134	111 (-17.2)	113 (-15.7)	123 (-8.2)	101 (-24.6)
Week 3	108	96 (-11.1)	95 (-12.0)	101 (-6.5)	90 (-16.7)
Week 4	94	93 (-1.1)	83 (-11.7)	101 (+7.4)	85 (-9.6)

3. Ophthalmoscopic examinations

There were no treatment-related ophthalmic lesions.

4. Neurotoxicity assessments

No treatment-related effects were observed.

5. Laboratory investigations

Haematology:

Males and females at 20000 ppm exhibited significantly increased platelet counts. In females at this dose a significantly reduced mean for the haemoglobin concentration was also observed.

In males the thromboplastin time was significantly shortened at 200 ppm and above when compared to corresponding controls. However, these are considered to be of no toxicological relevance, because the deviations to control values were very small, all individual values of compound treated rats were within the historical control range (5/10 of the control values were clearly above this range leading to a relative high control mean) and no corresponding findings were observed in females.

The partial thromboplastin time was significantly shortened from 2000 ppm in males; however, the differences are small and are within the range of the historical control data and are therefore not treatment related.

All other haematological parameters, which were significantly different from control values are considered to be of no toxicological relevance since either the differences from control were negligibly low or they did not show a correlation with the dose administered. There were no statistically significant differences among the differential blood parameters and no effects on the erythrocyte morphology.

Table 5.8.1- 96: Selected haematology findings

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Haemoglobin (g/L)	144	149*	151**	148	142
Haematocrit (L/L)	0.467	0.482	0.484*	0.479	0.459
Platelets / Thrombocytes (109/L)	1381	1413	1355	1386	1636**
Hepato - Quick (sec)	39.4	38.5	36.2**	36.4**	34.0**
Partial Thromboplastin Time (sec)	13.1	13.2	13.0	12.0*	11.9*
Females					
Haemoglobin (g/L)	149	146	151	148	142*
Haematocrit (L/L)	0.460	0.455	0.474	0.466	0.446
Platelets / Thrombocytes (109/L)	1451	1354	1349*	1422	1654**
Hepato - Quick (sec)	29.5	32.2	31.4	30.5	29.8
Partial Thromboplastin Time (sec)	16.3	12.0	13.2	13.9	13.0

* $p < 0.05$; statistically different to controls using Dunnett's or Kruskal-Wallis test
** $p < 0.01$; statistically different to controls using Dunnett's or Kruskal-Wallis test

Clinical chemistry:

With regard to clinical chemistry parameters, statistically significantly increased cholesterol concentrations were observed at 20,000 ppm in males and females. Statistically significant increases in several other parameters are considered to be of no toxicological relevance, because all individual values were within the historical control range (decreased creatinine at 20000 ppm males, increased calcium from 200 ppm in males and at 20000 ppm in females and increased chloride in males), the difference from controls are slight (increased protein at 20000 ppm in males) or a dose response is lacking. There were no remarkable changes concerning the Albumin/Globulin Quotients.

Table 5.8.1- 97: Selected clinical Chemistry Parameters

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Cholesterol (mmol/L)	1.59	1.61	1.55	1.68	2.23*
Creatinine (mmol/L)	49	50	48	49	46*
Urea (mmol/L)	5.64	6.42**	6.09	6.43**	6.10
Total Protein (g/L)	63.0	63.3	64.9*	64.0	65.1*
Albumin (g/L)	34.8	34.7	35.3	36.6**	35.3
Albumin Globulin Quotient	1.24	1.21	1.20	1.34**	1.18
Ca (mmol/L)	2.43	2.40	2.50**	2.52**	2.53**
Cl (mmol/L)	99	100	99	100	101*
Females					
Cholesterol (mmol/L)	1.48	1.55	1.60	1.69	2.12**
Creatinine (mmol/L)	48	49	49	50	47
Urea (mmol/L)	6.81	6.40	7.12	6.47	6.80
Total Protein (g/L)	62.0	62.9	63.7	64.1	64.9
Albumin (g/L)	34.3	34.5	34.6	35.3	34.8
Albumin Globulin Quotient	1.25	1.22	1.19	1.23	1.16
Ca (mmol/L)	2.35	2.34	2.39	2.40	2.45**
Cl (mmol/L)	102	103	103	104	103

* $p < 0.05$; statistically different to controls using Dunnett's, Kruskal-Wallis or Adjusted Welsh test
** $p < 0.01$; statistically different to controls using Dunnett's, Kruskal-Wallis or Adjusted Welsh test

Urinalysis:

Males at 20000 ppm exhibited statistically significantly lower means for urine volume and accordingly significantly higher urine density.

The semi-quantitatively determined blood, bilirubin, glucose, protein, urobilinogen, and ketone body concentrations and also the pH values were unremarkable in all groups. The sediments showed no abnormalities in males or females.

Table 5.8.1- 98: Selected urinalyses parameters

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Volume (mL)	7.0	7.8	7.9	7.0	4.9**
Density (g/L)	1026	1024	1024	1026	1033*
Protein (g/L)	1.28	1.06	1.11	1.00	1.58
Protein x Volume (mg)	8.6	8.1	8.5	6.9	7.8
Females					
Volume (mL)	2.7	3.7	2.9	3.0	2.6
Density (g/L)	1032	1034	1031	1032	1035
Protein (g/L)	0.22	0.17	0.14	0.19	0.26
Protein x Volume (mg)	0.5	0.6	0.4	0.5	0.7

* $p < 0.05$; statistically different to controls using Dunnett's, Kruskal-Wallis or Adjusted Welsh test
** $p < 0.01$; statistically different to controls using Dunnett's, Kruskal-Wallis or Adjusted Welsh test

6. Sacrifice and pathology

There was an increase in absolute (+25% in females) and relative (+14% in males and +23% in females) weights of the liver at 20000 ppm as well as of the heart in 20000 ppm females (about +13%).

In 20000 ppm males statistically significantly increased relative kidney weights (+15%) occurred. Furthermore, 20000 ppm males exhibited significantly reduced absolute weights of the epididymides (-18%).

The statistically significant increase of relative brain weights (+8%) found in 20000 ppm males is considered to be of no toxicological relevance, because the difference to the control mean is too small. Thus, this significance is considered to be incidental.

Table 5.8.1- 99: Absolute organ weights (% difference to control)

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Terminal body weight (g)	264	248 (-6.1)	265 (+0.4)	253 (-4.2)	239** (-9.5)
Brain abs. (mg)	1867	1843 (-1.3)	1833 (-1.8)	1832 (-1.9)	1831 (-1.9)
Brain rel.	709	744 (+4.9)	695 (-2.0)	726 (+2.4)	767* (+8.2)
Heart abs. (mg)	1074	1043 (-2.9)	1022 (-4.8)	982 (-8.6)	1052 (-2.0)
Heart rel.	408	421 (+3.2)	385 (-5.6)	389 (-4.7)	442 (+8.3)
Liver abs. (mg)	7721	7057 (-8.6)	7691 (-0.4)	7320 (-5.2)	7982 (+3.0)
Liver rel.	2915	2841 (-2.5)	2899 (-0.5)	2891 (-0.8)	3329** (+14.2)
Kidneys abs. (mg)	1882	1739 (-7.6)	1917 (+1.9)	1831 (-2.7)	1963 (+4.3)
Kidneys rel.	711	701 (-1.4)	723 (+1.7)	725 (+2.0)	821** (+15.5)
Epididymides abs. (mg)	1031	934 (-9.4)	939 (-8.9)	928 (-10.0)	846** (-17.9)
Epididymides rel.	393	377 (-4.1)	355 (-9.7)	367 (-6.6)	354 (-9.9)
Females					
Terminal body weight (g)	155	158 (+1.9)	153 (-1.3)	158 (+1.9)	157 (+1.3)
Brain abs. (mg)	1659	1719 (+3.6)	1705 (+2.8)	1704 (+2.4)	1722 (+3.8)
Brain rel.	1072	1091 (+1.8)	1120 (+4.5)	1090 (+1.7)	1096 (+2.2)
Heart abs. (mg)	654	707 (+8.1)	680 (+4.0)	647 (-1.1)	750* (+14.7)
Heart rel.	423	447 (+5.7)	443 (+4.7)	410 (-3.1)	477* (+12.8)
Liver abs. (mg)	4765	4888 (+2.6)	4681 (-1.8)	4887 (+2.6)	5941** (+24.7)
Liver rel.	3066	3087 (+0.7)	3044 (-0.7)	3113 (+1.5)	3774** (+23.1)
Kidneys abs. (mg)	1217	1215 (-0.2)	1200 (-1.4)	1179 (-3.1)	1254 (+3.0)
Kidneys rel.	784	768 (-2.0)	782 (-0.3)	750 (-4.3)	798 (+1.8)

* $p < 0.05$; statistically different to controls using Dunnett's test
** $p < 0.01$; statistically different to controls using Dunnett's test

7. Histopathology

Histopathology revealed the following treatment-related alterations at 20000 ppm:

Kidneys: Increased number of males showing basophilic cortical tubules with concurrent increase in severity score.

Thyroid Gland: Granular or clumpy alteration of the follicular colloid in both genders and flattened follicular epithelium in two often high dose males.

Liver: Minimal cytoplasmic change of periportal hepatocytes in high dose females with concurrent reduction of periportally stored fat.

No treatment-related findings were observed up to 2000 ppm.

III. Conclusion

The NOAEL in the 28-dietary study in rats with M-04 was considered to be 2000 ppm (equivalent to 159.2 and 230.6 mg/kg/day, in males and females respectively).

At the LOAEL of 20000 ppm (equivalent to 1775 & 1930.8 mg/kg bw/d in males and females respectively) reductions in body weight and body weight gain were observed in males in addition to increased kidney weights, urinalysis alterations and histopathology findings. Haematological and clinical chemistry parameters were altered, and increased liver weights and histopathological findings were observed in females.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 407 and is valid and acceptable to investigate the short-term toxicity of M04 in rats. A NOAEL of 2000 ppm (equivalent to 159.2 and 230.6 mg/kg bw/d in males and females) was determined from this study.

Data Point:	KCA 5.8.1/22
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE C657378 (Project AE C638206) - Unscheduled DNA synthesis test with rat liver cells in vivo
Report No:	M-230518-01-2
Document No:	M-230518-01-2
Guideline(s) followed in study:	OECD: 486
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted in the DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The potential of technical M-04 to induce DNA damage and repair as evidenced by unscheduled DNA synthesis in rat hepatocytes following a single oral dose was tested.

Two groups of four male Sprague Dawley rats were given a single oral dose of either 1000 or 2000 mg/kg bw M-04 in 0.5% aqueous Cremophor emulsion. The higher dose level corresponded to the international regulatory limit dose for such tests. A concurrent negative control group was treated with the vehicle and a positive control group was treated with DMH at 40 mg/kg bw (for the 4-hour expression) or 2-AAF at 100 mg/kg bw (for the 16-hour expression). Hepatocytes were isolated at 4- or 16-hours following exposure of the animals to the test substance. Four animals from the treated and vehicle control groups were assessed, whilst two animals from the positive control groups were assessed for each exposure period.

The isolated hepatocytes were allowed to attach to glass coverslips and were cultured *in vitro* with (methyl-^{3}H)thymidine at 10 µCi/mL for four hours to 'radiolabel' DNA undergoing repair replication. The hepatocytes were fixed and processed for autoradiography. DNA repair was assessed by comparing the labelling levels of hepatocyte nuclei from treated animals with control values and with the accompanying cytoplasmic labelling levels (grains from at least 100 cells per animal were examined).

M-04 did not cause any significant increases in either the gross nuclear grain count or the net nuclear grain count (i.e. the gross nuclear grain count minus the cytoplasmic grain count) at any dose level at either exposure period.

Positive control group animals showed a large statistically significant increase in the net nuclear grain count, which was accompanied by a large increase in the gross nuclear grain count, demonstrating the sensitivity of the assay.

It is therefore concluded that M-04 did not induce unscheduled DNA synthesis (DNA repair) in this *in vivo* rat liver test.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-04 (referred to as AE C657378 in the report)
Purity:	98.3%
Batch no.:	1119-DC/3

2. Vehicle and/or positive control

Vehicle:	0.5% aqueous Cremophor emulsion
Positive control:	N,N'-dimethylhydrazine (DMH; 4-hour sacrifice) 2-acetylaminofluorene (2-AAF; 16-hour sacrifice)

3. Test animals

Species:	Rat, male
Strain:	Wistar Crl:(WI)BR
Age:	7 to 9 weeks
Weight at start:	149.7-250.1 g
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Standard rodent diet
Water:	Provided *ad libitum*
Housing:	Housed individually in Makrolon type IIA cages
Temperature:	22.5-26 °C
Humidity:	40-42%
Air changes:	Ten times/hour
Photoperiod:	12 hours light/12 hours dark

4. Test substance doses

The selection of the doses was based on a pilot test, in which three male rats/group were orally dosed 500, 1000 or 2000 mg/kg bw of the test substance at a volume of 20 mL/kg. An observation period of 72 hours followed. The following clinical signs were recorded

Table 5.8.1- 100: results of the preliminary toxicity study

Dose (mg/kg bw)	Day 1*	Day 2	Day 3	Day 4
500	Roughened fur, rapid breathing (3/3)	Roughened fur (3/3)	None	None
1000	Semi-anaesthetised state (3/3)	Roughened fur (33)	Rapid breathing (1/3)	None
2000	Semi-anaesthetised state, roughened fur, flush (3/3)	Roughened fur (3/3), twitching (2/3), flush (1/3), rapid breathing (1/3)	Roughened fur (3/3)	Roughened fur (3/3)

*Day of application

Based on the results of the preliminary toxicity study, 2000 kg (limit dose in guideline) was selected as the high dose for the UDS assay and 1000 mg/kg bw (half the top dose) was selected as the lower dose.

B. Test performance

Experimental phase: January 12 to April 21, 2004

1. Treatment and sampling times

Each dose group comprised four evaluable rats (replacement groups were included to ensure the minimum number of evaluable animals was achieved). Each dose substance was administered once. Food was withdrawn 6-7 hours before treatment for the 16-hour exposure group and 14-hours before treatment for the 4-hour exposure group and was reintroduced one hour following treatment. Four animals were treated per day and at least one control animal was concurrently exposed.

The experimental design is shown below:

Table 5.8.1- 101: UDS *in vivo* test design

Experimental group	Dose in mg/kg bw	No. of animals	
		4-hour	16-hour
Negative control	0	4	4
M-04	1000	4	4
	2000	4	4
Positive control			
DMH	40	4	-
2-AAF	100	-	4

The test substance was suspended in 0.4% aqueous Cremophor emulsion using a magnetic stirrer until oral administration vi a stomach tube. The positive controls were administered the same way; 2-AAF was dissolved in corn oil and DMH was dissolved in 0.9% NaCl. The vehicle control animals received 0.5% aqueous Cremophor emulsion by the same method.

The vehicle control and the treated groups were administered a volume of 20 ml/kg bw and each positive control group was administered a volume of 10 mL/kg.

2. UDS test

Hepatocyte isolation:

Following exposure (4 or 16 hours), the animals were anaesthetised with an i.p injection of phenobarbital-sodium. The liver was exposed, and the vena cava was cannulated using an appropriate syringe. Perfusion was performed with two peristaltic pumps; the first pump (with perfusion solution I) was set at 5mL/min for 1.5 minutes then 10 mL/min for 2 minutes and the second pump (with perfusion solution II) was set at 15 mL/min until 200 mL solution was used.

Following perfusion, the liver was removed and stored in 30 mL of perfusion solution II for cell preparation. Primary hepatocytes were prepared under sterile conditions by making incisions in each lobe. Liver cells were then collected, and the obtained cell suspension filtered over gross gauze, filled up to 50 mL with cold Williams medium E (WEI). The resulting supernatant was discarded, and the pellet resuspended in WIE. The cell suspension was filtered again over a fine gauze and filled to 50 mL with WIE. Subsequent centrifugation for 3 minutes (50 x g and <15°C) followed, then resuspending and a second centrifugation. The resulting pellet was resuspended in 25 mL WEI and used for the determination of cell viability and cell concentration by trypan blue exclusion (used as a measure for substance induced cytotoxicity during in vivo exposure).

Hepatocyte culture:

Freshly isolated hepatocytes were established as primary cell culture in WEI supplemented with L-glutamine (2mM), gentamycin sulphate (50µg/mL), dexamethasone (2.4 µM) and 10% heat inactivated calf serum (designated culture medium 1).

To determine cell viability, attachment rate and morphology (1.5 hours following attachment) Cells for each animal were seeded in two 60mm petri dishes ($7.5x10^5$ viable cells/dish) precoated with collagen.

To determine the genotoxicity of the test substance in the UDS assay a round coverslip pre-coated with collagen was placed into each well of a 6-well culture dish. Approximately $3.75x10^5$ viable cells were seeded per well (in 2.5mL culture medium 1), whereby 4 wells/animal including controls were established.

All cultures were incubated for 90 minutes at 37°C in a humidified atmosphere containing 5% CO_2.

Cell harvest:

The cells designated for the viability assessment (petri dishes) were investigated after the attachment period for cell number and viability by the trypan blue exclusion method.

Cells of cultures for the genotoxicity assessment (after the attachment period), were washed with culture medium 2 (culture medium 1 without dexamethasone and containing 1% FCS) and incubated in the same medium. To each culture 10 µCi/mL ^{3}H-thymidine was added. The cultures were then incubated.

After 4-hour incubation the hepatocytes were washed twice with culture medium 3 (culture medium 2 without gentamycin sulphate) containing unlabelled thymidine and were cultured overnight in this medium.

Autoradiography:

The autoradiography procedure was performed in the dark. Air-dried coverslips were mounted cell side up on microscopic slides. In a dark-room these were dipped in NTB-2 photographic emulsion, diluted 1:1 with distilled water and air dried overnight. The following day the coated slides were stored in light-tight boxes in the presence of a drying agent for 14-days at -20˚C in order to reduce CG background and therefore to increase the sensitivity of the assay. The photographic emulsion was then developed for 3-4 minutes in Kodak D-19 developer at <15˚C. The slides were then rinsed in distilled water, fixed for 7-8 minutes, air dried, and stained with haematoxylin and eosin.

Examination of the slides:

Grain counting was carried out by eye using a Zeiss microscope (100x objective under oil immersion) interfaced to a flat screen with high resolution colour camera. To determine UDS, each coded slide was examined (50/cells/slide); three slides per animal were evaluated (total of 150 nuclei/rat). Only cells viable at the time of fixation were scored (isolated nuclei and abnormal cells were excluded). Cells were scored in a regular fashion, moving across the x-axis, from a randomly selected starting point on the lower third of the slide and continuing in a parallel fashion until 50 cells had been reached.

UDS was measured by counting nuclear grains and subtracting the average number of grains in 3 cytoplasmic areas of the same size as the corresponding nucleus (nuclear net grain count; NNG). The mean NNG per animal was determined from triplicate coverslips whilst the mean NNG per dose group was calculated from the mean NNG value of each animal in a respective dose. The number of cells in repair (i.e. nuclei with 5 or more net grains) was also determined.

The viability of the hepatocytes of the vehicle control animals collected by this process should normally exceed 70% or 50-70% for single animals if the cell preparation is of adequate quality; vehicle controls animals with less than 50% viability are unacceptable.

The viability of the monolayer cultures treated with vehicle controls should be ≥65%.

For each evaluated 50 cells per slide, the number of nuclear grains is scored as well as the numbers of three cytoplasmic grain counts from nuclear-sized areas adjacent to each nucleus.

Only cells with normal morphology at the time of fixation and with nuclei evenly coated with emulsion were scored. Abnormal cells (e.g. Those with pyknotic or lysed nuclei) and isolated nuclei were not counted; cells in S-phase were also excluded.

A cell with 5NNG or more is considered to be responding (in repair).

3. Evaluation

An assay is considered acceptable if the following crueria are met:

- Grain count data should be from at least 2 slides/animal and 100/cells/animal
- An experiment is invalid if cytoplasmic background counts in hepatocytes of vehicle controls exceed 30 grains per nuclear-sized area
- The average NNG value in hepatocytes of vehicle control animals should range from -8 and 0 and no more than 5% of the cells should be in repair.
- The positive controls should produce mean values greater than 2NNG per dose group with greater 10% cells in repair.

A test substance is considered positive if it yields +2 NNG or more (dose group average). Cells in repair are considered an addition.

A test substance is considered negative if ≤0 NNG.

A test substance is considered equivocal if the NNG is between 0 and 2 or a single animal shows mean NNG values above 0 but the dose group mean does not.

4. Statistical methods

The data are presented in tabular form. Descriptive statistical methods were used to calculate means and standard deviations. The means and standard deviation in the tables were calculated from the means calculated individually for each of the three coverslips/animal. Statistical evaluation of % cells in repair was performed using the Chi^2 test. The following terms were used in the evaluation.

- Net grains per nucleus (NNG) = Average no. of the mean NNG counts of each evaluable coverslip, 50 cells per coverslip.
- Mean grains per nucleus = Average no. of the mean nuclear grain counts of each evaluable coverslip, 50 cells per coverslip.
- Mean cytoplasmic grain count = Average no. of the mean cytoplasmic grain counts (3 areas per cell) of each evaluable coverslip, 50 cells per coverslip.
- % Nuclei with 5 or more grains = (No. of cells with 5 or more NNG counts per dose/no. evaluated cells per dose) x 100
- Absolute survival (%) = % viable cells after isolation

II. Results and Discussion

A. DNA repair test

There were no mortalities following a single oral dose of M-04 at 1000 or 2000 mg/kg bw; however, the following treatment-related clinical signs were noted:

Table 5.8.1- 102: Clinical observations

Dose (mg/kg bw)	16-hour sacrifice	4-hour sacrifice
1000	Roughened fur (3/4)	Roughened fur (3/4), rapid breathing (3/4)
2000	Roughened fur (4/4), pallor (2/4), shivering (1/4), rapid breathing (1/4)	Roughened fur (4/4), rapid breathing (4/4), flushed ears (1/4)

Some instances of roughened fur were also noted in the positive control groups.

The results of the DNA repair test with 4- and 16-hours exposure periods are summarised in the tables below:

Table 5.8.1- 103: Mean nuclear and cytoplasmic grain counts following 4-hour exposure

Treatment group	Dose (mg/kg bw)	Mean gross nuclear grain count (NG)	Mean cytoplasmic grain count (CG)	Mean net nuclear grain count (NNG)
M-04	2000	3.56±0.40	4.31±0.39	-0.74±0.13
	1000	3.52±0.83	4.49±0.60	-0.97±0.23
	0	2.49±0.39	4.45±0.37	-1.96±0.07
Positive control DMH	40	8.48±0.89*	3.81±0.17	4.67±0.80#

#Biologically relevant increase, *statistically significant increase p<0.05

Table 5.8.1- 104: Mean nuclear and cytoplasmic grain counts following 16-hour exposure

Treatment group	Dose (mg/kg bw)	Mean gross nuclear grain count (NG)	Mean cytoplasmic grain count (CG)	Mean net nuclear grain count (NNG)
M-04	2000	3.16±1.21	3.61±1.48	-0.45±0.36
	1000	2.81±0.91	3.30±1.01	-0.50±0.27
	0	1.75±0.79	3.03±1.00	-1.28±0.24
Positive control 2-AAF	100	7.15±1.37*	2.81±0.87	4.34±0.64#

#Biologically relevant increase, *statistically significant increase p<0.05

No relevant cytotoxic effects could be seen in hepatocytes of treated rats, including positive controls. Hepatocytes of the vehicle control groups showed good viability following isolation ranging from 73.1 to 82.1%; following attachment, viabilities between 73.1 and 89.9% were reached.

No biologically relevant increase in gross or net nuclear grain counts was seen at either the 4- or 16-hour exposure period. For animals sacrificed after 16-hours' exposure, NNG values of -1.28±0.24, -0.50±0.27 and -0.45±0.36 were seen in the 0 (vehicle control), 1000 and 2000 mg/kg bw groups, respectively. For animals sacrificed after 4-hours' exposure, mean NNG values were -1.96±0.07, -0.97±0.23 and -0.74±0.13 in the same respective dose groups. No cells in repair were observable in the vehicle control groups (one animal at 2000 mg/kg bw showed 0.67% cells in repair).

The positive controls 2-AAF and DMH, induced biologically relevant increases of the mean NNG values per animal, which were increased by 4.34±0.64 and 4.67±0.80, respectively. Furthermore, the number of cells in repair were increased by 26.7% to 49.3% for 2-AAF treated rats and by 31.3% to 53.3% for DMH treated rats; all increases were both statistically significant and biologically relevant.

III. Conclusion

M-04 did not induce unscheduled DNA synthesis (DNA repair) in this *in vivo* rat liver test.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 406 and is valid and acceptable to assess the potential of M-04 to induce DNA damage and repair *in vivo*. M-04 did not induce unscheduled DNA repair in the rat liver *in vivo* under the conditions of this study.

M-05 (AE 1344122)

The metabolite M-05 is not acutely toxic *via* the oral route; an LD_{50} of >2000 mg/kg bw/d was established in an acute oral toxicity study in rats.

A short-term (28-days) repeated-dose toxicity study conducted in rats is available and a NOAEL of 2000 ppm (equivalent to 152/167 mg/kg bw/d in M/F) was proposed from this study. At the LOAEL of 20000 ppm (equivalent to 1495/1615 mg/kg bw/d in M/F body weight gain and food consumption were reduced in males and females. An ADI of 1.6 mg/kg bw/d can be derived for M-05 from this NOAEL, with an applied safety factor of 1000.

M-05 was not genotoxic in a battery of *in vitro* tests. There was no evidence that M-05 was mutagenic in bacteria in an Ames test or in mammalian cells in a mutation test at the HPRT locus of Chinese hamster V79 cells. Furthermore, there was no evidence that M-05 could induce chromosome aberrations in cultured human peripheral blood lymphocytes. A recently conducted *in vitro* micronucleus test concluded that M-05 was not clastogenic nor aneugenic.

Overall, there is no toxicological concern for the metabolite M-05. The available studies are summarised below.

Data Point:	KCA 5.8.1/23
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE 1344122 - Acute toxicity in the rat after oral adminsitration
Report No:	AT00486
Document No:	M-235328-01-1
Guideline(s) followed in study:	OECD 423 (2001); Directive 67/548/EEC Annex IV B, Part B, B.1 (1967); US-EPA 712-C-98-190; OPPTS 870.1100 (1998).
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In an acute oral toxicity study conducted according to the acute toxic class method, six female fasted Wistar rats were each administered a single oral gavage dose of 2,000 mg/kg bw of M-10 (referred to as AE 1344122 in the report).

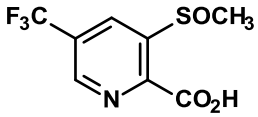
A dose of 2000 mg/kg bw was tolerated by rats without mortalities or clinical signs, effects on weight development or gross pathological findings.

In conclusion, the LD_{50} of M-10 is > 2000 mg/kg body weight.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M05 (referred to as AE 1344122 in the study)
Structure	
Chemical name:	3-methylsulfinyl-5-trifluoromethylpyridinc-2-carboxylic acid
Purity:	98.8%
Batch no.:	YG 3228
Appearance:	White powder
Expiry:	February 05, 2004

2. Vehicle and/or positive control

Vehicle:	demineralized water aided by 2% Cremphor EL

3. Test animals

Species:	Rat
Strain:	Female rats of Wistar origin (HsdCpb:Wu)
Age:	9 - 10 weeks
Weight at start:	148 g-166g
Source:	[redacted]
Acclimation period:	Yes
Diet:	Provimi Kliba 3883 0.15 Maus/Ratte Haltung, Kaiseraugst Switzerland
Water:	Water *ad libitum*
Housing:	In groups of three rats of the same sex in polycarbonate cages on lowdust wood granulate bedding (J. Rettenmaier & Sohne, 73479 Ellwangen-Holzmühle, Germany).
Temperature:	22 ±2 °C
Humidity:	55 ±5%
Air changes:	Approximately 10 changes per hour
Photoperiod:	12 hours: 12 hours

B. Study design

1. In-life dates: 8 to 24 April 2003

2. Animal assignment and treatment

Three animals were used for each step. The dose level to be used as the starting dose was selected from one of four fixed levels, 5, 50, 300 and 2000 mg/kg body weight. The starting dose level was that which was considered most likely to produce mortality in some of the dosed animals. The presence or absence of treatment-related mortality of the animals dosed at one step determined the next step as follows:

• no further testing is needed,

• dosing of three additional animals, with the same dose

• dosing of three additional animals at the next higher or the next lower dose level.

The substance was tested using a stepwise procedure, each step using three animals of a single sex (normally females) following the procedure described in the flow charts of Annex 2 of OECD guideline 423.

The appropriate dose volume of the test substance was administered to each rat by oral gavage. The day of dosing was designated Day 1.

C. Methods

1. Observations

Mortality:

Cages of rats were checked at least once daily for mortalities.

Clinical signs:

Animals were observed soon after dosing and at frequent intervals for the remainder of day 1. On subsequent days animals were observed at least once daily. The nature and severity of clinical signs and the time these were noted were recorded at each observation. Animals were observed for 14 days following dosing.

The body weights of each rat were recorded Days 1 (prior to dosing) and then weekly until the end of the study.

2. Necropsy

Animals which died or were killed in moribund state were weighed (except on day of application), dissected as soon as possible, and examined macroscopically. The surviving animals were sacrificed at the end of the study, dissected, and examined macroscopically.

II. Results and Discussion

A. Results

1. Dose-response table (LD_{50})

The LD50 was estimated according to OECD - Guideline for Testing of Chemicals No. 423 "Acute Oral Toxicity - Acute Toxic Class Method"; adopted: December 17, 2001.

The results of the study for acute oral toxicity in the fasted rat, including the LD_{50}, are summarized in the table below.

Table 5.8.1- 105: Dose response

Dose (mg/kg bw)	Toxicological result*	Duration of signs	Time of death	Mortality (%)
Females				
1st 2,000	0/3/3	-	-	0
2nd 2,000	0/3/3	-	-	0

* Number of animals which died and/or were sacrificed moribundly / number of animals with signs / total number of animals.

The LD_{50} is therefore **≥ 2000 mg/kg bw**

2. Clinical signs

No clinical signs were observed at 2000 mg/kg body weight.

3. Body weights

There were no toxicological effects on body weights or on body weight gain.

4. Necropsy findings

The necropsies performed at the end of the study revealed no particular findings.

III. Conclusion

The LD_{50} of AE 1344122 (M-05) is ≥ 2000 mg/kg bw. Thus, AE 1344122 (M05) is considered to be unclassified according to the European hazard classification (Globally Harmonised Classification System).

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 423 and is valid and acceptable to determine the acute oral toxicity of M-05 in rats. M-05 was not acutely toxic via the oral route; an LD50 of >2000 mg/kg bw was established from this study.

Data Point:	KCA 5.8.1/24
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE 1344122 - Salmonella/microsome test - Plate incorporation and preincubation method
Report No:	AT00599
Document No:	M-218257-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC, B13/14 ((2000); OECD 471 (1997); US-EPA 712-C-98-247, OPPTS 870.5100 1988)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

M-05 (referred to as AE 1344122 in the report) was initially investigated using the *Salmonella*/microsome plate incorporation test for point mutagenic effects in doses of up to and including 5000 µg per plate on five *Salmonella typhimurium* LT2 mutants. These comprised the histidine auxotrophic strains TA 1535, TA 100, TA 1537, TA 98, and TA 102.

Doses up to and including 158 µg/plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. At higher doses, the substance had only a weak, strain-specific bacteriotoxic effect. Due to the weakness of this effect this range could nevertheless be used up to 5000 ug per plate for assessment purposes.

Evidence of mutagenic activity of M-05 was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed.

The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C and 2-aminoanthracene had a marked mutagenic effect, as was seen by a biologically relevant increase in mutant colonies compared to the corresponding negative controls.

M-05 was investigated in an independent repeat using the *Salmonella*/microsome test for point mutagenic effects in doses up to 5000 µg/tube after preincubation for 20 minutes at 37°C on five *Salmonella typhimurium* LT2 mutants. These comprised the histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA102.

Doses up to and including 158 µg/tube did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. At higher doses, the substance had a strain-specific bacteriotoxic effect. Due to this effect 5000 µg/tube range could not be used for assessment purposes.

Evidence of mutagenic activity of M-05 was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed.

The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, cumene hydroperoxide and 2-aminoanthracene had a marked mutagenic effect, as was seen by a biologically relevant increase in mutant colonies compared to the corresponding negative controls.

Therefore M-05 was considered to be non-mutagenic without and with S9 mix in the plate incorporation as well as in the preincubation modification of the *Salmonella*/microsome test.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-05 (referred to as AE 1344122 in the report)
Purity:	98.8%
Batch no.:	YG3228

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	Without S9 mix:
	Na-azide: TA 1535
	Nitrofurantoin (NF) : TA 100
	4-Nitro-1,2-phenylene diamine (4-NPDA): TA 1537, TA 98
	Mitomycin C (MMC) : TA 102 (plate incorporation trials
	Cumene hydroperoxide (Cumene) : TA 102 (preincubation trials)
	With S9 mix:
	2-Aminoanthracene : TA 98, TA 100, TA 1535, TA 1537, TA 102

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

S9 mix was used to simulate the mammalian metabolism of the test substance. It was made from the livers of at least six adult male Sprague Dawley rats, of approximately 200 to 300 g in weight. For enzyme induction, the animals received a single intraperitoneal injection of Aroclor 1254, dissolved in corn oil, at a dose of 500 mg/kg body weight, five days prior to sacrifice. The animals were prepared unfasted, following the directions of Ames *et al.* (1975) and Maron and Ames (1983).

The rats were terminated. Livers were removed under sterile conditions immediately after sacrifice and kept at 4°C until all animals had been prepared. All the remaining steps were carried out under sterile conditions at 4°C.

The livers were washed with cold (4°C), 0.15 M KCl solution (approximately 1 mL KCl per 1 g liver), and then homogenized in fresh, cold (4°C), 0.15 M KCl (approximately 3 mL KCI per 1 g liver). The homogenate was then centrifuged in a cooling centrifuge at 4°C and 9000 g for 10 minutes. The supernatant (the S9 fraction) was stored at -80°C in small portions.

Cofactor solution (70 mL) was composed as follows:

$MgCl_2.6H_2O$	162.6 mg
KCl	246.0 mg
glucose-6-phosphate, disodium salt	179.1 mg
NADP, disodium salt	315.0 mg
phosphate buffer	100.0 mM

S9 mix consists of this cofactor solution, S9 fraction and, if needed, 0.15 M KCl. The amount of S9 fraction in S9 mix is indicated in the tables in percent. The S9 mix comprised the amount of S9 fraction (x%) indicated in the tables, 70% cofactor solution and (30-x)% 0.15 M KCl. The S9 fraction was derived from the preparation dated February 4, 2003 (protein content 25.6 mg per ml). Prior to first use, each batch was checked for its metabolizing capacity by using reference mutagens; appropriate activity was demonstrated. At the beginning of each experiment 4 aliquots of the S9 mix were plated (0.5 ml per plate) in order to assess its sterility. This was repeated after completion of test tube plating. The sterility control plates were then incubated for 48 hours at 37°C. No indication of contamination of S9 mix was found.

4. Test organisms:

Histidine-deficient mutants of *Salmonella typhimurium* LT2 served as indicators to demonstrate point mutagenic effects. These strains were selected specifically for the *Salmonella*/ microsome test. Since point mutations can be divided into two basic classes, base-pair substitutions and frameshift mutations, several strains were used which cover both types.

These included the strains selected by Ames *et al.* (1973b), *Salmonella typhimurium* TA1535 and TA 1537, as well as *Salmonella typhimurium* TA100, TA 98 and TA 102 developed by McCann *et al.* (1975b) and Levin *et al.* (1982), respectively.

TA 1535 and TA 100 bear the base-pair substitution his G 46, and TA 100 additionally contains the plasmid pKM 101. This R factor also contained in TA 98 and TA102, codes for an ampicillin resistance and should raise the sensitivity of the strains. TA 102 carries the ochre mutation his G 428 on the multicopy plasmid pAQ1, which codes in addition for tetracycline resistance. TA1537 and TA 98 bear frameshift markers. TA 1537 exhibits the +1 mutant, his C 3076, while TA 98 bears the +2 type, his D 3052.

Furthermore, the strains have other properties, which should increase their sensitivity. They are all deep rough, i.e. partly deficient in lipopolysaccharide side chains in their cell walls, enabling larger molecules to penetrate the bacterial cell wall and produce mutations. With the exception of TA 102, all strains have reduced capability to repair DNA-damage which increases the likelihood that such damage results in mutations.

The mutations of the bacterial strains used in this study are described in the table below.

Table 5.8.1- 106: *Salmonella typhimurium* strains

Salmonella typhimurium		
Strains	**Genotype**	**Type of mutations indicated**
TA 1537	his C 3076; rfa$^-$; uvrB$^-$	frame shift mutations
TA 98	his D 3052; rfa$^-$; uvrB$^-$; R-factor	" "
TA 1535	his G 46; rfa$^-$; uvrB$^-$	base-pair substitutions
TA 100	his G 46; rfa$^-$; uvrB$^-$; R-factor	" "
TA 102	his G 428; rfa$^-$; uvrB$^-$; R-factor	" "

Regular checking of the properties of the *Salmonella typhimurium* strains was performed.

The original strains were obtained from Prof. Bruce Ames and arrived at Toxicology, Bayer HealthCare, Bayer AG, on August 15, 1997.

5. Test substance concentrations used:

Test concentrations of 16, 50, 158, 500, 1581 and 5000 µg M-05/plate were used.

B. Test performance

Experimental phase: 22 to 28 July 2003

1. Pre-experiment for toxicity

No pre-experiment was performed.

2. Mutagenicity test

For each strain and dose level, including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

Plate Incorporation Method:

- 100 µL test solution at each dose level (solvent or reference mutagen solution (positive control)),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL bacteria suspension (cf. test system, pre-culture of the strains),
- 2000 µL overlay agar.

Preincubation Method:

In the pre-incubation assay 100 µL test solution (solvent or reference mutagen solution (positive control)), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspension were mixed in a test tube and incubated at 37 °C for 20 minutes. After pre-incubation 2.0 mL overlay agar was added to each tube. The mixture was poured on agar plates.

The plates were incubated for 48 hours at 37 °C prior to counting.

Data recording:

The colonies were counted automatically using an Artek counter, model 982B. Data were transferred to a PC and processed with the released and DQS 6.0 based BioSys software Ames- Test III (Rev. 3.106).

3. Statistics

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

4. Acceptance / assessment criteria:

The following criteria determined the acceptance of an assay:

a) The negative controls had to be within the expected range, as defined by published data (e.g. Maron and Ames, 1983) and/or the laboratories' own historical data.

b) The positive controls had to show sufficient effects, as defined by the laboratories' experience.

c) Titer determinations had to demonstrate sufficient bacterial density in the suspension.

Only trials which complied with all three of the above criteria were accepted for assessment. Even if the criteria for points (b) and (c) were not met, a trial was accepted if it showed mutagenic activity of the test compound. Furthermore, an unacceptable trial would have been repeated.

5. Evaluation of results

A reproducible and dose-related increase in mutant counts of at least one strain is considered to be a positive result. For TA1535, TA100 and TA 98 this increase should be about twice that of negative controls, whereas for TA 1537, at least a threefold increase should be reached. For TA 102 an increase of about 100 mutants should be reached. Otherwise, the result is evaluated as negative. However, these guidelines may be overruled by good scientific judgment.

In case of questionable results, investigations should continue, possibly with modifications, until a final evaluation is possible.

II. Results and Discussion

A. Mutation assays

The *Salmonella*/microsome plate incorporation test, employing doses of up to 5000 ug per plate, showed M-05 to produce bacteriotoxic effects, starting at 500 ug per plate.

Evaluation of individual dose groups, with respect to relevant assessment parameters (dose effect, reproducibility), revealed no biologically relevant variations from the respective negative controls.

In spite of the low doses used, positive controls increased the mutant counts to well over those of the negative controls, and thus demonstrated the system's high sensitivity.

Despite this sensitivity, no indications of mutagenic effects of M-05 could be found at assessable doses of up to 5000 ug per plate in any of the *Salmonella typhimurium* strains used in the plate incorporation assay.

The *Salmonella*/microsome test, using preincubation for 20 minutes at 37°C and employing doses of up to 5000 ug per tube, showed M-05 to produce bacteriotoxic effects at 500 ug per tube and above. Therefore 5000 ug per tube could not be used for assessment.

In agreement with the plate incorporation assay, evaluation of individual dose groups of the preincubation assay, with respect to relevant assessment parameters (dose effect, reproducibility), revealed no biologically relevant variations from the respective negative controls.

In spite of the low doses used, positive controls increased the mutant counts to well over those of the negative controls, and thus demonstrated the system's high sensitivity.

Despite this sensitivity, no indications of mutagenic effects of M-05 could be found at assessable doses of up to 1581 ug per tube in any of the *Salmonella typhimurium* strains used in the preincubation assay.

An overview of the results is given in the following tables.

Table 5.8.1- 107: Summary of plate incorporation test

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean)				
		TA 1535	TA 100	TA 1537	TA 98	TA 102
Without metabolic activation (-S9)						
M-05	0	18	165	7	22	205
	16	13	158	6	13	194
	50	15	159	7	17	191
	158	20	163	8	18	199
	500	16	165	8	16	224
	1581	16	173	6	22	204
	5000	16	152	6	20	234
Positive control						
Na-azide	10	755	-	-	-	-
NF	0.2	-	287	-	-	-
4-NPDA	10 / 0.5	-	-	109	172	-
MMC	0.2	-	-	-	-	592
Historical solvent control mean	-	9 (±2)	88 (±24)	7 (±1)	22 (±7)	201 (±17)
Historical control range	-	8 – 14	70 – 136	6 – 8	14 – 34	174 - 220
With metabolic activation (+S9)						
M-05	0	10	209	9	21	264
	16	12	194	9	26	260
	50	14	183	7	29	274
	158	10	214	10	30	271
	500	9	198	7	35	244
	1581	9	197	9	26	297
	5000	11	145	9	23	262
Positive control						
2-AA	3	185	1375	354	1272	694
Historical solvent control mean	-	10 (±1)	107 (±23)	9 (±1)	30 (±7)	272 (±13)
Historical control range	-	9 – 12	89 – 151	8 – 10	23 – 41	248 - 288

Na-azide = sodium azide, NF = Nitrofurantoin; 4-NPDA = 4-Nitro-1,2-phenylene diamine, MMC = Mitomycin C;
2-AA = 2-aminoanthracene
(±X): Standard deviation
Historical control data based on experiments from 1996 to 2002.

Table 5.8.1- 108: Summary of pre-incubation test

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean)				
		TA 1535	TA 100	TA 1537	TA 98	TA 102
Without metabolic activation (-S9)						
M-05	0	15	140	7	22	268
	16	18	141	8	28	292
	50	19	133	10	22	286
	158	19	[131]	6	27	270
	500	20	136	7	25	279
	1581	18	143	10	23	262
	5000	6	70	-	15	107
Positive control						
Na-azide	10	735	-	-	-	-
NF	0.2	-	483	-	-	-
4-NPDA	10 / 0.5	-	-	129	171	-
Cumene	50	-	-	-	-	493
Historical solvent control mean	-	9 (±2)	93 (±25)	7 (±1)	22 (±6)	245 (±17)
Historical control range	-	7 – 12	72 – 45	6 – 8	15 – 32	223 - 271
With metabolic activation (+S9)						
M-05	0	13	168	11	44	304
	16	12	162	14	50	282
	50	14	158	10	45	314
	158	16	162	9	37	301
	500	16	155	9	50	309
	1581	13	150	11	39	303
	5000	9	106	6	14	113
Positive control						
2-AA	3	227	1707	570	1171	641
Historical solvent control mean		10 (±1)	107 (±24)	9 (±1)	30 (±6)	312 (±21)
Historical control range	-	8 – 11	88 – 155	8 – 9	23 – 40	281 - 337

Na-azide = sodium azide; NF = Nitrofurantoin; 4-NPDA = 4-Nitro-1,2-phenylene diamine; Cumene = Cumene hydroperoxide; 2-AA = 2-aminoanthracene
(±X): Standard deviation
Historical control data based on experiments from 1996 to 2002.

III. Conclusion

No indications of mutagenic effects of M-05 could be found at assessable doses of up to 5000 ug per plate in any of the *Salmonella typhimurium* strains used in the plate incorporation assay, or at assessable doses of up to 1581 ug per tube in in the preincubation assay.

Therefore M-05 is not mutagenic *in vitro* in bacterial cells under the conditions of this assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 473 and is valid and acceptable to assess the mutagenic potential of M-05 in *S-typhimurium*.

M-05 is not mutagenic in bacterial cells under the conditions of this study when tested up to either the limit dose of 5000 µg/plate or cytotoxic concentrations (+/- S9), either by base pair changes or frameshift mutations.

Data Point:	KCA 5.8.1/25
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE 1344122 (metabolite of AE C638206): Induction of chromosome aberrations in cultured human peripheral blood lmphocytes
Report No:	C034338
Document No:	M-234746-01-1
Guideline(s) followed in study:	OECD 473 (1997), ICH Tripartite Harmonised Guideline (1995)
Deviations from current test guideline:	Cytotoxicity was not evaluated by the parameters of relative population doubling (RPD) or relative increase in cell count (RICC). Only 100 metaphases were analysed, rather than a minimum of 300 as recommended in the current guidance.
Previous evaluation:	yes, evaluated and accepted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

This study was performed to assess the ability of M-05 to induce chromosomal aberrations in human lymphocytes cultured *in vitro*.

Human lymphocytes, in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to the test substance both in the presence and absence of S9 mix derived from rat livers. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using Colcemid®, the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

In order to assess the toxicity of M-05 to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. On the basis of these data, the following concentrations were selected for metaphase analysis:

Experiment 1 without S9 (3 hours treatment, 17 hours recovery): 1296, 2026 & 2532 µg/mL

Experiment 1 with S9 (3 hours treatment, 17 hours recovery): 1620, 2026 & 2532 µg/mL

Experiment 2 without S9 (20 hours treatment,): 306.1, 498.5 & 1123 µg/mL

Experiment 2 with S9 (20 hours treatment,): 1829, 2152 & 2532 µg/mL

In the presence and absence of S9 mix (3+17-hour pulse treatment), experiments 1 & 2, the frequencies of cells with structural aberrations were similar to those seen in concurrent vehicle controls. Furthermore, the number of aberrant cells (excluding gaps) in all treated cultures fell within the normal historical control ranges.

In the absence of S9 mix (20-hour exposure), experiment 2, a small but significant increase in aberrant cell frequency was seen at the highest concentration examined (1123 µg/mL). At this concentration, a single culture exhibited an aberrant cell frequency that marginally exceeded the historical negative control (normal) range. However, the aberrant cell frequency of the replicate culture, and all other treated cultures fell within the normal range. Therefore, this observation is not considered to be biologically significant.

The quantitative analysis for polyploidy (numerical aberrations) revealed that normal frequencies (within negative historical control ranges) were observed for the majority of cultures in the presence and absence of S-9 mix in both experiments.

All positive control compounds caused statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

It is concluded that M-05 has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system, under the experimental conditions described.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-05 (referred to as AE 1344122 in the report)
Purity:	98.8%
Batch no.:	YG3228
Expiry date:	February 5, 2004

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: 4-Nitroquinoline 1-oxide (NQO); 5 µg/mL
	+S9: Cyclophosphamide (CPA); 6.25 µg/mL

3. Activation:

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male SD rats inducted with Aroclor 1254 (obtained from Molecular Toxicology Inc., USA). The batches of S9 were stored frozen at -80 C and thawed prior to use. Each batch was checked for sterility, protein content, ability to convert known pro-mutagens to bacterial mutagens and cytochrome P450-catalysed enzymes activities.

Preparation of S9-mix:

Glucose-6-phosphate (180 mg/mL), NAPD (25 mg/mL), KCl (150 nM) and rat liver S-9 were mixed in the ratio of 1:1:1:2. An aliquot of the resulting S9 mix was added to each required cell culture to achieve the final concentration in a total of 10 mL. the final concentration of liver homogenate in the test system was 2%. Cultures treated in the absence of S9 received an equal volume of 150 mM KCl.

4. Cell cultures and medium:

Blood was collected from 3 healthy non-smoking, female volunteers. Whole blood cultures were established in sterile tubes by placing 0.4 mL heparinised blood into 9 mL Hepes-buffered RPMI medium containing 20% (v/v) foetal calf serum and 50 µg gentamycin. Phytohaemagglutinin (PHA) was included at concentration of 2% of culture volumes, to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37˚C and rocked continuously.

5. Test substance concentrations used:

In experiment 1 the tested concentrations were 0 (solvent control), 5.02, 71.27, 89.09, 111.4, 139.2, 174, 217.5, 271.9, 339.8, 424.8, 531, 663.7, 829.7, 1037, 1296, 1620, 2026 & 2532 µg/mL in 3 hour treatments with 17 hours recovery (3+17) with and without metabolic activation.

In experiment 2 the tested concentrations were 221.2, 260.2, 306.1, 360.1, 423.7, 298.5, 586.4, 689.9, 811.7, 954.9, 1123, 1322, 1555, 1829, 2152 & 2532 µg/mL in a 20+0 hour treatment without metabolic activation and 811.7, 954.9, 1123, 1322, 1555, 1829, 2152 & 2532 µg/mL in a 3+17 hours treatment with metabolic activation.

The highest dose selected for chromosome analysis should be one at which at least 50% mitotic inhibition has occurred, or the highest dose tested; slides from highly toxic concentrations should be avoided.

Therefore, based on these criteria the following doses were selected for analysis (the highest selected dose and two lower doses):

Experiment 1 without S9 (3 hours treatment, 17 hours recovery): 1296, 2026 & 2532 µg/mL

Experiment 1 with S9 (3 hours treatment, 17 hours recovery): 1620, 2026 & 2532 µg/mL

Experiment 2 without S9 (20 hours treatment,): 306.1, 498.5 & 1123 µg/mL

Experiment 2 with S9 (20 hours treatment,): 1829, 2152 & 2532 µg/mL

6. Controls:

Sterile DMSO was added to negative control cultures. The positive control chemicals were dissolved in DMSO immediately prior to use at the following concentrations:

-S9: 4-Notroquinoline 1-oxide (NQO); 1.25, 2.50 & 5 µg/mL

+S9: Cyclophosphamide (CPA); 3.125, 6.25 & 12.5 µg/mL

Cells treated with 5.00 µg NQO/mL and 6.25 µg CPA/mL (both experiments) gave satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage. Therefore, these concentrations were selected for analysis.

B. Test performance

Experimental phase: 05 March to 01 May 2003

1. Preliminary Assay and second test

S-9 mix or KCl (0.5 mL) was added appropriately. One set of quadruple cultures (A, B, C and D) for each treatment regime was then treated with the solvent and one set of duplicate cultures with the test article (0.1 mL/culture). Additional duplicate cultures for treatment for treatment in the absence of S9 and in its presence were treated with 0.1 mL of the positive control chemicals. All cultures were then incubated at 37˚C.

Treatment media remained on cultures receiving the continuous treatment until sampling (20h after start of treatment). Cultures received pulse treatments (+/- S9) for 3 hours only and were then pelleted, washed twice with sterile saline, and resuspended in fresh treatment (with gentamycin and foetal calf serum). These cultures were incubated for a further 17 hours before harvesting.

Harvesting and fixation:

Approximately 2 hours prior to harvest, colchicine was added to give a final concentration of 1µg/mL to arrest dividing cells in metaphase. At the defined sampling time cultures were centrifuged, the supernatant carefully removed, and the cells resuspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37˚C for 15 minutes to allow cell swelling to occur. Cells were then fixed by dropping the KCl suspension into an equal volume of fresh methanol/glacial acetic acid (3:1, v/v). The fixative was changed by centrifugation and resuspension. The procedure was repeated many times until the pellets were clean. Lymphocytes were kept in fixative in the refrigerator before slides were prepared; slides were not prepared on the day of harvest to ensure cells were adequately fixed.

Slide preparation:

The cells were pelleted and resuspended in a minimal amount of fresh fixative to give a milky suspension. Several drops of aqueous acetic acid were added to each suspension to enhance chromosome spreading, and several drops of suspension were transferred to clean slides. Slides were flamed if necessary, to enhance metaphase spreading. After the slides had dried the cells were stained for 5 minutes in 4% (v/v) filtered Giemsa stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried, and mounted with coverslips.

Microscopic examination:

Slides were examined, uncoded for mitotic index (MI) or percentage of cells in mitosis. Slides from enough dose levels from each treatment regime were scored to determine if chemically induced mitotic inhibition had occurred. This is defined as a clear decrease in mitotic index compared with negative controls (based on at least 1000 cells counted), preferably dose related.

For each treatment regime, two solvent control cultures were initially to be analysed for chromosome aberrations. Slides form the remaining solvent control cultures and untreated controls were only analysed if necessary. A single positive control dose, which gave a satisfactory response was analysed.

100 metaphases from each slide were analysed. Only cells with 44-46 chromosomes were acceptable for analysis of structural aberrations.

3. Assessment of results

An assay is considered to be acceptable if the following criteria are met:

- The binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures, and

- The proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range, and

- At least 160 cells out of an intended 200 are analysable at each dose level, and

- The positive control chemicals induce statistically significant increases in the proportion of cells with structural aberrations

The test substance is considered to cause a positive response if the following conditions are met:

- The proportion of cells with structural aberrations at one or more concentrations exceeds the normal range in both replicate cultures, and
- A statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurs at the concentrations.

A negative response is claimed if no statistically significant increases in the number of aberrant cells above concurrent control frequencies are observed, at any dose level.

A further evaluation may be carried out if the above criteria for a positive or a negative response are not met.

6. Statistics

Aberrant cells in each culture were categorised as:

1) cells with structural aberrations including gaps

2) cells with structural aberrations excluding gaps

3) polyploid, endoreduplicated or hyperdiploid cells

Cells with structural aberrations excluding gaps in the negative control cultures were compared with historical control data to determine the acceptability of the assay and those in the treated cultures were compared with the normal ranges.

The statistical significance of any data set was only taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentrations exceeded the normal range. Fishers exact test would be used in this case. Probability values of $p < 0.05$ would be accepted as significant. The proportions of aberrant cells in each replicate were also used to establish heterogeneity between replicates by means of a binomial dispersion test. Probability values < 0.05 would be significant.

II. Results and Discussion

1. Experiment 1

Experiment I was performed using a pulse (3+17-hour) treatment in the presence and absence of S9 mix.

In the presence of S9 mix the reduction in mitotic index was 20% at the highest concentration tested of 2532 µg/mL. Concentrations of 1620, 2026 & 2532 µg/mL were selected for the metaphase analysis.

In the absence of S9 mix the reduction in mitotic index was 27% at the highest concentration tested of 2532 µg/mL. Concentrations of 1296, 2026 & 2532 µg/mL were selected for the metaphase analysis.

In the presence and absence of S9 mix, the frequencies of cells with structural aberrations were similar to those seen in concurrent vehicle controls. Furthermore, the number of aberrant cells (excluding gaps) in all treated cultures fell within the normal historical control ranges.

The quantitative analysis for polyploidy (numerical aberrations) revealed that normal frequencies (within negative historical control ranges) were observed for the majority of cultures in the presence and absence of S-9 mix in experiment 1.

2. Experiment 2

Experiment 2 comprised a continuous exposure (20+0-hour treatment) in the absence of S9 and a pulse exposure (3+17-hour treatment) in the presence of S9.

The reduction in mitotic index in the absence of S9 mix (20-hour exposure) was 48% at 2532µg/mL (the highest concentration tested). Concentrations of 306.1, 498.5 & 1123 µg/mL were selected for the metaphase analysis.

In the presence of S9 mix (3+17-hour pulse treatment) the reduction in mitotic index was 44% at the highest concentration tested (2532 µg/mL). Concentrations of 1829, 2152 & 2532 µg/mL were selected for metaphase analysis.

In the presence of S9 mix (3+17-hour pulse treatment) frequencies of cells with structural aberrations were similar to those seen in concurrent vehicle controls. Furthermore, the number of aberrant cells (excluding gaps) in all treated cultures fell within the normal historical control ranges.

In the absence of S9 mix (20-hour exposure), a small but significant increase in aberrant cell frequency was seen at the highest concentration examined (1123 µg/mL). At this concentration, a single culture exhibited an aberrant cell frequency that marginally exceeded the historical negative control (normal) range. However, the aberrant cell frequency of the replicate culture, and all other treated cultures fell within the normal range. Therefore, this observation is not considered to be biologically significant.

The quantitative analysis for polyploidy (numerical aberrations) revealed that normal frequencies (within negative historical control ranges) were observed for the majority of cultures in the absence of S-9 mix in experiment 2.

The positive control chemicals NQO and CPA induced statistically significant increases in the number of cells with structural aberrations, thus confirming the sensitivity of the assay for both experiments.

An overview of the results for experiments 1 and 2 is provided in the tables below.

Table 5.8.1- 109: Relative mitotic indices and mean percentage of aberrant human lymphocytes in experiment 1

Treatment	Concentration (µg/ml)	Treatment time (h)	Mitotic index (mean)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
Without metabolic activation (-S9)					
M-05	1296	3	11.2	3	2
	2026	3	9.4	6	6
	2532	3	9.6	3	3
Solvent control	-	3	13.1	3	2
Positive control NQO	5	3	-	51	49*
With metabolic activation (+S9)					
M-05	1159	3	9.8	0	0
	1545	3	8.6	3	1
	2060	3	7.8	5	3
Solvent control	-	3	9.8	3	3
Positive control CPA	6.25	3	-	66	63*

*statistically significant p<0.001

Table 5.8.1- 110: Relative mitotic indices and mean percentage of aberrant human lymphocytes in experiment 2

Treatment	Concentration (µg/ml)	Treatment time (h)	Mitotic index (mean)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
Without metabolic activation (-S9)					
M-05	306.1	20	8.0	10	4
	498.5	20	5.8	7	4
	1123	20	4.6	9	8
Solvent control	-	20	8.8	4	2
Positive control NQO	5	20	-	45	37*
With metabolic activation (+S9)					
M-05	306.1	3	11.7	4	4
	498.5	3	11.2	3	2
	1123	3	6.9	5	2
Solvent control	-	3	12.4	4	4
Positive control NQO	20	3	-	66	58*

*statistically significant p<0.001

III. Conclusion

M-05 did not induce chromosomal aberrations in cultured human peripheral blood lymphocytes following a continuous 20-hour treatment in the absence of S9 mix or in 4-hour pulse treatments in the presence or absence of S9 mix. The was no evidence of polyploidy (numerical aberrations).

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 473 and is valid and acceptable to assess the potential of M-05 to induce chromosome aberrations in human lymphocytes *in vitro*. M-05 was not clastogenic in human lymphocytes under the conditions of this study following a 20-hour continuous exposure in the absence of S9 or following a 4-hour pulse exposure in the presence or absence of S9 and there was no evidence of polyploidy.

Data Point:	KCA 5.8.1/26
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE 1344123 (metabolite of AE C628206) - V79/HPRT-test in vitro for the detection of induced forward mutations
Report No:	AT00591
Document No:	M-218169-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC, B.17 (2000); OECD 476 (1997); US-EPA EPA712-C-98-221, OPPTS 870.5300 (1998)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The study was performed to investigate the potential of M-05 to induce gene mutations at the HPRT locus in V 79 cells of the Chinese hamster *in vitro*.

The assay was performed in two independent experiments both with and without rat liver microsomal activation (S9-mix). The test article was dissolved in DMSO and tested at concentrations ranging from 75 to 2400 µg/mL. The concentration ranges were based on the results of preliminary testing for solubility and toxicity in which the pH of the medium was altered at 2000 µg/mL and the osmolality of the medium was slightly changed at 2400 µg/mL, no cytotoxicity was observed.

No relevant increase in mutant colony numbers was obtained in any experiment.

The positive controls showed distinct statistically significant increases in induced mutant colonies, thus indicating the sensitivity of the assay and the negative controls gave the expected results.

It was concluded that M-05 is not mutagenic in this HPRT test with V79 Chinese hamster cells.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-05 (referred to as AE 1344122 in the report)
Purity:	98.8%
Batch no.:	YG3228
Expiry date:	February 5, 2004

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: Ethyl methanesulphonate (EMS), 900 µg/mL +S9: Dimethylbenzanthracene (DMBA), 20µg/mL

3. Activation:

Metabolic activation was provided by S9 mix. The S9 fraction was isolated in house from the livers of Aroclor 1254 induced male SD rats and was kept frozen at -80˚C. Samples of the batch were tested for contamination and cytotoxicity prior to use. To prepare the S9 mix, two parts of the thawed S9 fraction were mixed three parts of freshly dissolved co-factors (in sodium phosphate buffer) to give the following final concentrations:

$MgCl_2$ x 6H2O (8mM)

KCl (33mM)

Glucose-6-phosphate (5mM)

NADP (1mM)

S9 fraction (40% v/v)

Sodium phosphate buffer (60% v/v)

The S9 mix was stored on ice until use the same day.

4. Cell cultures and medium:

Cell cultures

V79 cell stocks (derived from Chinese hamster lung cells) are stored in liquid nitrogen. Laboratory cultures are maintained in plastic tissue culture vessels at 37˚C in a humid atmosphere containing 5% CO_2. Exponential growth was maintained by twice weekly sub-culturing. The cells were checked for mycoplasma contamination of which there was no evidence. To reduce the number of spontaneous 6-TG resistant mutants cell cultures were sub-cloned by plating 1000 cells/culture vessel at least every two weeks. If necessary, the spontaneous frequency of HPRT-mutants was further reduced by the addition of thymidine (9µg/mL), hypoxanthine (10µg/mL), glycine (22.5µg/mL and methotrexate (0.3µg/mL). A 6-TG sensitive sub-clone was then used for the HPRT test.

In all parts of the study incubation was performed at 37˚C humidified air with 5% CO_2.

Media

Cells were maintained in hypoxanthine free Eagle's Minimal Essential medium (MEM, Gibco) which has been proven suitable for growth of V79 cells. The MEM was supplemented nonessential amino acids, L-glutamine (2mM), MEM-vitamins, $NaHCO_3$, penicillin (100 units/mL, streptomycin (100 units/mL) and heat activated foetal calf serum (10%; reduced to 2% in treated cultures).

For the selection of mutants, a hypoxanthine-free culture medium was used containing 10µg/mL of 6-thioguanine (6-TG)

5. Test substance concentrations used:

M-05 was tested in concentrations ranging from 75 µg/mL to 2400 µg/mL with and without metabolic activation, in the first mutation experiment and in the independent repeat.

B. Test performance

Experimental phase: May 20 to July 03, 2003

1. Preliminary assay

In order to select the dose levels for the mutation assays, a preliminary cytotoxicity assay was conducted with and without metabolic activation at concentrations ranging from 1 µg/mL to 2500 µg/mL.

Exponentially growing V79 cells were plated in 20 mL culture medium in a 250 mL flask ($4x10^6$ per flask) One culture was available for each concentration. Following attachment (16-24 hours), the cells were exposed without S9 mix to vehicle alone and a range of concentrations of the test substance for 5 hours in 20 ml culture medium containing 2% FCS. Thereafter, cell monolayers were washed with PBS, trypsinised and re-plated in 5 mL culture medium at a density of 200 cells into each of three petri dishes. The dishes were incubated for 6-8 days to allow colony development.

Thereafter, the colonies were fixed, stained, and counted. Cytotoxicity was expressed as comparison of colonies in treated cultures versus vehicle control cultures (relative cloning efficiency).

2. Main assay

Without S9

Exponentially growing V79 cells were plated in culture medium at a final volume of 20 mL in two 250 mL flasks/concentration ($4x10^6$ per flask) including controls. Following attachment (16-24 hours), the cells were exposed for 5 hours in 20 ml culture medium with reduced serum content (2%). Corresponding controls received the same treatment. Thereafter, cell monolayers were washed with PBS, trypsinised and re-plated in 20 mL culture medium using 200 cells per petri dish (one flask and three petri dishes were used per culture). Petri dishes were incubated for 6 days to allow colony development and determine the survival to treatment.

Cells in 250 mL flasks were incubated to permit growth and expression of 1 induced mutation. Cells were sub-cultured (=count 1, 3 days) by re-seeding 1.5 x 10^6 cells into 20 ml medium in 250 mL flasks. Following the expression period (=count 2, total 6 days) cultures were re-seeded in petri dishes at $3x10^5$ cells per dish (8 dishes per culture) in 20 mL culture medium without hypoxanthine but containing 10 µg/mL 6-TG for mutant selection. In addition, 200 cells per dish (3 per culture) were seeded in 5 mL culture medium to determine absolute cloning efficiency for each concentration.

After 6-8 days incubation, the colonies were fixed, stained, and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.

At least two trials were performed and at least four concentrations will be determined in each trial.

With S9

The activation assay was performed independently; the procedure was identical to the non-activation assay described above except for the addition of S9 mix. In the activation assay 19mL of culture medium with 1 mL of S9 was used (rather than 20 mL of culture medium) during the treatment period, resulting in a concentration of 5% S9 mix in the cultures.

3. Acceptance Criteria

The assay was considered valid if the following criteria were met:

- The average cloning efficiency of the negative and vehicle controls should be at least 50%
- The average of mutant frequency of the vehicle control should not exceed 25x10^{-6} cells
- The mutant frequency of the two cultures of the vehicle and negative controls should only differ to an acceptable extent (not greater than 5x10^{-6})
- The positive control should induce an average mutant frequency of at least 3 times the vehicle control
- If not limited by solubility, the highest concentration should induce cytotoxicity of about 80-90% or should be a precipitative concentration. The survival at the lowest concentration should be in the range of the negative control
- For the calculation of an acceptable mutant frequency, at least 5 dishes per culture should be available and relative survival to treatment, relative population growth and absolute cloning efficiency should be 10% or greater

4. Criteria for a positive response

The test substance is classified as mutagenic if:

- A concentration related increase in mutant frequencies is observed in parallel cultures.
- The increase in mutant frequencies should be 2-3 times higher than the highest negative or vehicle control in the respective trial
- The result should be reproduced in the second trial
- the increase should occur in the absence of a change in osmolality compared to the vehicle control

A test substance is considered negative if:

- there is no reproducible or relevant increase in mutant frequencies

A test substance will be considered equivocal if:

- There is no strict concentration related increase in mutant frequencies, but one or more concentrations induce a reproducible and biologically relevant increase in mutant frequencies

Sound scientific judgement should be used in implementing the above criteria.

5. Statistical analysis

The statistical analysis relies on the mutant frequencies which are submitted to a weighted analysis of variance and a weighted recursive regression, both with Poisson derived wights.

II. Results and Discussion

A. Preliminary cytotoxicity assay

No cytotoxicity was observed. However, the pH of the culture medium was altered at 2000 µg/mL and the osmolality was slightly altered at 2400 µg/mL. therefore 2400 µg/mL was selected as the top concentration in the main mutation assays (the limit of solubility).

B. Main assay

Without metabolic activation

Two trials were performed in the absence of S9 mix. In treated cultures, no concentration-related decreases were observed in relative survival to treatment or relative population growth.

No M-05 induced increases in mutant frequencies were observed in the absence of S9; furthermore, statistical analysis revealed no statistically significant increases.

The mutation frequencies of the negative and vehicle controls were all within the normal range and the positive control EMS induced a clear mutagenic and statistically significant effect in both trials, thus demonstrating the validity of the assay.

With metabolic activation

Two trials were performed in the presence of S9 mix. In treated cultures, no concentration-related decreases were observed in relative survival to treatment or relative population growth.

No M-05 induced increases in mutant frequencies were observed in the absence of S9; furthermore, statistical analysis revealed no statistically significant increases.

The mutation frequencies of the negative and vehicle controls were all within the normal range and the positive control DMBA induced a clear mutagenic and statistically significant effect in both trials, thus demonstrating the validity of the assay.

The results from both experiments with and without S9 are summarised in the tables below:

Table 5.8.1- 111: Relative survival and mutant frequencies in experiment 1 without metabolic activation

Treatment	Concentration (µg/ml)	Population growth (%)	Mutation frequency
M-05	75	99.2	2.8
	75	189.3	1.1
	150	122.8	4.6
	150	117.1	3.8
	300	111.8	0.5
	300	154.5	3.8
	600	126.1	4.7
	600	133.6	0.6
	1200	115.2	2.9
	1200	110.3	1.9
	2400	105.5	0.5
	2400	123.4	3.5
Negative control	-	102.7	1.2
	-	140.5	1.2
Solvent control	-	100.0	2.2
	-	100.0	2.9
Positive control EMS	900	44.9	612.1
	900	39.3	562.3

Table 5.8.1- 112: Relative survival and mutant frequencies in experiment 2 without metabolic activation

Treatment	Concentration (µg/ml)	Population growth (%)	Mutation frequency
M-05	75	140.7	6.6
	75	70.5	0.8
	150	102.1	5.1
	150	67.5	2.8
	300	117.4	1.4
	300	57.5	4.0
	600	129.4	7.7
	600	75.5	0.7
	1200	60.0	0.6
	1200	57.7	-
	2400	70.4	1.9
	2400	32.0	3.5
Negative control	-	157.4	5.4
	-	109.0	1.4
Solvent control		100.0	3.8
		100.0	-
Positive control EMS	900	52.5	701.3
	900	22.7	789.0

Table 5.8.1- 113: Relative survival and mutant frequencies in experiment 1 with metabolic activation

Treatment	Concentration (µg/ml)	Population growth (%)	Mutation frequency
M-05	75	154.6	2.5
	75	144.2	3.5
	150	168.5	1.9
	150	139.1	3.4
	300	124.8	0.6
	300	160.1	0.0
	600	115.4	0.0
	600	130.2	4.0
	1200	121.9	0.6
	1200	132.0	2.1
	2400	73.5	0.9
	2400	137.0	3.2
Negative control	-	122.8	0.7
	-	163.2	0.6
Solvent control	-	100.0	1.7
	-	100.0	1.8
Positive control DMBA	20	15.7	131.1
	20	18.1	198.7

Table 5.8.1- 114: Relative survival and mutant frequencies in experiment 2 with metabolic activation

Treatment	Concentration (µg/ml)	Population growth (%)	Mutation frequency
M-05	75	96.3	
	75	107.1	0.0
	150	76.5	1.9
	150	68.0	0.0
	300	107.1	3.2
	300	100.7	3.2
	600	142.6	1.6
	600	114.7	0.6
	1200	99.5	1.0
	1200	85.5	8.7
	2400	71.7	0.0
	2400	83.0	1.4
Negative control	-	127.6	2.4
	-	178.9	2.5
Solvent control		100.0	2.6
		100.0	5.4
Positive control EMS	20	86.7	87.7
	20	71.8	-

III. Conclusion

M-05 did not induce gene mutations in mammalian cells (Chinese hamster lung V79 cells) either in the presence or absence of metabolic activation provided by S9 mix. Appropriate responses exhibited by the concurrent negative, vehicle and positive controls confirmed the validity of the assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD 476 and is valid and acceptable to assess the potential of M-05 to induce gene mutations at the HPRT locus in Chines Hamster Lung V79 cells. M-05 is not mutagenic in mammalian cells under the conditions of this study when tested up to its limit of solubility.

Data Point:	KCA 5.8.1/62
Report Author:	[redacted]
Report Year:	2019
Report Title:	AE 1344122: Micronucleus test in human lymphocytes In vitro
Report No:	1969603
Document No:	M-673685-01-1
Guideline(s) followed in study:	OECD Test Guideline No. 487 (July 2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

M-05 (referred to as AE 1344122 in the report) was evaluated for its potential to induce micronuclei in human lymphocytes *in vitro*, in two independent experiments. Experiment I comprised a four hour exposure period in the presence and absence of metabolic activation (provided by S9) whilst experiment II provided a 20-hour exposure period in the absence of S9. Concentrations up to 2024 µg/mL were tested in duplicate and 1000 binucleated cells/culture were examined for cytogenetic damage.

In experiment I, no cytotoxicity or precipitation was observed (+/- S9) up to the highest evaluated concentration. Similarly, in experiment II (-S9), no cytotoxicity or precipitation was observed at the highest tested concentration. Therefore, concentrations up to the limit concentration of 2024 µg/mL were evaluated.

No relevant increases in the numbers of micronucleated cells were observed following treatment with the test item either in the presence or absence of S9 mix in either experiment.

Appropriate reference substances gave the expected statistically significant increase in the number of micronucleated cells.

The test substance M-05 can be considered neither clastogenic nor aneugenic under the conditions of this *in vitro* micronucleus test, when tested up to the highest possible concentration, both in the presence and absence of metabolic activation.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-05 (referred to as AE 1344122 in the report)
Purity:	98.8 % (w/w)
Batch no.:	BCS-BA50602
Expiry date:	18th February 2026

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive controls:	-S9
	Mitomycin C (MMC), 0.8 µg/mL (98% purity, dissolved in deionized water)
	Demecolcine, 75 ng/mL (purity ≥98%, dissolved in deionized water)
	+S9
	Cyclophosphamide (CPA), 17.5 µg/ml (purity 97-103%, dissolved in saline)

3. Activation:

Metabolic activation was provided by S9-mix derived from phenobarbital/ß-naphthoflavone induced rat liver homogenate; each batch of prepared S9 has been routinely evaluated for its ability to activate known mutagens.

An appropriate quantity of S9 was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation used for this study was 30.9 mg/mL.

4. Cell cultures and media:

Cells

Blood was drawn from healthy non-smoking donors, not receiving medication. Blood from a female donor (29 years old) and a male donor (29 years old) were used in experiments I and II, respectively. The lymphocytes have been shown to respond well to PHA and positive control substances; furthermore, both donors have an established low incidence of micronuclei in peripheral blood lymphocytes.

Media

Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hours following collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1), supplemented with 200 mM GlutaMAX™. The medium was further supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (foetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).

All incubations were carried out at 37 °C with 5.5 % CO2 in humidified air.

5. Test compound concentrations used:

Test concentrations for the first experiment (4-hour pulse treatment) were 0 (solvent control), 15.3, 26.8, 47, 82.2, 144, 252, 441, 771, 1349 & 2024 µg/mL, both with and without S9 mix.

In the second experiment a continuous (20 hour) treatment was used at test concentrations of 0 (solvent control), 144, 252, 441, 771, 1349 & 2024 µg/mL, in the absence of S9 mix.

B. Test Performance

Experimental phase: 21st August 2019 to 18th October 2019

1. Preliminary assay

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity (characterized by the percentages of reduction in the CBPI in comparison to the controls and expressed as % cytostasis) was determined from 500 cells/culture. The experimental conditions in this pre-experimental phase were identical to those described for the mutagenicity assay.

The pre-test was performed with 10 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hours (with and without S9 mix). The preparation interval was 40 hours after start of the exposure.

This preliminary test was designated Experiment I since the cultures fulfilled the criteria for cytogenetic evaluation and appropriate concentrations could be selected for evaluation.

2. Cytogenetic experiment

Cells were subject to either 4 hours pulse exposure (with and without S9; experiment I) or 20 hours continuous exposure (without S9; experiment II). The succeeding procedure for cell preparation was the same for both exposure periods.

Pulse exposure

Approximately 48 hours following seeding, two blood cultures of 10 mL each, were set up in parallel in 25 cm² cell culture flasks (for each test item concentration). The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H2O, 192 mg/L Na2HPO4 • 2 H2O and 150 mg/L KH2PO4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

Continuous exposure

Approximately 48 hours following seeding, two blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells re-suspended in and washed with "saline G". The washing procedure was repeated once as described. Following washing, the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

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

Preparation of cells

Following the procedures above (approximately 40 hours from the start of treatment), the cultures were harvested by centrifugation. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37°C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

3. Acceptance Criteria

The micronucleus assay is deemed acceptable if it fulfils the following criteria:

- The concurrent solvent control will normally be within the laboratory historical solvent control data range. The average mutant frequency of the vehicle controls should not exceed $25x10^{-6}$ cells
- The concurrent positive controls should produce a statistically significant increase in the micronucleus frequency and should be within the laboratory historical positive control data range. The positive controls should induce a mutant frequency at least 3 times that of the controls
- Cell proliferation criteria in the solvent control are considered acceptable.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- The quality of the slides should allow the evaluation of an adequate number of cells and concentrations
- The criteria for the selection of top concentrations should be met

Any mutant frequencies not meeting these criteria were excluded from the statistical analyses.

4. Assessment criteria

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative if, in all of the experimental conditions examined:

- None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase
- The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- The increase is concentration-related in at least one experimental condition
- The results are outside the range of the laboratory historical solvent control data (95% control limit realized as 95% confidence interval)

When all of the criteria are met, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system.

There is no requirement for verification of a clear positive or negative response.

In case the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. narrow concentration spacing, other metabolic activation conditions, i.e. S9 concentration or S9 origin) could be useful.

5. Statistical analysis

Statistical significance was confirmed by the Chi square test ($p < 0.05$), using a validated test script of "R", a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those values that indicated an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression was performed using a validated test script of "R", to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Both, biological and statistical significance were considered together.

II. Results and discussion

In experiment I neither precipitation nor cytotoxicity was observed up to the highest applied concentration, either in the presence or absence of S9, and no relevant influence on osmolarity or pH was observed. Similarly, in experiment II, no precipitation or cytotoxicity was observed up to the highest applied concentration. The highest concentration applied (2024µg/ml) was therefore the limit concentration.

The results of both experiments, with and without metabolic activation, are summarised in the table below:

Table 5.8.1- 115: Summary of results of experiment I and II

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	Historical control data	
						95% Ctrl limit	Min - Max
Exposure period 4 hrs without S9 mix							
I	40 hrs	Solvent control[1]	1.73		0.25	0.01 – 1.20	0.00 – 1.55
		Positive control[2]	1.76	n.c	**6.45**^{S}	2.66 – 22.74	1.95 – 28.60
		771	1.78	n.c	0.30		
		1349	1.79	n.c	0.45		
		2024	1.79	n.c	0.45		
Exposure period 20 hrs without S9 mix							
II	40 hrs	Solvent control[1]	1.74		0.20	0.00 – 1.14	0.05 – 1.60
		Positive control[3]	1.82	n.c	**2.85**^{S}	1.15 – 6.44	1.95 – 8.80
		771	1.65	12.2	0.30		
		1349	1.71	4.8	0.30		
		2024	1.74	0.5	0.20		
Exposure period 4 hrs with S9 mix							
I	40 hrs	Solvent control[1]	1.92		0.30	0.00 – 1.24	0.10 – 1.30
		Positive control[3]	1.77	15.5	**2.85**^{S}	1.01 – 7.34	1.80 – 8.85
		771	1.85	6.9	0.40		
		1349	1.84	8.4	0.15		
		2024	1.84	8.8	0.35		

* For the positive control groups and the test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
S The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c Not calculated as the CBPI is equal to or higher than the solvent control value
1 DMSO 0.5 % (v/v)
2 MMC 0.8 µg/mL
3 Demecolcine 75 ng/mL
4 CPA 17.5 µg/mL

In both independent experiments, neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells were observed following treatment with the test item either in the presence or absence of S9 mix.

Demecolcine (100 ng/mL), MMC (0.8 µg/mL) and CPA (15 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei, thus confirming the validity of the assay to detect clastogens/aneugens.

III. Conclusions

The test substance M-05 did not induce micronuclei in this *in vitro* micronucleus test in human lymphocytes, when tested up to precipitative concentrations.

Therefore M-05 is considered to be neither clastogenic nor aneugenic under the conditions of this assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 487 and is valid and acceptable to assess the clastogenicity of M-05 *in vitro*. M-05 is neither clastogenic nor aneugenic under the conditions of this study.

Data Point:	KCA 5.8.1/27
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE 1344122 - 28-day toxicity study in the rat by dietary administration
Report No:	C037198
Document No:	M-222343-01-1
Guideline(s) followed in study:	EC Directive 92/69/EEC, Annex V, Method B7 (1992), OECD 407 (1995)
Deviations from current test guideline:	Seminal vesicles were not weighed at necropsy
Previous evaluation:	yes, evaluated and accepted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

M-05 (AE 1344122) was administered continuously *via* the diet to groups of Wistar rats (10/sex/group) for 28 days at concentrations of 20, 200, 2000 and 20000 ppm (equivalent to 0, 1.5, 14.9, 151.6, 1495.3 & 1.7, 16.8, 167.1 & 1615.5 mg/kg bw/d in males and females respectively). Animals were observed daily for mortality and clinical signs, with physical examinations performed weekly. In addition, grasping, righting, corneal, pupillary, auditory startle and head shaking reflexes were examined once during the acclimatization phase and during Week 4 of the study. Body weight and food consumption were recorded weekly. During the acclimatisation phase all animals were subjected to an ophthalmic examination, all animals in the control and 20000 ppm groups were re-examined during week 4. Haematology, clinical chemistry and urine parameters were determined at the end of the study. Additional urine samples were collected once per week for future analysis of two of the metabolites of the test substance, P2 and P4. All animals were necropsied, selected organs weighed, and a range of tissues were taken, fixed and examined microscopically.

No mortalities occured during the course of the study.

At 20000 ppm, clinical signs consisted of scabs around the nose/head region observed in 3 males together with soiling around the eye in one male and chromodacryorrhea (recorded at the ophthalmological examination) in two other males. One female had anogenital soiling. Absolute body weight in males was reduced by 7%, with overall mean body weight gain reduced by 18% by day 28, compared with controls. In females, absolute body weight was reduced by 4% on Day 28, whilst overall body weight gain was 18% lower than the control group. Food consumption was reduced by 13% during week one of the treatment period in males. Over the course of the entire treatment period food consumption was reduced by 9% in females.

At clinical chemistry examination, organic phosphorus concentration was reduced by 10% by Day 28 in males. Urinalysis revealed coarsely granular casts in the urine of 1/10 males and 9/10 females. In addition, slightly lower pH values and ketone levels were noted in males whilst mean urinary volume was higher than control levels for both sexes.

At microscopic examination, minimal to moderate tubular degeneration/regeneration and single cell necrosis of minimal to moderate severity was observed in the kidney of 8/10 females. This change was correlated with the coarsely granular casts observed in the urine.

At 2000 ppm, clinical findings were limited to scabs around the nose/head region in 2/10 males and nasal soiling noted in one male. In the absence of any other treatment-related effects in either sex at this dose level, these findings were considered to have no toxicological relevance.

There were no treatment related findings in either sex at either 200 or 20 ppm.

In conclusion, the No Observed Adverse Effect Level (NOAEL) was considered to be 2000 ppm in males, equivalent to 152/167 mg/kg bw/day in M/F.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-05 (referred to as AE C344122 in the report)
Purity:	98.8% w/w
Batch no.:	YG3228

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rats
Strain:	Wistar rats Rj: WI (IOPS HAN)
Age:	Approx. 7 weeks of age at start of treatment
Weight at start:	236 - 273 g for males and 169 - 204 g for females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 supplied by Scientific Animal Food and Engineering (S.A.F.E.), Epinay-sur-Orge, France Animal diet fasted overnight prior to blood sampling, urine sampling and prior to sacrifice
Water:	Water ad lib, except during weekly overnight urine collection when water was withheld
Housing:	Individually in suspended stainless-steel wire mesh cages
Temperature:	20 - 24 °C
Humidity:	40 to 70%
Air changes:	10 – 15 air changes/hour (average, not monitored)
Photoperiod:	12 hours

B. Study design

1. In-life dates: 3 March to 30 March, 2003

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 5.8.1- 116: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	10	10
2	20	10	10
3	200	10	10
4	2,000	10	10
5	20,000	10	10

3. Diet preparation and analysis

The test substance was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required dietary concentrations. There was one preparation for each concentration. When not in use, the diet formulations were stored at approximately -18°C.

The homogeneity of test substance in diet was verified before the study for the dose levels of 20, 200 and 20 000 ppm to demonstrate adequate formulation procedures. Dietary levels of the test substance were verified for each concentration.

The stability of the frozen dietary formulation was determined at the 20, 200 and 20 000 ppm dose levels over a duration which covered the period of storage and use for the study.

Routine analyses of food and water indicated that there was no contamination which could have been expected to have compromised the study.

4. Statistics

The following variables were analysed:

- Body weight parameters
- Body weight gain/day parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Haematology parameters (except eosinophils, basophils, monocytes and large unstained cells)
- Clinical chemistry parameters
- Quantitative urinalysis parameters (only pH, volume and refractive index)
- Terminal body weight, absolute and relative organ weights parameters,

Mean and standard deviation were calculated for each group. All statistical analyses were conducted separately for males and females.

Body weight gain/day: Mean and standard deviation were calculated for each group and per time period. Bartlett test was performed to compare the homogeneity of group variances.

If the Bartlett test was not significant (α = 0.05), means were compared using analysis of variance (ANOVA). If the ANOVA was not significant (α = 0.05), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant (α = 0.05), means of the exposed groups were compared to the mean of the control group using Dunnett's test (2-sided).

If the Bartlett test was significant (α = 0.05), group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant (α = 0.05), the group means were considered to be homogeneous and no further analysis was performed.

If the Kruskal-Wallis test was significant (α = 0.05), means of the exposed groups were compared to the mean of the control group using the Dunn test (2-sided).

Body weight and average food consumption/day parameters: Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters. Bartlett test was performed to compare the homogeneity of group variances.

If the Bartlett test was not significant (α = 0.05), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant (α = 0.05), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant (α = 0.05), means of the exposed groups were compared to the mean of the control group using Dunnett's test (2-sided).

If the Bartlett test was significant (α = 0.05), data were transformed using log transformation. If the Bartlett test on log transformed data was not significant (α = 0.05), means were compared using the ANOVA on log transformed data. If the ANOVA on log transformed data was not significant (α = 0.05), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log transformed data was significant (α = 0.05), means of the exposed groups were compared to the mean of the control group using Dunnett's test (2-sided) on log transformed data.

If the Bartlett test was significant (α = 0.05) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant (α = 0.05), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant (α = 0.05), means of the exposed groups were compared to the mean of the control group using the Dunn test (2-sided).

Haematology parameters: Bartlett test was performed to compare the homogeneity of group variances.

If the Bartlett test was not significant (α = 0.05), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant (α = 0.05), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant (α = 0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (2-sided).

If the Bartlett test was significant (α = 0.05), data were transformed using the square root transformation. If the Bartlett test on square root transformed data was not significant (α = 0.05), means were compared using the ANOVA on square root transformed data. If the ANOVA on square root transformed data was not significant (α = 0.05), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on square root transformed data was significant (α = 0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (2-sided) on square root transformed data.

If the Bartlett test was significant (α = 0.05) even after square root transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant (α = 0.05), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant (o=0.05), means of the exposed groups were compared to the mean of the control group using the Dunn test (2-sided).

Quantitative urinalysis parameter (pH): Group means were compared using the non-parametric Kruskal Wallis test. If the Kruskal-Wallis test was not significant (α = 0.05), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant (α = 0.05), means of the exposed groups were compared to the mean of the control group using the Dunn test (2-sided).

If one or more group variance(s) equalled 0, means were compared using non-parametric procedures.

Group means were compared at the 5% and 1% level of significance.

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

C. Methods

1. Observations

Animals were observed for mortality and morbundity twice daily (once daily at weekends and public holidays). Clinical observations were made at least once daily for all animals. Detailed physical examinations were conducted at least weekly during treatment period. Records of observations included the nature, time of onset, severity and duration of any abnormal behaviour or condition.

Cages and cage trays were inspected daily for evidence of ill-health.

2. Body weight and food intake

The weight of the animals was recorded three times during acclimatisation, on the first day of test substance administration and then at weekly intervals throughout the treatment and before necropsy.

The amount of food consumed for each animal was measured at weekly intervals throughout the treatment period and the weekly mean achieved dose intake in mg/kg/day was calculated. Any food spillage was noted.

3. Ophthalmoscopic examination

Ophthalmological examinations were conducted on each animal prior to the start of treatment (acclimatisation). During week 4 animals from the control and higher dose groups were re-examined. Both eyes of each animal were examined using an indirect ophthalmoscope after instillation of an atropinic agent (Mydriaticum supplied by Merch, Sharp and Dohme).

4. Neurotoxicity assessments

Prior to the commencement of treatment (acclimatisation) and during week 4 the following reflexes were tested:

- Grasping reflex (by pulling the animal across a textured surface)
- Righting reflex (by putting the animal on its back)
- Corneal reflex (following touching of the cornea with a fine nylon string)
- Pupillary reflex (by covering the eyes of the animal for a few seconds and then observing pupillary reflex by focusing a light in the eyes)
- Auditory startle reflex (by observing the animal's response to a clap of the hands)
- Head shaking reflex (head shaking response of the animal due to blowing on the ear)

5. Laboratory investigations (haematology, clinical chemistry, urinalysis)

On study Days 24, 25 or 26, blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus. An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were fasted overnight prior to bleeding and anesthetized by inhalation of isoflurane. Blood was collected on EDTA for haematology (0.5 ml), on lithium heparin (for plasma, 2.5 ml) and clot activator (for serum, 0.6 ml) for clinical chemistry and on sodium citrate for coagulation parameters (0.9 ml).

The following parameters were determined:

Haematology
Haematocrit
Haemoglobin
Red blood cells
Mean corpuscular volume
Mean corpuscular haemoglobin
Mean corpuscular haemoglobin concentration
Platelets
Reticulocyte count
White blood cells and differential count evaluation

Clinical chemistry
Total protein
Albumin
Total globulin
A/G ratio (A/G)
Calcium
Inorganic phosphorus
Sodium
Potassium
Urea
Creatinine
Glucose
Total cholesterol
Total bilirubin
Triglycerides
Chloride
Aspartate aminotransferase
Alanine aminotransferase
Alkaline phosphatase
G-glutamyl transpeptidase

Urinalysis:

Urine samples were collected overnight from all animals once per week during the four-week study. Any significant change in general appearance of the urine was recorded. Urine volume and pH was determined. The urinary refractive index was measured using a refractometer. After centrifugation, microscopic examination of urinary sediment was conducted and the presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was graded.

The following parameters were determined:

Table 5.8.1- 117: Urinalysis

Quantitative measures	Semi-quantitative measures
Urine volume pH Urinary refractive index	Glucose Bilirubin Ketone bodies Occult blood Protein Urobilinogen

6. Sacrifice and pathology

Animals were killed by exsanguination via the abdominal aorta under deep anaesthesia. All animals were examined thoroughly, including examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically.

Samples were fixed by immersion in neutral buffered 10% formalin (except the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative). With the exception of larynx exorbital gland, pharynx and nasal cavities, all samples were embedded in paraffin wax. Histological sections, stained with haematoxylin and eosin, were prepared for all the organs from all the animals.

The following organs from all animals were weighed at necropsy:

Adrenal gland	Kidneys	Spleen
Brain	Liver	Testis
Heart	Ovaries	Thyroid gland (with parathyroid)
Epididymides	Pituitary gland	Prostate
Uterus (including cervix)		

The following organs and tissues from all animals were fixed in 10% neutral buffered formalin:

Adrenal gland	Eye and optic nerve
Aorta	Exorbital (lachrymal) gland
Articular surface (femoro-tibial)	Harderian (lachrymal) gland
Bone (Sternum)	Kidneys
Brain	Larynx/pharynx
Epididymides	Liver
Oesophagus	Lungs
Heart	Mammary gland
Lymph nodes (submaxillary, mesenteric)	Ovaries
Nasal cavities	Pituitary gland
Pancreas	Nerve (sciatic)
Prostate gland	Skeletal muscle
Seminal vesicle	Spinal cord (cervical, thoracic, lumbar)
Skin	Stomach
Spleen	Testes
Submaxillary (salivary) gland	Thyroid (with parathyroid)
Thymus	Trachea
Tongue	Uterus (including cervix)
Urinary bladder	Uterus (including cervix)
Vagina	
Intestine (duodenum, jejunum, ileum, caecum, colon, rectum)	

A bone marrow smear was prepared from one femur but was not examined.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities. Clinical signs considered to be treatment related consisted of scabs around the nose/head region noted in 3/10 males at 20000 ppm and 2/10 males at 2000 ppm. In addition, soiling around the eye/nose was observed in one male at both 20000 ppm and 2000 ppm. One female had anogenital soiling at 20000 ppm.

2. Body weight and food consumption

At 20000 ppm absolute body weight in males was slightly reduced throughout treatment with a 7% reduction by day 28 which was statistically significant ($p<0.01$), compared with the controls. Overall body weight gain in the males was 18% lower than in the control group.

In the high dose females, absolute body weight was reduced by 4% by day 28, whilst overall body weight gain was 18% lower than in the control group.

Table 5.8.1- 118: Mean body weights and body weight gains per day

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Mean body weight [g]					
Day 1	254	252	252	253	253
Day 8	307	306	305	309	294
Day 15	351	350	350	355	336
Day 22	379	366	368	371	352
Day 28	395	387	389	394	369* [a]
Body weight gain per day [g] (std dev)					
Day 1-8	7.5 (0.4)	7.8 (1.2)	7.5 ((1.2)	8.0 (1.2)	5.9 (1.1)* [a]
Day 8-15	6.4 (0.4)	6.3 (0.5)	6.5 (0.6)	6.6 (0.9)	6.0 (0.9)
Day 15-22	2.9 (2.1)	2.3 (2.8)	2.7 (2.6)	2.2 (2.7)	2.2 (3.0)
Day 22-28	4.0 (1.8)	3.4 (2.6)	3.4 (2.5)	3.8 (3.4)	2.9 (2.5)
Females					
Mean body weight [g]					
Day 1	187	186	185	185	186
Day 8	206	204	209	207	202
Day 15	225	221	225	226	219
Day 22	226	222	226	228	217
Day 28	240	235	242	239	230

	Dose level (ppm)				
	0	20	200	2,000	20,000
Body weight gain per day [g] (std dev)					
Day 1-8	2.7 (0.6)	2.5 (0.6)	3.5 (0.6)	3.1 (1.1)	2.3 (0.8)
Day 8-15	2.8 (1.2)	2.4 (0.8)	2.3 (0.8)	2.7 (0.8)	2.5 (0.8)
Day 15-22	0.1 (1.8)	0.1 (1.6)	0.1 (1.7)	0.2 (1.7)	-0.4 (1.3)
Day 22-28	2.4 (2.3)	2.3 (2.4)	2.7 (1.7)	2.0 (1.2)	2.2 (2.0)

Note: Mean of 10 individuals

* $p < 0.01$; statistically different to control

Values in parenthesis = standard deviation

a Bart; NSg – 05 / ANOVA; NSg – 05 / Dunnett's

During the first week of the treatment, food consumption was reduced by 13% ($p \leq 0.05$) in males dosed at 20000 ppm, thereafter food consumption was comparable with the controls.

In the high dose females, there was an overall reduction in food consumption of 9% compared with controls the effect being most pronounced at Weeks 1 and 3 when there was a 12% reduction.

Food consumption was unaffected by treatment at 2000 ppm or below in either sex.

Table 5.8.1- 119: Mean food consumption (g/animal/day)

	Dose level (ppm)				
	0	20	200	2,000	20,000
Males					
Day 8	26.2 (1.0)	26.1 (1.0)	25.9 (1.3)	26.8 (1.7)	22.8 (4.2)* [a]
Day 15	27.5 (0.7)	27.5 (1.5)	27.5 (1.5)	28.1 (1.8)	26.9 (1.4)
Day 22	27.1 (1.5)	26.5 (1.5)	26.9 (1.9)	27.3 (1.7)	26.8 (1.8)
Day 28	24.9 (1.3)	23.8 (1.7)	24.1 (1.2)	25.1 (2.1)	24.1 (1.5)
Females					
Day 8	19.0 (1.5)	18.4 (1.3)	18.8 (0.8)	18.8 (1.5)	16.8 (1.5)* [a]
Day 15	19.9 (1.9)	19.4 (1.8)	19.3 (1.0)	19.5 (1.3)	18.6 (1.5)
Day 22[b]	19.8 (1.8)	19.1 (2.5)	19.1 (1.8)	19.2 (1.6)	17.5 (1.6)
Day 28	18.0 (1.8)	17.7 (1.5)	18.1 (1.3)	17.4 (1.1)	17.1 (1.5)

Note: Mean of 10 individuals

* $p < 0.05$; statistically different to control

Values in parenthesis = standard deviation

a Bart;Sig-05/Transformations not helpful/NP-KW;Sig-05/Dunn's Rank Sum

3. Ophthalmoscopic examinations

Chromodacryorrhea was observed in two animals at the highest dose level of 20000 ppm, one of the males also had a mat aspect of the cornea.

4. Neurotoxicity assessments

No treatment-related effects were observed.

5. Laboratory investigations

There were no treatment-related haematological findings. The few statistically significant differences were considered not to be toxicologically relevant in view of their low amplitude and the variation of individual values.

Table 5.8.1- 120: Selected haematology findings

	Units	Dose level (ppm)				
		0	20	200	2,000	20,000
		Males				
Red blood cell (RBC)	10E12/L	8.57 (0.30)	8.67 (0.23)	8.55 (0.26)	8.79 (0.35)	8.26 (0.43)
Haemoglobin (HGB)	(g/dL)	15.7 (0.5)	15.8 (0.7)	15.6 (0.4)	16.2 (0.5)	15.2 (0.8)
Haematocrit (HCT)	L/L	0.500 (0.014)	0.0503 (0.023)	0.495 (0.012)	0.514 (0.020)	0.477 (0.024)*
		Females				
Red blood cell (RBC)	10E12/L	8.25 (0.24)	8.41 (0.24)	8.60 (0.21)*[a]	8.58 (0.29)*[a]	8.35 (0.32)
Haemoglobin (HGB)	(g/dL)	15.1 (0.5)	15.3 (0.3)	15.6 (0.4)	15.5 (0.4)	15.0 (0.5)
Haematocrit (HCT)	L/L	0.466 (0.016)	0.471 (0.013)	0.481 (0.012)	0.478 (0.009)	0.466 (0.019)

* $p < 0.05$; statistically different to control

a Bart; NSg – 05 / ANOVA; Sig – 05 / Dunnett's

With regard to clinical chemistry, lower mean inorganic phosphorus concentrations were seen in males at 20000 ppm (-10%; $p<0.01$). Statistically significant lower mean bilirubin concentration was seen in females at 20 000 ppm. This was considered not to be toxicologically relevant in view of the range of individual values observed.

Table 5.8.1- 121: Selected mean clinical chemistry findings

	Units	Dose level (ppm)				
		0	20	200	2,000	20,000
		Males				
Total bilirubin (TBIL)	µmol/L	1.1 (0.3)	1.2 (0.2)	1.3 (0.2)	0.9 (0.4)	1.1 (0.3)
Inorganic phosphorus (PHOS)	mmol/l	2.56 (0.14)	2.41 (0.14)	2.36 (0.17)	2.41 (0.19)	2.13 (0.10)*[a]
		Females				
Total bilirubin (TBIL)	µmol/L	1.7 (0.2)	1.4 (.4)	1.4 (0.3)	1.6 (0.3)	1.2 (0.3)*[b]
Inorganic phosphorus (PHOS)	mmol/L	2.02 (0.16)	1.99 (0.14)	2.01 (0.20)	2.11 (0.24)	1.91 (0.16)

* $p < 0.05$; statistically different to control

a Bart;NSg-05/Anova;Sig-05/Dunnett's

b Bart;NSg-05/Anova;Sig-05/ No unplanned test performed

Urinalysis:

Coarsely granular casts were observed in the urine from both sexes at 20000 ppm (1/10 males and 9/10 females). A tendency towards lower pH values ($p<0.05$) and ketone levels were noted in males at 20000 ppm compared to the control group, in addition normal urinary crystals were observed in lower number, especially in male animals. At 20 000 ppm, mean urinary volume of both sexes was higher than in control group, the effect being statistically significant for females (+100%, $p<0.01$).

Table 5.8.1- 122: Selected urinalysis findings

	Units	Dose level (ppm)				
		0	20	200	2,000	20,000
		Males				
Volume (VOL)	mL	4.2 (2.0)	4.5 (1.7)	4.7 (1.7)	3.4 (1.3)	5.9 (1.6)
Refractive index (RI)	unit	1.349 (0.005)	1.345 (0.002)	1.346 (0.004)	1.349 (0.005)	1.348 (0.003)
pH	-	6.8 (0.3)	7.0 (0.3)	6.9 (0.3)	6.6 (0.4)	6.2 (0.3)*[b]
		Females				
Volume (VOL)	mL	2.1 (1.0)	1.8 (0.9)	2.5 (1.3)	2.3 (1.3)	4.2 (1.9)*[a]
Refractive index (RI)	unit	1.350 (0.005)	1.346 (0.006)	1.345 (0.004)	1.349 (0.007)	1.347 (0.004)
pH	-	6.2 (0.4)	6.3 (0.5)	6.2 (0.3)	6.2 90.4)	5.8 (0.4)

* $p < 0.01$; statistically different to control

Values in parenthesis = standard deviation

a Bart;NSg-05/Anova;Sig-05/Dunnett's

b NP-KW; Sig – 05 / Dunn's rank sum

6. Sacrifice and pathology

Terminal body and organ weight: Mean terminal body weight was statistically significantly lower in males at 20000 ppm, when compared to controls (-7%, $p<0.01$). Mean liver weights were statistically significantly lower in males at 20000 ppm, when compared to controls. However, this change was considered not to be relevant since it was not correlated with histological observation. Any other statistically significant changes were also considered as incidental.

Table 5.8.1- 123: Selected organ weights

	Units	Dose level (ppm)				
		0	20	200	2,000	20,000
		Males				
Total body weight (TBW)	g	376.8 (17.8)	367.2 (13.7)	370.5 (17.7)	375.2 (22.4)	351.2 (12.9)*[a]
Brain	g	2.05 (0.03)	2.08 (0.08)	2.02 (0.07)	2.06 (0.09)	2.02 (0.07)
Heart	g	1.32 (0.12)	1.33 (0.06)	1.30 (0.08)	1.35 (0.11)	1.20 (0.07)*[a]
Liver	g	10.23 (0.65)	9.89 (0.76)	10.07 (0.95)	10.25 (0.83)	8.87 (0.38)*[a]
		Females				
Volume (VOL)	mL	2.1 (1.0)	1.8 (0.9)	2.5 (1.3)	2.3 (1.3)	4.2 (1.9)*[a]
Refractive index (RI)	unit	1.350 (0.005)	1.346 (0.006)	1.345 (0.004)	1.349 (0.007)	1.347 (0.004)
pH	-	6.2 (0.4)	6.3 (0.5)	6.2 (0.3)	6.2 90.4)	5.8 (0.4)

* $p < 0.05$; statistically different to control

Values in parenthesis = standard deviation

a Bart;NSg-05/Anova;Sig-05/Dunnett's

Gross pathology:

Black to dark red soiled fur around nose and/or eyes was found in 3/10 males at 20 000 ppm. All other gross pathology changes were considered as incidental and not treatment-related as they occurred in isolation or not in a dose-related manner.

Microscopic pathology:

Terminal sacrifice: Treatment related findings were observed in kidney of females dosed at 20 000 ppm. At this dose level, tubular degeneration/regeneration and single cell necrosis of tubular epithelial cells were seen in 8/10 females. The severity varied between grade 1 (minimal) and grade 3 (moderate). Affected segments of the proximal tubule were located in the inner cortex. Tubular regeneration/degeneration was characterized by slight cytoplasmic basophilia, hypertrophic nuclei, and nuclear crowding. Several tubules contained eosinophilic cellular debris. This change was correlated with coarsely granular casts observed in the urine. In addition, single cell necrosis with eosinophilic cytoplasm and pyknotic nuclei were found in the same areas. No treatment-related microscopic findings were observed in any treatment group in males or at 2000 ppm and below for females.

Table 5.8.1- 124: Histopathology findings in the kidneys

Severity	Dose (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Tubular degeneration / re-generation in males					
Minimal	0	0	0	0	0
Slight	0	0	0	0	0
Moderate	0	0	0	0	0
Total	**0**	**0**	**0**	**0**	**0**
Single cell necrosis in males					
Minimal	0	0	0	0	0
Slight	0	0	0	0	0
Moderate	0	0	0	0	0
Total	**0**	**0**	**0**	**0**	**0**
Tubular degeneration / re-generation in females					
Minimal	0	0	0	0	2
Slight	0	0	0	0	3
Moderate	0	0	0	0	3
Total	**0**	**0**	**0**	**0**	**8**
Single cell necrosis in females					
Minimal	0	0	0	0	2
Slight	0	0	0	0	5
Moderate	0	0	0	0	1
Total	**0**	**0**	**0**	**0**	**8**

A variety of spontaneous changes were noted in control and treated animals with no indication of a treatment-related effect. The spectrum of changes were consistent with changes commonly encountered in laboratory rats of this age and strain

8. Diet analysis

Homogeneity of diet formulations at 20, 200 and 20 000 ppm were found to be within acceptable ranges (88-95% of nominal concentrations). The dietary levels of the test substance verified at each of the dose levels were within acceptable target range (90-96% of nominal concentrations). Hence all homogeneity and concentration samples analysed were within the (in-house) target range of 85-115% of nominal concentration. Diet mixtures were found to be stable (85-98% of nominal concentrations) over 104 days (94 days frozen storage followed by 10 days at ambient temperature) which covered the conditions of storage and usage for this study.

Table 5.8.1- 125: Achieved dosage (group mean values)

Nominal test conc. (ppm)	Study week				
	1	2	3	4	1-4
	Dietary conc. (mg/kg/day)				
	Males				
20	1.7	1.6	1.4	1.2	1.5
200	17.0	15.7	14.6	12.4	14.9
2000	173.5	158.3	147.2	127.4	151.6
20000	1551.0	1601.2	1522.7	1306.3	1495.3
	Females				
20	1.8	1.8	1.7	1.5	1.7
200	18.0	17.2	16.9	15.0	16.8
2000	181.6	172.6	168.4	145.6	167.1
20000	1663.4	1698.6	1612.9	1487.0	1615.5

III. Conclusion

At 20000ppm treatment-related clinical signs consisted of scabs around the nose/head region observed in 3 males together with soiling around the eye in one male and chromodacryorrhea (recorded at the ophthalmological examination) in two other males. One female had anogenital soiling. Absolute body weight in males was reduced by 7%, with overall mean body weight gain reduced by 18% by Day 28, compared to the controls. In females, absolute body weight was reduced by 4% on Day 28 whilst overall body weight gain was 18% lower than the control group.

Food consumption was reduced by 13% during week one of the treatment in males whilst over the course of the study food consumption was reduced by 9% in females.

At clinical chemistry examination, organic phosphorus concentration was reduced by 10% by Day 28 in males. Urinalysis revealed coarsely granular casts in the urine of 1/10 males and 9/10 females. In addition, slightly lower pH values and ketone levels were noted in males whilst mean urinary volume was higher than control levels for both sexes.

At microscopic examination, minimal to moderate tubular degeneration/regeneration and single cell necrosis of minimal to moderate severity was observed in the kidney of 8/10 females. This change was correlated with the coarsely granular casts observed in the urine.

At 2000ppm treatment related clinical findings were limited to scabs around the nose/head region in 2/10 males and nasal soiling noted in one male. In the absence of any other treatment-related effects in either sex at this dose level, these findings were considered to have no toxicological relevance.

There were no treatment related findings in either sex at 200 or 20 ppm.

In conclusion, 2000 ppm of M-05 was considered to be the No Observed Adverse Effect Level (NOAEL). (equivalent to 152mg/kg/day in males and 167mg/kg/day in females) when administered to rats via the diet over a 28-day period.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 407 and is valid and acceptable to assess the short-term oral toxicity of M-05 in the rat. A NOAEL of 200 ppm (equivalent to 152/167 mg/kg bw/d was established from this study.

M-09 (AE B102859)

The metabolite M-09 has been tested in a battery of *in vitro* genotoxicity tests. M-09 did not induce chromosome aberrations *in vitro* in rat lymphocytes, whilst a recently conducted *in vitro* micronucleus test (MMT) confirmed that M-09 was not clastogenic or aneugenic.

There was no evidence that M-09 was mutagenic *in vitro* in mammalian cells when tested at the HPRT locus in chines hamster ovary cells; however, a slight but reproducible increase in the number of revertant colonies/plate was observed in an Ames test with tester strain TA 1535 in the presence of metabolic activation (+S9) and in the absence of metabolic activation in tester strain WP2*uvr*A. This positive *in vitro* result for mutagenicity in bacterial cells is being followed up *in vivo* with a mammalian alkaline comet assay, which is currently ongoing and will be submitted in November 2020.

With the proviso of the *in vitro* result in bacterial cells not being reflected in the follow up *in vivo* comet assay, then there is no toxicological concern for the metabolite M-09.

Genotoxicity

The letter of access for the following studies M-685650-01-1, M-685979-01-1 and M-685978-01-1 is provided as M-683828-01-1.

Data Point:	KCA 5.8.1/64
Report Author:	[redacted]
Report Year:	2005
Report Title:	Salmonella-Escherichia coli/mammalian-microsome reverse mutation assay preincubation method with a confirmatory assay with pyridinol metabolite of haloxyfop (3-chloro-5-(trifluoromethyl)-2-pyridinol)
Report No:	6736-149
Document No:	M-685650-01-1
Guideline(s) followed in study:	OECD 471 (1997); US-EPA OPPTS 870.5100, (1998); Directive 2000/32fEC, (2000); JMAFF (1988)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

M-09 was tested for mutagenic potential in four histidine-requiring strains (TA 98, TA 100, TA 1535 and TA 1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA) of *Escherichia coli*, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S9), in two independent experiments. Test substance concentrations in the range of 6.67 to 5000 µg/plate were tested in a range finding study using tester strains TA100 and WP2*uvr*A (+/- S9). Based on the results of the range-finding study, the following concentrations were selected for the main mutation assays:

Experiment I: 50, 100, 250, 1000, 2500 & 5000 µg/plate

Experiment II: 50, 100, 250, 500, 1000, 2000, 3000, 4000 & 5000 µg/plate,

Each concentration (plus negative and positive controls) was tested in triplicate.

In the range-finding study, concentration-related decreases were observed at 5000 µg/plate with TA100 (+S9) and from 3300 µg/plate with strain WP2*uvr*A (+/- S9). Reduced background lawns were observed in the presence of S9 from 3300 µg/plate in both strains (+S9). In the absence of S9 reduced bacterial background lawns were observed from 667 µg/plate with WP2*uvr*A and at 5000 µg/plate with TA 100.

In the initial mutation assay, positive increases in the mean number of revertants/plate were observed with tester strain TA 1535 in the presence (4.2-fold) and absence (4.5-fold) of S9 mix. This was confirmed in a confirmatory mutation assay in which positive increases in the mean number of revertants/plate were observed with tester strain TA 1535 in the presence of S-9 mix (4.3-fold) and with tester strain WP2*urv*A in the presence (8.6-fold) and absence (10.8-fold) of S9 mix.

Positive controls gave the expected response, thus confirming the validity of the assay.

It was concluded that M-09 caused a reproducible, positive increase in the mean number of revertant colonies/plate with the tester strain TA1535 in the presence of S9 and with the tester strain WP2*urv*A in the presence and absence of S9. M-09 is mutagenic in bacterial cells under the conditions of this assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-09 (AEB102859), referred to as Pyridinol Metabolite of Haloxyfop (3-Chloro-5-(trifluoromethyl)-2-Pyridinol) in the report
Purity:	Not provided
Batch no.:	AGR218257
Expiry date:	11 May 2008

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	Without S9 mix:
	sodium azide: TA 100, TA 1535
	2-nitrofluorene : TA 98
	ICR-191 TA 1537
	4-nitroquinoline-N-oxide: WP2*urv*A
	With S9 mix:
	2-aminoanthracene : TA 100, TA 1535, TA 1537, WP2*urv*A

3. Activation:

Metabolic activation was provided by S9, prepared from liver homogenate of Aroclor induced male SD rats. The protein concentrations of the S9 preparation was 40, 39.1 and 40.3 mg/mL for lot nos. 1753, 1776 and 1820, respectively.

The S9 mix was prepared immediately prior to use and contained the following components:

0.70 mL H_20

0.10 mL 1M NaH_2PO_4/na_2HPO_4, pH 7.4

0.02 mL 0.25M Glucose-6-phosphate

0.04 mL 0.10M NADP

0.04 mL 0.825M KCl/0.2M $MgCl_2$

0.10 mL S9 Homogenate

4. Test organisms:

In addition to a mutation in either the histidine or tryptophan operons, the tester strains contain additional mutations that enhance their sensitivity to some mutagenic compounds. Mutations of either the *urv*A gene (*Escherichia coli*) or the *uvr*B gene (*Salmonella typhimurium*) results a deficient DNA excision repair system, which greatly enhances the sensitivity of the strain to certain mutagens.

Salmonella typhimurium strains also contain the *rfa* wall mutation which results in the loss of an enzyme responsible for synthesis of the cell wall, resulting in cell wall deficiency and increasing permeability.

Tester strains TA98 and TA100 also contain the pKM101 plasmid, which further increases the sensitivity of these strains.

The mutations of the bacterial strains used in this study are described in the table below.

Table 5.8.1- 126: *Salmonella typhimurium* and *Escherichia coli* strains

Tester strain	*His/trp* Mutation	Additional mutations		Plasmid
		Repair	LPS	
TA98	*his*D3052	*uvr*B	*rfa*	pKM101
TA100	*his*G46	*uvr*B	*rfa*	pKM101
TA1535	*his*G46	*uvr*B	*rfa*	-
TA1537	*his*C3076	*uvr*B	*rfa*	-
WP2*urv*A	*trp*	*urv*A	-	-

Tester strains were checked for confirmation of genotype on the day of use. The integrity of the tester strains was confirmed in order for the test to be considered valid (mutations, spontaneous revertants and culture density).

Salmonella typhimurium strains were obtained directly from Dr. Ames, Berkley and Escherichia coli strains were received from the national collection of industrial bacteria, Scotland.

5. Test substance concentrations used:

Test substance concentrations in the range of 6.67 to 5000 µg/plate were tested in a range finding study using tester strains TA100 and WP2*urv*A (+/- S9).

Based on the results of the range-finding study, the following concentrations were selected for the main mutation assay:

Experiment I: 50, 100, 250, 1000, 2500 & 5000 µg/plate

Experiment II:50, 100, 250, 500, 1000, 2000, 3000, 4000 & 5000 µg/plate

B. Test performance

Experimental phase: March 29 to May 12, 2005

1. Pre-experiment for toxicity

To determine the concentrations for the main study, a range-finding study was conducted with tester strains TA100 and WP2*urv*A in the presence and absence of S9 mix. Ten concentrations of the test article were tested at one plate per concentration and the cytotoxicity, as an increase in the number of revertant colonies/plate and/or thinning of the background lawn, was determined up to 5000 µg/plate.

2. Mutagenicity test

In an initial mutagenicity assay, the test substance was tested in all tester strains both in the absence and presence of S9, along with appropriate positive and vehicle controls. The results of the initial mutagenicity test were confirmed in an independent confirmatory experiment.

The preincubation method was used, in which S9 mix (or phosphate buffer), tester strain and test article are preincubated prior to the addition of molten agar, before being overlaid onto minimal agar. Following incubation, revertant colonies were counted.

The test article, vehicle controls and positive controls were all tested in triplicate.

Plating procedures

The same procedure was used for the all three assays. 500µL of S9 mix (+S9) or 0.1M phosphate buffer (-S9) was added to 13 x 100 mm pre-heated, glass culture tubes. The mixture was vortexed and incubated for 20±2 minutes at 37±1˚C. 2mL of molten selective top agar was then added to each tube and the mixture vortexed and overlaid onto the surface of 25 mL of minimal bottom agar contained in a 15 x 100 mm petri dish. When solidified the plates were inverted and incubated at 52±4 hours at 37±1˚C. Positive control articles were plated using a 50 µL plating aliquot.

Data recording

The background lawn was examined macroscopically and microscopically for signs of cytotoxicity and precipitation. Cytotoxicity was scored relative to the vehicle control plate and recorded with the revertant colony counts for all plates at that concentration.

Revertant colonies were counted automated colony counter or by hand.

For all replicates, mean revertants/plate and standard deviation were calculated.

3. Statistics

Statistical analysis is not required and was not carried out.

4. Acceptance / assessment criteria:

The *Salmonella typhimurium* reverse mutation assay is considered acceptable if it meets the following criteria:

- The integrity of the tester strains has been demonstrated
- The mean value of a positive control for a respective tester strain exhibited at least a 3-fold increase over the mean value of the vehicle control for that strain
- A minimum of one non-toxic concentration was tested and cytotoxicity was demonstrable as a decrease in revertant/colonies or reduction in background lawn (thinning of background lawn alone is not evidence of cytotoxicity)

5. Evaluation of results

For a result to be considered positive, it should produce a 2-fold concentration related increase in the mean revertant colonies/plate over the respective vehicle control for tester strain TA 100 and a 3-fold increase for all other strains.

The response should be reproducible.

II. Results and Discussion

A. Mutation assays

Range finding assay

In the range-finding study, concentration-related decreases were observed at 5000 µg/plate with TA100 (+S9) and from 3300 µg/plate with strain WP2*uvr*A (+/- S9). Reduced background lawns were observed in the presence of S9 from 3300 µg/plate in both strains (+S9). In the absence of S9 reduced bacterial background lawns were observed from 667 µg/plate with WP2*uvr*A and at 5000 µg/plate with TA 100.

Initial mutation assay

All data in the initial mutation assay were acceptable; positive increases in the mean number of revertants/plate were observed with tester strain TA 1535 in the presence (4.2-fold) and absence (4.5-fold) of S9 mix. Increases in the mean number of revertants/plate were also seen with TA 100 in the presence (1.9-fold) and absence (1.7-fold) of S9 mix but did not meet the criteria for a 2-fold increase. No increases were seen in any other strain either in the presence or absence of metabolic activation. Positive controls gave the expected response, thus confirming the validity of the assay.

Confirmatory mutagenicity assay

All data in the confirmatory mutation assay were acceptable; positive increases in the mean number of revertants/plate were observed with tester strain TA 1535 in the presence of S-9 mix (4.3-fold) and with tester strain WP2*uvr*A in the presence (8.6-fold) and absence (10.8-fold) of S9 mix. In addition, increases were observed with TA 1535 in the absence of S9 mix (2.5-fold) but without meeting the criteria for a 3-fold increase for this strain. No further increases were seen in any other strain in the absence or presence of S9 mix. Positive controls gave the expected response, thus confirming the validity of the assay.

The results of the initial and confirmatory mutation assays are summarised in the tables below:

Table 5.8.1- 127: Summary of the initial mutation experiment

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ± SD)				
		TA 98	TA 100	TA 1535	TA 1537	WP2*uvr*A
With metabolic activation (+S9)						
M-09	50	32±3	126±8	21±2	10±2	27±4
	100	27±4	131±5	22±6	10±3	43±11
	250	28±2	143±8	27±6	7±2	60±10
	500	22±4	156±6	33±4	13±2	118±10
	1000	30±10	185±7	20±5	9±2	117±12
	2500	19±1	221±17	75±5	7±1	150±4
	5000	2±3	19±30	41±41	1±1	0±1
Vehicle control	-	27±10	117±4	18±1	14±2	20±7
Positive control*	-	497±3	1485±142	182±6	249±7	422±10
Historical solvent control mean	-	23.9±8.4	91.7±15.6	13.2±3.7	9.3±3.9	21.8±5.5
Historical control range	-	11-53	62-132	5-21	2-13	15-32
Without metabolic activation (-S9)						
M-09	50	14±5	101±15	19±5	6±3	24±10
	100	19±6	109±5	17±9	6±1	31±5
	250	17±3	117±4	21±2	7±2	46±9
	500	31±29	119±28	27±1	5±2	67±14
	1000	12±2	151±6	47±19	5±5	85±9
	2500	13±9	179±17	63±6	6±0	138±17
	5000	0±0	2±4	0±1	0±0	0±0
Vehicle control	-	21±5	103±8	14±3	10±3	13±2
Positive control**	-	2403±96	1072±70	838±22	3984±171	575±38
Historical solvent control mean	-	16.7±5	90.4±41.6	14.3±6.5	7.4±3.2	16±3.7
Historical control range	-	7-26	49-264	7-33	3-14	10-25

*+S9: benzo[a]pyrene TA 98 (2.5 µg/plate), 2-aminoanthracene TA 100, TA 1535 & TA 1537 (2.5 µg/plate) & WP2*uvr*A (25 µg/plate)

**-S9: 2-nitrofluorene TA 98 (10 µg/plate), sodium azide TA 100 & TA 1535 (2 µg/plate), ICR-191 TA1537 (2 µg/plate) & 4-nitroquinoline-N-oxide WP2*uvr*A (0.4 µg/plate)

Table 5.8.1- 128: Summary of the confirmatory mutation experiment

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ± SD)				
		TA 98	TA 100	TA 1535	TA 1537	WP2*urv*A
With metabolic activation (+S9)						
M-09	50	26±7	109±7	14±4	7±2	26±5
	100	22±3	112±2	18±0	8±2	31±1
	250	20±4	108±7	23±6	8±5	39±10
	500	19±1	121±9	23±3	7±1	98±13
	1000	17±2	149±15	38±8	12±5	128±28
	2000	19±4	172±13	47±5	3±3	147±17
	3000	11±1	143±48	29±7	4±3	119±27
	4000	3±1	13±11	21±14	2±1	11±3
	5000	5±2	6±6	18±7	1±1	10±6
Vehicle control	-	23±4	101±18	11±4	8±2	17±1
Positive control*	-	377±56	1326±42	916±73	196±3	718±190
Historical solvent control mean	-	23.9±8.4	91.7±15.1	13.2±3.5	9.3±3.9	21.8±5.5
Historical control range	-	11-53	62-132	5-21	2-17	15-32
Without metabolic activation (-S9)						
M-09	50	17±8	92±5	13±2	3±2	21±3
	100	15±1	91±18	17±2	3±2	35±13
	250	18±2	113±8	17±2	6±1	43±20
	500	16±1	112±3	26±7	7±3	110±11
	1000	15±1	130±21	39±3	5±2	116±12
	2000	9±1	170±20	45±4	5±6	141±7
	3000	8±1	101±48	27±7	3±1	107±10
	4000	6±1	6±11	18±2	0±0	5±1
	5000	0±0	5±9	2±3	0±0	1±1
Vehicle control	-	12±0	87±13	18±1	6±1	13±4
Positive control**	-	335±76	1158±83	916±73	3821±170	718±190
Historical solvent control mean	-	16.7±5	90.4±41.6	14.3±6.5	7.4±3.2	16±3.7
Historical control range	-	7-26	49-264	7-33	3-14	10-25

*+S9: benzo[a]pyrene TA 98 (2.5 µg/plate), 2-aminoanthracene TA 100, TA 1535 & TA 1537 (2.5 µg/plate) & WP2*urv*A (25 µg/plate)

**-S9: 2-nitrofluorene TA 98 (10 µg/plate), sodium azide TA 100 & TA 1535 (2 µg/plate), ICR-191 TA1537 (2 µg/plate) & 4-nitroquinoline-N-oxide WP2*urv*A (0.4 µg/plate)

III. Conclusion

M-09 caused a reproducible, positive increase in the mean number of revertant colonies/plate with the tester strain TA1535 in the presence of S9 and with the tester strain WP2*wvr*A in the presence and absence of S9. M-09 is mutagenic in bacterial cells under the conditions of this assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of M-09 in *S-typhimurium* and *E. Coli*. Fluopicolide is mutagenic *in vitro* in bacterial cells under the conditions of this study when tested up to precipitating concentrations (+/- S9).

Data Point:	KCA 5.8.1/65
Report Author:	Seidel, S. D.; Schisler, M.R.; Klemert, K.M.
Report Year:	2005
Report Title:	Evaluation of pyridinol metabolite of haloxyfop (3-chloro-5-(trifluoromethyl)-2-pyridinol) in the Chinese hamster ovary cell hypoxanthine-guanine-phosphoribosyl transferase (CHO/HGPRT) forward mutation assay
Report No:	051060
Document No:	M-685979-01-1
Guideline(s) followed in study:	US-EPA 712- C-98-221, OPPTS 870.5300(1998); OECD, 476 (1997); EC, B.17 (2000)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The study was performed to investigate the potential of M-09 to induce gene mutations at the HPRT locus in ovary cells of the Chinese hamster *in vitro*.

The assay was performed in two experiments with and two without rat liver microsomal activation (S9-mix). The test article was dissolved in DMSO and tested at the concentrations ranging from 125 to 2000 µg/mL.

The concentration ranges were based on the results of preliminary testing for solubility and toxicity.

No relevant increase in mutant colony numbers was obtained in any experiment.

The positive controls showed distinct statistically significant increases in induced mutant colonies, thus indicating the sensitivity of the assay and the negative controls gave the expected results.

It was concluded that M-09 was not mutagenic in this HPRT test with Chinese hamster ovary cells.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-09 (AEB102859), referred to as Pyridinol Metabolite of Haloxyfop (3-Chloro-5-(trifluoromethyl)-2-Pyridinol) in the report
Purity:	99.9%
Batch no.:	ACPR 1-95

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: Ethyl methanesulphonate (EMS), 621 µg/mL +S9: 20-methylcholanthrene (20-MCA), 4µg/mL

3. Activation:

Metabolic activation was provided by S9, prepared from liver homogenate of Aroclor induced male SD rats.

The S9 mix was prepared immediately prior to use and contained the following components:

10 mM $MgCl_26H_2O$

4 mM nicotinamide adenine dinucleotide phosphate

10 mM $CaCl_2$

30 mM KCl

50 mM sodium phosphate (pH 8.0)

The reconstituted mix was added to the culture medium to obtain the desired final concentration of S9 in the culture (2%). Hence the final concentration of the co-factors was 1/5 of those above.

4. Cell cultures and media:

Cell cultures

Cell stock cultures of the cell line CHO-K1-BH_4 were stored at <80˚C and periodically checked for mycoplasma contamination. The cells were grown as monolayer cultures under standard conditions (5% CO^2 in 37˚C humidified incubator).

Media

The cells were routinely maintained in Ham's F-12 nutrient mix, supplemented with 5% (v/v) heat inactivated foetal calf serum, antibiotics and antimycotics and an additional 2 mM L-glutamine. The selection medium used for the detection of $HGPRT^-$ mutants was Ham's F-12 nutrient mix without hypoxanthine, supplemented with antibiotics, 10 µM 6-thioguanine and 5% serum.

5. Test substance concentrations used:

The following concentrations of test substance were tested in the presence and absence of S9:

Preliminary assay (A1): 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 & 2000 µg/mL

Initial mutation assay (B1): 125, 250, 500, 1000, 1500 & 2000 µg/mL

Confirmatory mutation assay (C1): 125, 250, 500, 1000 & 2000 µg/mL

B. Test performance

Experimental phase: Not provided

1. Preliminary assay

A cytotoxicity assay was conducted to aid in the selection of concentrations for the main mutation assay; cytotoxicity was determined from the ability of the treated cells to form colonies.

Cells were seeded into T-25 flasks (10×10^6 cells/flask) approximately 24-hours prior to treatment. Treatment was for 4-hours and included one replicate/dose in the absence and presence of S9 mix. Following termination of treatment, the cells were trypsinised and replated at a density of 200 cells/dish into 60 mm dishes (3/dose). The dishes were incubated for 6-10 days to allow colony formation and the colonies were then fixed and stained. The number of colonies per dish were counted, and the mean colonies/treatment expressed relative to the solvent control value. The highest concentration was that which did not increase the osmolality of the medium and the lowest concentration should give survival comparable to the solvent control. The test substance was freely soluble and was therefore tested up to the limit equivalent to 10 mM.

2. Main mutation assays

Each dose level was tested in duplicate. The number of cells treated at each dose level was adjusted to yield at least 1×10^8 surviving cells. The cells were trypsinised at the end of the treatment and replated at a density of 1×10^6 /100 mm dish (at least 2 dishes/replicate) for phenotypic expression. In addition, 200 cells/60mm dish (3 dishes/replicate) were also plated to determine the toxicity and incubated for approximately 6-8 days to permit colony formation. During the phenotypic expression period (7-9 days), cells in the larger petri dishes were sub-cultured every 2-3 days and plated (at least 2 dishes/replicate) at a density of approximately 1×10^6 cells/10 mm petri dish. At each subculture, cells from various dishes within each replicate were pooled prior to replating.

At the end of the expression period, the cultures were trypsinised and plated at a density of 2×10^5 cells/100 mm dish (a total of 10 dishes/treatment) in the selection media for the determination of HGPRT mutants and 200 cells/60 mm dish (3 dishes/treatment) in Ham's F-12 medium without hypoxanthine for determination of cloning efficiency. Treatments resulting in less than approximately 10% relative cell survival (in concurrent toxicity assays) were not used for determining cloning efficiency or mutation frequencies. The dishes were incubated for about 6-10 days and the colonies were fixed and stained. The mutant frequency expressed as mutants per 10^6 cloneable cells for each replica was calculated.

3. Statistical analysis

The frequencies of mutants per 10^6 cloneable cells was evaluated using a weighted analysis of variance; weights were derived from the inverse of the mutation frequency variance. The actual plate counts are assumed follow a Poisson distribution; therefore, the mean plate count was used as an estimate of variance.

If the analysis of variance was significant at alpha=0.05, a Dunnett's t-test was conducted, comparing each treated group and the positive control to the negative control (alpha=0.05, one sided). An additionals comparison of the positive control to the negative control (alpha=0.05) was conducted using a linear contrast statement. Linear dose-related trend tests were performed if any of the pairwise comparisons of test material with the negative control yielded significant differences.

II. Results and Discussion

There was no appreciable change in the pH and osmolality of the treatment medium containing approximately 2010 µg/mL of the test material, when compared with medium containing solvent alone (1% DMSO).

A. Preliminary cytotoxicity assay (Assay A1)

A preliminary toxicity assay was conducted at concentrations of 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 & 2000 µg/mL in the presence and absence of S9.

In the absence of S9 little or no toxicity was observed, the relative cell survival (RCS) values ranged from 67.6 to 124.7%. In the presence of S9, moderate toxicity was observed, RCS values ranged from 22.1 to 119%.

Therefore, concentrations of 125, 250, 500, 1000, 1500 and 2000 µg/mL were selected for the initial gene mutation assay (+/- S9).

B. Mutation assays

Initial mutation assay (Assay B1)

In the absence of S9, the relative cell survival (RCS) ranged from 84.8 to 102%. In the presence of S9, the RCS values ranged from 64.7 to 114%.

The mutant frequencies observed in the cultures treated with the test material in the presence and absence of S9 were not statistically different from the concurrent solvent control values; furthermore, all mutant frequencies were within the range of the historical control data.

Confirmatory mutation assay

In the absence of S9 no toxicity was observed, RCS values ranged from 80.2 to 131.5%. In the presence of S9, moderate toxicity was observed at the highest concentration (2000 µg/mL) with an average value of 59.6%. the remaining cultures had RCS values ranging from 96.5 to 125.9%.

The mutant frequencies observed in the cultures treated with the test material in the presence and absence of S9 were not statistically different from the concurrent solvent control values; furthermore, all mutant frequencies were within the range of the historical control data.

In both mutation assays, the positive controls induced significant increases in mutant frequencies, this confirming the sensitivity of the assay. The results from both experiments with and without S9 are summarised in the tables below:

Table 5.8.1- 129: Relative survival and mutant frequencies in the initial mutation assay (B1) with and without metabolic activation

Treatment	Concentration (µg/ml)	Relative cell survival[a]	Mutation frequency
Without metabolic activation (-S9)			
M-09	125	91.2	9.0
	125	97.3	1.5
	250	89.6	10.2
	250	94.1	5.2
	500	86.1	6.7
	500	92.9	14.0
	1000	84.8	5.4
	1000	85.0	4.7
	1500	86.4	4.5
	1500	91.4	1.1
	2000[b]	93.4	12.3
	2000[b]	102.0	8.5
Negative control	-	95.2	15.0
	-	104.8	3.9
Positive control		32.4	296.1[c]
		28.0	375.0[c]
With metabolic activation (+S9)			
M-09	125	79.2	9.6
	125	77.3	8.8
	250	100.1	19.1
	250	114.0	11.0
	500	100.5	6.8
	500	87.1	9.9
	1000	77.1	9.8
	1000	91.8	6.7
	1500	73.0	6.9
	1500	76.4	13.2
	2000[b]	69.6	11.3
	2000[b]	64.7	9.4
Negative control	-	102.7	1.2
	-	97.3	5.4
Positive control		59.6	245.1[c]
		69.6	270.7[c]

[a]RCS (%) = mean no. colonies/dish in treated/100/mean no. colonies/dish in negative control

[b]Precipitation in the medium

[c]Statistically significant from controls (alpha=0.05)

Table 5.8.1- 130: Relative survival and mutant frequencies in the initial mutation assay (C1) with and without metabolic activation

Treatment	Concentration (µg/ml)	Relative cell survival[a]	Mutation frequency
Without metabolic activation (-S9)			
M-09	125	96.3	1.8
	125	96.3	21.0
	250	131.5	10.8
	250	103.9	5.2
	500	95.6	9.8
	500	92.8	11.1
	1000	116.9	13.8
	1000	102.1	12.6
	2000[b]	84.7	5.5
	2000[b]	80.2	17.1
Negative control	-	93.6	3.2
	-	106.4	26.3
Positive control		33.8	345.2[c]
		27.2	391.2[c]
With metabolic activation (+S9)			
M-09	125	125.9	3.2
	125	98.4	5.0
	250	100.9	6.5
	250	114.0	6.8
	500	97.2	10.4
	500	96.5	8.7
	1000	105.2	6.4
	1000	105.7	6.1
	2000[b]	66.7	14.5
	2000[b]	52.4	8.9
Negative control	-	103.0	9.2
		97.0	6.6
Positive control		70.4	358.4[c]
		56.3	420.5[c]

[a]RCS (%) = mean no. colonies/dish in treated x 100/mean no. colonies/dish in negative control

[b]Precipitation in the medium

[c]Statistically significant from controls (alpha=0.05)

III. Conclusion

M-09 did not induce gene mutations in mammalian cells (Chinese hamster ovary cells) either in the presence or absence of metabolic activation provided by S9 mix. Appropriate responses exhibited by the concurrent negative, vehicle and positive controls confirmed the validity of the assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD 476 and is valid and acceptable to assess the potential of M-09 to induce gene mutations at the HPRT locus in Chines Hamster Ovary cells. M-09 is not mutagenic in mammalian cells under the conditions of this assay.

Data Point:	KCA 5.8.1/66
Report Author:	[redacted]
Report Year:	2005
Report Title:	Evaluation of pyridinol metabolite of haloxyfop (3-chloro-5-(trifluoromethyl)-2-pyridinol) in an in vitro chromosomal abberation assay utilizing rat lymphocytes
Report No:	051059
Document No:	M-685978-01-1
Guideline(s) followed in study:	US-EPA OPPTS 870.5375 (1998); OECD 473 (1997); EC, B.10 (2000); JMAFF, Mutagenicity Guidelines (2001)
Deviations from current test guideline:	Cytotoxicity was not evaluated by the parameters of relative population doubling (RPD) or relative increase in cell count (RICC). Only 100 metaphases were analysed, rather than a minimum of 300 as recommended in the current guidance.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

M-09 was evaluated in an *in vitro* chromosome aberration assay in rat lymphocytes. Cells were treated in the absence or presence of S9 activation with concentrations of the test substance ranging from 0 to 2000 µg/mL, including solvent and positive controls. The duration of treatment was 24-hours (-S9) and 4 hours (+/-S9). Based upon mitotic indices, cultures treated with 0, 250, 500 & 2000 µg/mL (4h, -S9), 0, 250, 500 & 100 µg/ml (4h, +S9) and 0.62.5, 125 & 250 µg/mL (24h, -S9) were selected for determining the frequency of chromosomal aberrations.

A statistically significant increase in the percentage of aberrant cells was observed in cultures treated for 4 hours in the absence of S9 (~6.5% at 2000 µg/ml compared with 1% in controls). There were no significant increases in aberrant cell frequencies at the remaining concentrations either in the absence or presence of S9 following 4- or 24-hours' exposure. A confirmatory assay to examine the reproducibility of the single increase observed at the only precipitative concentration. No significant increases were seen in the confirmatory assay.

There was no evidence of polyploidy and the positive controls gave the expected responses, thus confirming the sensitivity of the assay.

It was concluded that M-09 is not clastogenic under the conditions if this assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-09 (AE B102859), referred to as Pyridinol Metabolite of Haloxyfop (3-Chloro-5-(trifluoromethyl)-2-Pyridinol) in the report
Purity:	99.9%
Batch no.:	ACPR 1-95

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: Mitomycin C (MMCO); 0.5 µg/mL (4h treatment) or 0.95 & 0.075 µg/mL (24h treatment) +S9: Cyclophosphamide monohydrate (CP); 4 & 6 µg/mL (4h treatment)

3. Activation:

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male SD rats inducted with Aroclor 1254 (obtained from Molecular Toxicology Inc., USA). The batches of S9 were stored frozen at -100˚C and thawed prior to use.

Preparation of S9-mix:

Thawed S9 was reconstituted at a final concentration of 10% (v/v) to form the S9 mix containing the following:

10 mM $MgCl_2 6H_2O$

4 mM nicotinamide adenine dinucleotide phosphate

10 mM $CaCl_2$

5 mM glucose-6-phosphate

The reconstituted mix was added to the culture medium to obtain the desires final concentration of the S9 in the culture. Hence the final concentration of the co-factors in the culture medium was 1/5 of the above concentrations.

4. Cell cultures and medium:

Lymphocytes were obtained from male SD rats aged 11-13 weeks at the start of the study. The rats were sacrificed by CO_2 and the blood collected by cardiac puncture. The blood samples from individual rats were pooled and whole blood cultures set up in RPMI 1640 medium (with 25 mM HEPES) supplemented with 10% heat-activated foetal bovine serum, antibiotics and antimycotics, 30 µg/mL PHA and an additional 2mM L-glutamine. Cultures were initiated by inoculating approximately 0.5 mL of whole blood/5mL of culture medium.

Cultures were set up in duplicate at each dose level in T-25 plastic tissue culture flasks and incubated at 37˚C.

5. Test substance concentrations used:

The following concentrations were used:

Experiment 1 (assay A1), 4-hour exposure: 0, 31.3, 62.5, 125, 250, 500, 1000 & 2000 µg/mL in the presence and absence of S9

Experiment 1 (assay A1), 24-hour exposure: 0, 15.6, 31.3, 62.5, 125, 250, 500, 1000 & 2000 µg/mL in the absence of S9

Experiment 2 (assay B1), 4-hour exposure: 0, 125, 250, 500, 1000 & 2000 µg/mL in the presence of S9 (repeat of the 4-hour exposure owing to the failure of the positive control, CP, in experiment A1)

Experiment 3 (assay C1), 4-hour exposure: 0, 125, 250, 500, 750, 1000, 1500 & 2000 µg/mL

B. Test performance

Experimental phase: Not provided

1. Treatment procedure without metabolic activation

Short treatment

48-hours after culture initiation, the cell suspension was dispensed into 15 mL centrifuge tubes (5.5 mL/tube, 2 cultures/dose). The cells were sedimented by centrifugation and the culture medium was removed and saved. The cells were exposed to medium containing the test substance, positive or solvent controls for 4 hours at 37˚C and the exposure terminated by washing the cells with culture medium. The cells were then placed in individual tissue culture flasks along with 4.5 mL of the original culture medium until harvest. The cultures were harvested 24 hours after treatment initiation (i.e. 20 hours after treatment termination).

Continuous treatment

Cultures were treated for 1.5 times the normal cell cycle length. The solvent, positive control, or test material were added directly to the culture flasks 48-hours after culture initiation and the cultures were harvested 24-hours later.

2. Treatment procedure with metabolic activation

Only the short treatment was used in the presence of S9; 48-hours after culture initiation, the cell suspension was dispensed into 15 ml centrifuge tubes (5.5 mL/tube, 2 cultures/dose). The cells were sedimented by centrifugation and the culture medium was removed and saved. The cells were exposed to medium (with the addition of S9 mix) containing the test substance, positive or solvent controls for 4 hours at 37˚C and the exposure terminated by washing the cells with culture medium. The cells were then placed in individual tissue culture flasks along with 4.5 mL of the original culture medium until harvest. The cultures were harvested 24 hours after treatment initiation (i.e. 20 hours after treatment termination).

3. Toxicity and chromosomal aberration assay

Colcemid was added to the system 2-3 hours prior to cell harvest. The cells were swollen by hypotonic treatment, fixed, dropped onto slides and stained. Mitotic indices were determined as the number of cells in metaphase among 1000 cells/replicate and expressed as percentage.

One hundred metaphases/replicate were examined. (reduced to 50 metaphases/replicate when high numbers of aberrations were observed); 100 metaphases/replicate were examined for polyploidy.

4. Assessment of results

An assay is considered to be acceptable if the following criteria are met:

- The chromosomal aberration frequency in the positive control cultures is significantly higher than the solvent controls
- The aberration frequency in the solvent control is with the historical control data

The test substance is considered to cause a positive response if the following conditions are met:

- A significant increase in the frequency of cells with aberrations is seen
- The increase is dose-related
- The increase is reproducible

5. Statistics

The proportion of cells with aberrations (excluding gaps) were compared by the following statistical methods:

Data from each replicate in a dose level were pooled. A two-way contingency table was constructed to analyse the frequencies of aberrant cells. An overall Chi-squared test based on the table was partitioned into components of interest. Statistics were generated to test the global hypotheses:

- No difference in the average number of cells with aberrations among the dose groups
- No linear trend of increasing number of cells with aberrations with increasing dose

An ordinal metric was used for the doses in the statistical evaluation. If either statistic was found to be significant at alpha = 0.05) versus a one-sided increasing alternative, pairwise tests were performed at each dose level again versus a one-sided alternative.

Polyploid cells were analysed by the fisher's exact test. The number of polyploid cells were pooled across replicates for the analysis and evaluated at alpha = 0.05. the data was analysed separately based on the presence or absence of 89.

II. Results and Discussion

There were no significant increases in the incidence of polyploid cells in any treated cultures, compared with the solvent control values. The pH and osmolality of the treatment media containing 2010 µg/mL (10mM limit) or 1% DMSO were not affected.

1. Experiment 1 (assay A1)

An initial experiment was performed with a 4-hour exposure period at concentrations of 0 (solvent control), 31.3, 62.5, 125, 250, 1000 and 2000 µg/mL, n the presence and absence of metabolic activation (S9). Additional cultures were treated continuously for 24 hours in the absence of S9, at the same concentrations with an additional lower concentration of 15.6 µg/mL.

Toxicity

In the absence of S9 (4h), moderate to no toxicity was observed; the relative mitotic indices ranged from 32 to 104%. Therefore, concentrations of 250, 500 and 2000 µg/mL were selected for determining chromosomal aberration frequencies.

Similarly, in the presence of S9 (4h), the relative mitotic indices ranged from 55.9 to 105.9%; however, examination of the slides revealed that the positive control chemical (CP) had not induced the expected in chromosomal damage at any concentration. Hence this part of the assay (4h treatment, -S9) was repeated as assay B1 (see below).

In the absence of S9 (24h), excessive toxicity was seen at the top three concentrations, whilst the lower concentrations had relative mitotic indices of 28 to 100%. Therefore, cultures treated with 62.5, 125 and 250 µg/mL were selected for the determination of chromosomal aberration frequencies.

MMC at concentrations of 0.5 µg/mL (4h) and 0.05 µg/mL (20h) was selected as the positive control for evaluation in the absence of S9.

Chromosomal aberrations

In the absence of S9 (4h) the frequency of cells with aberrations were 1, 1, 1.5 and 6.5% at 0 (solvent control), 250, 500 and 2000 µg/mL respectively; statistical analysis revealed a statistical difference between the control and the high concentration. A confirmatory assay (C1) was performed to verify these results.

In the absence of S9 (24h) the frequency of cells with aberrations were 1.5, 1, 1.5 and 2% at 0 (solvent control), 62.5, 125 and 250 µg/mL; no statistical differences were identified, and all values were within the range of the historical control data.

Significant increases in the frequency of cells with aberrations were observed in cultures treated with the positive control; aberrant cell frequencies in MMC cultures were 25.1% and 31% after 4- and 24-hours' exposure, respectively.

2. Experiment 2 (assay B1)

A repeat assay was conducted with a 4-hour exposure period in the presence of S9 (owing to the lack of response of the positive control in the previous assay. In the repeat assay, cultures were treated with 0 (solvent control), 125, 250, 500, 1000 and 2000 µg/mL of test material.

Toxicity

Excessive toxicity was seen at the top concentration of 2000 µg/mL, evidenced by the absence of mitotic figures, and precipitation was seen at this concentration only. The relative mitotic indices of the remaining cultures ranged from 44.5 to 85.5%. Therefore, concentrations treated with 250, 500 and 1000 µg/mL were selected for determining the chromosomal aberration frequencies.

The positive control, CP, at 4 µg/mL was selected for the evaluation of aberrations.

Chromosomal aberrations

In the absence of S9 (4h), aberrant cell frequencies were 3, 1.5 and 4% at 0 (solvent control), 250, 500 and 1000 µg/mL respectively; statistical analyses did not reveal a significant difference between the solvent control and the treated cultures and all values were within the range of the historical control data.

Significant increases in the frequency of cells with aberrations were observed in cultures treated with the positive control; the aberrant cell frequency in CP treated cultures was 26.4%.

3. Experiment 3 (assay C1)

A confirmatory assay was conducted with a 4-hour exposure period at concentrations of 0 (solvent control), 125, 250, 500, 750, 1000, 1500 and 2000 µg/mL, both in the presence and absence of S9.

Toxicity

Excessive toxicity was seen at the top concentration of 2000 µg/mL (+/- S9), evidenced by the absence of mitotic figures. The relative mitotic indices of the remaining cultures ranged from 27.5 to 68.6% in the absence of S9 and from 44.8 to 89.7% in the presence of S9. Therefore, concentrations treated with 0, 250, 500, 750, 1000 and 1500 µg/mL in the absence of S9 were selected for determining the chromosomal aberration frequencies.

The positive control, MMC, at 0.5 µg/mL was selected for the evaluation of aberrations for the 4-hour assay in the absence of S9.

Chromosomal aberrations

In the absence of S9 (4h), aberrant cell frequencies were 1.5, 1, 2, 1, 3 and 2.5% at 0 (solvent control), 250, 500, 750, 1000 and 1500 µg/mL respectively; statistical analyses did not reveal a significant difference between the solvent control and the treated cultures and all values were within the range of the historical control data.

Significant increases in the frequency of cells with aberrations were observed in cultures treated with the positive control; the aberrant cell frequency in MMC treated cultures was 49%.

The relative mitotic indices and the % aberrant cells in all three experiments ae summarised in the tables below.

Table 5.8.1- 131: Relative mitotic indices and mean percentage of aberrant cells in experiment 1 (A1)

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index[a]	Percent aberrant cells Excluding gaps	HCD (range)**
Without metabolic activation (-S9)					
M-09	31.3	4	96.8	-	
	62.5	4	98.4		
	125	4	104	-	
	250	4	99.2	1	
	500	4	91.2	1.5	
	1000	4	32	-	
	2000[b]	4	37.6	6.5*	
Solvent control	-	4	100	1	0-6
MMC	0.5	4	68.8	25.1*	
Without metabolic activation (-S9)					
M-09	15.6	24	99.2	-	
	31.3	24	100	-	
	62.5	24	91.2	1	
	125	24	87.2	1.5	
	250	24	28	2	
	500	24	0		
	1000	24	0	-	
	2000[b]	24	0	-	
Solvent control		24	100	1.5	0-6
MMC	0.05	24	65.6	31*	
MMC	0.075	24	68	-	
With metabolic activation (+S9)					
M-09	31.3	4	102.5	-	
	62.2	4	103.4	-	
	125	4	105.9	-	
	250	4	104.2	-	
	500	4	95.8	-	
	1000	4	88.1	-	
	2000[b]	4	55.9	-	
Untreated control		4		-	0-4
CP	4	4	105.9	-	
CP	6	4	84.7	-	

[a]RMI = Average of treated x100/Average of solvent control, [b]recipitation in medium, *statistically different from control ($p<0.05$), **average aberration frequencies from 2000-2005 (15/15, 11/11, 9/8, 14/10, 14/7 & 15/7 studies respectively +/-S9)

Table 5.8.1- 132: Relative mitotic indices and mean percentage of aberrant cells in experiment 2 (B1)

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index[a]	Percent aberrant cells Excluding gaps	HCD (range)**
With metabolic activation (+S9)					
M-09	125	4	85.5	-	
	250	4	82.7	1	
	500	4	70	3	
	1000	4	44.5	4	
	2000[b]	4	0	-	
Solvent control	-	4	100	3	0-4
CP	4	4	47.3	26.4*	
CP	6	4	27.3	-	

[a]RMI = Average of treated x100/Average of solvent control, [P]recipitation in medium, *statistically different from control (p<0.05), **average aberration frequencies from 2000-2005 (15/15, 11/11, 9/8, 14/10, 14/7 & 15/7 studies respectively +/-S9)

Table 5.8.1- 133: Relative mitotic indices and mean percentage of aberrant cells in experiment 3 (C1)

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index[a]	Percent aberrant cells Excluding gaps	HCD (range)**
Without metabolic activation (-S9)					
M-09	125	4	68.6	-	
	250	4	51	1	
	500	4	43.1	2	
	750	4	35.3	1	
	1000	4	27.5	3	
	1500	4	29.4	2.5	
	2000[b]	4	0	-	
Solvent control	-	4	100	1.5	0-6
MMC	0.5	4	19.6	49*	
With metabolic activation (+S9)					
M-09	125	4	89.7	-	
	250	4	58.6	-	
	500	4	62.1	-	
	750	4	44.8	-	
	1000	4	58.6	-	
	1500	4	82.8	-	
	2000[b]	4	0	--	
Solvent control	-	4	100	-	0-4
CP	4	4	31	-	
CP	6	4	27.6	-	

[a]RMI = Average of treated x100/Average of solvent control, [P]recipitation in medium, *statistically different from control (p<0.05), **average aberration frequencies from 2000-2005 (15/15, 11/11, 9/8, 14/10, 14/7 & 15/7 studies respectively +/-S9)

III. Conclusion

M-09 induced structural aberrations in rat lymphocytes *in vitro* following 4-hours' exposure at 2000µg/mL in the absence of metabolic activation (S9). A similar effect was not apparent after 4-hours exposure in the presence of S9 or after 24 hours' exposure in the absence of S9. A confirmatory assay at 4h (-S9) did not produce a reproducible result. Therefore, M-09 is not clastogenic under the conditions of this *in vitro* assay in rat lymphocytes. There was no evidence of polyploidy.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 473 and is valid and acceptable to assess the potential of M-09 to induce chromosome aberrations in rat lymphocytes *in vitro*. M-09 is not clastogenic under the conditions of this assay either in the presence or absence of S9 and there was no evidence of polyploidy.

Data Point:	KCA 5.8.1/67
Report Author:	[redacted]
Report Year:	2020
Report Title:	AE B102859: Micronucleus test in human lymphocytes in vitro
Report No:	1969604
Document No:	M-677035-01-1
Guideline(s) followed in study:	OECD 487 (2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

M-09 (referred to as AE B102859 in the report) was evaluated for its potential to induce micronuclei in human lymphocytes *in vitro*, in two independent experiments; Experiment I comprised a four hour exposure period in the presence and absence of metabolic activation (provided by S9) whilst experiment II provided a 20-hour exposure period in the absence of S9. Concentrations up to 1975 µg/mL were tested in duplicate and 1000 binucleated cells/culture were examined for cytogenetic damage. Experiment II was repeated owing to cytotoxicity and the repeat experiment was designated experiment IIb.

In Experiment I in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration, which showed precipitation. In Experiment IIB in the absence of S9 mix after continuous treatment moderate cytotoxicity was observed at the highest evaluated concentration. The next higher tested concentration, however, which was separated by a factor smaller than requested by the guideline could not be evaluated for cytogenetic damage due to strong cytotoxicity.

In this study, neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells was observed after treatment with the test item.

Appropriate reference substances gave the expected statistically significant increase in the number of micronucleated cells.

The test substance M-09 can be considered neither clastogenic nor aneugenic under the conditions of this *in vitro* micronucleus test, when tested up to the highest possible concentration, both in the presence and absence of metabolic activation.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-09 (referred to as AE B102859 in the report)
Purity:	99.2 % (w/w)
Batch no.:	1356440
Expiry date:	29th February 2020

2. Vehicle and/or positive control

Vehicle:	0.5% DMSO
Positive controls:	-S9
	Mitomycin C (MMC), 0.8 µg/mL (98% purity, dissolved in deionized water)
	Demecolcine, 50 ng/mL (purity ≥98%, dissolved in deionized water)
	+S9
	Cyclophosphamide (CPA), 17.5 µg/ml (purity 97-103%, dissolved in saline)

3. Activation:

Metabolic activation was provided by S9-mix derived from phenobarbital/ß-naphthoflavone induced rat liver homogenate; each batch of prepared S9 has been routinely evaluated for its ability to activate known mutagens.

An appropriate quantity of S9 was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained MgCl2 (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation used for this study was 30.9 mg/mL.

4. Cell cultures and media:

Cells

Blood was drawn from healthy non-smoking donors, not receiving medication. Blood from a female donor (29 years old) and a female donor (34 years old) were used in experiments I and II, respectively. The lymphocytes have been shown to respond well to PHA and positive control substances; furthermore, both donors have an established low incidence of micronuclei in peripheral blood lymphocytes.

Media

Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hours following collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1), supplemented with 200 mM GlutaMAX™. The medium was further supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (foetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).

All incubations were carried out at 37 °C with 5.5 % CO2 in humidified air.

5. Test compound concentrations used:

Test concentrations for the first experiment (4-hour pulse treatment) were 0 (solvent control), 15, 26.2, 45.8, 80.2, 140, 246, 430, 752, 1317 & 1975 µg/mL, both with and without S9 mix.

In the second experiment a continuous (20 hour) treatment was used at test concentrations of 0 (solvent control), 140, 246, 430, 752, 1317 & 1975 µg/mL, in the absence of S9 mix.

B. Test Performance

Experimental phase: 21st August 2019 to 20th November 2019

1. Preliminary assay

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity (characterized by the percentages of reduction in the CBPI in comparison to the controls and expressed as % cytostasis) was determined from 500 cells/culture. The experimental conditions in this pre-experimental phase were identical to those described for the mutagenicity assay.

The pre-test was performed with 10 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hours (with and without S9 mix). The preparation interval was 40 hours after start of the exposure.

This preliminary test was designated Experiment I since the cultures fulfilled the criteria for cytogenetic evaluation and appropriate concentrations could be selected for evaluation.

No cytotoxic effects were observed in Experiment I after 4 hours treatment in the absence and presence of S9 mix. Therefore, 1975 µg/mL were chosen as top treatment concentration for Experiment IIA. Due to the steep increase in cytotoxicity no evaluable concentrations in a cytotoxic range were available. Therefore, the experiment was chosen to be repeated with a modified concentration range with a top concentration of 800 µg/mL (Exp. IIB).
The cytogenetic evaluation of concentrations in Experiment IIB without S9 mix higher than indicated in Table 1 was impossible due to strong test item-induced toxic effects (low cell numbers).

2. Cytogenetic experiment

Cells were subject to either 4 hours pulse exposure (with and without S9; experiment I) or 20 hours continuous exposure (without S9; experiment IIa or IIb). The succeeding procedure for cell preparation was the same for both exposure periods.

Pulse exposure

Approximately 48 hours following seeding, two blood cultures of 10 mL each, were set up in parallel in 25 cm² cell culture flasks (for each test item concentration). The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H2O, 192 mg/L Na2HPO4 • 2 H2O and 150 mg/L KH2PO4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

Continuous exposure

Approximately 48 hours following seeding, two blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells re-suspended in and washed with "saline G". The washing procedure was repeated once as described. Following washing, the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

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

Preparation of cells

Following the procedures above (approximately 40 hours from the start of treatment), the cultures were harvested by centrifugation. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

3. Acceptance Criteria

The micronucleus assay is deemed acceptable if it fulfils the following criteria:

- The concurrent solvent control will normally be within the laboratory historical solvent control data range. The average mutant frequency of the vehicle controls should not exceed $25x10^{-6}$ cells
- The concurrent positive controls should produce a statistically significant increase in the micronucleus frequency and should be within the laboratory historical positive control data range. The positive controls should induce a mutant frequency at least 3 times that of the controls
- Cell proliferation criteria in the solvent control are considered acceptable.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- The quality of the slides should allow the evaluation of an adequate number of cells and concentrations
- The criteria for the selection of top concentrations should be met

Any mutant frequencies not meeting these criteria were excluded from the statistical analyses.

4. Assessment criteria

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative if, in all of the experimental conditions examined:

- None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase
- The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- The increase is concentration-related in at least one experimental condition
- The results are outside the range of the laboratory historical solvent control data (95% control limit realized as 95% confidence interval

When all of the criteria are met, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system.

There is no requirement for verification of a clear positive or negative response.

In case the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. narrow concentration spacing, other metabolic activation conditions, i.e. S9 concentration or S9 origin) could be useful.

5. Statistical analysis

Statistical significance was confirmed by the Chi square test ($p < 0.05$), using a validated test script of "R", a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those values that indicated an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression was performed using a validated test script of "R", to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Both, biological and statistical significance were considered together.

II. Results and discussion

In Experiment I, precipitation of the test item in the culture medium was observed at 1975 µg/mL in the absence and presence of S9 mix. In all other experimental parts, no precipitation occurred.

No relevant influence on osmolarity or pH was observed.

In Experiment I in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration, which showed precipitation. In Experiment IIB in the absence of S9 mix after continuous treatment moderate cytotoxicity was observed at the highest evaluated concentration. The next higher tested concentration, however, which was separated by a factor of 1.1, much smaller than requested by the guideline could not be evaluated for cytogenetic damage due to strong cytotoxicity.

The results of both experiments, with and without metabolic activation, are summarised in the table below.

Table 5.8.1- 134: Summary of results of experiment I and IIb

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %	Micronucleated cells in %**	Historical control data	
						95% Ctrl limit	Min - Max
Exposure period 4 hrs without S9 mix							
I	40 hrs	Solvent control[1]	1.80		0.30	0.01 – 1.20	0.00 – 1.55
		Positive control[2]	1.79	1.1	**4.85**S	2.66 – 22.74	3.95 – 28.60
		752	1.69	14.3	0.10		
		1317	1.70	12.9	0.10		
		1975P	1.65	19.0	0.30		
Exposure period 20 hrs without S9 mix							
II	40 hrs	Solvent control[1]	1.99		0.70	0.00 – 1.14	0.05 – 1.60
		Positive control[3]	1.64	35.8	**4.20**	1.15 – 6.44	1.95 – 8.80
		215	1.93	6.4	0.25		
		452	1.69	30.5	0.20		
		546	1.64	35.2	0.20		
		601	n.e	n.e	n.e		
Exposure period 4 hrs with S9 mix							
I	40 hrs	Solvent control[1]	1.67		0.35	0.00 – 1.24	0.10 – 1.30
		Positive control[4]	1.51	23.7	**2.75**S	1.01 – 7.34	1.80 – 8.85
		752	1.48	28.5	0.50		
		1317	1.44	35.2	0.65		
		1975P	1.49	27.8	0.55		

* For the positive control groups and the test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
P Precipitation occurred at the end of treatment
S The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c. Not calculated as the CBPI is equal or higher than the solvent control value
n.e. Not evaluable due to strong cytotoxic effects
1 DMSO 0.5 % (v/v)
2 MMC 0.8 µg/mL
3 Demecolcine 50 ng/mL
4 CPA 17.5 µg/mL

In both independent experiments, neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells were observed following treatment with the test item either in the presence or absence of S9 mix.

Demecolcine (100 ng/mL), MMC (0.8 µg/mL) and CPA (15 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei, thus confirming the validity of the assay to detect clastogens/aneugens.

III. Conclusions

The test substance M-09 did not induce micronuclei in this *in vitro* micronucleus test in human lymphocytes, when tested up to precipitative concentrations.

Therefore M-09 is considered to be neither clastogenic nor aneugenic under the conditions of this assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 487 and is valid and acceptable to assess the clastogenicity of M-09 in vitro. M-09 is neither clastogenic nor aneugenic under the conditions of this study.

The following study is currently ongoing for M-09 and will be submitted in November 2020.

Dossier node	Draft title	Study ID	Planned submission
KCA 5.8.1	*In vivo* mammalian Alkaline Comet Assay	TXAC0063	November 2020

M-10 (AE 1344123)

M-10 was not acutely toxic *via* the oral route. In an acute oral toxicity study according to the acute toxic class method an LD_{50} of >2000 was established.

The short-term toxicity of M-10 was investigated in rats in a 28-day oral toxicity study. There were no adverse effects observed up to and including the highest dose tested. Therefore, a NOAEL of 20000 ppm (equivalent to 1748 and 2299 mg/kg bw/d in males and females respectively) was established from this study. A separate ADI of 1.7 mg/kg bw/d can thus be derived from this NOAEL using a safety factor of 1000.

M-10 has been tested in a battery of *in vitro* and *in vivo* genotoxicity tests. M-10 was not mutagenic *in vitro* in bacterial cells (Ames test) in the presence and absence of metabolic activation. However, in mammalian cells *in vitro*, a weak but statistically significant increase in mutant colonies was observed, which was dose-related and reproducible in the presence of metabolic activation (S9); therefore it is considered that M-10 is weakly mutagenic *in vitro* in mammalian cells under the conditions of this assay. This weakly positive *in vitro* result is being followed up *in vivo* in a mammalian alkaline comet assay, which is currently ongoing and will be submitted in November 2020. With regard to clastogenicity, M-10 induced a weak increase in chromosome aberrations in human lymphocytes at high doses, in which an alteration to a slightly more acidic pH was apparent (pH 7 to pH6), the slight increase in chromosome aberrations was seen in the absence of S9 after 20 hours exposure. This *in vitro* result was followed up with an appropriate *in vivo* test (MMT) in which no evidence of clastogenicity or aneugenicity was seen. Although exposure of the target tissue was not explicitly shown there was no alteration in the NCE/PCE ratio and no clinical signs of toxicity, exposure of the bone-marrow is likely, owing to the route of exposure (i.p injection) and the highest dose tested being the limit dose (2000 mg/kg bw/d). The lack of clastogenic potential for M-10 was confirmed in a recently conducted *in vitro* MNT in which no evidence of clastogenicity or aneugenicity was seen.

There is no evidence that the metabolite M-10 is clastogenic, M-10 is also not mutagenic, on the proviso that the *in vivo* Comet assay (currently ongoing) confirms the lack of mutagenic potential for M-10. Overall, there is no toxicological concern for the metabolite M-10.

The available studies are summarised below.

Acute toxicity

Data Point:	KCA 5.8.1/28
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C638206-AE 1344123 - Acute toxicity in the rat after oral administration
Report No:	AT00666
Document No:	M-221555-01-1
Guideline(s) followed in study:	Directive 67/548/EEC, Annex IV B, Part B, B. 1 tris (1967); OECD 423 (2001); US-EPA 712-C-98-190, OPPTS 870.1100 (1998)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In an acute toxicity study conducted according to the acute toxic class method, six female fasted Wistar rats (3/step) were each administered a single oral gavage dose of 2,000 mg/kg bw of M-10 (referred to as AE C638206-AE 1344123 in the report).

A dose of 2000 mg/kg bw was tolerated by rats without mortalities, effects on weight development and gross pathological findings.

The only clinical sign was diarrhoea.

In conclusion, according to OECD guideline 423 the LD_{50} cut-off of M-10 is ≥ 5000 mg/kg body weight.

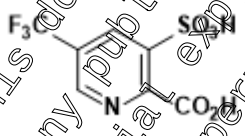
I. Materials and Methods

A. Materials

1. Test material

Test substance: AE C638206-AE 1344123
also known as M-10; BCS-AT35976; and P4

Structure



Chemical name: 2-pyridinecarboxylic acid, 3-sulfo-5-(trifluoromethyl)-
Purity: 98.5%
Batch no.: NLL7333-9a
Appearance: White powder
Expiry: November 26, 2003

2. Vehicle and/or positive control

Vehicle:	demineralized water, buffered to pH 6 by NaHCOs

3. Test animals

Species:	Rat
Strain:	Female rats of Wistar origin (HsdCpb:Wu)
Age:	10-11 weeks
Weight at start:	168 g-188g
Source:	[redacted]
Acclimation period:	Yes
Diet:	Provimi Kliba 3883.0.15 Maus/Ratte Haltung, Kaiseraugst Switzerland
Water:	Water *ad libitum*
Housing:	In groups of three rats of the same sex in polycarbonate cages on lowdust wood granulate bedding (J. Rettenmaier & Sohne, 73479 Ellwangen-olzmuhle, Germany).
Temperature:	22 ± 2 °C
Humidity:	55 ±5%
Air changes:	Approximately 10 changes per hour
Photoperiod:	12 hours: 12 hours

B. Study design

1. In-life dates: July 16 to August 5, 2003

2. Animal assignment and treatment

Two groups of three female rats were treated at 2000 mg/kg bw. The appropriate dose volume of the test substance was administered to each rat by oral gavage. The day of dosing was designated Day 1.

C. Methods

1. Observations

Mortality:

Cages of rats were checked at least once daily for mortalities.

Clinical signs:

Animals were observed soon after dosing and at frequent intervals for the remainder of Day 1. On subsequent days animals were observed at least once daily. The nature and severity of clinical signs and the time these were noted were recorded at each observation. Animals were observed for 14 days respectively following dosing.

The body weights of each rat were recorded Days 1 (prior to dosing), 8 and 15.

2. Necropsy

The surviving animals were sacrificed by carbon dioxide at the end of the study, dissected and examined macroscopically.

II. Results and Discussion

A. Results

1. Dose-response table (LD_{50})

The results of the study for acute oral toxicity in the fasted rat, including the LD_{50}, are summarized in the table below.

Table 5.8.1- 135: Dose response

Dose (mg/kg bw)	Toxicological result*	Occurrence of signs	Time of death	Mortality (%)
Females				
1st 2,000	0/3/3	4 – 5 hours	-	0
2nd 2,000	0/3/3	5 hours	-	0

* Number of animals which died and/or were sacrificed moribundly / number of animals with signs / total number of animals.

The LD_{50} is therefore **>2000 mg/kg bw**

2. Clinical signs

The only clinical sign observed at 2000 mg/kg body weight was diarrhoea.

3. Body weights

There were no toxicological effects on body weights or on body weight gain.

4. Necropsy findings

The necropsies performed at the end of the study revealed no unusual findings.

III. Conclusion

An LD_{50} of > 2000 mg/kg bw was derived from this study for the metabolite M-10.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 423 and is valid and acceptable to investigate the acute oral toxicity of M-10 in the rat. M-10 is not acutely toxic *via* the oral route under the conditions of this study. An LD_{50} of > 2000 mg/kg bw was established.

Genotoxicity

Data Point:	KCA 5.8.1/29
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE 1344123 - Salmonella/microsome test - Plate incorporation and preincubation method
Report No:	AT00743
Document No:	M-223117-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC, B. 13/14. (2000); OECD 471 (1997); US-EPA 712-C-98-247, OPPTS 870.5100 (1998)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

M-10 (referred to as AE 1344123 in the report) was initially investigated using the *Salmonella*/microsome plate incorporation test for point mutagenic effects at doses of up to and including 5000 µg per plate on five *Salmonella typhimurium* LT2 mutants. These comprised the histidine auxotrophic strains TA 1535, TA 100, TA 1537, TA 98, and TA 102. The independent repeat was performed as a preincubation assay for 20 minutes at 37°C. All other conditions remained unchanged.

Doses up to and including 50 µg per plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. At higher doses, the substance had only a strain-specific bacteriotoxic effect. Due to the weakness of this effect this range could nevertheless be used for assessment purposes. Substance precipitation occurred at 5000 µg per plate.

Evidence of mutagenic activity of M-10 was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed.

The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C, cumene hydroperoxide and 2-aminoanthracene had a marked mutagenic effect, as evidenced by a biologically relevant increase in mutant colonies compared to the corresponding negative controls.

Therefore, M-10 was considered to be non-mutagenic without and with S9 mix in the plate incorporation as well as in the preincubation modification of the *Salmonella*/microsome test.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE 1344123 also known as M-10
Purity:	98.5%
Batch no.:	NLL7333-9a

2. Vehicle and/or positive control

Vehicle:	M-10 and Mitomycin C: deionized water Other positive controls: DMSO
Positive control:	Without S9 mix: Na-azide: TA 1535 Nitrofurantoin (NF): TA 100 4-Nitro-1,2-phenylene diamine (4-NPDA): TA 1537, TA 98 Mitomycin C (MMC): TA 102 (plate incorporation trials) Cumene hydroperoxide (Cumene): TA 102 (preincubation trials) With S9 mix: 2-Aminoantaracene: TA 98, TA 100, TA 1535, TA 1537, TA 102

3. Activation:

Owing to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

S9 mix was used to simulate the mammalian metabolism of the test substance. It was derived from the livers of at least six adult male Sprague Dawley rats of approximately 200 to 300 g in weight. For enzyme induction, the animals received a single intraperitoneal injection of Aroclor 1254, dissolved in corn oil, at a dose of 500 mg/kg body weight, five days prior to sacrifice. The animals were prepared unfasted, following the directions of Ames *et al.* (1975) and Maron and Ames (1983).

The rats were terminated. Livers were removed under sterile conditions immediately after sacrifice and kept at 4°C until all animals had been prepared. All the remaining steps were carried out under sterile conditions at 4°C.

The livers were washed with cold (4°C) 0.15 M KCl solution (approximately 1 mL KCl per 1 g liver), and then homogenized in fresh, cold (4°C) 0.15 M KCl (approximately 3 mL KCl per 1 g liver). The homogenate was then centrifuged in a cooling centrifuge at 4°C and 9000 g for 10 minutes. The supernatant (the S9 fraction) was stored at -80°C in small portions.

Cofactor solution (70 mL) was composed as follows:

162.6 mg $MgCl_2 \cdot 6H_2O$

246.0 mg KCl

179.1 mg glucose-6-phosphate, disodium salt

315.0 mg NADP, disodium salt

100.0 mM phosphate buffer

S9 mix consists of this cofactor solution, S9 fraction and, if needed, 0.15 M KCl. The amount of S9 fraction in S9 mix is indicated in the tables in percent. The S9 mix comprised the amount of S9 fraction (x%) indicated in the tables, 70% cofactor solution and (30-x)% 0.15 M KCl. The S9 fraction was derived from the preparation dated February 4, 2003 (protein content 25.6 mg per ml). Prior to first use, each batch was checked for its metabolizing capacity by using reference mutagens; appropriate activity was demonstrated. At the beginning of each experiment 4 aliquots of the S9 mix were plated (0.5 ml per plate) in order to assess its sterility. This was repeated after completion of test tube plating. The sterility control plates were then incubated for 48 hours at 37°C. No indication of contamination of S9 mix was found.

4. Test organisms:

Histidine-deficient mutants of *Salmonella typhimurium* LT2 served as indicators to demonstrate point mutagenic effects. These strains were selected specifically for the *Salmonella*/ microsome test. Since point mutations can be divided into two basic classes, base-pair substitutions and frameshift mutations, several strains were used which cover both types.

These included the strains selected by Ames *et al.* (1973b), *Salmonella typhimurium* TA1535 and TA 1537, as well as *Salmonella typhimurium* TA100, TA 98 and TA 102

TA 1535 and TA 100 bear the base-pair substitution, his G 46, and TA 100 additionally contains the plasmid pKM 101. This R factor, also contained in TA 98 and TA102, codes for an ampicillin resistance and should raise the sensitivity of the strains. TA 102 carries the ochre mutation his G 428 on the multicopy plasmid pAQ1, which codes in addition for tetracycline resistance. TA1537 and TA 98 bear frameshift markers. TA 1537 exhibits the -1 mutant, his C 3076, while TA 98 bears the +2 type, his D 3052.

Furthermore, the strains have other properties, which should increase their sensitivity. They are all deep rough, i.e. partly deficient in lipopolysaccharide side chains in their cell walls, enabling larger molecules to penetrate the bacterial cell wall and produce mutations. With the exception of TA 102, all strains have reduced capability to repair DNA-damage which increases the likelihood that such damage results in mutations.

The mutations of the bacterial strains used in this study are described in the table below.

Table 5.8.1- 136: ***Salmonella typhimurium* strains**

Salmonella typhimurium		
Strains	**Genotype**	**Type of mutations indicated**
TA 1537	his C 3076; rfa$^-$; uvrB$^-$	frame shift mutations
TA 98	his D 3052; rfa$^-$; uvrB$^-$; R-factor	" "
TA 1535	his G 46; rfa$^-$; uvrB$^-$	base-pair substitutions
TA 100	his G 46; rfa$^-$; uvrB$^-$; R-factor	" "
TA 102	his G 428; rfa$^-$; uvrB$^+$; R-factor	" "

Regular checking of the properties of the *Salmonella typhimurium* strains was performed.

The original strains were obtained from Prof. Bruce Ames and arrived at Toxicology, Bayer HealthCare, Bayer AG, on August 15, 1997.

5. Test substance concentrations used:
Test concentrations of 16, 50, 158, 500, 1581 and 5000 µg M-10/plate were used.

B. Test performance

Experimental phase: 17 September to 02 October, 2003

1. Pre-experiment for toxicity

No pre-experiment was performed.

2. Mutagenicity test

For each strain and dose level, including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

Plate Incorporation Method:

- 100 µL test solution at each dose level (solvent or reference mutagen solution (positive control)),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL bacteria suspension (cf. test system, pre-culture of the strains),
- 2000 µL overlay agar.

Preincubation Method:

In the pre-incubation assay 100 µL test solution (solvent or reference mutagen solution (positive control)), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspension were mixed in a test tube and incubated at 37 °C for 20 minutes. After pre-incubation 2.0 mL overlay agar was added to each tube. The mixture was poured on agar plates.

The plates were incubated for 48 hours at 37 °C prior to counting.

Data recording:

The colonies were counted automatically using an Artek counter, model 982B. Data were transferred to a PC and processed with the released and BQS 6.0 based BioSys software Ames- Test III (Rev. 3.106).

3. Statistics

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

4. Acceptance / assessment criteria:

The following criteria determined the acceptance of an assay:

a) The negative controls had to be within the expected range, as defined by published data (e.g. Maron and Ames, 1983) and/or the laboratories' own historical data.

b) The positive controls had to show sufficient effects, as defined by the laboratories' experience.

c) Titer determinations had to demonstrate sufficient bacterial density in the suspension.

Only trials which complied with all three of the above criteria were accepted for assessment. Even if the criteria for points (b) and (c) were not met, a trial was accepted if it showed mutagenic activity of the test compound. Furthermore, an unacceptable trial would have been repeated.

5. Evaluation of results

A reproducible and dose-related increase in mutant counts of at least one strain is considered to be a positive result. For TA1535, TA100 and TA 98 this increase should be about twice that of negative controls, whereas for TA 1537, at least a threefold increase should be reached. For TA 102 an increase of about 100 mutants should be reached. Otherwise, the result is evaluated as negative. However, these guidelines may be overruled by good scientific judgment.

In case of questionable results, investigations should continue, possibly with modifications, until a final evaluation is possible.

II. Results and Discussion

A. Mutation assays

The *Salmonella*/microsome test, employing doses of up to 5000 µg per plate, showed M-10 to produce bacteriotoxic effects, starting at 158 µg per plate. Substance precipitation occurred at 5000 µg per plate.

Evaluation of individual dose groups, with respect to relevant assessment parameters (dose effect, reproducibility), revealed no biologically relevant variations from the respective negative controls.

In spite of the low doses used, positive controls increased the mutant counts to well over those of the negative controls, and thus demonstrated the system's high sensitivity.

Despite this sensitivity, no indications of mutagenic effects of M-10 could be found at assessable doses of up to 5000 µg per plate in any of the *Salmonella typhimurium* strains used.

An overview of the results is given in the following tables.

Table 5.8.1- 137: Summary of plate incorporation test

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean)				
		TA 1535	TA 100	TA 1537	TA 98	TA 102
Without metabolic activation (-S9)						
M-10	0	11	150	11	29	414
	16	14	151	11	26	406
	50	18	151	5	30	410
	158	10	157	11	37	433
	500	14	171	10	28	399
	1581	12	156	10	36	440
	5000	14	153	7	29	356
Positive control						
Na-azide	10	572	-	-	-	-
NF	0.2	-	754	2	-	-
4-NPDA	10 / 0.5	-	-	90	167	-
MMC	0.2					728
Historical control mean	-	10 (±2)	93 (±18)	8 (±1)	26 (±8)	209 (±16)
Historical control range	-	8-14	81-130	6-9	17-39	185-228
With metabolic activation (+S9)						
M-10	0	19	181	19	46	453
	16	20	185	18	46	455
	50	21	181	21	40	455
	158	17	183	19	40	426
	500	21	197	20	47	470
	1581	15	120	16	43	334
	5000	29	123	15	16	211
Positive control						
2-AA	3	161	1822	309	1551	816
Historical control mean	-	11 (±1)	114 (±17)	9 (±1)	36 (±10)	282 (±14)
Historical control range	-	9-11	102-151	7-10	26-50	260-300

Na-azide = sodium azide; NF = Nitrofurantoin, 4-NPDA = 4-Nitro-1,2-phenylene diamine, MMC = Mitomycin C;
2-AA = 2-aminoanthracene
(±X): Standard deviation
Historical control data based on experiments from 1996 to 2002.

Table 5.8.1- 138: Summary of pre-incubation test

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean)				
		TA 1535	TA 100	TA 1537	TA 98	TA 102
Without metabolic activation (-S9)						
M-10	0	17	121	6	26	435
	16	14	121	5	19	417
	50	20	136	5	21	386
	158	19	151	6	22	400
	500	20	130	8	27	401
	1581	10	103	5	15	309
	5000	10	98	4	17	280
Positive control						
Na-azide	10	685	-	-	-	-
NF	0.2	-	290	-	-	-
4-NPDA	10 / 0.5	-	-	91	164	-
Cumene	50					547
Historical control mean	-	11 (±3)	126 (±17)	9 (±1)	27 (±8)	259 (±16)
Historical control range	-	10-17	100-153	8-11	17-40	239-285
With metabolic activation (+S9)						
M-10	0	13	148	10	34	539
	16	11	149	10	31	527
	50	14	153	10	28	534
	158	13	168	10	42	443
	500	9	133	8	38	528
	1581	10	134	7	25	239
	5000	12	71	5	13	221
Positive control				636		
2-AA	3	64	1765	86	774	636
Historical control mean	-	12 (±2)	144 (±15)	11 (±1)	34 (±8)	317 (±25)
Historical control range	-	10-15	117-167	9-12	26-45	294-364

Na-azide = sodium azide; NF = Nitrofurantoin, 4-NPDA = 4-Nitro-1,2-phenylene diamine; Cumene = Cumene hydroperoxide; 2-AA = 2-aminoanthracene
(±X): Standard deviation
Historical control data based on experiments from 1996 to 2002.

III. Conclusion

No indications of mutagenic effects of M-10 could be found at assessable doses of up to 5000 µg per plate in any of the *Salmonella typhimurium* strains used in the plate incorporation or preincubation assays.

Due to these results M-10 has to be regarded as non-mutagenic

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of M-10 in *S-typhimurium*.

M-10 is not mutagenic in bacterial cells under the conditions of this study when tested up to 5000 µg/plate (+/- S9), either by base pair changes or frameshift mutations.

Data Point:	KCA 5.8.1/30
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE 1344123: Induction of chromosome aberrations in cultured human peripheral blood lymphocytes
Report No:	C038437
Document No:	M-224986-01-1
Guideline(s) followed in study:	OECD 473 (1997), ICH Tripartite Harmonised Guideline (1995)
Deviations from current test guideline:	Only 100 metaphases were analysed rather than the 300 recommended in the current guidance; cytotoxicity was not evaluated by relative population doubling or relative increase in cell count as recommended in the current guidance.
Previous evaluation:	yes, evaluated and accepted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

M-10 (referred to as AE 1344123 in the report) was tested in an *in vitro* cytogenetics assay using duplicate human lymphocyte cultures prepared from the pooled blood of three male donors in two independent experiments. Treatments covering a broad range of doses, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9). The test item was dissolved in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) and the highest dose level used, 5500 µg/mL, was in excess of the solubility limit in culture medium.

In Experiment I, treatment in the absence and presence of S-9 was for 3 hours followed by a 17-hour recovery period prior to harvest (3+17). The test item dose levels for chromosome analysis were limited by changes in pH of the post treatment medium. Chromosome aberrations were analysed at three dose levels and the highest concentration chosen for analysis, 2240 µg/mL, induced approximately 11% and 27% mitotic inhibition in the absence and presence of S9, respectively. At this concentration, a decrease in pH of the post treatment medium by approximately 1 pH unit (compared to concurrent controls) was also evident both in the absence and presence of S-9. Higher concentrations (2800 µg/mL and above) reduced the pH of the post treatment medium by more than 1 pH unit.

In Experiment 2, treatment in the absence of S-9 was continuous for 20 hours. Treatment in the presence of S-9 was for 3 hours only followed by a 17-hour recovery period prior to harvest (3+17). Chromosome aberrations were analysed at three dose levels. The highest concentrations chosen for analysis, 1147 µg/mL for 20+0-hour treatment in the absence of S-9 induced approximately 58% mitotic inhibition. The highest concentration (3500 µg/mL) selected for analysis following 3+17-hour treatment in the presence of S-9, was limited by changes in pH of the post treatment medium. A decrease of approximately 1 pH unit (compared to concurrent controls) was observed at this concentration.

No increases in the frequency of cells with numerical aberrations, which exceeded the historical negative control range, were observed in the majority of cultures treated with M-10 in the absence and presence of S-9. The one exception to this was observed in a single culture at the highest concentration (3500 µg/mL) analysed following 3+17 hour treatment in the presence of S-9 in Experiment 2, where the numerical aberration frequency marginally exceeded the historical control (normal) range. However, the numerical aberration frequency of the replicate culture at this concentration and all other test article treated cultures fell within the normal ranges. This observation was therefore not considered to be of biological relevance.

It is concluded that M-10 did not induce chromosome aberrations in cultured human peripheral blood lymphocytes, when tested up to its limit of cytotoxicity (20+0 hour, -S-9) or up to the maximum practicable concentration limited by changes in pH changes of -1 pH unit compared to concurrent solvent controls (3+17 hour, +/-S-9).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE 1344123 also known as AE C638206-AE 1344123 and M-10
Purity:	98.5%
Batch no.:	NLL7333-9A

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	Without S9 mix: 4-Nitroquinoline 1-oxide (NQO)
	With S9 mix: Cyclophosphamide (CPA)

3. Activation:

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. The batches of MolTox™ S-9 were stored frozen in aliquots at -80°C and thawed just prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufm-O-dealkylase activities).

Preparation of S-9 mix:

Glucose-6-phosphate (180 mg/mL), NADP (25 mg/mL), 150 mM KC1 and rat liver S-9 were mixed in the ratio 1:1:1:2. An aliquot of the resulting S-9 mix was added to each cell culture designated for treatment in the presence of S-9 to achieve the required final concentration in a total of 10 mL. The final concentration of liver homogenate in the test system was 2%. Cultures treated in the absence of S-9 received an equal volume of 150 mM KC1.

4. Blood cultures:

Blood from three healthy, non-smoking male volunteers was used for each experiment of this study.

No donor was suspected of any virus infection nor had been exposed to high levels of radiation or hazardous chemicals. For each experiment, an appropriate volume of whole blood was drawn from the peripheral circulation within two days prior to culture initiation. Blood was stored refrigerated and pooled prior to use. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL heparinised blood into 9.0 mL Hepes-buffered RPMI medium containing 20% (v/v) foetal calf serum and 50 µg/mL gentamycin. Phytohaemagglutinin (PHA, reagent grade) was included at a concentration of approximately 2% of culture volume to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37°C and rocked continuously.

5. Test substance concentrations used:

Experiment 1:	78.81, 98.52, 123.1, 153.9, 192.4, 240.5, 300.6, 375.8, 469.8, 587.2, 734.0, 917.5, 1147, 1434, 1792, 2240, 2800 & 3500 µg/mL
Experiment 2, Trial 1:	63.05, 78.81, 98.52, 123.1, 153.9, 192.4, 240.5, 300.6, 375.8, 469.8, 587.2, 734.0, 917.5, 1147, 1434, 1792, 2240 & 2800 µg/mL
Experiment 2, Trial 2:	469.8, 587.2, 734.0, 917.5, 1147, 1434, 1792, 2240, 2800 & 3500 µg/mL

It should be noted that an initial of Experiment 2 was performed. However, it was not possible to select a suitable top dose for chromosome aberration analysis from 3+17, +S-9 treatment due to insufficient mitotic inhibition or decreases in pH. This part of the study was therefore repeated using a more targeted dosing regime. The structural aberration frequencies from solvent control treated cultures following 20+0-hour, treatment in the absence of S-9 exceeded the historical control range. Therefore, the data generated from this part of the study was rejected and the experiment repeated. The data generated from this initial Experiment 2 (Experiment 2, Trial 1) are not further reported.

B. Test performance

Experimental phase: 30 June – 29 September 2003

1. Pre-experiment for toxicity

No pre-experiment was performed.

2. Methods

Treatment:

S-9 mix or KCl (0.5 mL) was added appropriately. One set of quadruplicate cultures (A, B, C and D) for each of the treatment regimes was then treated with the solvent and one set of duplicate cultures with the test item (0.1 mL per culture). Additional duplicate cultures for treatments in the absence of S-9 and in its presence, were treated with 0.1 mL of the positive control chemicals. All cultures were then incubated at 37°C.

Treatment media remained on cultures receiving the continuous treatment until sampling, that is, 20 hours after the beginning of treatment. Cultures received pulse treatments (both in the absence and presence of S-9) for 3 hours only. They were then pelleted (approximately 300 x 'g', 10 minutes), washed twice with sterile saline (prewarmed at 37°C) and resuspended in fresh medium containing foetal calf serum and gentamycin. Cultures were incubated for a further 17 hours before harvesting.

Harvesting:

Approximately 2 hours prior to harvest, colchicine was added to give a final concentration of approximately 1 µg/mL to arrest dividing cells in metaphase. At the defined sampling time cultures were centrifuged at approximately 300 x 'g' for 10 minutes; the supernatant was carefully removed and cells were resuspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37°C for 15 minutes to allow cell swelling to occur. Cells were then fixed by dropping the KCl suspension into an equal volume of fresh, ice-cold methanol/glacial acetic acid (3:1 v/v).The fixative was changed by centrifugation (approximately 300 x 'g', 10 minutes) and resuspension. This procedure was repeated several times (centrifuging at approximately 1250 x 'g1, 2-3 minutes) until the cell pellets were clean.

Preparation of metaphase spreads:

Lymphocytes were kept in fixative in the refrigerator before slides were prepared but slides were not made on the day of harvest to ensure cells were adequately fixed. Cells were pelleted and resuspended in a minimal amount of fresh fixative (if required) so as to give a milky suspension. Several drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance chromosome spreading, and several drops of suspension were transferred to clean microscope slides labelled with the appropriate study details. Slides were flamed to improve metaphase spreading.

After the slides had dried the cells were stained for 5 minutes in 4% (v/v) filtered Giemsa stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried and mounted with coverslips.

Selection of doses for cytogenetic analysis:

Slides were examined, uncoded, for mitotic index (MI) or percentage of cells in mitosis. Slides from enough dose levels from each treatment regime were scored to determine if chemically induced mitotic inhibition had occurred. This is defined as a clear decrease in mitotic index compared with negative controls (based on at least 1000 cells counted), preferably dose-related.

Rationale for dose selection:

The highest dose for chromosome analysis from cultures sampled at 20 hours should be one at which at least 50% mitotic inhibition (approximately) has occurred or should be the highest dose tested. Analysis of slides from highly cytotoxic concentrations is avoided, if possible. Slides from cultures treated with heavily precipitating doses are checked to confirm that the presence of precipitate does not preclude analysis. Slides from the highest selected dose and two lower doses, such that a range of cytotoxicity from maximum to little or none is covered, were taken for microscope analysis.

For each treatment regime, two solvent control cultures were initially to be analysed for chromosome aberrations. Slides from the remaining solvent control cultures were only to be analysed if considered necessary, for example, to help resolve an equivocal result. A single positive control dose level, which gives satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage, was analysed.

Scoring of aberrations:

Slides from NQO and CPA positive control treatments were checked to ensure that the system was operating satisfactorily. Slides from the selected treatments and from solvent and positive controls were coded using randomly generated letters by a person not connected with the scoring of the slides. Labels bearing only the study reference number, experiment number, the sex of the donor and the code were used to cover treatment details on the slides.

Where possible, one hundred metaphases from each code were analysed for chromosome aberrations. Only cells with 44-46 chromosomes were considered acceptable for analysis of structural aberrations. Any cell with more than 46 chromosomes, that is polyploid, endoreduplicated and hyperdiploid cells, observed during this search was noted and recorded separately. Classification of structural aberrations was based on the scheme described by ISCN. Under this scheme, a gap is defined as a discontinuity less than the width of the chromatid and no evidence of displacement of the fragment and a deletion is defined as a discontinuity greater than the width of the chromatid and/or evidence of displacement of the fragment.

3. Treatment of data

After completion of microscopic analysis, data were decoded. The aberrant cells in each culture were categorised as follows:

1. cells with structural aberrations including gaps
2. cells with structural aberrations excluding gaps
3. polyploid, endoreduplicated or hyperdiploid cells.

The totals for category 2 in negative control cultures were compared with the current laboratory negative control (normal) ranges to determine whether the assay was acceptable or not. The proportion of cells in category 2 in test item treated cultures were also compared with normal ranges. The statistical significance of any data set was only to be taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentration exceeded the normal range. Under this condition, the statistical method used would be Fisher's exact test. Probability values of $p < 0.05$ were to be accepted as significant. The proportions of cells in categories 1 and 3 were also examined in relation to historical negative control (normal) ranges and statistical analysis by Fisher's exact test may be used.

4. Acceptance / assessment criteria:

The human lymphocyte assay is considered valid if the following criteria are met:

1. the binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures, and
2. the proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range, and
3. at least 160 cells out of an intended 200 are analysable at each dose level, and
4. the positive control chemicals induce statistically significant increases in the proportion of cells with structural aberrations.

5. Evaluation criteria

A test item is considered as positive in this assay if:

1. the proportions of cells with structural aberrations at one or more concentration exceeds the normal range in both replicate cultures, and
2. a statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurs at these doses.

Increased incidence of cells with gaps or increased proportions of cells with structural aberrations not exceeding the normal range or occurring only at very high or very toxic concentrations are likely to be concluded as "equivocal". Full assessment of the biological importance of such increases is likely only to be possible with reference to data from other test systems. Evidence of a dose-related effect is considered useful but not essential in the evaluation of a positive result. Cells with exchange aberrations or cells with greater than one structural aberration occur very infrequently in negative control cultures. Their appearance is therefore considered to be of particular biological significance.

II. Results and Discussion

Selection of doses for cytogenetic analysis

The results of mitotic index determinations for the treatments in Experiment 1 were as follows:

Table 5.8.1- 139: Summary of mitotic index determinations from Experiment 1

Treatment (µg/mL)	Mitotic index (%)					
	A/C	3+17, -S-9 B/D	MIH*	A/C	3+17, +S-9 B/D	MIH*
Solvent	4.6/5.3	7.2/6.1	-	4.6/4.7	5.6/5.6	-
78.81	NS	NS	-	NS	NS	-
98.52	NM	NM	-	NM	NM	-
123.1	NM	NM	-	NM	NM	-
153.9	NM	NM	-	NM	NM	-
192.4	NM	NM	-	NM	NM	-
240.5	NM	NM	-	NS	NS	-
300.6	NM	NM	-	NS	NS	-
375.8	NM	NM	-	NS	NS	-
469.8	NS	NS	-	NS	NS	-
587.2	NS	NS	-	NS	NS	-
734.0	NS	NS	-	NS	NS	-
917.5	NS	NS	-	5.3	4.7	2
1147	NS	NS	-	4.2	5.2	8
1434	**4.9**	**6.7**	**0**	**3.7**	**5.3**	**12**
1792	**5.4**	**5**	**10**	**3.8**	**5.1**	**13**
2240	**5.1**	**5.2**	**11**	**3.2**	**4.3**	**27**
2800	3.7	5.7	19	3.7	4.8	17
3500	3.1	4.0	33	4.4	3	28

NM = slides not made; NS = not scored
*Mitotic inhibition (%) = [1 - (mean MIT/mean MIC)] x 100%
(where T = treatment and C = negative control)
Doses in **bold** selected for analysis

The results of mitotic index determinations for the treatments in Experiment 2 were as follows:

Table 5.8.1- 140: Summary of mitotic index determinations from Experiment 2, Trial 2

Treatment (µg/mL)	Mitotic index (%)		
	A/C	20+0, -S-9 B/D	MIH*
Solvent	9.8/8.5	10.1/10.6	-
63.05	NS	NS	-
78.81	NS	NS	-
98.52	NS	NS	-
123.1	NS	NS	-
153.9	NS	NS	-
192.4	NS	NS	-
240.5	NS	NS	-
300.6	NS	NS	-
375.8	NS	NS	-
469.8	10.6	10.9	0
587.2	5.6	4.3	49
734.0	**6.9**	**8.0**	**24**
917.5	**6.1**	**6.7**	**34**
1147	**3.8**	**4.4**	**58**
1434	3.0	3.3	68
1792	3.4	4.1	62
2240	2.6	3.6	68
2800	1.4	1.7	84

NS = not scored
*Mitotic inhibition (%) = [1 - (mean MIT/mean MIC)] x 100%
(where T = treatment and C = negative control)
Doses in **bold** selected for analysis

Table 5.8.1- 141: Summary of mitotic index determinations from Experiment 2, Trial 3

Treatment (µg/mL)	Mitotic index (%)		
	A/C	20+0, -S-9 B/D	MIH*
Solvent	6.8/9.8	9.5/8.1	-
469.8	NS	NS	-
587.2	NS	NS	-
734.0	NS	NS	-
917.5	10.0	9.0	0
1147	8.1	8.4	4
1434	6.8	8.8	9
1792	**8.2**	**7.0**	**11**
2240	5.6	8.4	18
2800	**7.1**	**6.0**	**23**
3500	**5.1**	**4.4**	**44**

NS = not scored
*Mitotic inhibition (%) = [1 - (mean MIT/mean MIC)] x 100%
(where T = treatment and C = negative control)
Doses in **bold** selected for analysis

Chromosome aberration analysis

Validity of study:

The data confirmed that:

1. no evidence of significant heterogeneity between replicate cultures was obtained in the binomial dispersion test, and
2. the proportion of cells with structural aberrations (excluding gaps) in negative control cultures fell within the normal range, and
3. at least 160 cells out of an intended 200 were analysed at each dose level, and
4. the positive control chemicals NQO and CPA induced statistically significant increases in the number of cells with structural aberrations.

The study was therefore considered to be valid.

Analysis of data:

Historical control data:

Historical negative (solvent) control data, calculated in October 2002, is summarized in the following table:

Table 5.8.1- 142: Historical ranges for solvent controls

Sex and S-9 treatment	Category	Total number of cells scored	Aberrant cells scored per 100 cells		
			Mean	Calculated normal range*	Observed Range
Male -S-9	Structural aberrations including gaps	9600	2.09	3-7	0-8
	Structural aberrations excluding gaps	9600	1.26	2-5	0-6
	Polyploid cells	8342	0.25	1-2	0-2
	Numerical aberrations	8342	0.38	1-2	0-2
Male +S-9 (Aroclor 1254)	Structural aberrations including gaps	9200	1.58	3-6	0-9
	Structural aberrations excluding gaps	9200	0.97	2-4	0-6
	Polyploid cells	8237	0.31	1-2	0-2
	Numerical aberrations	8237	0.45	1-2	0-3

*Calculated range = 99% confidence interval

Structural aberrations:

Treatment of cultures with M-10 in the absence and presence of S-9 resulted in frequencies of cells with structural aberrations which were similar to those in concurrent negative controls. A slight increase in aberrations (excluding gaps) was seen at 1792 µg/mL in the absence of S9 (20 hour exposure); however, the increase in the numbers of aberrant cells (excluding gaps) in all treated cultures fell within historical negative control ranges.

Table 5.8.1- 143: M-10: Summary of numbers and types of structural aberrations observed

Treatment (µg/mL)	Cells*	G	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
3 hour treatment -S-9,17 hour recovery (3+17), Experiment 1									
Solvent	200	2	0	0	2	1	0	5	3
1434	200	2	0	0	2	0	0	4	2
1792	200	2	0	0	3	0	0	5	3
2240	200	4	0	0	3	0	0	7	3
NQO, 5.00	196	8	9	1	55	25	4	102	94
3 hour treatment +S-9,17 hour recovery (3+17), Experiment 1									
Solvent	200	0	0	0	1	0	0	3	3
1434	200	1	0	0	2	0	0	3	2
1792	200	4	0	0	5	1	0	10	6
2240	200	3	0	0	0	0	0	3	0
CPA, 6.25	200	10	10	0	69	6	0	95	85
20 hour treatment -S-9, 0 hour recovery (20+0), Experiment 2, Trial 2									
Solvent	200	2	0	0	1	0	0	3	1
734.0	200	2	0	1	1	1	0	5	3
917.5	200	2	3	0	0	0	0	5	3
1147	200	0	1	0	3	0	0	4	4
NQO, 2.50	200	10	9	0	44	25	5	93	83
3 hour treatment +S-9,17 hour recovery (3+17), Experiment 2, Trial 3									
Solvent	200	0	0	0	1	0	0	1	1
1792	200	0	0	0	2	1	0	3	3
2800	200	1	1	0	3	0	0	5	4
3500	200	2	2	0	1	0	0	5	3
CPA, 6.25	196	3	6	0	129	20	2	160	157

*: Total cells examined for structural aberrations
NQO: 4-Nitroquinoline 1-oxide
CPA: Cyclophosphamide

Numerical aberrations:

No increases in the frequency of cells with numerical aberrations, which exceeded the historical negative control range, were observed in the majority of cultures treated with M-10 in the absence and presence of S-9. The one exception to this was observed in a single culture at the highest concentration (3500 µg/mL) analysed following 3+17 hour treatment in the presence of S-9 in Experiment 2, where the numerical aberration frequency marginally exceeded the historical control (normal) range. However, the numerical aberration frequency of the replicate culture at this concentration and all other test article treated cultures fell within the normal ranges. This observation was therefore not considered to be of biological relevance.

Table 5.8.1- 144: M-10: Summary of numbers and types of numerical aberrations observed

Treatment (µg/mL)	Cells*	H	E	P	Tot abs	% with num abs
3-hour treatment -S-9,17 hour recovery (3+17), Experiment 1						
Solvent	200	0	0	0	0	0
1434	201	0	0	1	1	0.5
1792	201	0	1	0	1	0.5
2240	201	0	0	1	1	0.5
NQO, 5.00	196	0	0	0	0	0
3-hour treatment +S-9,17 hour recovery (3+17), Experiment 1						
Solvent	200	0	0	0	0	0
1434	200	0	0	0	0	0
1792	202	0	0	2	2	1.0
2240	200	0	0	0	0	0
CPA, 6.25	201	1	0	0	1	0.5
20-hour treatment -S-9, 0 hour recovery (20+0), Experiment 2, Trial 2						
Solvent	200	0	0	0	0	0
734.0	200	0	0	0	0	0
917.5	200	0	0	0	0	0
1147	201	0	0	1	1	0.5
NQO, 2.50	201	1	0	0	1	0.5
3-hour treatment +S-9,17 hour recovery (3+17), Experiment 2, Trial 3						
Solvent	201	0	0	1	1	0.5
1792	200	0	0	0	0	0
2800	202	0	1	1	2	1.0
3500	205	0	2	3	5	2.4
CPA, 6.25	198	1	0	1	2	1.0

*: Total cells examined for structural aberrations
NQO: 4-Nitroquinoline 1 -oxide
CPA: Cyclophospliamide
H: hyperdiploid (47-68 chromosomes)
E: endoreduplicated
P: polyploid (greater than 68 chromosomes)

III. Conclusion

M-10 did not induce chromosome aberrations in cultured human peripheral blood lymphocytes, when tested up to its limit of cytotoxicity (20+0 hour, -S-9) or up to the maximum practicable concentration limited by changes in pH changes of >1 pH unit compared to concurrent solvent controls (3+17 hour, +/-S-9).

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 473 and is valid and acceptable to assess the potential of M-10 to induce chromosome aberrations in cultured human peripheral blood lymphocytes.

M-10 did not induce structural chromosome aberrations in cultured human peripheral blood lymphocytes under the conditions of this study when tested up to its limit of cytotoxicity (20+0 hour, -S-9) or up to the maximum practicable concentration limited by changes in pH (3+17 hour, +/-S-9).

Data Point:	KCA 5.8.1/31
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE C638206-AE 1344123 - V79/HPRT-test in vitro for the detection of induced forward mutations
Report No:	AT00947
Document No:	M-227048-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC, B.17. (2000); OECD 476 (1997); US-EPA 712-C-98-221, OPPTS 870.5300 (1998)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The study was performed to investigate the potential of M-10 to induce gene mutations at the HPRT locus in V 79 cells of the Chinese hamster *in vitro*.

The assay was performed in three experiments with and two without rat liver microsomal activation (S9-mix). The test article was dissolved in deionized water and tested at the following concentrations:

85, 170, 340, 680, 1360 & 2720 µg/mL (+/- S9)

The concentration ranges were based on the results of preliminary testing for solubility and toxicity. No toxicity was observed in the preliminary study, but a change in the pH of the media determined the highest dose.

A weak but statistically increase in mutant frequencies was observed both with and without S9; however reproducible and concentration related increases were only seen in the presence of S9.

The positive controls showed distinct statistically significant increases in induced mutant colonies, thus indicating the sensitivity of the assay and the negative controls gave the expected results.

It is concluded that M-10 is weakly mutagenic in the presence of metabolic activation, in this HPRT test with V79 Chinese hamster cells.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-10 (reffered to as AE C638206-AE 1344123 in the report)
Purity:	98.5%
Batch no.:	NLL733-9a

2. Vehicle and/or positive control

Vehicle:	Deionised water
Positive control:	-S9: Ethyl methanesulphonate (EMS), 900 µg/mL +S9: Dimethylbenzanthracene (DMBA), 20µg/mL

3. Activation:

Metabolic activation was provided by S9 mix. The S9 fraction was isolated in house from the livers of Aroclor 1254 induced male SD rats and was kept frozen at -80°C. Samples of the batch were tested for contamination and cytotoxicity prior to use. To prepare the S9 mix, two parts of the thawed S9 fraction were mixed with three parts of freshly dissolved co-factors (in sodium phosphate buffer) to give the following final concentrations:

$MgCl_2$ x 6H2O (8mM)

KCl (33mM)

Glucose-6-phosphate (5mM)

NADP (1mM)

S9 fraction (40% v/v)

Sodium phosphate buffer (60% v/v)

The S9 mix was stored on ice until use the same day.

4. Cell cultures and media:

Cell cultures

V79 cell stocks (derived from Chinese hamster lung cells) are stored in liquid nitrogen. Laboratory cultures are maintained in plastic tissue culture vessels at 37°C in a humid atmosphere containing 5% CO_2. Exponential growth was maintained by twice weekly sub-culturing. The cells were checked for mycoplasma contamination of which there was no evidence. To reduce the number of spontaneous 6-TG resistant mutants cell cultures were sub-cloned by plating 1000 cells/culture vessel at least every two weeks. If necessary the spontaneous frequency of HPRT-mutants was further reduced by the addition of thymidine (9µg/mL), hypoxanthine (10µg/mL), glycine (22.5µg/mL and methotrexate (0.3µg/mL). A 6-TG sensitive sub-clone was then used for the HPRT test.

In all parts of the study incubation was performed at 37°C humidified air with 5% CO_2.

Media

Cells were maintained in hypoxanthine free Eagle's Minimal Essential medium (MEM, Gibco) which has been proven suitable for growth of V79 cells. The MEM was supplemented nonessential amino acids, L-glutamine (2mM), MEM-vitamins, $NaHCO_3$, penicillin (100 units/mL, streptomycin (100 µg/mL) and heat activated foetal calf serum (10%; reduced to 2% in treated cultures).

For the selection of mutants, a hypoxanthine-free culture medium was used containing 10µg/mL of 6-thioguanine (6-TG)

5. Test substance concentrations used:

The following test concentrations were used for the mutation assays (with and without metabolic activation provided by S9 mix):

Preliminary cytotoxicity assay: 12.5, 25, 50, 125, 250, 500, 1250, 2500 & 5000 µg/mL (+/- S9)

Mutation assays: 85, 170, 340, 680, 1360 & 2720 µg/mL (+/-S9)

B. Test performance

Experimental phase: August 13 to December 17, 2003

1. Preliminary assay

A preliminary cytotoxicity assay was conducted with and without metabolic activation at the following concentrations in order to select the dose levels for the mutation assays:

12.5, 25, 50, 125, 250, 500, 1250, 2500 & 5000 µg/mL

Cell cultures for the preliminary toxicity assay received the same treatment as in the mutation assays. For each concentration one culture was used.

2. Main assay

Without S9

Exponentially growing V79 cells were plated in culture medium at a final volume of 20 mL in two 250 mL flasks/concentration ($4x10^6$ per flask) including controls. Following attachment (16-24 hours), the cells were exposed for 5 hours in 20 ml culture medium with reduced serum (2%). Corresponding controls received the same treatment. Thereafter, cell monolayers were washed with PBS, trypsinised and re-plated in 20 mL culture medium using 200 cells per petri dish (one flask and three petri dishes were used per culture). Petri dishes were incubated for 6 days to allow colony development and determine the 'survival to treatment).

Cells in 250 mL flasks were incubated to permit growth and expression of 1 induced mutation. Cells were sub-cultured (=count 1, 3 days) by re-seeding 1.5 x 10^6 cells into 20 ml medium in 250 mL flasks. Following the expression period (=count 2, total 6 days) cultures were re-seeded in petri dishes at $3x10^5$ cells per dish (8 dishes per culture) in 20 mL culture medium without hypoxanthine but containing 10 µg/mL 6-Tg for mutant selection. In addition, 200 cells per dish (3 per culture) were seeded in 5 mL culture medium to determine absolute cloning efficiency for each concentration.

After 6-8 days incubation, the colonies were fixed, stained, and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes. Two non-activation trials were performed.

With S9

The activation assay was performed independently; the procedure was identical to the non-activation assay described above except for the addition of S9 mix. In the activation assay 19mL of culture medium with 1 mL of S9 was used (rather than 20 mL of culture medium) during the treatment period, resulting in a concentration of 5% S9 mix in the cultures.

3. Acceptance Criteria

The assay was considered valid if the following criteria were met:

- The average cloning efficiency of the negative and vehicle controls should be at least 50%
- The average of mutant frequency of the vehicle control should not exceed $25x10^{-6}$ cells
- The mutant frequency of the two cultures of the vehicle and negative controls should only differ to an acceptable extent (not greater than $5x10^{-6}$)
- The positive control should induce an average mutant frequency of at least 3 times the vehicle control
- If not limited by solubility, the highest concentration should induce cytotoxicity of about 80-90% or should be precipitative concentration. The survival at the lowest concentration should be in the range of the negative control
- For the calculation of an acceptable mutant frequency at least 5 dishes per culture should be available and relative survival to treatment, relative population growth and absolute cloning efficiency should be 10% or greater

4. Criteria for a positive response

The test substance is classified as mutagenic if:

- A concentration related increase in mutant frequencies is observed in parallel cultures.
- The increase in mutant frequencies should be 2-3 times higher than the highest negative or vehicle control in the respective trial
- The result should be reproduced in the second trial
- the increase should occur in the absence of a change in osmolality compared to the vehicle control

A test substance is considered negative if:

- there is no reproducible or relevant increase in mutant frequencies

A test substance will be considered equivocal if:

- There is no strict concentration related increase in mutant frequencies, but one or more concentrations induce a reproducible and biologically relevant increase in mutant frequencies

Sound scientific judgement should be used in implementing the above criteria.

5. Statistical analysis

The statistical analysis relies on the mutant frequencies which are submitted to a weighted analysis of variance and a weighted recursive regression, both with Poisson derived wights.

II. Results and Discussion

A. Preliminary cytotoxicity assay

There was no effect on the osmolality of the medium up to the highest concentration tested (5000 µg/mL); there was no effect on the pH of the medium up to 625 µg/mL. No precipitation or cytotoxicity was observed up to the highest tested concentration tested, either in the presence or absence of S9 mix. Therefore, based on the changes in pH in the media, concentrations of 85 to 2020 µg/mL were selected for the first main mutation assay; the same concentrations were used in the independent repeats.

B. Main mutation assays

Without metabolic activation

Two trials were performed in the absence of S9 mix. Concentration related decreases in relative population growth were observed in all treated cultures.

M-10 induced statistically significant increases in mutant frequencies in the absence of S9; however, the increase was neither concentration related nor reproducible.

The mutation frequencies of the negative and vehicle controls were all within the normal range and the positive control EMS induced a clear mutagenic and statistically significant effect in both trials, thus demonstrating the validity of the assay.

With metabolic activation

Three trials were performed in the absence of S9 mix. Cytotoxic effects were seen in all trials; concentration related decreases in relative population growth in all treated cultures were observed.

M-10 induced weak but statistically significant increases in mutant frequencies in the presence of S9; therefore, M-10 is considered to be a weak mutagen in the presence of metabolic activation.

The mutation frequencies of the negative and vehicle controls were all within the normal range and the positive control DMBA induced a clear mutagenic and statistically significant effect in both trials, thus demonstrating the validity of the assay.

The results from three experiments with S9 and two experiments without S9 are summarised in the tables below:

Table 5.8.1- 145: Relative survival and mutant frequencies in experiment 1 without metabolic activation

Treatment	Concentration (µg/ml)	Relative population growth (%)	Mutation frequency $10E^{-6}$
M-10	85	ne	ne
	85	ne	ne
	170	176.0	1.7
	170	84.3	1.8
	340	134.8	0.5
	340	113.9	0.4
	680	118.5	1.4
	680	49.1	0.5
	1360	96.1	2.4
	1360	75.4	4.6*
	2720	71.6	1.5
	2720	55.9	0.5
Negative control	-	121.4	0.5
	-	84.1	1.3
Solvent control	-	100.0	1.1
	-	100.0	0.7
Positive control EMS	900	53.7	590.6*
	900	37.6	889.5*

*statistically significant from controls (α=0.05) in one-sided dunnett test

Ne= not evaluated due to cytotoxicity

Table 5.8.1- 146: Relative survival and mutant frequencies in experiment 2 without metabolic activation

Treatment	Concentration (µg/ml)	Relative population growth (%)	Mutation frequency $10E^{-6}$
M-10	85	128.3	12.5
	85	103.2	7.2
	170	123.0	2.4
	170	72.2	1.0
	340	123.5	1.0
	340	94.8	0.8
	680	74.2	2.2
	680	74.7	2.1
	1360	79.1	6.6
	1360	69.6	7.9
	2720	51.7	6.0
	2720	54.6	10.4*
Negative control	-	89.1	4.2
		76.2	1.5
Solvent control		100.0	5.3
	-	100.0	1.6
Positive control EMS	900	44.0	566.1*
	900	31.8	467.2*

*statistically significant from controls (α=0.05) in one-sided dunnett test

Table 5.8.1- 147: Relative survival and mutant frequencies in experiment 1 with metabolic activation

Treatment	Concentration (µg/ml)	Relative population growth (%)	Mutation frequency $10E^{-6}$
M-10	85	60.0	0.8
	85	42.5	0.7
	170	ne	ne
	170	ne	ne
	340	34.4	0.9
	340	30.2	0.9
	680	32.5	0.8
	680	24.2	1.8
	1360	98.7	5.9
	1360	11.7	7.5*
	2720	20.1	4.5
	2720	6.9	10.0
Negative control	-	158.3	0.5
	-	134.7	0.6
Solvent control	-	100.0	1.3
	-	100.0	1.0
Positive control DMBA	20	25.0	105.3*
	20	28.0	92.8*

*statistically significant from controls (α=0.05) in one-sided dunnett test

Ne= not evaluated due to cytotoxicity

Table 5.8.1- 148: Relative survival and mutant frequencies in experiment 2 with metabolic activation

Treatment	Concentration (µg/ml)	Relative population growth (%)	Mutation frequency $10E^{-6}$
M-10	85	133.9	9.9
	85	113.0	8.0
	170	91.1	4.8
	170	124.3	6.6
	340	161.1	15.7
	340	72.7	13.6*
	680	71.2	8.8
	680	82.6	10.3
	1360	89.1	8.8
	1360	51.6	11.0
	2720	61.9	33.2*
	2720	46.4	17.7
Negative control	-	106.3	6.0
		99.5	3.1
Solvent control		100.0	4.7
	-	100.0	6.1
Positive control DMBA	20	41.9	79.4*
	20	41.9	121.2*

*statistically significant from controls (α=0.05) in one-sided dunnett test

Table 5.8.1- 149: Relative survival and mutant frequencies in experiment 3 with metabolic activation

Treatment	Concentration (µg/ml)	Relative population growth (%)	Mutation frequency $10E^{-6}$
M-10	85	43.1	5.9
	85	28.2	4.1
	170	43.9	3.3
	170	28.2	0.4
	340	26.4	2.5
	340	24.9	0.4
	680	19.2	3.1
	680	18.1	7.4
	1360	8.9	6.2
	1360	14.8	20.5*
	2720	ne	9.7
	2720	ne	11.3
Negative control	-		4.5
	-		4.7
Solvent control	-		1.1
	-		1.3
Positive control DMBA	20		226.7*
	20		197.3*

*statistically significant from controls (α=0.05) in one-sided dunnett test

Ne= not evaluated due to cytotoxicity

III. Conclusion

M-10 induced weak but statistically significant and concentration related increases in mutant frequencies in mammalian cells (Chinese hamster lung V79 cells) in the presence of metabolic activation provided by S9 mix. Appropriate responses exhibited by the concurrent negative, vehicle and positive controls confirmed the validity of the assay.

Therefore, M-10 is considered weakly mutagenic under the conditions of this assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD 476 and is valid and acceptable to assess the potential of M-10 to induce gene mutations at the HPRT locus in Chines Hamster Lung V79 cells. M-10 is weakly mutagenic in mammalian cells under the conditions of this study.

Data Point:	KCA 5.8.1/32
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE 1344123: Induction of micronuclei in the bone marrow of treated mice
Report No:	C041698
Document No:	M-231208-01-1
Guideline(s) followed in study:	OECD 474 (1997); ICH Tripartite Harmonised Guideline on Genotoxicity (1995).
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no calibration plot or calibration equation presented, however the calibration range and correlation coefficient (0.9999) are reported. For the accuracy and precision data, there is only 1 concentration level prepared in matrix (LOQ samples). However, there are 6 determinations at this level with a mean recovery between 70-110% and RSD <20%. Considering that this analytical method is validated in support of a toxicological cold study, the method validation is considered fit for purpose. Study: 2000 rather than 4000 immature erythrocytes were analysed
Previous evaluation:	yes, evaluated and accepted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The micronucleus test was employed to investigate M-10 in male CD-1 mice for a possible clastogenic effect on the chromosomes of bone marrow erythroblasts.

Cyclophosphamide (a known clastogen and cytostatic agent) served as the positive control.

Male mice treated with M-10 received two intraperitoneal administrations of 1000 mg/kg bw, separated by 24 hours. Males of the positive control received a single intraperitoneal treatment of 40 mg/kg bw cyclophosphamide. Doses were based on a preliminary dose-range finding assay in which no deaths or clinical signs were seen up to and including 1000 mg/kg bw/d. Therefore, this was selected as the limit dose in the micronucleus test.

The femoral marrow of all groups was prepared 24 hours after the last administration.

Males treated twice with M-10 in doses up to and including 1000 mg/kg bw did not show any signs of toxicity and there was no difference in the ratio of polychromatic to normochromatic erythrocytes between the control and treated groups; however, the route of administration (i.p) means that the test substance is highly likely to have reached the target tissue, particularly at the high-dose (limit dose) of 1000 mg/kg bw/d) employed in this study.

Following two intraperitoneal treatments of males with doses of 1000 mg/kg bw no indications of a clastogenic effect of M-10 were found.

The positive control produced a clear clastogenic effect, as demonstrated by a biologically relevant increase in polychromatic erythrocytes with micronuclei.

It was concluded that M-10 is neither clastogenic nor aneugenic under the conditions of this assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-10 (referred to as AE 1344123 in the report)
Purity:	98.2%
Batch no.:	NLL 7333-9A

2. Vehicle and/or positive control

Vehicle:	Purified water
Positive control:	2 mg/mL cyclophosphamide (CPA) in saline

3. Test animals

Species:	Mice
Strain:	Crl:CD-1 (ICR)BR
Age:	5-6 weeks
Weight at start:	22-33 g
Source:	Charles River UK Ltd, Margate, UK
Acclimation period:	At least five days
Diet:	Special diets services Ltd. Provided *ad libitum*
Water:	Provided *ad libitum*
Housing:	Housed in groups of ≤3 of the same sex in solid floored cages with wood shavings
Identification	Cage markings and ear tags
Temperature:	19-25°C
Humidity:	40-70%
Air changes:	15/hour
Photoperiod:	12 hours light/12 hours dark

4. Test substance doses

The selection of doses was based on initial range-finding study using animals of the same source, strain, and age. In the range-finding study groups of 4 sex mice were treated with the test substance at a dose of 2000 mg/kg bw/d. Animals were dosed for two consecutive days and observations were made for 2 days following the second dose. Clinical signs and body weights were recorded, and a maximum acceptable dose selected for use as the high dose in the main study.

There were no deaths or clinical signs of toxicity; therefore, the main study was conducted with a single limit dose of 2000 mg/kg bw/d. There were no significant sex differences and so only males were included.

B. Test performance

Experimental phase: December 8, 2003 to February 16, 2004

1. Treatment and sampling times

The study design of the main study was as follows:

Table 5.8.1- 150: Study design

Experimental group	Dose in mg/kg bw	No. of animals	Route and number of applications	
Negative control	0	6	i.p.	2
M-10	1000	6	i.p.	2
Positive control (Cyclophosphamide)	40	6	i.p.	1

Male mice (six/group) received two intraperitoneal administrations of 1000 mg/kg bw, separated by 24 hours. Males of the positive control received a single intraperitoneal treatment with 40 mg/kg bw cyclophosphamide, whilst two injections of the negative control (1% MC) were administered the same way as the treated groups. In all groups the administered volume was 20 mL/kg.

2. Tissues and cells examined

Mice were killed in groups, 24-hours following the final dose, by CO_2 asphyxiation. Both femurs from each animal were exposed, removed, cleaned of adherent tissue and the end removed from the shanks. Using a syringe and needle, bone marrows were flushed from the marrow cavity with 1mL foetal bovine serum into centrifuge tubes. The tubes were centrifuged (1250 x g, 2-3 minutes) and the serum was aspirated to leave 1-2 drops and the cell pellet. The pellet was mixed into the small volume of serum in each tube and a smear prepared from a small volume of the suspension on of each of 2 slides. Slides were air dried, fixed and rinsed. One slide from each set of 2 was then taken, the other was reserved. Following a second fixing/rinsing slides were stained for 10 minutes in filtered Giesma stain diluted 1:6 (v/v) in distilled water. Stained slides were rinsed and allowed to dry thoroughly before clearing in xylene for 3 minutes. When dry the slides were mounted with cover slips.

3. Evaluation

Initially the relative proportions of polychromatic erythrocytes (PCE), seen as pale blue or blue/grey enucleate cells, and normochromatic erythrocytes, seen as smaller yellow/orange stained enucleate cells, were determined until a total of 1000 (PCE plus NCE) had been analysed.

Counting of PCE cells continued until 2000 PCE had been observed.

The ratio of PCE/NCE for each animal and the mean for each group was calculated. The individual and group mean frequency of micronucleated PCE/1000 cells (+/- SD) was also established. A reduction in the PCE/NCE ratio would provide evidence of bone marrow toxicity (exposure).

4. Evaluation criteria

The assay is considered acceptable if the following criteria are met:

- The incidence and distribution of micronucleated PCE in the concurrent vehicle control group is comparable with the historical negative controls
- At least 5/males/group are available for analysis
- The positive control chemical (CPA) induces statistically significant increases in the frequency of micronucleated PCE

A test item is considered positive if:

- A statistically significant increase in the frequency of micronucleated PCE occurs in at least one dose
- Any increase is outside the range of the historical control data

5. Statistical methods

The frequencies of micronucleated PCE in the vehicle control animals were compared to the historical negative control data to determine the acceptability of the assay. For each group, inter-individual variation in the numbers of micronucleated PCE was estimated by means of a heterogeneity chi^2 test.

The numbers of micronucleated PCE in each treated group were then compared with the numbers in the vehicle control groups by using a 2 X 2 contingency table to determine chi^2. Probability values of $p = \leq 0.05$ were to be accepted as significant. A further statistical test (for linear trend) was used to evaluate possible dose-response relationships.

If the heterogeneity chi^2 test provides evidence of significant ($p=\leq 0.05$) variability between animals within at least one group then non-parametric analysis will be more appropriate, a Wilcoxon rank sum test will then be used.

II. Results and Discussion

A. Micronucleus assay

Following two intraperitoneal administrations of 1000 mg/kg bw there were no deaths or clinical signs of toxicity.

Treated mice exhibited group mean ratios of PCE to NCE which were similar to the concurrent negative controls and fell within the historical negative control range.

The frequency of micronucleated PCE in the treated groups were not significantly different from the vehicle controls. Some vehicle and tested mice exhibited micronuclei frequencies that were outside the range of the historical control data; a subsequent evaluation found that the MN frequencies were likely to be the result of misclassification of micronuclei by the analyst, as a precaution the slides were re-scored in a peer review and the negative result was confirmed.

An overview of the genotoxicity evaluation is provided below.

Table 5.8.1- 151: Group mean PCE/NCE ratios and incidences of micronucleated PCE and NCE (initial analysis)

Treatment group	Dose (mg/kg bw)	No. of animals	Mean PCE/NCE	No. micronucleated PCE/2000 ± SD
Vehicle control	0	6	1.04	2.08±0.38
M-10	250	6	0.93	1.67±0.52
Positive control (Cyclophosphamide)	40	6	1.10	13.42±4.50

Table 5.8.1- 152: Group mean PCE/NCE ratios and incidences of micronucleated PCE and NCE (peer review analysis)

Treatment group	Dose (mg/kg bw)	No. of animals	Mean PCE/NCE	No. micronucleated PCE/2000 ± SD
Vehicle control	0	6	1.02	1.33±1.40
M-10	250	6	1.04	0.33±0.26
Positive control (Cyclophosphamide)	40	6	1.00	14.25±6.82

III. Conclusion

There was no indication of a clastogenic effects in male mice following two intraperitoneal treatments with doses of 2000 mg/kg bw.

Bone marrow exposure could not be demonstrated by a depression of the ratio of polychromatic to normochromatic erythrocytes (PCE/NCE ratio at 1000 mg/kg bw/d was comparable to controls) and there were no clinical signs of toxicity; however, M-10 was tested up to the limit dose and the method of dose administration (i.p) is likely to lead to exposure of the target tissue.

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered M-10 in the micronucleus test in male mice, i.e. in a somatic test system *in vivo*.

Assessment and conclusion by applicant:

This study was conducted according to OECD TG 474 and is valid and acceptable to determine the clastogenic potential of M-10 *in vivo*. M-10 was not clastogenic under the conditions of this study and there was no evidence of aneugenicity.

Data Point:	KCA 5.8.1/33
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE 1344123: Measurement of unscheduled DNA synthesis in rat liver using an in vivo/in vitro procedure Final report
Report No:	C041629
Document No:	M-231091-01-1
Guideline(s) followed in study:	OECD: 486 1997); UKEMS guidelines (1993)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

M-10 (reported as AE 1344123) was tested for its ability to induce unscheduled DNA synthesis (UDS) in the livers of male rats dosed via oral gavage using an *in vivo/in vitro* procedure.

Groups of four male rats were treated once with the vehicle (purified water), M-10 (at 800 mg/kg or 2000 mg/kg) or the required positive control via oral gavage, at a dose volume of 10 mL/kg. The positive controls used were 75 mg/kg 2-acetamidofluorene (2-AAF) suspended in corn oil (12-14 hour experiment) and 10 mg/kg dimethyl nitrosamine (DMN) dissolved in purified water (2-4 hour experiment). No clinical signs of toxicity were observed in the main study.

No clinical signs were observed in the main study.

Approximately 12-14 hours (Experiment 1) or 2-4 hours (Experiment 2) after dosing, animals were sacrificed, and their livers perfused with collagenase to provide a primary culture of hepatocytes. Cultures were made from three animals in each dose group and were treated with [^{3}H] thymidine. Six slides (where available) from each animal were prepared with fixed hepatocytes and of these, three were dipped in photographic emulsion to prepare autoradiograms. Slides were examined microscopically after development of the emulsion and staining, and the net grain count (NNG), the number of grains present in the nucleus minus the mean number of grains in three equivalent areas of cytoplasm, was determined for each of two of the three slides, each animal and dose group.

Negative (vehicle) control animals gave a group mean NNG value 0.6 or less with no more than 0.3% cells in repair. Group mean NNG values were increased by 2-AAF and DMN treatment to more than 6.6 and more than 60% cells found to be in repair. In this study the vehicle control NNG value was consistent with both published and historical control data, and the system was shown to be sensitive to two known DNA damaging agents requiring metabolism for their action. The assay was therefore accepted as valid.

Treatment with 800 or 2000 mg/kg M-10 did not produce a group mean NNG value greater than 0.4 and no cells were found in repair at either dose.

When treated once via oral gavage with M-10 at doses up to 2000 mg/kg male rats showed no induction of UDS in hepatocytes isolated *ex vivo* approximately 12-14 or 2-4 hours after dosing. It is concluded that M-10 had no genotoxic activity detectable in this test system under the experimental conditions employed.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-10 (referred to as AE 1344123 in the report)
Purity:	98.2%
Batch no.:	NLL 7333-9A

2. Vehicle and/or positive control

Vehicle:	Purified water
Positive control:	2-Acetamidofluorene (2-AAF; 12-14 hour experiment) Dimethymitrosamine (DMN; 2-4 hour experiment)

3. Test animals

Species:	Rat, male
Strain:	Wistar Crl:WI (Glx/BRL/Han)
Age:	7 to 8 weeks
Weight at start:	206 - 254 g
Source:	[redacted]
Acclimation period:	6 – 9 days
Diet:	Standard rodent diet
Water:	Provided *ad libitum*
Housing:	Groups of four in solid-floored cages
Temperature:	19.5-20.4°C
Humidity:	57-68%
Air changes:	Fifteen times/hour
Photoperiod:	12 hours light/12 hours dark

4. Test substance doses

The Sponsor indicated that in an acute oral toxicity study in rats the LD_{50} of M-10 was higher than 2000 mg/kg. For non-toxic compounds, the maximum recommended dose (according to current test guidelines) is 2000 mg/kg. The maximum recommended dose for the UDS experiments was therefore 2000 mg/kg and a lower dose of 40% of the maximum dose was also tested. As there was no evidence of substantial inter-sex differences in toxicity or likely sex-specific human exposure, only male animals were tested in this study.

B. Test performance

Experimental phase: 5 January to 2 March 2004

1. Treatment and sampling times

Animals were weighed before dosing and the volume of vehicle, test article preparation or positive control solution to be administered was calculated based on a dose volume of 10 mL/kg. Animals were not fasted prior to dosing. All treatments were given via oral gavage to maximise exposure of the test article to the target organ.

In the UDS experiments, groups of four male rats were treated as detailed in the following table.

Table 5.8.1- 153: UDS *in vivo* test design

Experimental group	Dose (mg/kg)	No. of animals	
		Experiment 1 (12-14 hour)	Experiment 2 (2-4 hour)
Negative control (purified water)	0	4	4
M-10	800	4	4
	2000	4	4
Positive control			
2-AAF	75	4	-
DMN	10	-	4

Information supplied by the Sponsor confirmed test item formulations in water in the range 20 to 20000 mg/mL were homogenous and stable for up to 8 days when stored at room temperature.

Dosing preparations were made by dissolving M-10 in purified water with the aid of stirring to give concentrations of 80 and 200 mg/mL. The pH of each formulation was adjusted to approximately pH 6. The test item preparations were protected from light, stored at room temperature and used within 3 days of initial formulation.

Samples were taken from each test item formulation and analysed to determine achieved concentration.

The negative (vehicle) control was purified water.

2-Acetamidofluorene (2-AAF) was freshly suspended in corn oil using a Silverson homogeniser at 7.5 mg/mL to serve as the positive control for the 12-14 hour experiment. Dimethylmitrosamine (DMN) dissolved in purified water at 1.0 mg/mL was used as the positive control for the 2-4 hour experiment.

2. UDS test

Killing of animals and preparation of hepatocyte cultures.

For Experiments 1 and 2, animals were sacrificed a nominal 12-14 hours and 2-4 hours after dosing, respectively.

Individual animals were anaesthetised with halothane and maintained under deep anaesthesia to prevent any likelihood of recovery. The liver was surgically exposed, the hepatic portal vein and superior vena cava cannulated with suitable cannula and the liver perfused with suitable buffers. Approximately 400 mL of calcium free

Buffer 1 was pumped at a flow rate of approximately 40 mL/min to wash the liver free of blood. The liver was then perfused with Buffer 2 also at a flow rate of 40 mL/min for approximately 5 minutes. Both buffers were gassed with 5% CO_2 in air (v/v) prior to use and Buffer 2 throughout perfusion.

Calcium and collagenase were added to the reservoir and after one to two minutes the waste line was placed in the Buffer 2 reservoir so that the perfusate recirculates and the flow rate was reduced to 20 mL/min. When the reticular pattern of the liver had begun to break up and the liver became 'spongy', the perfusion was stopped.

The liver was cut free into a suitable container with Buffer 2. The liver was transferred to a sterile dish, cut open and the hepatocytes carefully teased out. The resulting hepatocyte suspension was gently washed through 190 µm nylon mesh with Williams E Medium-Complete (WE-C) to a volume of approximately 100 mL. Of this suspension, approximately 50 mL was taken and centrifuged at approximately 40 x 'g' for two to three minutes. The resultant pellet was resuspended in WE-C. The centrifugation and resuspension procedure was repeated at least twice and the pellet resuspended finally in approximately 20 mL WE-C. A sample (0.5 mL) of this suspension was taken, diluted with an equal

volume of 0.4% (w/v) trypan blue in phosphate buffered saline (PBS) and the proportion of viable cells (those with unstained nuclei) determined using an haemocytometer. The culture was diluted where possible to provide approximately 1.5 x 10^5 viable cells/mL.

Three mL of hepatocyte suspension was added to each well of a six-well multiplate containing 25mm round plastic coverslips and incubated at 37°C ± 1°C in a 5% CO_2 in air (v/v) atmosphere for at least 90 minutes to allow cells to attach.

Radiolabelling of hepatocyte cultures:

Medium was removed from the cells and the monolayers washed with 2mL Williams E Medium-Incomplete (WE-I) which was then replaced with 2 mL WE-I containing 10 µCi/mL [^{3}H] thymidine. After approximately 4 hours incubation at 37°C in a 5% CO_2 in air (v/v) atmosphere the medium was removed, and the cells washed with three changes of WE-I containing 0.25 mM thymidine. Cultures were then incubated overnight with 3 mL of the same medium.

To prepare for autoradiography, coverslips were washed with 2mL phosphate buffered saline (PBS) and the cells fixed with three changes of 2 mL glacial acetic acid:ethanol (1:3 v/v). The coverslips were then washed four times with purified water, allowed to dry and mounted onto previously labelled microscope slides, cells side up, with DPX.

Autoradiography:

Three of the five or six slides from each animal were coated in Ilford K2 liquid emulsion using a dipping technique. The slides were then packed in light tight boxes containing desiccant, sealed with tape and refrigerated for 14 days. At the end of this time, the emulsion was developed in Kodak D19 developer and fixed using Ilford Hypam fixer. The cell nuclei and cytoplasm were then stained with Meyers haemalum/eosin Y. Slides were then dehydrated in ethanol, cleared in xylene and mounted with coverslips for microscopic examination. The spare, duplicate sets of slides were not required.

Grain counting:

Grain counting was performed using a microscope with a video camera connected to an image analysis system (Perceptive Instruments) and a computer programmed for automatic data capture.

Each slide was examined to ensure that the culture was viable. A patch of cells was selected as a starting point and cells were scored in a regular fashion by bringing new cells into the field of view, moving only in one axis. If the desired number of cells had not been scored before coming to the edge of the slide, the stage was moved one or two fields on the other axis and counting resumed. The circular field was centred over the nucleus of a suitable cell and the grains counted. The field of view was moved and counts obtained for three separate adjacent areas of cytoplasm. Nuclear and mean cytoplasmic grain counts were then recorded, and the net grains/nucleus (NNG) determined. 100 cells were analysed per animal, where possible using two of the three slides in each case.

The following criteria were used for cell analysis:

1. only cells with normal morphology were scored
2. isolated nuclei with no surrounding cytoplasm were not scored
3. cells without nuclear and/or cytoplasmic graining were not scored
4. cells with unusual staining artefacts were not scored
5. heavily labelled cells in S-phase were not scored
6. all other normal cells, 100 per animal were scored
7. all slides were analysed blind (coded).

Treatment of data:

The following were calculated for each slide, animal and dose point:

1. the population average NNG and standard deviation (SD)
2. the percent of cells responding or in repair (ie ≥ NNG)
3. the population average cytoplasmic and nuclear grain count.

3. Evaluation

An assay is considered acceptable if the following criteria are met:

- the negative control animals had a group mean NNG value that did not exceed the upper limit of the historical range
- The positive control treatments should have group mean values of five or more NNG with 50% or more cells having NNG counts of five or greater.

The test item would be considered as positive in this assay if, at any dose and at either time point:

1. the test item yielded group mean NNG values greater than 0 NNG and 20% or more of cells responding (mean NNG values ≥ 5)
2. an increase above solvent control levels was seen in both NNG and the percentage of cells in repair.

Cytoplasmic and nuclear grain count values as well as the concurrent negative control data would be considered in relation to the overall NNG values of cultures from treated animals.

If the test article failed to induce UDS at any dose tested after both 2-4 and 12-14 hours exposure, it would be considered clearly negative in this system.

4. Statistical methods

No statistical analysis of the generated data was undertaken.

II. Results and Discussion

A. UDS assay

Chemical analysis of the dose preparations of M-10 used in the main UDS experiments confirmed that the achieved concentrations of M-10 were within 100%±10% of nominal and were therefore acceptable for dosing.

No clinical signs of toxicity were observed in the main study experiments.

The results of the DNA repair test with 12-14 and 2-4 hour exposure periods are summarised in the tables below:

Table 5.8.1- 154: Mean nuclear and cytoplasmic grain counts following 12-14 hour exposure

Treatment group	Dose (mg/kg bw)	Mean gross nuclear grain count (NG)	Mean cytoplasmic grain count (CG)	Mean net nuclear grain count (NNG)
Negative control Water	0	1.69±0.02	1.15±0.40	0.55±0.00
M-10	800	2.18±0.23	1.78±0.11	0.40±0.12
	2000	1.95±0.18	1.87±0.31	0.08±0.49
Positive control 2-AAF	75	8.45±0.59	1.84±0.31	6.61±0.88

Table 5.8.1- 155: Mean nuclear and cytoplasmic grain counts following 2-4 hour exposure

Treatment group	Dose (mg/kg bw)	Mean gross nuclear grain count (NG)	Mean cytoplasmic grain count (CG)	Mean net nuclear grain count (NNG)
Negative control Water	0	1.85±0.08	2.93±0.26	-1.08±0.19
M-10	800	1.73±0.13	3.08±0.03	-1.35±0.09
	2000	1.75±0.15	2.98±0.15	-1.22±0.04
Positive control DMN	10	14.57±0.98	2.47±0.20	12.09±0.83

Treatment with M-10 at doses up to 2000 mg/kg yielded NNG values less than the concurrent negative control and which also fell within the laboratory's historical control range. Group mean NNG values over the two experiments were in the range -1.2 to 0.4 with no cells seen in repair at any dose of M-10.

The data obtained in this study indicate that treatment *via* oral gavage of male rats dosed once with 800 or 2000 mg/kg M-10 did not result in increased UDS in hepatocytes isolated approximately 12-14 or 2-4 hours after dosing.

The group mean net grain count for vehicle-treated animals was less than the upper limit of the historical control range (0.6 and -1.1 for 12-14 hour and 2-4 hour experiments respectively). The positive control chemicals 2-AAF and DMN induced increases in group mean net grain count of five or more (6.6 and 12.1 respectively), and 50% or more of cells (69% and 100% respectively) had net grain counts of five or more. these results showed that the test system was sensitive to two known DNA damaging agents requiring metabolism for their action and that the experiment was valid.

III. Conclusion

When treated once *via* oral gavage with M-10 at doses up to 2000 mg/kg male rats showed no induction of UDS in hepatocytes isolated *ex vivo* approximately 12-14 or 2-4 hours after dosing. It is concluded that M-10 had no genotoxic activity detectable in this test system under the experimental conditions employed.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 486 and is valid and acceptable to assess the potential of M-10 to induce DNA damage and repair *ex vivo*. M-10 did not induce unscheduled DNA repair in the rat liver *ex vivo* under the conditions of this study.

Data Point:	KCA 5.8.1/68
Report Author:	[redacted]
Report Year:	2019
Report Title:	AE 1344123: Micronucleus test in human lymphocytes In vitro
Report No:	1969605
Document No:	M-673682-01-1
Guideline(s) followed in study:	OECD Test Guideline No. 487 (July 2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

M-10 (referred to as AE 1344123 in the report) was evaluated for its potential to induce micronuclei in human lymphocytes *in vitro*, in two independent experiments. Experiment I comprised a four hour exposure period in the presence and absence of metabolic activation (provided by S9) whilst experiment II provided a 20-hour exposure period in the absence of S9. Concentrations up to 2030 µg/mL were tested in duplicate and 1000 binucleated cells/culture were examined for cytogenetic damage.

In experiment I, no cytotoxicity or precipitation was observed (+/- S9) up to the highest evaluated concentration. Similarly, in experiment II (-S9), no cytotoxicity or precipitation was observed at the highest tested concentration.

No relevant increases in the numbers of micronucleated cells were observed following treatment with the test item either in the presence or absence of S9 mix in either experiment; however, a statistically significant increase was observed in experiment II in the absence of S9 mix at the highest applied concentration (2030µg/mL). The value of 0.75% micronucleated cells is clearly within the range of the historical control data range (0.00-1.14% micronucleated cells) and no dose dependency was observed via a trend test. Therefore, this finding can be regarded as biologically irrelevant.

Appropriate reference substances gave the expected statistically significant increase in the number of micronucleated cells.

The test substance M-10 can be considered neither clastogenic nor aneugenic under the conditions of this *in vitro* micronucleus test, when tested up to the highest possible concentration, both in the presence and absence of metabolic activation.

I. Materials and methods

A. Materials

1. Test material

Test substance:	M-10 (referred to as AE 1344123 in the report)
Purity:	98.5 % (w/w)
Batch no.:	NLL7333-9a
Expiry date:	18th February 2026

2. Vehicle and/or positive control

Vehicle:	10% deionized water
Positive controls:	-S9
	Mitomycin C (MMC), 0.8 µg/mL (98% purity, dissolved in deionized water)
	Demecolcine, 100 ng/mL (purity ≥98%, dissolved in deionized water)
	+S9
	Cyclophosphamide (CPA), 17.5 µg/ml (purity 97-103%, dissolved in saline)

3. Activation:

Metabolic activation was provided by S9-mix derived from phenobarbital/ß-naphthoflavone induced rat liver homogenate; each batch of prepared S9 has been routinely evaluated for its ability to activate known mutagens.

An appropriate quantity of S9 was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation used for this study was 30.9 mg/mL.

4. Cell cultures and media:

Cells

Blood was drawn from healthy non-smoking donors, not receiving medication. Blood from a male donor (20 years old) and a male donor (34 years old) were used in experiments I and II, respectively. The lymphocytes have been shown to respond well to PHA and positive control substances; furthermore, both donors have an established low incidence of micronuclei in peripheral blood lymphocytes.

Media

Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hours following collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1), supplemented with 200 mM GlutaMAX™. The medium was further supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (foetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).

All incubations were carried out at 37 °C with 5.5 % CO2 in humidified air.

5. Test compound concentrations used:

Test concentrations for the first experiment (4-hour pulse treatment) were 0 (solvent control), 15.4, 26.9, 4.17, 82.5, 144, 253, 442, 773, 1353 & 2030 µg/mL both with and without S9 mix.

In the second experiment a continuous (20 hour) treatment was used at test concentrations of 0 (solvent control), 144, 253, 442, 773, 1353 & 2030 µg/mL, in the absence of S9 mix.

B. Test Performance

Experimental phase: 21st August 2019 to 25th October 2019

1. Preliminary assay

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity (characterized by the percentages of reduction in the CBPI in comparison to the controls and expressed as % cytostasis) was determined from 500 cells/culture. The experimental conditions in this pre-experimental phase were identical to those described for the mutagenicity assay.

The pre-test was performed with test item concentrations ranging from 15.4 to 2030 µg/mL, along with solvent and positive controls. All cell cultures were set up in duplicate. Exposure time was 4 hours (with and without S9 mix). The preparation interval was 40 hours after start of the exposure.

This preliminary test was designated Experiment I since the cultures fulfilled the criteria for cytogenetic evaluation and appropriate concentrations could be selected for evaluation.

No cytotoxic effects were observed; therefore, 2030 µg/mL was chosen as the top concentration for experiment II.

2. Cytogenetic experiment

Cells were subject to either 4 hours pulse exposure (with and without S9; experiment I) or 20 hours continuous exposure (without S9; experiment II). The succeeding procedure for cell preparation was the same for both exposure periods.

Pulse exposure

Approximately 48 hours following seeding, two blood cultures of 10 mL each, were set up in parallel in 25 cm² cell culture flasks (for each test item concentration). The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hours the cells were spun down by gentle centrifugation for 5 minutes. The superinatant was discarded, and the cells resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H2O, 192 mg/L Na2HPO4 • 2 H2O and 150 mg/L KH2PO4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

Continuous exposure

Approximately 48 hours following seeding, two blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells re-suspended in and washed with "saline G". The washing procedure was repeated once as described. Following washing, the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for a further 20 hours until preparation.

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

Preparation of cells

Following the procedures above (approximately 40 hours from the start of treatment), the cultures were harvested by centrifugation. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

3. Acceptance Criteria

The micronucleus assay is deemed acceptable if it fulfils the following criteria:

- The concurrent solvent control will normally be within the laboratory historical solvent control data range.
- The concurrent positive controls should produce a statistically significant increase in the micronucleus frequency and should be within the laboratory historical positive control data range.
- Cell proliferation criteria in the solvent control are considered acceptable.
- All experimental conditions described were tested unless one exposure condition resulted in a clearly positive result.
- The quality of the slides should allow the evaluation of an adequate number of cells and concentrations
- The criteria for the selection of top concentrations should be met

Any mutant frequencies not meeting these criteria were excluded from the statistical analyses.

4. Assessment criteria

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative if, in all of the experimental conditions examined:

- None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase
- The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- The increase is concentration-related in at least one experimental condition
- The results are outside the range of the laboratory historical solvent control data (95% control limit realized as 95% confidence interval)

When all of the criteria are met, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system.

There is no requirement for verification of a clear positive or negative response.

In case the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. narrow concentration spacing, other metabolic activation conditions, i.e. S9 concentration or S9 origin) could be useful.

5. Statistical analysis

Statistical significance was confirmed by the Chi square test ($p < 0.05$), using a validated test script of "R", a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those values that indicated an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression was performed using a validated test script of "R", to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Both, biological and statistical significance were considered together.

II. Results and discussion

In experiment I neither precipitation nor cytotoxicity was observed up to the highest applied concentration, either in the presence or absence of S9, and no relevant influence on osmolarity or pH was observed. Similarly, in experiment II, no precipitation or cytotoxicity was observed up to the highest applied concentration. The highest concentration applied (2030µg/ml) was therefore the limit concentration.

The results of both experiments, with and without metabolic activation, are summarised in table 5.8.6.1-22 below:

Table 5.8.1- 156: Summary of results of experiment I and II

Exp	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	Historical control data	
						95% Ctrl limit	Min - Max
Exposure period 4 hrs without S9 mix							
I	40 hrs	Solvent control[1]	1.78		0.20	0.01 – 1.20	0.00 – 1.55
		Positive control[2]	1.69	11.6	**10.15[S]**	2.66 – 22.74	1.95 – 28.60
		773	1.82	n.c	0.10		
		1353	1.79	n.c	0.15		
		2030	1.84	n.c	0.30		
Exposure period 20 hrs without S9 mix							
II	40 hrs	Solvent control[1]	1.93		0.20	0.00 – 1.14	0.05 – 1.60
		Positive control[2]	1.54	41.5	**6.50[S]**	1.15 – 6.44	1.95 – 8.80
		773	1.95	n.c	0.25		
		1353	1.85	8.6	0.15		
		2030	1.94	n.c	**0.75[S]**		
Exposure period 4 hrs with S9 mix							
I	40 hrs	Solvent control[1]	1.83		0.25	0.00 – 1.24	0.10 – 1.30
		Positive control[#4]	1.72	13.9	**2.18S**	1.01 – 7.34	1.80 – 8.85
		773	1.78	6.8	0.40		
		1353	1.82	1.6	0.10		
		2030	1.77	8.9	0.15		

* For the positive control groups and the test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
\# The number of micronucleated cells was determined in a sample of 4000 binucleated cells
S The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c. Not calculated as the CBPI is equal or higher than the solvent control value
[1] Deion. water 10.0 % (v/v)
[2] MMC 0.8 µg/mL
[3] Demecolcine 100 ng/mL
[4] CPA 17.5 µg/mL

In both independent experiments, no biologically relevant increases in the number of micronucleated cells were observed following treatment with the test item either in the presence or absence of S9 mix. However, a statistically significant increase was observed in experiment II in the absence of S9 mix at the highest applied concentration (2030µg/mL). The value of 0.75% micronucleated cells is clearly within the range of the historical control data range (0.00-1.14% micronucleated cells) and no dose dependency was observed via a trend test. Therefore, this finding can be regarded as biologically irrelevant.

Demecolcine (100 ng/mL), MMC (0.8 µg/mL) and CPA (15 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei, thus confirming the validity of the assay to detect clastogens/aneugens.

III. Conclusions

The test substance M-10 did not induce micronuclei in this *in vitro* micronucleus test in human lymphocytes, when tested up to precipitative concentrations.

Therefore M-10 is considered to be neither clastogenic nor aneugenic under the conditions of this assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 487 and is valid and acceptable to assess the clastogenicity of M-10 *in vitro*. M-10 is neither clastogenic nor aneugenic under the conditions of this study.

An *in vivo* Comet assay with M-10 is currently ongoing and will be submitted in November 2020.

Dossier node	Draft title	Study ID	Planned submission
KCA 5.8.1	*In vivo* mammalian Alkaline Comet Assay	TXA0099	November 2020

Short-term toxicity

Data Point:	KCA 5.8.1/34
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE 1344123 (Metabolite of AE C638206) - Subacute toxicity study in rats (Administration in the diet for 4 weeks)
Report No:	C038970
Document No:	M-226127-01-1
Guideline(s) followed in study:	JMAFF 12 Nousan No. 8147 (2000); OECD 407; US-EPA OPPTS 870.3100 (2001)
Deviations from current test guideline:	Kidneys, thyroid and bone/bone marrow were not examined histopathologically at sacrifice.
Previous evaluation:	yes, evaluated and accepted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Groups of 10 male and 10 female Wistar rats were administered M-10 (reported as AE C657378) in the diet at dose levels of 0, 20, 200, 2,000 or 20,000 ppm (corresponding to actual doses of 1.71,16.34,163.81 and 1748.17 mg/kg body weight per day in males and 2.24,23.52,232.99 and 2298.89 mg/kg body weight per day in females) for 28 consecutive days.

There were no mortalities or clinical signs of toxicity. Daily observations of animals showed diarrhoea in all 20000 ppm animals from week two on. Functional Observational Battery investigations revealed no indications of neurotoxicity.

The body weights were not affected in males and females up to and including 20000 ppm.

The food intake was not adversely influenced up to and including 20000 ppm.

There was no compound related effect on eyes.

Haematological investigations revealed no toxicologically significant effects on red and white blood cells, and on coagulation.

Clinical laboratory tests revealed no toxicologically significant effects on the activity of enzymes, and on the concentrations of substrates, and electrolytes in blood.

Urinalyses revealed no remarkable effects up to and including 20000 ppm.

Organ weights as well as gross and histopathological investigations of organs and tissues gave no indication of test compound related functional or morphological changes in both sexes.

Although diarrhoea was observed in all animals at 20000 ppm from week 2 up to final sacrifice, this clinical sign was not associated with any body weight or food intake changes. In addition, no effects were seen in the gastrointestinal tract at the macroscopic and microscopic examinations of these tissues. Therefore, although this sign was considered as clearly related to the treatment with M-10, due to the lack of additional changes, it was not considered as adverse effect under the conditions of this study.

In conclusion, under the conditions described, the dietary administration of M-10 to male and female rats was tolerated without adverse effects up to and including 20000 ppm. Thus, the NOAEL for M-10 was set at the highest dose tested of 20000 ppm (equivalent to 1748.17 and 2298.89 mg/kg/day, in males and females respectively).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-10 (referred to as AE 1344123 in the report)
Purity:	98.5%
Batch no.:	NLL7333-9a
Expiry:	26/11/2003

2. Vehicle and/or positive control

Vehicle:	Orally via diet

3. Test animals

Species:	Rats
Strain:	Wistar Hsd.Cpb:WU
Age:	5-6 weeks of age
Weight at start:	140 g to 164 g for males and 104 g to 132 g for females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Provimi Kliba 3883.9.25 supplied by Provimi Kliba AG, CH-4303 Kaiseraugst, Switzerland

Water:	Water ad lib
Housing:	Caged in polycarbonate cages Type III h in groups of five or six, by sex during the acclimation period and individually in Type IIA cages during the treatment period
Temperature:	22 ± 2 °C
Humidity:	55 ± 5%
Air changes:	> 10 passages per hour
Photoperiod:	12:12 hours

B. Study design

1. In-life dates: July 31 to September 3, 2003

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 5.8.1- 157: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	10	10
2	20	10	10
3	200	10	10
4	2,000	10	10
5	20,000	10	10

Animals were killed on Study Day 33/34 (males) and Study Day 34/35 (females).

3. Diet preparation and analysis

Prior to the start of treatment, a procedure was developed to prepare homogeneous and stable mixtures of the test material in the laboratory rodent diet at the required nominal concentrations.

Test diets were prepared weekly throughout the study. The test item was mixed into the diet intended for the following week using a mixing granulator (manufactured by the company Lodige, Paderborn, Germany) with the appropriate quantity of laboratory rodent diet.

During the study, the correct concentration in the formulations were checked analytically once including the homogeneity in 20 and 20000 ppm formulations.

The mean results for the test-diet samples, analysed and prepared for dosing, were within the range 94% to 103% of nominal.

Stability was satisfactory over the time of use of the diet (8 days), i.e. % nominal levels declined by a maximum of 12% over 8 days storage at room temperature.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

The following convention has been used to indicate statistical significance:

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

C. Methods

1. Observations

On working days, the experimental animals were inspected twice a day for morbidity and mortality (once on weekends and public holidays). General clinical examinations (in the home cage) were made daily, detailed clinical examinations were performed weekly. Once before the start of treatment and once weekly thereafter animals were placed into a standard arena (open field) for behavioral observations. Any clinical signs (findings) and abnormalities were recorded. Body surfaces and orifices, posture, general behaviour, breathing and excretory products were assessed.

If animals became ill, they were marked (with cage labels) or set apart, observed more frequently and sacrificed prematurely, if death seemed imminent.

2. Body weight and food intake

The weight of the animals was recorded at receipt. The body weights of the individual experimental animals were determined before the beginning of the study and weekly thereafter up to scheduled necropsy. On the day of necropsy body weights were measured for calculation of relative organ weights.

Food intake of each individual animal was determined at comparable periodical intervals (e.g. weekly). These primary data were then used to calculate the group means for each period of approximately 7 days.

3. Ophthalmoscopic examination

Ophthalmological examinations were conducted on each animal prior to the start of treatment and near terminal sacrifice on the control and high dose rats. The pupillary reflex of both eyes was first tested in a darkened room and the anterior regions of the eye were inspected. After dilating the pupils with Mydriaticum Roche® or Mydriaticum Stulln® drops, the refractive elements of the eye as well as iris and fundus were examined using an indirect ophthalmoscope. In addition, the optical media were examined with a ZEISS slit lamp.

4. Neurotoxicity assessments

Near terminal sacrifice the appearance, behaviour and functional integrity of each animal was assessed using a Functional Observation Battery (FOB). The FOB was comprised of a combination of examinations that include observations in the home cage, assessment of the of the animals and their reaction to handling on removal from the cage and observations in an open field standard arena. In addition to the FOB, following manipulative tests were additionally determined: approach response, touch response, auditory response, tail pinch response, righting reflex, grip strength, landing footsplay, body temperature, body weight.

5. Laboratory investigations (haematology, clinical chemistry, urinalysis)

Clinical laboratory investigations on blood and urine samples were performed on all animals per group near terminal sacrifice.

The blood samples for determination of glucose concentrations were directly taken after the urine sampling period from the caudal vein of fasted (for about 16 hrs), non-anesthetized animals.

The blood samples used for determining the other parameters in peripheral blood were collected in the morning from the retro-orbital venous plexus of fasted (for about 16 hrs) animals anesthetized with CO2/O2 (80:20).

Urine samples were collected at room temperature during a period of 16 hours. Prior to the urine collection all animals were given 5 ml tap water/animal by stomach tube. Feed and water were not supplied

The following parameters were determined:

Table 5.8.1- 158: Haematology

Leukocytes	Mean corpuscular volume (MCV)
Erythrocytes	Mean corpuscular haemoglobin (MCH)
Haemoglobin	Mean corpuscular haemoglobin concentration (MCHC)
Haematocrit	
Reticulocytes	
Differential blood count	HQUICK (thromboplastin time)
	Platelets

Table 5.8.1- 159: Clinical Chemistry

Alkaline phosphatase	Creatinine
Aspartate aminotransferase	Total protein
Alanine aminotransferase	Albumin/globulin ratio
Glutamate dehydrogenase	Albumin
Gamma glutamyl transferase	Sodium
Cholesterol	Potassium
Triglyceride	Chloride
Urea nitrogen	Calcium
Total bilirubin	Glucose

Table 5.8.1- 160: Urinalyses

Semi-quantitatively:	Quantitatively:
pH	Volume
Glucose	Specific gravity
Ketones	Protein x Vol
Bilirubin	Protein
Urobilinogen	
Blood	
Microscopy of sediment	

4. Sacrifice and pathology

Animals were killed by exsanguination *via* the abdominal aorta under deep diethyl ether anaesthesia, necropsied and their organs and tissues subjected to thorough gross pathological examination.

The following organs from all animals were weighed at necropsy:

Adrenals	Liver	Testes
Brain	Ovaries/ oviducts	Thymus
Epididymides	Pituitary (fixed)	Thyroids/parathyroids
Heart	Prostate	Uterus were recorded
Kidneys	Spleen	

Representative tissue samples of the following organs were preserved in 10% formalin except for the eyes, kidneys and testes which were fixed in Davidson's solution:

Adrenal glands	Aorta	Brain (3 regions)
Epididymides	Oesophagus	Extra orbital lacrimal glands
Eye lids	Femur	Harderian glands
Head	Heart	Intestine
Larynx	Liver	Lungs (with bronchi)
Mandibular lymph nodes	Mesenteric lymph node	Optic nerves
Ovaries (with oviducts)	Pancreas	Payer's patches
Pituitary gland	Prostate gland	Salivary glands
Sciatic nerve	Seminal vesicles with coagulation glands	Skeletal muscle
Skin with mammary region	Spinal cord (3 samples) with vertebrae	Spleen
Sternum (with bone marrow)	Stomach	Thymus
Thyroid gland (including parathyroid glands)	Tongue	Trachea
Ureters	Urethra	Urinary bladder
Uterus	Vagina	Zymbal's glands
Remaining intestines	Physical identifier	All organs or tissues with macroscopic findings.

5. Histopathology

Following fixation, nominal 5 µm sections were prepared and stained with haematoxylin and eosin. All tissues were examined from animals in the control and highest dose groups, as well as the liver, lung and kidneys from animals in all dose groups.

In addition, a Cryostat section of the liver from all animals was stained with Oil Red O to demonstrate lipid.

Tissues were examined for histopathological change with a light microscope.

II. Results and Discussion

A. Results

1. Clinical results

At 200 ppm one female died prematurely due to blood sampling. Thus, no treatment-related mortality was recorded throughout the study. At 20000 ppm, in all animals, diarrhoea was observed from week two on.

2. Body weight and food and water intake

Body weight

There was no treatment-related effect on body weight development in either sex at any dose.

Compared to controls, there were no statistically significant differences with regard to mean body weight in males or females. Decreased body weight gains were observed in females only during the last week of treatment; however, there was no clear dose response and the finding was only significant at the mid-dose of 200 ppm, this effect was therefore not related to treatment with M-10.

Table 5.8.1- 161: Mean body weights and body weight gains per day

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Body weight [g] (% difference to control)					
Day 1	154	153 (-0.6)	152 (-1.3)	152 (-1.3)	154 (0)
Day 8	205	208 (1.5)	208 (1.5)	209 (2)	208 (1.5)
Day 15	247	251 (1.6)	252 (2)	255 (3.2)	247 (0)
Day 22	276	286 (3.6)	285 (3.3)	290 (5.1)	282 (2.2)
Day 29	285	295 (3.5)	297 (4.2)	303 (6.3)	292 (2.5)
Body weight gain per day [g] (% difference to control)					
Day 1-8	51	55 (7.8)	56 (9.8)	57 (11.8)*	55 (7.8)
Day 8-15	42	43 (2.4)	44 (4.8)	47 (11.9)	39 (-7.1)
Day 15-22	29	35 (20.7)	33 (13.8)	35 (20.7)	34 (17.2)
Day 22-29	8	9 (12.5)	12 (50)	13 (62.5)	10 (25)
Females					
Body weight [g] (% difference to control)					
Day 1	119	114 (-4.2)	115 (-3.4)	118 (-0.8)	121 (1.7)
Day 8	141	138 (-2.1)	137 (-2.8)	143 (1.4)	143 (1.4)
Day 15	159	154 (-3.1)	158 (-0.6)	161 (1.3)	162 (1.9)
Day 22	176	172 (-2.3)	170 (-3.4)	176 (0)	177 (0.6)
Day 29	165	161 (-2.4)	168 (1.8)	166 (0.6)	168 (1.8)

	Dose level (ppm)				
	0	20	200	2,000	20,000
Body weight gain per day [g] (% difference to control)					
Day 1-8	22	24 (9.1)	22 (0)	24 (9.1)	22 (0)
Day 8-15	18	16 (-11.1)	20 (11.1)	18 (0)	19 (5.6)
Day 15-22	17	17 (0)	13 (-23.5)	15 (-11.8)	15 (-11.8)
Day 22-29	-11	-11 (0)	-5 (-54.5)*	-10 (-9.1)	-9 (-18.2)

* $p < 0.05$; statistically different to controls using Dunnett's test

Food intake

No toxicologically significant effects on food intake were observed at 20000 ppm and below in both sexes.

Table 5.8.1- 162: Mean food consumption (g/animal/day) (% difference to control)

	Dose level (ppm)				
	0	20	200	2,000	20,000
Males					
Week 1	21 (10.5)	19 (0)	20 (5.3)	21 (10.5)	21 (10.5)
Week 2	22 (0)	22 (0)	22 (0)	22 (0)	22 (0)
Week 3	24 (9.1)	23 (4.5)	23 (4.5)	25 (13.6)	24 (9.1)
Week 4	21 (10.5)	20 (5.3)	20 (5.3)	21 (10.5)	21 (10.5)
Females					
Week 1	18 (-14.3)	19 (-9.5)	19 (-9.5)	19 (-9.5)	18 (-14.3)
Week 2	18 (-14.3)	18 (-14.3)	19 (-9.5)	18 (-14.3)	18 (-14.3)
Week 3	18 (-5.3)	19 (0)	20 (5.3)	20 (5.3)	18 (-5.3)
Week 4	15 (-11.8)	17 (0)	17 (0)	16 (-5.9)	15 (-11.8)

3. Ophthalmoscopic examinations

The ophthalmological examinations performed near terminal sacrifice on the control and the high dose group animals revealed no evidence of treatment related effects.

4. Neurotoxicity assessments

No treatment-related effects were observed.

The functional observations showed no treatment-related or relevant differences in both sexes in comparison to the controls.

Reflex testing did not provide indications of treatment-related effects.

Grip strength measurements, determinations of landing foot-splay, and determinations of body weight temperature showed no treatment-related or relevant differences in all dose groups compared to controls.

5. Laboratory investigations

Haematology:

Males and females exhibited decreased monocyte counts. This effect became statistically significant at 200 and 2000 ppm in males (not dose-related) and at 20000 ppm in females. Since all individual values lay in the historical range (2s-range) of control values, they do not reflect any adverse effect on monocyte counts.

There were no toxicologically significant effects on the erythrocyte parameters and erythrocyte morphology, as well as on coagulation.

All other means of haematological parameters, which were significantly different from control values are considered to be of no toxicological relevance because either the differences from control were negligibly low or they did not show a correlation with the dose administered.

Clinical chemistry:

No significant differences occurred among the plasma enzyme activities up to 20000 ppm. Amongst the results of plasma substrate measurements statistically significant increased glucose concentrations were obvious at 200 ppm and above in females. These differences were not dose-related, and all individual values were within the historical control range (except animal no.90). Thus, this result was considered as incidental. At 20000 ppm the concentrations of creatinine and urea were decreased in females. Except the creatinine values of 2 females at 20000 ppm which lay slightly above the historical 2s range, all individual values were within the historical control range. They were considered as incidental.

Table 5.8.1- 163: Clinical chemistry substrates

	Glucose (mmol/l)	Chol. (mmol/l)	Triglycerides (mmol/l)	Creatinine (µmol/l)	Urea (mmol/l)	Total bilirubin (µmol/l)	Total protein (g/l)	Albumin (g/l)	Albumin globulin
Males									
0	3.77	2.04	0.8	57	7.17	1.3	68.2	36.7	1.18
20	3.93	1.86	0.69	58	7.45	1.3	70.3	38	1.18
200	3.67	1.99	0.72	58	7.1	1.2	71.6 *	39.0 *	1.2
2000	3.81	1.9	1.01	57	7.59	1.4	72.1 **	39.4 **	1.21
20000	3.92	1.82	0.68	60	7.12	1.2	69	38.2	1.24
Females									
0	3.61	1.81	0.52	67	7.91	1.5	70.3	39.4	1.28
20	3.89	1.78	0.55	64	7.16	1.3	69	39.1	1.31
200	3.94 *	1.84	0.54	60**	7.41	1.3	67.5	38.2	1.31
2000	4.25 **	1.81	0.4	62	7.25	1.1	69.8	38.9	1.26
20000	3.97 *	1.82	0.49	55**	5.89 **	1.2	69	38.3	1.26

* $p < 0.05$; statistically different to controls using Dunnett's test
** $p < 0.01$; statistically different to controls using Dunnett's test

Table 5.8.1- 164: Clinical chemistry electrolytes

	Na	K	Ca	Cl
Males				
0	147	6.3	2.47	98
20	148	6.9	2.51	96
200	147	8.0 **	2.61	96
2000	147	7.6 *	2.76 **	97
20000	147	6.6	2.46	96
Females				
0	147	6.8	2.79	97
20	147	7.5	2.82	97
200	146	6.6	2.79	99
2000	147	6.7	2.84	100 *
20000	146	6.9	2.9	99

* $p < 0.05$; statistically different to controls using Dunnett's test
** $p < 0.01$; statistically different to controls using Dunnett's test

Other statistically significant effect for the remaining substrates are of no toxicological relevance, because a dose correlation is lacking, and/or all individual values were within the historical control range, and/or the effects were observed in one sex only.

Urinalysis:

No treatment-related effects were observed.

6. Sacrifice and pathology

Macroscopic examination at necropsy revealed no treatment-related gross lesions up to 20000 ppm.

No statistically significant and/or dose dependent organ weight deviations occurred in either sex up to the concentration of 20000 ppm.

7. Histopathology

Histopathology revealed that up to 20000 ppm no treatment-related microscopic findings were observed.

III. Conclusion

The dietary administration of M-10 to male and female rats was tolerated without adverse effects up to and including 20000 ppm. There was no effect on body weight development or food consumption and there were no treatment related mortalities or clinical signs or unusual findings at necropsy. There were no apparent differences in organ weights in comparison to controls and no unusual treatment related findings with regard to haematology or clinical chemistry. Thus, the NOAEL for M-10 was set at the highest dose tested of 20000 ppm (equivalent to 1748.17 and 2298.89 mg/kg/day, in males and females respectively).

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 407 and is valid and acceptable to investigate the short-term toxicity of M-10. A NOAEL of 20000 ppm (the highest dose tested) was established for M-10 from the study; equivalent to 1748 and 2299 in males and females, respectively.

M-14 (AE 1388273)

No acute or short term toxicity studies are available for M-14 and are not required.

The metabolite M-14 has been tested in a battery of *in vitro* and *in vivo* genotoxicity tests. M-14 was not mutagenic *in vitro* in bacterial as evidenced in a negative Ames test when tested up to the highest required concentrations in the presence and absence of metabolic activation (provided by S9 mix), or in mammalian cells when tested at the HPRT locus of the Chinese hamster V79 cells. However, M-14 induced chromosome aberrations *in vitro* in cultured human peripheral blood lymphocytes, following both a 20-hour continuous exposure in the absence of metabolic activation (S9) and a 3-hour pulse exposure in the presence of S9. This *in vitro* result was followed up with an appropriate *in vivo* test (mouse micronucleus test), in which no evidence of clastogenicity or aneugenicity was seen and exposure of the target tissue was demonstrated by a depression of the ratio between the polychromatic and normochromatic erythrocytes. Furthermore, the route of administration (i.p.) is highly likely to have resulted in systemic exposure of the test substance (and thus exposure of the highly perfuse bone marrow tissue). Overall, there is no evidence that M-14 is clastogenic or aneugenic *in vivo*. A negative *in vivo* UDS assay showed that M-14 did not induce DNA repair (damage) in isolated rat hepatocytes.

Considering the negative results for mutagenicity *in vitro* and the negative *in vivo* MMT and UDS assay, it is concluded that the metabolite M-14 is not genotoxic and there is no toxicological concern for this metabolite.

Genotoxicity

Data Point:	KCA 5.8.1/35
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE 1388273 - Salmonella/microsome test - Plate incorporation and preincubation method
Report No:	AT00655
Document No:	M-221023-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC, B.13/14 (2000); OECD 471 (1997); US-EPA 712-C-98-247, OPPTS 870.5100 (1998)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

M-14 (referred to as AE 1388273 in the report) was initially investigated using the *Salmonella* microsome plate incorporation test for point mutagenic effects in doses of up to and including 5000 µg per plate on five *Salmonella typhimurium* LT2 mutants. These comprised the histidine auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102. The independent repeat was performed as preincubation for 20 minutes at 37°C. Other conditions remained unchanged.

Doses up to and including 1581 µg per plate did not cause any bacteriotoxic effects. Total bacteria counts remained unchanged and no inhibition of growth was observed. At 5000 µg per plate, the substance had only a weak, strain-specific bacteriotoxic effect. Due to the weakness of this effect this dose could nevertheless be used for assessment purposes.

Evidence of mutagenic activity of M-14 was not seen. No biologically relevant increase in the mutant count, in comparison with the negative controls, was observed.

The positive controls sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, mitomycin C, cumene hydroperoxide and 2-aminoanthracene had a marked mutagenic effect, as was seen by a biologically relevant increase in mutant colonies compared to the corresponding negative controls.

Therefore, M-14 was considered to be non-mutagenic without and with S9 mix in the plate incorporation as well as in the preincubation modification of the *Salmonella*/microsome test.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-14 (referred to as AE 1388273 in the report)
Purity:	99.2%
Batch no.:	LP0653F

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	Without S9 mix:
	Na-azide: TA 1535
	Nitrofurantoin (NF) : TA 100
	4-Nitro-1,2-phenylene diamine (4-NPDA) : TA 1537, TA 98
	Mitomycin C (MMC) : TA 102 (plate incorporation trials
	Cumene hydroperoxide (Cumene) : TA 102 (preincubation trials)
	With S9 mix:
	2-Aminoanthracene : TA 98, TA 100, TA 1535, TA 1537, TA 102

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

S9 mix was used to simulate the mammalian metabolism of the test substance. It was made from the livers of at least six adult male Sprague Dawley rats, of approximately 200 to 300 g in weight. For enzyme induction, the animals received a single intraperitoneal injection of Aroclor 1254, dissolved in corn oil, at a dose of 500 mg/kg body weight, five days prior to sacrifice. The animals were prepared unfasted, following the directions of Ames *et al.* (1975) and Maron and Ames (1983).

The rats were terminated. Livers were removed under sterile conditions immediately after sacrifice and kept at 4°C until all animals had been prepared. All the remaining steps were carried out under sterile conditions at 4°C.

The livers were washed with cold (4°C), 0.15 M KCl solution (approximately 1 mL KCl per 1 g liver), and then homogenized in fresh, cold (4°C), 0.15 M KCl (approximately 3 mL KCI per 1 g liver). The homogenate was then centrifuged in a cooling centrifuge at 4°C and 9000 g for 10 minutes. The supernatant (the S9 fraction) was stored at -80°C in small portions.

Cofactor solution (70 mL) was composed as follows:

$MgCl_2.6H_2O$	162.6 mg
KCl	246.0 mg
glucose-6-phosphate, disodium salt	179.1 mg
NADP, disodium salt	315.0 mg
phosphate buffer	100.0 mM

S9 mix consists of this cofactor solution, S9 fraction and, if needed, 0.15 M KCl. The amount of S9 fraction in S9 mix is indicated in the tables in percent. The S9 mix comprised the amount of S9 fraction (x%) indicated in the tables, 70% cofactor solution and (30-x)% 0.15 M KCl. The S9 fraction was derived from the preparation dated February 4, 2003 (protein content 25.6 mg per ml). Prior to first use, each batch was checked for its metabolizing capacity by using reference mutagens; appropriate activity was demonstrated. At the beginning of each experiment 4 aliquots of the S9 mix were plated (0.5 mL per plate) in order to assess its sterility. This was repeated after completion of test tube plating. The sterility control plates were then incubated for 48 hours at 37°C. No indication of contamination of S9 mix was found.

4. Test organisms:

Histidine-deficient mutants of *Salmonella typhimurium* LT2 served as indicators to demonstrate point mutagenic effects. These strains were selected specifically for the *Salmonella*/ microsome test. Since point mutations can be divided into two basic classes, base-pair substitutions and frameshift mutations, several strains were used which cover both types.

These included the strains selected by Ames *et al.* (1973b), *Salmonella typhimurium* TA1535 and TA 1537, as well as *Salmonella typhimurium* TA100, TA 98 and TA 102 developed by McCann *et al.* (1975b) and Levin *et al.* (1982), respectively.

TA 1535 and TA 100 bear the base-pair substitution, his G 46, and TA 100 additionally contains the plasmid pKM 101. This R factor, also contained in TA 98 and TA102, codes for an ampicillin resistance and should raise the sensitivity of the strains. TA 102 carries the ochre mutation his G 428 on the multicopy plasmid pAQ1, which codes in addition for tetracycline resistance. TA1537 and TA 98 bear frameshift markers. TA 1537 exhibits the +1 mutant, his C 3076, while TA 98 bears the +2 type, his D 3052.

Furthermore, the strains have other properties, which should increase their sensitivity. They are all deep rough, i.e. partly deficient in lipopolysaccharide side chains in their cell walls, enabling larger molecules to penetrate the bacterial cell wall and produce mutations. With the exception of TA 102, all strains have reduced capability to repair DNA-damage which increases the likelihood that such damage results in mutations.

The mutations of the bacterial strains used in this study are described in table 5.4.1-1.

Table 5.8.1- 165: *Salmonella typhimurium* strains

Salmonella typhimurium		
Strains	**Genotype**	**Type of mutations indicated**
TA 1537	his C 3076; rfa^-; $uvrB^-$	frame shift mutations
TA 98	his D 3052; rfa^-; $uvrB^-$; R-factor	" "
TA 1535	his G 46; rfa^-; $uvrB^-$	base-pair substitutions
TA 100	his G 46; rfa^-; $uvrB^-$; R-factor	" "
TA 102	his G 428; rfa^-; $uvrB^+$; R-factor	" "

Regular checking of the properties of the *Salmonella typhimurium* strains was performed.

The original strains were obtained from Prof. Bruce Ames and arrived at Toxicology, Bayer HealthCare, Bayer AG, on August 15, 1997.

5. Test substance concentrations used:
Test concentrations of 16, 50, 158, 500, 1581 and 5000 µg M-05/plate were used.

B. Test performance

Experimental phase: 19 August – 21 September 2003

1. Pre-experiment for toxicity

No pre-experiment was performed.

2. Mutagenicity test

For each strain and dose level, including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

Plate Incorporation Method:

- 100 µL test solution at each dose level (solvent or reference mutagen solution (positive control)),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL bacteria suspension (cf. test system, pre-culture of the strains),
- 2000 µL overlay agar.

Preincubation Method:

In the pre-incubation assay 100 µL test solution (solvent or reference mutagen solution (positive control)), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspension were mixed in a test tube and incubated at 37 °C for 20 minutes. After pre-incubation 2.0 mL overlay agar was added to each tube. The mixture was poured on agar plates.

The plates were incubated for 48 hours at 37 °C prior to counting.

Data recording:

The colonies were counted automatically using an Artek counter, model 982B. Data were transferred to a PC and processed with the released and DOS 6.0 based BioSys software Ames- Test III (Rev. 3.106).

3. Statistics

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

4. Acceptance / assessment criteria:

The following criteria determined the acceptance of an assay:

a) The negative controls had to be within the expected range, as defined by published data (e.g. Maron and Ames, 1983) and/or the laboratories' own historical data.

b) The positive controls had to show sufficient effects, as defined by the laboratories' experience.

c) Titer determinations had to demonstrate sufficient bacterial density in the suspension.

Only trials which complied with all three of the above criteria were accepted for assessment. Even if the criteria for points (b) and (c) were not met, a trial was accepted if it showed mutagenic activity of the test compound. Furthermore, an unacceptable trial would have been repeated.

5. Evaluation of results

A reproducible and dose-related increase in mutant counts of at least one strain is considered to be a positive result. For TA1535, TA100 and TA 98 this increase should be about twice that of negative controls, whereas for TA 1537, at least a threefold increase should be reached. For TA 102 an increase of about 100 mutants should be reached. Otherwise, the result is evaluated as negative. However, these guidelines may be overruled by good scientific judgment.

In case of questionable results, investigations should continue, possibly with modifications, until a final evaluation is possible.

II. Results and Discussion

A. Mutation assays

The *Salmonella*/microsome test, employing doses of up to 5000 µg per plate, showed M-14 to produce bacteriotoxic effects at 5000 µg per plate.

Evaluation of individual dose groups, with respect to relevant assessment parameters (dose effect, reproducibility), revealed no biologically relevant variations from the respective negative controls.

In spite of the low doses used, positive controls increased the mutant counts to well over those of the negative controls, and thus demonstrated the system's high sensitivity.

Despite this sensitivity, no indications of mutagenic effects of M-14 could be found at assessable doses of up to 5000 µg per plate in any of the *Salmonella typhimurium* strains used.

An overview of the results is given in the tables below.

Table 5.8.1- 166: Summary of plate incorporation test

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean)				
		TA 1535	TA 100	TA 1537	TA 98	TA 102
Without metabolic activation (-S9)						
M-14	0	15	169	8	20	391
	16	14	161	6	27	354
	50	17	177	7	17	366
	158	15	176	6	18	374
	500	17	193	8	28	321
	1581	16	178	6	18	310
	5000	17	192	4	20	302
Positive control						
Na-azide	10	657	-	-	-	-
NF	0.2	-	398	-	-	-
4-NPDA	10 / 0.5	-	-	100	166	-
MMC	0.2					599
Historical solvent control mean	-	9 (±2)	88 (±24)	7 (±1)	22 (±7)	201 (±17)
Historical control range	-	8 – 14	70 – 136	6 – 8	14 – 34	174 - 220
With metabolic activation (+S9)						
M-14	0	11	206	7	33	435
	16	9	206	8	38	393
	50	11	192	8	36	417
	158	8	216	8	37	417
	500	8	214	9	33	387
	1581	11	202	8	33	388
	5000	15	218	7	23	291
Positive control						
2-AA	3	193	1414	418	1099	706
Historical solvent control mean	-	10 (±1)	107 (±23)	9 (±1)	30 (±7)	272 (±13)
Historical control range	-	9 – 12	89 – 151	8 – 10	23 – 41	248 - 288

Na-azide = sodium azide, NF = Nitrofurantoin; 4-NPDA = 4-Nitro-1,2-phenylene diamine, MMC = Mitomycin C;
2-AA = 2-aminoanthracene
(±X): Standard deviation
Historical control data based on experiments from 1996 to 2002.

Table 5.8.1- 167: Summary of pre-incubation test

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean)				
		TA 1535	TA 100	TA 1537	TA 98	TA 102
Without metabolic activation (-S9)						
M-14	0	25	118	6	26	240
	16	26	123	5	23	248
	50	24	116	4	23	262
	158	17	126	6	24	262
	500	26	117	5	21	249
	1581	31	128	4	25	264
	5000	24	139	4	24	212
Positive control						
Na-azide	10	724	-	-	-	-
NF	0.2	-	859	-	-	-
4-NPDA	10 / 0.5	-	-	80	165	-
Cumene	50	-	-	-	-	472
Historical solvent control mean	-	9 (±2)	93 (±25)	7 (±1)	22 (±6)	245 (±17)
Historical control range	-	7 – 12	72 – 45	6 – 8	15 – 32	223 - 271
With metabolic activation (+S9)						
M-14	0	12	119	9	34	326
	16	10	103	10	44	319
	50	12	116	10	42	351
	158	12	120	8	41	355
	500	12	119	8	40	332
	1581	9	113	7	39	301
	5000	15	133	7	33	224
Positive control						
2-AA	3	229	1646	399	1383	518
Historical solvent control mean	-	10 (±1)	107 (±24)	9 (±1)	30 (±6)	312 (±21)
Historical control range	-	8 – 11	88 – 155	8 – 9	23 – 40	281 - 337

Na-azide = sodium azide; NF = Nitrofurantoin; 4-NPDA = 4-Nitro-1,2-phenylene diamine; Cumene = Cumene hydroperoxide; 2-AA = 2-aminoanthracene
(±X): Standard deviation
Historical control data based on experiments from 1996 to 2002.

III. Conclusion

No indications of mutagenic effects of M-14 could be found at assessable doses of up to 5000 ug per plate in any of the *Salmonella typhimurium* strains used in the plate incorporation or preincubation assays.

Due to these results M-14 has to be regarded as non-mutagenic

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of M-14 in *S-typhimurium*.

M-14 is not mutagenic in bacterial cells under the conditions of this study when tested up to 5000 µg/plate (+/- S9), either by base pair changes or frameshift mutations.

Data Point:	KCA 5.8.1/36
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE 1388273: Induction of chromosome aberrations in cultured human peripheral blood lymphocytes
Report No:	C038438
Document No:	M-224988-01-1
Guideline(s) followed in study:	OECD 473 (1997), ICH Tripartite Harmonised Guideline (1995)
Deviations from current test guideline:	Cytotoxicity was not evaluated by the parameters of relative population doubling (RPD) or relative increase in cell count (RICC). Only 100 metaphases were analysed, rather than a minimum of 300 as recommended in the current guidance.
Previous evaluation:	yes, evaluated and accepted in DAR 2005
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

This study was performed to assess the ability of M-14 to induce chromosomal aberrations in human lymphocytes cultured *in vitro*.

Human lymphocytes, in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to the test substance both in the presence and absence of S9 mix derived from rat livers. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using Colcemid®, the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

In order to assess the toxicity of M-14 to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. On the basis of these data, the following concentrations were selected for metaphase analysis:

Experiment 1

Without S9 (3 hours treatment, 17 hours recovery): 2048, 3200 & 5000 µg/mL

With S9 (3 hours treatment, 17 hours recovery): 3200, 4000 & 5000 µg/mL

Experiment 2

Without S9 (20 hours treatment): 300.5, 415.9 & 489.3 µg/mL

With S9 (3 hours treatment, 17 hours recovery): 2560, 4000 & 5000 µg/mL

In the presence of S9 mix (3+17-hour pulse treatment) frequencies of cells with structural aberrations were statistically significantly elevated at 5000 µg/mL compared with the concurrent vehicle controls furthermore, the numbers of aberrant cells in the majority of treated cultures in experiment 2 exceeded the historical negative control ranges.

In the absence of S9 mix (20h continuous treatment) frequencies of cells with structural aberrations were statistically significantly elevated at all analysed treated concentrations compared with the concurrent vehicle controls; furthermore, the numbers of aberrant cells in the majority of treated cultures exceeded the historical negative control ranges and there was clear evidence of a dose related increase in the frequency of cells with structural aberrations.

In the absence of S9 mix (3+17-hour pulse treatment) frequencies of cells with structural aberrations were similar to those seen in concurrent vehicle controls. Furthermore, the number of aberrant cells (excluding gaps) in all treated cultures fell within the normal historical control ranges.

The quantitative analysis for polyploidy (numerical aberrations) revealed that normal frequencies (within negative historical control ranges) were observed for the majority of cultures in the presence and absence of S-9 mix in experiment 1. Sporadic increases in some cultures marginally exceeded the historical control ranges and were not considered to of biological relevance.

All positive control compounds caused statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

It is concluded that M-14 has shown evidence of clastogenic activity following a 20-hour continuous exposure in the absence of S9 mix and a 3-hour pulse treatment in the presence of S9 mix, in this *in vitro* cytogenetic test system, under the experimental conditions described.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-14 (referred to as AE 1388273 in the report)
Purity:	99.2%
Batch no.:	LP0653F

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: 4-Nitroquinoline-1-oxide (NQO); 5.0 & 2.50 µg/mL (exp. 1& 2) +S9: Cyclophosphamide (CPA); 6.25 µg/mL

3. Activation:

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male SD rats inducted with Aroclor 1254 (obtained from Molecular Toxicology Inc., USA). The batches of S9 were stored frozen at -80˚C and thawed prior to use. Each batch was checked for sterility, protein content, ability to convert known pro-mutagens to bacterial mutagens and cytochrome P450-catalysed enzymes activities.

Preparation of S9-mix:

Glucose-6-phosphate (180 mg/mL), NAPD (25 mg/mL), KCl (150 mM) and rat liver S9 were mixed in the ratio of 1:1:1:2. An aliquot of the resulting S9 mix was added to each required cell culture to achieve the final concentration in a total of 10 mL. the final concentration of liver homogenate in the test system was 2%. Cultures treated in the absence of S9 received an equal volume of 150 mM KCl.

4. Cell cultures and medium:

Blood was collected from three healthy non-smoking, female volunteers within one day of culture initiation. Whole blood cultures were established in sterile tubes by placing 0.4 mL heparinised blood into 9.6 mL (exp. 1) or 9.0 mL (exp. 2) Hepes-buffered RPMI medium containing 20% (v/v) foetal calf serum and 50 µg gentamycin. Phytohaemagglutinin (PHA) was included at concentration of 2% and 4% of culture volumes (experiments 1 and 2 respectively) to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37˚C and rocked continuously.

5. Test substance concentrations used:

Owing to irregular toxicity in experiment 2 (trial 1), it was not possible to select doses for analysis from the 3+17h treatment +S9; therefore, this treatment was repeated in a separate trial (designated experiment 1 trial 2).

In experiment 1 the tested concentrations were 0 (solvent control), 112.6, 140.7, 175.9, 219.9, 274.9, 343.6, 429.5, 536.9, 671.1, 838.9, 1049, 1311, 1638, 2048, 2560, 3200, 4000 & 5000 µg/mL with and without metabolic activation.

In experiment 2 (trial 1) the tested concentrations were 0 (solvent control), 300.5, 353.5, 415.9, 489.3, 575.6, 677.2, 796.7, 937.3, 1103, 1297, 1526, 1796, 2112, 2610, 3070, 3612, 4250 & 5000 µg/mL with and without metabolic activation.

In experiment 2 (trial 2) the tested concentrations were 0 (solvent control), 112.6, 140.7, 175.9, 219.9, 274.9, 343.6, 429.5, 536.8, 671, 838.8, 1049, 1311, 1638, 2048, 2560, 3200, 4000 & 5000 µg/mL with metabolic activation.

The highest dose selected for chromosome analysis should be one at which at least 50% mitotic inhibition has occurred, or the highest dose tested; slides from highly toxic concentrations should be avoided.

Therefore, based on these criteria the following doses were selected for analysis (the highest selected dose and two lower doses):

Experiment 1 without S9 (3 hours treatment, 17 hours recovery): 2048, 3200 & 5000 µg/mL

Experiment 1 with S9 (3 hours treatment, 17 hours recovery): 3200, 4000 & 5000 µg/mL

Experiment 2 without S9 (20 hours treatment): 300.5, 415.9 & 489.3 µg/mL

Experiment 2 with S9 (3 hours treatment, 17 hours recovery): 2560, 4000 & 5000 µg/mL

B. Test performance

Experimental phase: July 7 to October 1, 2003

1. Experiments 1 & 2

S-9 mix or KCl (0.5 mL) was added appropriately. One set of quadruple cultures (A, B, C and D) for each treatment regime was then treated with the solvent, and one set of duplicate cultures with the test article (8.5 mL/culture). Additional duplicate cultures for treatment for treatment in the absence of S9 and its presence were treated with 0.1 mL of the positive control chemicals. All cultures were then incubated at 37˚C.

Treatment media remained on cultures receiving the continuous treatment until sampling (20h after start of treatment). Cultures received pulse treatments (+/- S9) for 3 hours only and were then pelleted, washed twice with sterile saline, and resuspended in fresh treatment (with gentamycin and foetal calf serum). These cultures were incubated for a further 17 hours before harvesting.

Harvesting and fixation

Approximately 2 hours prior to harvest, colchicine was added to give a final concentration of 1µg/mL to arrest dividing cells in metaphase. At the defined sampling time cultures were centrifuged, the supernatant carefully removed, and the cells resuspended in 4 mL pre warmed hypotonic (0.075 M) KCl and incubated at 37˚C for 15 minutes to allow cell swelling to occur. Cells were then fixed by dropping the KCl suspension into an equal volume of fresh methanol/glacial acetic acid (3:1, v/v). the fixative was changed by centrifugation and resuspension. The procedure was repeated several times until the pellets were clean. Lymphocytes were kept in fixative in the refrigerator before slides were prepared; slides were not prepared on the day of harvest to ensure cells were adequately fixed.

Slide preparation

The cells were pelleted and resuspended in a minimal amount of fresh fixative to give a milky suspension. Several drops of aqueous acetic acid were added to each suspension to enhance chromosome spreading, and several drops of suspension were transferred to clean slides. Slides were flamed if necessary, to enhance metaphase spreading. After the slides had dried the cells were stained for 5 minutes in 4% (v/v) filtered Giemsa stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried, and mounted with coverslips.

Microscopic examination

Slides were examined uncoded for mitotic index (MI) or percentage of cells in mitosis. Slides from enough dose levels from each treatment regime were scored to determine if chemically induced mitotic inhibition had occurred. This is defined as a clear decrease in mitotic index compared with negative controls (based on at least 1000 cells counted), preferably dose related.

For each treatment regime two solvent control cultures were initially to be analysed for chromosome aberrations. Slides form the remaining solvent control cultures and untreated controls were only analysed if necessary. A single positive control dose, which gave a satisfactory response was analysed.

100 metaphases from each slide were analysed. Only cells with 44-46 chromosomes were acceptable for analysis of structural aberrations.

3. Assessment of results

An assay is considered to be acceptable if the following criteria are met:

- The binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures, and

- The proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range, and

- At least 160 cells out of an intended 200 are analysable at each dose level, and

- The positive control chemicals induce statistically significant increases in the proportion of cells with structural aberrations

The test substance is considered to cause a positive response if the following conditions are met:

- The proportion of cells with structural aberrations at one or more concentrations exceeds the normal range in both replicate cultures, and
- A statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurs at the concentrations.

A negative response is claimed if no statistically significant increases in the number of aberrant cells above concurrent control frequencies are observed, at any dose level.

A further evaluation may be carried out if the above criteria for a positive or a negative response are not met.

6. Statistics

Aberrant cells in each culture were categorised as:

1) cells with structural aberrations including gaps

2) cells with structural aberrations excluding gaps

3) polyploid, endoreduplicated or hyperdiploid cells

Cells with structural aberrations excluding gaps in the negative control cultures were compared with historical control data to determine the acceptability of the assay and those in the treated cultures were compared with the normal ranges.

The statistical significance of any data set was only taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentrations exceeded the normal range. Fishers exact test would be used in this case. Probability values of $p < 0.05$ would be accepted as significant. The proportions of aberrant cells in each replicate were also used to establish heterogeneity between replicates by means of a binomial dispersion test. Probability values < 0.05 would be significant.

II. Results and Discussion

1. Experiment 1

Experiment 1 was performed using pulse (3-hour) treatments in the absence and presence of S-9 mix.

Mitotic inhibition

In the presence of S9 mix (3+17-hour pulse treatment) mitotic inhibition was 44% at the highest concentration tested of 5000 µg/mL. Concentrations of 2048, 3200 & 5000 µg/mL were therefore selected for the metaphase analysis.

In the absence of S9 mix (3+17-hour pulse treatment) mitotic inhibition was 41% at the highest concentration tested of 5000 µg/mL. Concentrations of 3200, 4000 & 5000 µg/mL were therefore selected for the metaphase analysis.

Structural aberrations

In the presence of S9 mix (3+17-hour pulse treatment) frequencies of cells with structural aberrations were statistically significantly elevated at 5000 µg/mL compared with the concurrent vehicle controls but fell within the range of the historical control data.

In the absence of S9 mix (3+17-hour pulse treatment) frequencies of cells with structural aberrations were similar to those seen in concurrent vehicle controls. Furthermore, the number of aberrant cells (excluding gaps) in all treated cultures fell within the normal historical control ranges.

The quantitative analysis for polyploidy (numerical aberrations) revealed that normal frequencies (within negative historical control ranges) were observed for the majority of cultures in the presence and absence of S-9 mix in experiment 1. Sporadic increases in some cultures marginally exceeded the historical control ranges and were not considered to of biological relevance.

2. Experiment 2

Experiment 2 comprised continuous exposure (20h treatment) in the absence of S9 and a pulse exposure (3+17-hour treatment) in the presence of S9. An irregular toxicity profile in experiment 1 meant that the doses for analysis from the 3+17h treatment +S9 could not be selected; therefore, this portion of the assay was repeated.

Mitotic inhibition

In the presence of S9 mix (3+17-hour pulse treatment) mitotic inhibition was 52% at the highest concentration tested of 5000 µg/mL. Concentrations of 2560, 4000 & 5000 µg/mL were therefore selected for the metaphase analysis.

In the absence of S9 mix (20-hour continuous treatment) mitotic inhibition was 52% at the highest concentration tested of 5000 µg/mL. Concentrations of 300.5, 415.9 & 489.3 µg/mL were therefore selected for the metaphase analysis.

Structural aberrations

In the presence of S9 mix (3+17-hour pulse treatment), similarly to experiment 1, frequencies of cells with structural aberrations were statistically significantly elevated at 4000 and 5000 µg/mL compared with the concurrent vehicle controls; furthermore, the numbers of aberrant cells in the majority of treated cultures in experiment 2 exceeded the historical negative control ranges.

In the absence of S9 mix (20h continuous treatment) frequencies of cells with structural aberrations were statistically significantly elevated at all analysed treated concentrations compared with the concurrent vehicle controls; furthermore, the numbers of aberrant cells in the majority of treated cultures exceeded the historical negative control ranges and there was clear evidence of a dose related increase in the frequency of cells with structural aberrations.

The quantitative analysis for polyploidy (numerical aberrations) revealed that normal frequencies (within negative historical control ranges) were observed for the majority of cultures in the presence and absence of S-9 mix in experiment 2. Sporadic increases in some cultures marginally exceeded the historical control ranges and were not considered to of biological relevance.

An overview of the results for experiments 1 and 2 is provided in the tables below.

Table 5.8.1- 168: Relative mitotic indices and mean percentage of aberrant human lymphocytes in experiment 1

Treatment	Concentration (µg/ml)	Treatment time (h)	Mitotic index (mean)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
Without metabolic activation (-S9)					
M-14	2048	3	7.0	3	1
	3200	3	6.2	1	0
	5000	3	5.0	4	3
Solvent control		3	8.9	3	0
NQO	5	3		37	36*
With metabolic activation (+S9)					
M-14	3200	3	6.9	2	1
	4000	3	5.9	2	0
	5000	3	5.0	15	14*
Solvent control		3	8.4	1	0
CPA	6.25	3	-	52	50*
Historical control ranges#				3-8 (-S9) 2-6 (+S9)	3-6 (-S9) 2-4 (+S9)

* statistically different from controls ($p<0.001$)
# Calculated normal range of aberrant cells scored per 100 cells (99% confidence interval, October 2002)

Table 5.8.1- 169: Relative mitotic indices and mean percentage of aberrant human lymphocytes in experiment 2

Treatment	Concentration (µg/ml)	Treatment time (h)	Mitotic index (mean)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
Without metabolic activation (-S9)					
M-14	300.5	20	9.8	18	8**
	415.9	20	6.8	34	24*
	489.3	20	5.4	51	38*
Solvent control	-	20	11.1	3	1
NQO	2.5	20	-	53	47*
With metabolic activation (+S9)3					
M-14	2560	3	8.0	11	9
	4000	3	5.7	14	13
	5000	3	4.5	55	53*
Solvent control	-	3	9.2	3	6
CPA	6.25	3	-	74	67*
Historical control ranges#				3-8 (-S9) 2-6 (+S9)	3-6 (-S9) 2-4 (+S9)

* statistically different from controls ($p < 0.001$); ** statistically different from controls ($p < 0.005$)
#Calculated normal range of aberrant cells scored per 100 cells (99% confidence interval, October 2002)

III. Conclusion

M-14 induced structural chromosome aberrations in cultured human peripheral blood lymphocytes following continuous 20-hour treatment (+/- S9) when tested up to cytotoxic concentrations and following a 3-hour pulse treatment (+S9) when tested up to 5000 µg/mL. A similar effect was not apparent following 3+17-hour treatment in the absence of S9 mix. There was no evidence of numerical aberrations (polyploidy).

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 479 and is valid and acceptable to assess the potential of M-14 to induce chromosome aberrations in human lymphocytes *in vitro*. M-14 was clastogenic in human lymphocytes under the conditions of this study following a 20-hour continuous exposure in the absence of S9 and a 3-hour pulse treatment in the presence of S9. There was no evidence of clastogenicity following a 3-hour pulse exposure in the absence of S9 and there was no evidence of polyploidy.

Data Point:	KCA 5.8.1/37
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE 1388273 - V79/HPRT-test in vitro for the detection of induced forward mutations
Report No:	AT01045
Document No:	M-228554-01-1
Guideline(s) followed in study:	Commission Directive 2000/32/EC B.17. (2000); OECD 476 (1997); US-EPA 712-C-98-221, OPPTS 870.5300 (1998)
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The study was performed to investigate the potential of M-14 to induce gene mutations at the HPRT locus in V 79 cells of the Chinese hamster *in vitro*.

The assay was performed in two experiments with and two without rat liver microsomal activation (S9-mix). The test article was dissolved in DMSO and tested at the following concentrations:

80, 160, 320, 640, 1280 & 2560 µg/mL -/- S9

The concentration ranges were based on the results of preliminary testing for solubility and toxicity. The highest concentration was neither toxic nor precipitative either in the presence or absence of metabolic activation.

No relevant increase in mutant colony numbers was obtained in any experiment.

The positive controls showed distinct statistically significant increases in induced mutant colonies, thus indicating the sensitivity of the assay and the negative controls gave the expected results.

M-14 was not mutagenic in this HPRT test with V79 Chinese hamster cells.

A. Materials

1. Test material

Test substance:	M-14 (referred to as AE 1388273 in the report)
Purity:	99.2%
Batch no.:	LP0653F

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: Ethyl methanesulphonate (EMS), 900 µg/mL +S9: Dimethylbenzanthracene (DMBA), 20µg/mL

3. Activation:

Metabolic activation was provided by S9 mix. The S9 fraction was isolated in house from the livers of Aroclor 1254 induced male SD rats and was kept frozen at -80˚C. Samples of the batch were tested for contamination and cytotoxicity prior to use. To prepare the S9 mix, two parts of the thawed S9 fraction were mixed three parts of freshly dissolved co-factors (in sodium phosphate buffer) to give the following final concentrations:

$MgCl_2$ x 6H2O (8mM)

KCl (33mM)

Glucose-6-phosphate (5mM)

NADP (1mM)

S9 fraction (40% v/v)

Sodium phosphate buffer (60% v/v)

The S9 mix was stored on ice until use the same day.

4. Cell cultures and media:

Cell cultures

V79 cell stocks (derived from Chinese hamster lung cells) are stored in liquid nitrogen. Laboratory cultures are maintained in plastic tissue culture vessels at 37˚C in a humid atmosphere containing 5% CO_2. Exponential growth was maintained by twice weekly sub-culturing. The cells were checked for mycoplasma contamination of which there was no evidence. To reduce the number of spontaneous 6-TG resistant mutants cell cultures were sub-cloned by plating 1000 cells/culture vessel at least every two weeks. If necessary, the spontaneous frequency of HPRT-mutants was further reduced by the addition of thymidine (9µg/mL), hypoxanthine (10µg/mL), glycine (22.5µg/mL and methotrexate (0.3µg/mL). A 6-TG sensitive sub-clone was then used for the HPRT test.

In all parts of the study incubation was performed at 37˚C humidified air with 5% CO_2.

Media

Cells were maintained in hypoxanthine free Eagle's Minimal Essential medium (MEM, Gibco) which has been proven suitable for growth of V79 cells. The MEM was supplemented nonessential amino acids, L-glutamine (2mM), MEM-vitamins, $NaHCO_3$, penicillin (100 units/mL, streptomycin (100 µ/mL) and heat activated foetal calf serum (10%; reduced to 2% in treated cultures).

For the selection of mutants, a hypoxanthine-free culture medium was used containing 10µg/mL of 6-thioguanine (6-TG).

5. Test substance concentrations used:

The following test concentrations were used for the mutation assays (with and without metabolic activation provided by S9 mix):

Preliminary toxicity assay: 25.78, 51.56, 103.13, 206.25, 412.50, 825, 1650 & 3300 µg/mL (+/- S9)

Main mutation assays: 80, 160, 320, 640, 1280 & 2560 µg/mL (+/- S9)

B. Test performance

Experimental phase: September 23, 2003 to January 26, 2004

1. Preliminary assay

A preliminary cytotoxicity assay was conducted with and without metabolic activation at the following concentrations in order to select the dose levels for the mutation assays:

25.78, 51.56, 103.13, 206.25, 412.50, 825, 1650 & 3300 µg/mL (+/- S9) µg/mL

Cell cultures for the preliminary toxicity assay received the same treatment as in the mutation assays. For each concentration one culture was used.

2. Main assay

Without S9

Exponentially growing V79 cells were plated in culture medium at a final volume of 20 mL in two 250 mL flasks/concentration ($4x10^6$ per flask) including controls. Following attachment (16-24 hours), the cells were exposed for 5 hours in 20 ml culture medium with reduced serum (2%). Corresponding controls received the same treatment. Thereafter, cell monolayers were washed with PBS, trypsinised and re-plated in 20 mL culture medium using 200 cells per petri dish (one flask and three petri dishes were used per culture). Petri dishes were incubated for 6 days to allow colony development and determine the 'survival to treatment).

Cells in 250 mL flasks were incubated to permit growth and expression of 1 induced mutation. Cells were sub-cultured (=count 1, 3 days) by re-seeding 1.5×10^6 cells into 20 ml medium in 250 mL flasks. Following the expression period (=count 2, total 6 days) cultures were re-seeded in petri dishes at $3x10^5$ cells per dish (8 dishes per culture) in 20 mL culture medium without hypoxanthine but containing 10 µg/mL 6-Tg for mutant selection. In addition, 200 cells per dish (3 per culture) were seeded in 5 mL culture medium to determine absolute cloning efficiency for each concentration.

After 6-8 days incubation, the colonies were fixed, stained, and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.

Two trials were performed without metabolic activation.

With S9

The activation assay was performed independently; the procedure was identical to the non-activation assay described above except for the addition of S9 mix. In the activation assay 19mL of culture medium with 1 mL of S9 was used (rather than 20 mL of culture medium) during the treatment period, resulting in a concentration of 5% S9 mix in the cultures.

Two trials were performed with metabolic activation.

3. Acceptance Criteria

The assay was considered valid if the following criteria were met:

- The average cloning efficiency of the negative and vehicle controls should be at least 50%

- The average of mutant frequency of the vehicle control should not exceed $25x10^{-6}$ cells

- The mutant frequency of the two cultures of the vehicle and negative controls should only differ to an acceptable extent (not greater than $5x10^{-6}$)

- The positive control should induce an average mutant frequency of at least 3 times the vehicle control

- If not limited by solubility, the highest concentration should induce cytotoxicity of about 80-90% or should be precipitative concentration. The survival at the lowest concentration should be in the range of the negative control

- For the calculation of an acceptable mutant frequency, at least 5 dishes per culture should be available and relative survival to treatment, relative population growth and absolute cloning efficiency should be 10% or greater

4. Criteria for a positive response

The test substance is classified as mutagenic if:

- A concentration related increase in mutant frequencies is observed in parallel cultures.
- The increase in mutant frequencies should be 2-3 times higher than the highest negative or vehicle control in the respective trial
- The result should be reproduced in the second trial
- the increase should occur in the absence of a change in osmolality compared to the vehicle control

A test substance is considered negative if:

- there is no reproducible or relevant increase in mutant frequencies.

A test substance will be considered equivocal if:

- There is no strict concentration-related increase in mutant frequencies, but one or more concentrations induce a reproducible and biologically relevant increase in mutant frequencies

Sound scientific judgement should be used in implementing the above criteria.

5. Statistical analysis

The statistical analysis relies on the mutant frequencies which are submitted to a weighted analysis of variance and a weighted recursive regression, both with Poisson derived wights.

II. Results and Discussion

A. Preliminary cytotoxicity assay

The pH of the media was altered from 1650 µg/mL and the osmolality was slightly changed at 3300 µg/mL; no cytotoxicity or precipitation were observed up to the highest concentration tested. Therefore, concentrations of 80 to 2560 µg/mL were selected for the initial main mutation assay (slightly above the maximum recommended concentration of 10 mM). The same concentrations were used in the independent repeats.

B. Main mutation assays

The mean absolute cloning efficiency of vehicle controls were 80.8% and 83.4% without S9; one trial could not be used for assessment owing to high mutant counts in the negative and vehicle controls and a second trial was terminated following bad cloning of the cells. The mean absolute cloning efficiency of the vehicle controls with S9 were 71.9 and 85.3%, demonstrating good cloning conditions for the experiments.

Without metabolic activation

Two trials were performed in the absence of S9 mix. Concentration related decreases in relative survival to treatment and/or relative population growth were observed in all treated cultures.

No M-14 induced increases in mutant frequencies were observed in the absence of S9; furthermore, statistical analysis revealed no statistically significant increases.

The mutation frequencies of the negative and vehicle controls were all within the normal range and the positive control EMS induced a clear mutagenic and statistically significant effect in both trials, thus demonstrating the validity of the assay.

With metabolic activation

Two trials were performed in the presence of S9 mix. Cytotoxic effects were seen in both trials; concentration related decreases in relative survival to treatment and/or relative population growth in all treated cultures were observed.

No M-14 induced increases in mutant frequencies were observed in the presence of S9; furthermore, statistical analysis revealed no statistically significant increases.

The mutation frequencies of the negative and vehicle controls were all within the normal range and the positive control EMS induced a clear mutagenic and statistically significant effect in both trials, this demonstrating the validity of the assay.

The results from both experiments with and S9 and both experiments without S9 are summarised in the tables below:

Table 5.8.1- 170: Relative survival and mutant frequencies in experiment 1 without metabolic activation

Treatment	Concentration (µg/ml)	Relative population growth (%)	Mutation frequency $10E^{-6}$
M-14	80	132.9	2.8
	80	132.3	8.0
	160	114.1	7.1
	160	107.8	2.9
	320	132.3	7.3
	320	131.0	6.1
	640	97.3	3.6
	640	105.3	13.4
	1280	84.1	9.1
	1280	143.9	2.9
	2560	70.1	5.0
	2560	76.1	3.5
Negative control	-	102.5	3.6
	-	78.6	3.5
Solvent control	-	100.0	8.0
	-	100.0	1.6
Positive control EMS	900	37.2	538.9*
	900	58.9	588.5*

*statistically different from controls (α=0.05) one sided Dunnett test

Table 5.8.1- 171: Relative survival and mutant frequencies in experiment 2 without metabolic activation

Treatment	Concentration (µg/ml)	Relative population growth (%)	Mutation frequency $10E^{-6}$
M-14	80	188.9	2.4
	80	98.0	10.3
	160	100.2	10.9
	160	124.9	11.6
	320	124.2	7.1
	320	127.1	11.9
	640	86.8	8.7
	640	73.5	9.5
	1280	92.0	7.8
	1280	81.3	11.4
	2560	51.3	7.7
	2560	38.9	21.3
Negative control	-	114.5	8.1
	-	73.6	5.4
Solvent control	-	100.0	7.3
	-	100.0	13.9
Positive control EMS	900	88.3	295.1*
	900	85.4	358.6*

*statistically different from controls (α=0.05) one sided Dunnett test

Table 5.8.1- 172: Relative survival and mutant frequencies in experiment 1 with metabolic activation

Treatment	Concentration (µg/ml)	Relative population growth (%)	Mutation frequency $10E^{-6}$
M-14	80	81.3	7.8
	80	56.3	4.3
	160	92.3	5.9
	160	107.4	7.0
	320	76.7	3.4
	320	80.1	1.7
	640	70.5	6.5
	640	38.7	3.7
	1280	61.9	2.8
	1280	47.0	6.3
	2560	30.4	2.7
	2560	37.3	0.5
Negative control	-	111.8	0.5
	-	76.4	5.4
Solvent control	-	100.0	6.3
	-	100.0	7.5
Positive control EMS	900	53.1	47.6*
	900	58.2	33.8*

*statistically different from controls (α=0.05) one sided Dunnett test

Table 5.8.1- 173: Relative survival and mutant frequencies in experiment 2 with metabolic activation

Treatment	Concentration (µg/ml)	Relative population growth (%)	Mutation frequency $10E^{-6}$
M-14	80	70.1	15.0
	80	57.1	12.0
	160	68.2	1.6
	160	62.6	6.6
	320	67.5	7.5
	320	59.2	5.7
	640	58.1	7.5
	640	58.1	12.1
	1280	69.5	10.3
	1280	43.5	11.3
	2560	37.1	13.4
	2560	28.9	13.0
Negative control	-	87.3	8.1
	-	60.5	11.9
Solvent control	-	100.0	14.5
	-	100.0	9.3
Positive control EMS	900	42.0	86.2*
	900	39.6	76.3*

*statistically different from controls (α=0.05) one sided Dunnett test

III. Conclusion

M-14 did not induce gene mutations in mammalian cells (Chinese hamster lung V79 cells) either in the presence or absence of metabolic activation provided by S9 mix. Appropriate responses exhibited by the concurrent negative, vehicle and positive controls confirmed the validity of the assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD 476 and is valid and acceptable to assess the potential of M-14 to induce gene mutations at the HPRT locus in Chines Hamster Lung V79 cells. M-14 is not mutagenic in mammalian cells under the conditions of this study.

Data Point:	KCA 5.8.1/38
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE 1388273: Induction of micronuclei in the bone marrow of treated mice
Report No:	C041632
Document No:	M-231098-01-1
Guideline(s) followed in study:	OECD 474 (1997); ICH Tripartite Harmonised Guideline on Genotoxicity (1995)
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no calibration plot or calibration equation presented, however the calibration range and correlation coefficient (1.0000) are reported. For the accuracy and precision data, there is only 1 concentration level prepared in matrix (LOQ samples). However, there are 6 determinations at this level with a mean recovery between 70-110% and RSD <20%. Considering that this analytical method is validated in support of a toxicological cold study, the method validation is considered fit for purpose. Study: 2000 rather than 4000 immature erythrocytes were analysed
Previous evaluation:	yes, evaluated and accepted in DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The micronucleus test was employed to investigate M-14 in male CD-1 mice for a possible clastogenic effect on the chromosomes of bone marrow erythroblasts.

Cyclophosphamide (a known clastogen and cytostatic agent) served as the positive control.

Male mice treated with M-14 received two intraperitoneal administrations of 250, 500 or 1000 mg/kg bw, separated by 24 hours. Males of the positive control received a single intraperitoneal treatment with 2 mg/mL cyclophosphamide. 5/6 animals at 1000 mg/kg bw/d unexpectedly dies during the main study and so these animals were not examined for micronuclei. A second dose-range finding study conducted at 750 mg/kg bw/d confirmed that 500 mg/kg bw/d was close to the MTD and so a third dose group of 125 mg/kg bw/d was added to achieve the required number of dose groups.

The femoral marrow of all groups was prepared 24 hours after the last administration.

Males treated twice with M-14 in doses up to and including 500 mg/kg bw did not show any signs of toxicity, however the highest dose of 500 mg/kg bw/d has been demonstrated to be very close to the MTD. There was an altered ratio between polychromatic and normochromatic erythrocytes at 500 mg/kg bw/d, further demonstrating the systemic exposure of the animals to the test substance and the route of administration (i.p) means that the test substance is likely to have reached the target tissue.

Following two intraperitoneal treatments of males with doses up to and including 500 mg/kg bw no indications of a clastogenic effect of M-14 were found.

The positive control produced a clear clastogenic effect, as demonstrated by a biologically relevant increase in polychromatic erythrocytes with micronuclei.

It was concluded that M-14 is not clastogenic under the conditions of this assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-14 (referred to as AE 1388273 in the report)
Purity:	99.5%
Batch no.:	RSS1923LJ31633

2. Vehicle and/or positive control

Vehicle:	1% aqueous methylcellulose (1% MC)
Positive control:	2 mg/mL cyclophosphamide (CPA) in saline

3. Test animals

Species:	Mice
Strain:	Crl:CD-1 (ICR)BR
Age:	4-7 weeks
Weight at start:	21-33 g
Source:	[redacted]
Acclimation period:	At least five days
Diet:	Special diets services Ltd. Provided *ad libitum*
Water:	Provided *ad libitum*
Housing:	Housed in groups of ≤ 3 of the same sex in solid floored cages with wood shavings
Identification	Cage markings and ear tags
Temperature:	19-25 °C
Humidity:	40-70%
Air changes:	15/hour
Photoperiod:	12 hours light/12 hours dark

4. Test substance doses

The selection of doses was based on a pilot test using animals of the same source, strain, and age. In the pilot study up to 3/mice/sex received the test substance via intraperitoneal injections at doses of 500 (1/sex), 1000 (1/sex), 1250 (3/sex), 1500 (3/sex) and 2000 (1/sex) mg/kg bw; 24 hours later a second injection at the same dose was administered.

At 2000 mg/kg bw/d both animals died 0.5-hours after the first dose and at 1500 mg/kg bw/d 2 males and 3 females were found dead after the first dose preceded by clinical signs comprising lethargy, splayed gait, irregular breathing and unkempt fur. At 1250 mg/kg bw/d 2 males and 3 females were found dead 1 hour after the first dose with clinical signs characterized by lethargy, decreased rate of breathing, eye closure and abnormal gait. There were no deaths at 1000 or 500 mg/kg bw/d and the only clinical signs were lethargy in both sexes at 100 mg/kg bw/d. Based on this data, only males were used in the main study and a high dose of 1000 mg/kg bw/d was selected with a mid- and low-dose of 500 and 250 mg/kg bw/d.

However, 5/6 animals at this dose died in the main study prior to the scheduled sampling time. A further range-finding study was conducted with 3 males dosed at 750 mg/kg bw/d. No clinical signs were seen on the first day; however, on the second day 1 male died 0.5 hours following dosing, another was lethargic and cold to the touch 4 hours after dosing and was killed in extremis on day 3 after being found comatose. The remaining animal showed no clinical signs. As 500 mg/kg bw/d appeared to be close to the MTD, an additional main experiment was conducted with a low dose of 125 mg/kg bw (6 males).

B. Test performance

Experimental phase: December 8, 2003 to March 9, 2004

1. Treatment and sampling times

The study design of the main study was as follows:

Table 5.8.1- 174: Study design of main study

Experimental group	Dose in mg/kg bw	No. of animals	Route and number of applications	
Negative control	0	6	i.p.	2
M-14	250	6	i.p.	2
	500	6	i.p.	2
	1000	6	i.p.	2
Positive control (Cyclophosphamide)	20	6	i.p.	1

Male mice (six/group) received two intraperitoneal administrations of 250, 500 or 1000 mg/kg bw, separated by 24 hours. Males of the positive control received a single intraperitoneal treatment with 20 mg/kg bw cyclophosphamide, whilst two injections of the negative control (1% MC) were administered the same way as the treated groups. In all groups the administered volume was 20 mL/kg.

As 5/6 animals dosed at 1000 mg/kg bw/d died and following an additional range-finding study, a main further experiment was conducted to provide a total of 3 suitable dose levels. Animals of the additional main study were treated in the same way as the first main study as follows:

Table 5.8.1- 175: Additional main study

Experimental group	Dose in mg/kg bw	No. of animals	Route and number of applications	
Negative control	0	6	i.p.	2
M-14	125	6	i.p.	2
Positive control (Cyclophosphamide)	20	6	i.p.	1

2. Tissues and cells examined

Mice were killed in groups, 24-hours following the final dose, by CO_2 asphyxiation. Both femurs from each animal were exposed, removed, cleaned of adherent tissue and the end removed from the shanks. Using a syringe and needle, bone marrows were flushed from the marrow cavity with 1mL foetal bovine serum into centrifuge tubes. The tubes were centrifuged (1250 x g, 2-3 minutes) and the serum was aspirated to leave 1-2 drops and the cell pellet. The pellet was mixed into the small volume of serum in each tube and a smear prepared from a small volume of the suspension on of each of 2 slides. Slides were air dried, fixed and rinsed. One slide from each set of 2 was then taken, the other was reserved. Following a second fixing/rinsing slides were stained for 10 minutes in filtered Giesma stain diluted 1:6 (v/v) in distilled water. Stained slides were rinsed and allowed to dry thoroughly before clearing in xylene for 3 minutes. When dry the slides were mounted with cover slips.

3. Evaluation

Initially the relative proportions of polychromatic erythrocytes (PCE), seen as pale blue or blue/grey enucleate cells, and normochromatic erythrocytes, seen as smaller yellow/orange stained enucleate cells, were determined until a total of 1000 (PCE plus NCE) had been analysed.

Counting of PCE cells continued until 2000 PCE had been observed.

The ratio of PCE/NCE for each animal and the mean for each group was calculated. The individual and group mean frequency of micronucleated PCE/a000 cells (+/- SD) was also established. A reduction in the PCE/NCE ratio would provide evidence of bone marrow toxicity (exposure).

4. Evaluation criteria

The assay is considered acceptable if the following criteria are met:

- The incidence and distribution of micronucleated PCE in the concurrent vehicle control group is comparable with the historical negative controls
- At least 5/males/group are available for analysis
- The positive control chemical (CPA) induces statistically significant increases in the frequency of micronucleated PCE

A test item is considered positive if:

- A statistically significant increase in the frequency of micronucleated PCE occurs in at least one dose
- Any increase is outside the range of the historical control data

5. Statistical methods

The frequencies of micronucleated PCE in the vehicle control animals were compared to the historical negative control data to determine the acceptability of the assay. For each group, inter-individual variation in the numbers of micronucleated PCE was estimated by means of a heterogeneity chi^2 test.

The numbers of micronucleated PCE in each treated group were then compared with the numbers in the vehicle control groups by using a 2 X 2 contingency table to determine chi^2. Probability values of p = ≤0.05 were to be accepted as significant. A further statistical test (for linear trend) was used ot evaluate possible dose-response relationships.

If the heterogeneity chi^2 test provides evidence of significant (p=≤0.05) variability between animals within at least one group then non-parametric analysis will be more appropriate, a Wilcoxon rank sum test will then be used.

II. Results and Discussion

A. Micronucleus assay

Following two intraperitoneal administrations of 1000 mg/kg bw M-14, 5/6 males died on day 2 after exhibiting clinical signs comprising lethargy, hunched posture, and piloerection. This dose group was therefore excluded from the micronucleus assay. Following an additional range-finding study at 75 mg/kg bw/d it was determined that 500 mg/kg bw/d was close to the MTD and so an additional dose group at 125 mg/kg bw/d was tested. There were no deaths at 125, 250 and 500 mg/kg bw/d and no clinical signs were observed.

Treated mice exhibited group mean ratios of PCE to NCE which were similar to the concurrent negative controls and fell within the historical negative control range for most concentrations; the PCE/NCE ratio at 500 mg/kg bw/d, however, was depressed thus indicating exposure of the bone marrow to the test substance.

The frequency of micronucleated PCE in the treated groups were not significantly different from the vehicle controls.

An overview of the genotoxicity evaluation is provided below.

Table 5.8.1- 176:: Group mean PCE/NCE ratios and incidences of micronucleated PCE and NCE (main study)

Treatment group	Dose (mg/kg bw)	No. of animals	Mean PCE/NCE	No. micronucleated PCE/2000 ± SD
M-14	0	6	0.77	1.42±0.86
	250	6	0.88	0.67±0.61
	500	6	0.61	1.42±0.66
Positive control (Cyclophosphamide)	40	5	1.16	13.00±2.95

Table 5.8.1- 177: Group mean PCE/NCE ratios and incidences of micronucleated PCE and NCE (additional experiment)

Treatment group	Dose (mg/kg bw)	No. of animals	No. NCE/ 2000 PCE ± SD	No. micronucleated PCE/2000 ± SD
M-14	0	6	0.75	0.08±0.20
	125	6	0.86	0.33±0.26
Positive control (Cyclophosphamide)	40	6	1.31	9.0±1.70

III. Conclusion

There was no indication of a clastogenic effects in male mice following two intraperitoneal treatments with doses of up to and including 500 mg/kg bw.

The ratio of polychromatic to normochromatic erythrocytes was altered by treatment at the high dose of 500 mg/kg bw/d and thus confirmed relevant systemic bone marrow exposure; furthermore, a second dose range-finding study confirmed that 500 mg/kg bw/d was very close to the MTD and intraperitoneal administration of the test substance is likely to achieve exposure of the target tissue.

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered M-14 in the micronucleus test in male mice, i.e. in a somatic test system *in vivo*.

Assessment and conclusion by applicant:

This study was conducted according to OECD TG 474 and is valid and acceptable to determine the clastogenic potential of M-14 *in vivo*. M-14 was not clastogenic under the conditions of this study.

Data Point:	KCA 5.8.1/39
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE 1388273: Measurement of unscheduled DNA synthesis in rat liver using an in vivo/in vitro procedure
Report No:	C041627
Document No:	M-231087-01-1
Guideline(s) followed in study:	OECD 486 (1997); UKEMS guidelines (1993)
Deviations from current test guideline:	Minor deviations from the protocol relating to animal husbandry and slide preparation occurred which were considered not to have affected the validity or integrity of the study.
Previous evaluation:	yes, evaluated and accepted in DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

M-14 (reported as AE 1388273) was tested for its ability to induce unscheduled DNA synthesis (UDS) in the livers of male rats dosed via oral gavage using an *in vivo/in vitro* procedure.

Groups of four male rats were treated once with the vehicle 1% (w/v) aqueous methyl cellulose (1% MC), M-14 (at 800 mg/kg or 2000 mg/kg) or the required positive control, via oral gavage. The positive controls used were 75 mg/kg 2-acetamidofluorene (2-AAF) suspended in corn oil (12-14 hour experiment) and 10 mg/kg dimethylnitrosamine (DMN) dissolved in purified water (2-4 hour experiment). No clinical signs of toxicity were observed in the main study.

Approximately 12-14 hours (Experiment 1) or 2-4 hours (Experiment 2) after dosing, animals were sacrificed, and their livers perfused with collagenase to provide a primary culture of hepatocytes. Cultures were made from three animals in each dose group and were treated with [^{3}H] thymidine. Six slides (where available) from each animal were prepared with fixed hepatocytes and of these, three were dipped in photographic emulsion to prepare autoradiograms. Slides were examined microscopically after development of the emulsion and staining, and the net grain count (NNG), the number of grains present in the nucleus minus the mean number of grains in three equivalent areas of cytoplasm, was determined for each of two of the three slides, each animal and dose group.

Negative (vehicle) control animals gave a group mean NNG value of less than zero with no cells in repair. Group mean NNG values were increased by 2-AAF and DMN treatment to 9.1 or more with more than 70% of cells found to be in repair. In this study the vehicle control NNG value was consistent with both published and historical control data, and the system was shown to be sensitive to two known DNA damaging agents requiring metabolism for their action. The assay was therefore accepted as valid.

Treatment with 800 or 2000 mg/kg M-14 did not produce a group mean NNG value greater than -0.6 nor were any more than 1% cells found in repair at either dose.

When treated once via oral gavage with M-14 at doses up to 2000 mg/kg male rats showed no induction of UDS in hepatocytes isolated *ex vivo* approximately 12-14 or 2-4 hours after dosing. It is concluded that M-14 had no genotoxic activity detectable in this test system under the experimental conditions employed.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-14 (referred to as AE 1388273 in the report)
Purity:	99.5%
Batch no.:	RSS1923/LJ31633

2. Vehicle and/or positive control

Vehicle:	1% (w/v) aqueous methylcellulose (1% MC)
Positive control:	2-Acetamidofluorene (2-AAF; 12-14 hour experiment) Dimethymitrosamine (DMN; 2-4 hour experiment)

3. Test animals

Species:	Rat, male
Strain:	Wistar Crl:WI (Glx/BRL/Han)
Age:	7 to 8 weeks
Weight at start:	193 - 262 g
Source:	[redacted]
Acclimation period:	At least 5-days
Diet:	Standard rodent diet
Water:	Provided *ad libitum*
Housing:	Groups of four in solid-floored cages
Temperature:	19.5-20.1 °C
Humidity:	52-63%
Air changes:	Fifteen times/hour
Photoperiod:	12 hours light/12 hours dark

4. Test substance doses

The selection of the doses was based on a range-finding test, in which groups of three males and three females were orally dosed 1000 or 2000 mg/kg bw of the test substance at a volume of 10 mL/kg. An observation period of 48 hours followed. The following clinical signs were recorded

Table 5.8.1- 178: results of the range-finding test

Dose (mg/kg bw)	Day 1*	Day 2	Day 3
1000	Exopthalmus (3M 3F)	Normal (3M 3F)	Normal (3M 3F)
2000	Exopthalmus (3M 3F), piloerection (3M 3F)	Normal (3M 3F)	Normal (3M 3F)

*Day of application
M = Male
F Female

These treatments confirmed that a single oral dose of 2000 mg/kg (the maximum dose recommended by the current test guidelines) was well tolerated and was therefore selected as the maximum dose for the UDS experiments. A lower dose of 800 mg/kg (~40% of the maximum dose) was also selected. As no substantial inter-sex difference in toxicity was observed in the range-finder experiment, male animals only were treated in the main study.

B. Test performance

Experimental phase: 9 December 2003 to 5 March 2004

1. Treatment and sampling times

Animals were weighed before dosing and the volume of vehicle, test item preparation or positive control solution to be administered was calculated based on a dose volume of 20 mL/kg for the test item and negative control or 10 mL/kg for the positive controls. Animals were not fasted prior to dosing. All treatments were given via oral gavage to maximise exposure of the target organ to the test article.

The experimental design is shown below:

Table 5.8.1- 179: UDS *in vivo* test design

Experimental group	Dose (mg/kg)	No. of animals	
		Experiment 1 (12-14 hour)	Experiment 2 (2-4 hour)
Negative control (1% MC)	0	4	4
M-14	800	4	4
	2000	4	4
Positive control			
2-AAF	75	4	-
DMN	10		4

Dosing preparations were made by suspending M-14 in 1% (w/v) aqueous methylcellulose (1% MC) with the aid of stirring to give concentrations of 40 and 100 mg/mL. The test item preparations were protected from light and used within 26 hours of initial formulation.

On completion of the range-finder experiment and selection of main study dose levels, formulations of M-14 at 12.5 and 200 mg/mL were analysed for achieved concentration, homogeneity and stability over 4 days.

In the main study, each test item dose preparations was analysed for achieved concentration and homogeneity prior to dosing and for achieved concentration only after dosing. Samples were taken from the bulk formulations used for dosing. In addition, samples of the vehicle (taken before and after dosing) were also assayed for M-14 content.

The negative (vehicle) control was 1% (w/v) aqueous methylcellulose (1% MC).

2-Acetamidofluorene (2-AAF) was freshly suspended in corn oil using a Silverson homogeniser at 7.5 mg/mL to serve as the positive control for the 12-14 hour experiment. Dimethymitrosamine (DMN) dissolved in purified water at 1.0 mg/mL was used as the positive control for the 2-4 hour experiment.

2. UDS test

Killing of animals and preparation of hepatocyte cultures:

For Experiments 1 and 2, animals were sacrificed a nominal 12-14 hours and 2-4 hours after dosing, respectively.

Individual animals were anaesthetised with halothane and maintained under deep anaesthesia to prevent any likelihood of recovery. The liver was surgically exposed, the hepatic portal vein and superior vena cava cannulated with suitable cannulars and the liver perfused with suitable buffers. Approximately 400 mL of calcium free

Buffer 1 was pumped at a flow rate of approximately 40 mL/min to wash the liver free of blood. The liver was then perfused with Buffer 2 also at a flow rate of 40 mL/min for approximately 5 minutes. Both buffers were gassed with 5% CO_2 in air (v/v) prior to use and Buffer 2 throughout perfusion.

Calcium and collagenase was added to the reservoir and after one to two minutes the waste line was placed in the Buffer 2 reservoir so that the perfusate recirculates and the flow rate was reduced to 20 mL/min. When the reticular pattern of the liver had begun to break up and the liver became 'spongy', the perfusion was stopped.

The liver was cut free into a suitable container with Buffer 2. The liver was transferred to a sterile dish, cut open and the hepatocytes carefully teased out. The resulting hepatocyte suspension was gently washed through 150 µm nylon mesh with Williams E medium-Complete (WE-C) to a volume of approximately 100 mL. Of this suspension, approximately 50 mL was taken and centrifuged at approximately 40 x 'g' for two to three minutes. The resultant pellet was resuspended in WE-C. The centrifugation and resuspension procedure was repeated at least twice and the pellet resuspended finally in approximately 20 mL WE-C. A sample (0.5 mL) of this suspension was taken, diluted with an equal volume of 0.4% (w/v) trypan blue in phosphate buffered saline (PBS) and the proportion of viable cells (those with unstained nuclei) determined using an haemocytometer. The culture was diluted where possible to provide approximately 1.5 x 10^5 viable cells/mL.

Three mL of hepatocyte suspension was added to each well of a six-well multiplate containing 25mm round plastic coverslips and incubated at 37°C ± 1°C in a 5% CO_2 in air (v/v) atmosphere for at least 90 minutes to allow cells to attach.

Radiolabelling of hepatocyte cultures:

Medium was removed from the cells and the monolayers washed with 2mL Williams E Medium-Incomplete (WE-I) which was then replaced with 2 mL WE-I containing 10 µCi/mL [^{3}H] thymidine. After approximately 4 hours incubation at 37°C in a 5% CO_2 in air (v/v) atmosphere, the medium was removed, and the cells washed with three changes of WE-I containing 0.25 mM thymidine. Cultures were then incubated overnight with 2 mL of the same medium.

To prepare for autoradiography, coverslips were washed with 2mL phosphate buffered saline (PBS) and the cells fixed with three changes of 2 mL glacial acetic acid:ethanol (1:3 v/v). The coverslips were then washed four times with purified water, allowed to dry and mounted onto previously labelled microscope slides, cells side up, with DPX.

Autoradiography:

Three of the five or six slides from each animal were coated in Ilford K2 liquid emulsion using a dipping technique. The slides were then packed in light-tight boxes containing desiccant, sealed with tape and refrigerated for 14 days. At the end of this time, the emulsion was developed in Kodak D19 developer and fixed using Ilford Hypam fixer. The cell nuclei and cytoplasm were then stained with Meyers haemalum/eosin Y. Slides were then dehydrated in ethanol, cleared in xylene and mounted with coverslips for microscopic examination. The spare, duplicate sets of slides were not required.

Grain counting:

Grain counting was performed using a microscope with a video camera connected to an image analysis system (Perceptive Instruments) and a computer programmed for automatic data capture.

Each slide was examined to ensure that the culture was viable. A patch of cells was selected as a starting point and cells were scored in a regular fashion by bringing new cells into the field of view, moving only in one axis. If the desired number of cells had not been scored before coming to the edge of the slide, the stage was moved one or two fields on the other axis and counting resumed. The circular field was centred over the nucleus of a suitable cell and the grains counted. The field of view was moved, and

counts obtained for three separate adjacent areas of cytoplasm. Nuclear and mean cytoplasmic grain counts were then recorded, and the net grains/nucleus (NNG) determined. 100 cells were analysed per animal, where possible using two of the three slides in each case.

The following criteria were used for cell analysis:

8. only cells with normal morphology were scored
9. isolated nuclei with no surrounding cytoplasm were not scored
10. cells without nuclear and/or cytoplasmic graining were not scored
11. cells with unusual staining artefacts were not scored
12. heavily labelled cells in S-phase were not scored
13. all other normal cells, 100 per animal were scored
14. all slides were analysed blind (coded)

Treatment of data:

The following were calculated for each slide, animal and dose point:

4. the population average NNG and standard deviation (SD)
5. the percent of cells responding or in repair (ie ≥ NNG)
6. the population average cytoplasmic and nuclear grain count.

3. Evaluation

An assay is considered acceptable if the following criteria are met:

- the negative control animals had a group mean NNG value that did not exceed the upper limit of the historical range
- The positive control treatments should have group mean values of five or more NNG with 50% or more cells having NNG counts of five or greater

The test item would be considered as positive in this assay if, at any dose and at either time point:

3. the test item yielded group mean NNG values greater than 0 NNG and 20% or more of cells responding (mean NNG values ≥ 5)
4. an increase above solvent control levels was seen in both NNG and the percentage of cells in repair.

Cytoplasmic and nuclear grain count values as well as the concurrent negative control data would be considered in relation to the overall NNG values of cultures from treated animals.

If the test article failed to induce UDS at any dose tested after both 2-4 and 12-14 hours exposure, it would be considered clearly negative in this system.

4. Statistical methods

No statistical analysis of the generated data was undertaken.

II. Results and Discussion

A. UDS assay

Dose preparations of M-14 used in the main UDS experiments had achieved concentrations of 100-108% of nominal and were homogenous. Analysis of the same formulations after dosing confirmed that dose preparations were stable from the time of formulation to completion of animal dosing. Analysis therefore confirmed the acceptability of all dose preparations used in the UDS experiments.

No clinical signs of toxicity were observed in the main study experiments.

The results of the DNA repair test with 12-14 and 2-4 hour exposure periods are summarised in the tables below:

Table 5.8.1- 180: Mean nuclear and cytoplasmic grain counts following 12-14 hour exposure

Treatment group	Dose (mg/kg bw)	Mean gross nuclear grain count (NG)	Mean cytoplasmic grain count (CG)	Mean net nuclear grain count (NNG)
Negative control 1% MC	0	2.34±0.38	2.91±0.59	-0.57±0.49
M-14	800	2.15±0.29	2.75±0.37	-0.60±0.13
	2000	2.66±0.06	3.35±0.13	-0.75±0.41
Positive control 2-AAF	75	12.82±1.76	3.75±0.54	9.07±1.61

Table 5.8.1- 181: Mean nuclear and cytoplasmic grain counts following 2-4 hour exposure

Treatment group	Dose (mg/kg bw)	Mean gross nuclear grain count (NG)	Mean cytoplasmic grain count (CG)	Mean net nuclear grain count (NNG)
Negative control 1% MC	0	1.45±0.27	2.45±0.44	-0.77±0.19
M-14	800	1.30±0.09	1.97±0.13	-0.68±0.20
	2000	1.78±0.04	3.14±0.19	-1.36±0.22
Positive control DMN	10	16.54±5.90	2.12±0.36	14.42±5.54

Treatment with M-14 at doses up to 2000 mg/kg yielded NNG values less than zero, producing group mean NNG values over the two experiments in the range -0.6 to -1.4, well below the threshold value of 0 NNG required for a positive response. No more than 1% cells were seen in repair at any dose of M-14.

The data obtained in this study indicate that oral gavage treatment of male rats dosed once with 800 or 2000 mg/kg M-14 did not result in increased UDS in hepatocytes isolated approximately 12-14 or 2-4 hours after dosing.

The group mean net grain count for vehicle-treated animals was less than the upper limit of the historical control range (-0.6 and -0.8 for 12-14 hour and 2-4-hour experiments respectively). The positive control chemicals 2-AAF and DMN induced increases in group mean net grain count of five or more (9.1 and 14.4 respectively), and 50% or more of cells (79% and 100% respectively) had net grain counts of five or more. these results showed that the test system was sensitive to two known DNA damaging agents requiring metabolism for their action and that the experiment was valid.

III. Conclusion

When treated once via oral gavage with M-14 at doses up to 2000 mg/kg male rats showed no induction of UDS in hepatocytes isolated *ex vivo* approximately 12-14 or 2-4 hours after dosing.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 486 and is valid and acceptable to assess the potential of M-14 to induce DNA damage and repair *ex vivo*. M-14 did not induce unscheduled DNA repair in the rat liver *ex vivo* under the conditions of this study.

M-15 (AE 1413903)

No acute toxicity or short-term toxicity studies are available for M-15 and are not required.

The metabolite M-15 has been investigated for mutagenicity *in vitro* in bacterial cells and for mutagenicity and clastogenicity in mammalian cells. M-15 was not mutagenic *in vitro* in bacteria, as evidenced by a negative Ames test when tested in appropriate strains of *S typhimurium* in the presence and absence of metabolic activation. Neither was any mutagenic potential seen in mammalian cells *in vitro* when M-15 was tested at the HPRT locus of Chinese hamster V79 cells, with and without metabolic activation.

In an *in vitro* micronucleus test in cultured human peripheral blood lymphocytes M-15 did not induce any biologically relevant increases in the numbers of micronucleated cells either in the presence or absence of metabolic activation up to the highest required concentration; thus it can be concluded that M-15 is not clastogenic or aneugenic.

Overall, there is no evidence that M-15 is genotoxic and there is no toxicological concern for this metabolite. The available studies are summarised below.

Data Point:	KCA 5.8.1.36
Report Author:	[redacted]
Report Year:	2018
Report Title:	AE 1413903, pure: Salmonella typhimurium reverse mutation assay
Report No:	1876301
Document No:	M-614800-01-1
Guideline(s) followed in study:	OECD 471 (1997); EU Commission Regulation (EC) No 440/2008 (2008) US-EPA 712-C-98-247, OCSPP 870.5100 (1998)
Deviations from current test guideline:	Deviations from the current OECD guideline 471 (1997): none
Previous evaluation:	yes, evaluated and accepted by RMS Austria in 2018
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In this study compliant with the current OECD guideline 471 (1997) the potential of the fluopicolide metabolite M-15 (AE 1413903; batch AE 1413903, 93.2%) to induce gene mutations in bacterial cells was assessed in two independent experiments (plate incorporation test (experiment I) and pre-incubation test (experiment II)) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102 with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate.

The test substance was tested at the following concentrations:

Pre-experiment/experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

No precipitation of the test substance occurred up to the highest investigated dose.

The plates incubated with the test substance showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with M-15, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test substance did not induce gene mutations by base pair changes or frameshifts in the genome of the bacterial strains used.

Therefore, M-15, pure is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-15 (referred to as AE 1413903 in the report)
Purity:	93.2%
Batch no.:	SES 13490-2-4

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	Without S9 mix:
	sodium-azide ($Na\text{-}N_3$): TA 100, TA 1535
	4-nitro-o-phenylene diamine (4-NOPD) : TA 98, TA 1537
	Methyl methane sulfonate (MMS): TA 102
	With S9 mix:
	2-aminoanthracene (2-AA): TA 100, TA 1535, TA 1537, TA 98, TA 102

3. Activation:

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test. Furthermore, for each S9 batch a sterility test and the determination of the protein concentration were performed. The protein concentration of the S9 preparation was 32.9 mg/mL (Lot. No.: 010617K) in the pre-experiment / experiment I and 32.6 mg/mL (Lot No.: 010617E) in experiment II.

An appropriate quantity of S9 fraction was thawed and mixed with S9 cofactor solution, to result in a final concentration of approx. 10% v/v in the S9 mix. Cofactors were added to the S9 mix to reach the following concentrations in the S9 mix:

$MgCl_2$	8 mM
KCl	33 mM
Glucose-6-phosphate, disodium salt	5 mM
NADP	4 mM
Sodium-ortho-phosphate buffer (pH 7.4)	100.0 mM

4. Test organisms:
Five strains of *Salmonella typhimurium* TA 1537, TA 98, TA 1535, TA 100, and TA 102 were used. Strains TA 1537 and TA 98 were included to detect frame-shift mutations, whilst the remaining strains detected base-pair mutations.

Regular checking of the properties of the *Salmonella typhimurium* strains regarding the membrane permeability, ampicillin resistance; UV sensitivity, and amino acid requirement as well as normal spontaneous mutation rates was performed by the laboratory.

5. Test substance concentrations used:

The following test substance concentrations were used in the plate incorporation and pre-incubation assays:

Plate incorporation assay:	0, 3, 10, 33, 100, 333, 1000, 2500, 5000 µg fluopicolide/plate
Pre-incubation assay:	0, 33, 100, 333, 1000, 2500, 5000 µg fluopicolide/tube

B. Test performance

Experimental phase: 28 November to 21 September 2003

1. Salmonella/microsome test

The thawed bacterial suspension was transferred into 250 mL Erlenmeyer flasks containing 50 mL nutrient medium. A solution of 50 µL ampicillin (25 µg/mL) was added to strains TA 98, TA 100, and TA 102. This nutrient medium contained per litre:

8 g Nutrient Broth
5 g NaCl

The bacterial cultures were incubated in a shaking water bath for 4 hours at 37 °C. The optical density of the bacteria was determined by absorption measurement and the obtained values indicated that the bacteria were harvested at the late exponential or early stationary phase (10^8-10^9 cells/mL).

For each strain and dose level, including the controls, three plates were used. The pre-experiment is reported as experiment I, since evaluable plates (>0 colonies) were obtained at five concentrations or more in all strains used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

Experiment I / Plate incorporation assay

100 µL	Test solution at each dose level, solvent or positive
500 µL	S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
100 µL	Bacteria suspension (test system, pre-culture of the strains),
2000 µL	Overlay agar

Experiment II / Pre-incubation assay

In the pre-incubation assay 100 µL test solution (solvent or reference mutagen solution (positive control)), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspension were mixed in a test tube and incubated at 37 °C for 60 minutes. After pre-incubation 2.0 mL overlay agar (45 °C) was added to each tube. The mixture was poured on minimal agar plates.

After solidification, the plates were incubated upside down for at least 48 hours at 37 °C in the dark.

In parallel to each test a sterile control of the test item was performed and documented in the raw data. Therefore, 100 µL of the stock solution, 500 µL S9 mix / S9 mix substitution buffer were mixed with 2.0 mL overlay agar and poured on minimal agar plates.

2. Statistics

Descriptive statistical methods were used to calculate means and standard deviations.

3. Acceptance / assessment criteria:

The *Salmonella typhimurium* reverse mutation assay is considered acceptable if it meets the following criteria:

- regular background growth in the negative and solvent control.
- the spontaneous reversion rates in the negative and solvent control are in the range of our historical data.
- the positive control substances should produce an increase above the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control.
- a minimum of five analysable dose levels should be present with at least three dose levels showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5.

6. Evaluation criteria

Solubility: Precipitation of the test substance was recorded if observed.

Toxicity: Toxic effects evident as a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn.

Mutagenicity: A reproducible and dose-related increase in mutant counts of at least one strain was considered to be a positive result, if:

- for TA 102, TA 100 and TA 98 this increase was about twofold compared to negative controls
- for TA 1535 and TA 1537 this increase was at least threefold compared to negative controls

A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration. A dose dependent increase in the number of revertant colonies below the threshold was regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remained within the historical range of negative and solvent controls such an increase was not considered biologically relevant.

Otherwise, the result was evaluated as negative.

II. Results and Discussion

A. Toxicity and Solubility

No precipitation of the test substance occurred up to the highest investigated dose.

The plates incubated with the test substance showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used. No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.

B. Mutation Assay

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with M-15, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

An overview of the results is given in the following tables.

Table 5.8.1- 182: Summary of experiment I

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	TA 102
Without Activation	DMSO		11 ± 1	10 ± 1	24 ± 5	173 ± 15	508 ± 26
	Untreated		8 ± 1	9 ± 2	29 ± 3	182 ± 9	528 ± 4
	M-15	3 µg	12 ± 4	11 ± 1	26 ± 1	195 ± 20	510 ± 13
		10 µg	8 ± 2	10 ± 3	26 ± 6	179 ± 17	505 ± 24
		33 µg	10 ± 5	10 ± 1	26 ± 4	176 ± 8	505 ± 32
		100 µg	11 ± 0	10 ± 6	25 ± 3	186 ± 7	478 ± 12
		333 µg	11 ± 4	7 ± 2	29 ± 3	171 ± 7	510 ± 26
		1000 µg	11 ± 3	8 ± 3	30 ± 6	178 ± 5	515 ± 21
		2500 µg	13 ± 2	8 ± 3	23 ± 3	179 ± 20	514 ± 34
		5000 µg	12 ± 3	8 ± 3	29 ± 3	173 ± 12	491 ± 19
	NaN3	10 µg	1290 ± 45			2036 ± 73	
	4-NOPD	10 µg			294 ± 25		
	4-NOPD	50 µg	146 ± 10				
	MMS	2.0 µL					3775 ± 376
With Activation	DMSO		10 ± 3	12 ± 2	28 ± 6	154 ± 12	649 ± 3
	Untreated		13 ± 5	14 ± 7	36 ± 3	180 ± 20	667 ± 10
	M-15	3 µg	8 ± 1	13 ± 1	37 ± 1	156 ± 31	716 ± 26
		10 µg	13 ± 2	13 ± 2	30 ± 9	166 ± 11	687 ± 39
		33 µg	11 ± 3	14 ± 3	35 ± 4	159 ± 10	723 ± 14
		100 µg	8 ± 2	11 ± 4	26 ± 8	163 ± 22	664 ± 24
		333 µg	7 ± 0	10 ± 4	37 ± 2	157 ± 4	707 ± 51
		1000 µg	8 ± 2	13 ± 3	30 ± 8	162 ± 5	688 ± 37
		2500 µg	8 ± 1	14 ± 2	32 ± 5	159 ± 8	701 ± 31
		5000 µg	12 ± 4	16 ± 2	30 ± 7	182 ± 25	678 ± 25
	2-AA	2.5 µg	500 ± 35	108 ± 6	3530 ± 275	4472 ± 214	
	2-AA	10.0 µg					1829 ± 6

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
MMS	methyl methane sulfonate
4-NOPD	4-nitro-o-phenylene-diamine

Table 5.8.1- 183: Summary of experiment II

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	TA 102
Without Activation	DMSO		10 ± 4	12 ± 3	25 ± 8	160 ± 15	527 ± 6
	Untreated		9 ± 3	16 ± 8	26 ± 8	195 ± 20	553 ± 8
	M-15	33 µg	11 ± 4	11 ± 1	21 ± 7	182 ± 24	535 ± 23
		100 µg	11 ± 5	11 ± 6	23 ± 2	162 ± 18	509 ± 10
		333 µg	8 ± 2	10 ± 1	26 ± 4	169 ± 4	513 ± 14
		1000 µg	9 ± 2	11 ± 3	26 ± 2	176 ± 12	550 ± 16
		2500 µg	9 ± 3	11 ± 2	29 ± 7	160 ± 14	528 ± 26
		5000 µg	6 ± 1	12 ± 2	33 ± 5	166 ± 4	487 ± 31
	NaN3	10 µg	1153 ± 124			1853 ± 121	
	4-NOPD	10 µg			342 ± 24		
	4-NOPD	50 µg		201 ± 19			
	MMS	2.0 µL					4506 ± 168
With Activation	DMSO		13 ± 4	13 ± 3	30 ± 4	157 ± 9	652 ± 22
	Untreated		14 ± 4	17 ± 4	30 ± 7	202 ± 13	659 ± 7
	M-15	33 µg	12 ± 5	11 ± 1	34 ± 8	175 ± 11	757 ± 34
		100 µg	13 ± 5	15 ± 3	34 ± 11	164 ± 3	744 ± 61
		333 µg	10 ± 1	13 ± 3	26 ± 5	168 ± 17	728 ± 35
		1000 µg	9 ± 3	15 ± 3	34 ± 5	182 ± 11	731 ± 28
		2500 µg	11 ± 5	13 ± 6	36 ± 7	184 ± 26	714 ± 13
		5000 µg	13 ± 3	9 ± 5	32 ± 5	176 ± 4	661 ± 42
	2-AA	2.5 µg	533 ± 89	101 ± 14	4177 ± 290	3738 ± 244	
	2-AA	10.0 µg					2287 ± 78

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
MMS	methyl methane sulfonate
4-NOPD	4-nitro-o-phenylene-diamine

III. Conclusions

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test substance M-15 (AE 1413903) did not induce gene mutations by base pair changes or frameshifts in the genome of the bacterial strains used.

Assessment and conclusion by applicant:

The study was conducted in accordance with OECD TG 471 and is valid and acceptable to investigate the potential of M-15 to induce gene mutation *in vitro* in bacterial cells. M-15 was not mutagenic *in vitro* under the conditions of this assay either by means of base-pair substitutions or frameshift mutations, in appropriate strains of *S-typhimurium* in the presence or absence of metabolic activation (provided by S9 mix).

Data Point:	KCA 5.8.1/52
Report Author:	[redacted]
Report Year:	2018
Report Title:	AE 1413903, pure: Mammalian cell gene mutation assay in Chinese hamster V79 cells in vitro (V79-HPRT)
Report No:	1876303
Document No:	M-616550-01-1
Guideline(s) followed in study:	OECD 476 (2016); Commission Regulation (EC) No. 440/2008 B.17 (2008); US-EPA 712-C-98-221, OPPTS 870.5300 (1998); Japanese Guidelines, Kanpoan No. 287, Eisei No. 127
Deviations from current test guideline:	Deviations from the current OECD guideline 476 (2016): none
Previous evaluation:	yes, evaluated and accepted by RMS Austria in 2018
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In this study the potential of the fluopicolide metabolite M-15 (AE 1413903, batch-no. SES 13490-2-4, purity: 93.2%) to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster was investigated according to OECD guideline 476 (2016). The treatment period was 4 hours with and without metabolic activation. The maximum test substance concentration of the pre-experiment and the main experiment (2146 µg/mL) was chosen with respect to the current OECD guideline 476 (2016).

No relevant cytotoxic effects indicated by an adjusted cloning efficiency I below 20% occurred up to the highest concentration in the presence and absence of metabolic activation. No substantial and dose dependent increase of the mutation frequency was observed in the main experiment in the tested concentrations range from 67.1 to 2146.0 µg/mL.

In the main experiment the mean mutant frequency of the solvent controls was 12.7 mutants per 10^6 cells in the absence of metabolic activation and 27.5 mutants per 10^6 cells in the presence of metabolic activation. The mean mutant frequencies of the groups treated with the test substance was in the range of 8.1 up to 22.1 mutants per 10^6 cells. The values were well within the 95% confidence interval of our laboratory's historical negative control data and, thus, fulfilled the requirements of the current OECD Guideline 476.

Appropriate reference mutagens, used as positive controls, induced a distinct increase in mutant colonies and thus, showed the sensitivity of the test system and the activity of the metabolic activation system.

In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells. Therefore, M-15 is considered to be non-mutagenic in this HPRT assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-15 (referred to as AE 1413903 in the report)
Purity:	93.2%
Batch no.:	SES 13490-2-4

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	-S9: Ethyl methanesulphonate (EMS), 300 µg/mL +S9: Dimethylbenzanthracene (DMBA), 2.3µg/mL

3. Activation:

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as metabolic activation system. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test. The protein concentration of the S9 preparation was 32.7 mg/mL (Lot. No.: 270717) in the pre-experiment and in the main experiment.
Immediately prior to use, an appropriate quantity of S9 fraction to result in a final protein concentration of 0.75 mg/mL in the cultures was thawed at room temperature and mixed with the freshly prepared cofactor solution in a sodium phosphate buffer pH 7.4. The mixture of S9 fraction with cofactor solution is designated S9 mix. The S9 mix was kept on ice until use and contained the following components:

$MgCl_2$	8 mM
KCl	33 mM
Glucose-6-phosphate	5 mM
NADP	4 mM

4. Cell cultures and media:

V79 cells of the Chinese hamster were used (HPGRT locus was examined) under the following culture conditions:

Culture medium:	MEM (minimal essential medium) containing Hank's salts supplemented with 10% foetal bovine serum (FBS), neomycin (5 µg/mL) and amphotericin B (1%)
Treatment medium:	Culture medium without FBS
Selection medium:	Culture medium with 11 µg/mL 6-thioguanine (6-TG)
Environmental conditions:	All incubations were done at 37 °C with 1.5% carbon dioxide (CO_2) in humidified air.

5. Test substance concentrations used:

The following test substance concentrations were used:

Pre-test for cytotoxicity: 0, 16.8, 33.5, 67.1, 134.1, 268.3, 536.5, 1073, 2146 µg/mL (-/+S9)
Mutation assay: 0, 67.1, **134.1, 268.5, 536.5, 1073, 2146** µg/mL (-/+S9)

Concentrations in bold were used for mutation analysis.

B. Test performance

Experimental phase: December 05, 2017 to January 03, 2018

1. Test substance preparation

On the day of the experiment (immediately before treatment), the test substance was dissolved in DMSO. The final concentration of DMSO in the culture medium was 0.5% (v/v). The solvent was chosen according to its solubility properties and its relative non-toxicity to the cell cultures.

All formulations were prepared freshly before treatment and used within two hours of preparation. The formulation was assumed to be stable for this period unless specified otherwise by the Sponsor.

The osmolarity and the pH-value were determined in culture medium of the solvent control and at the maximum concentration of the pre-experiment without metabolic activation.

2. Pre-test for cytotoxicity

A pre-test was performed in order to determine the toxicity of the test substance. In addition, the pH-value and the osmolality were measured. The general culturing and experimental conditions in this pre-test were the same as described below for the mutagenicity experiment.

In this pre-test approx. 1.5 million cells were seeded in 25 cm^2 flasks 24 hours prior to treatment. After approx. 24 hours the test substance was added, and the treatment proceeded for 4 hours (duplicate cultures per concentration level). Immediately after treatment the test item was removed by rinsing with PBS. Subsequently, the cells were trypsinised and suspended in complete culture medium. After an appropriate dilution, the cell density was determined with a cell counter. Toxicity of the test substance is evident as a reduction of the cell density compared to a corresponding solvent control. A cell density of approx. 1.5 million cells in 25 cm^2 flasks is about the same as approx. 10 million cells seeded in 175 cm^2 bottles 24 hours prior to treatment with the main experiment.

3. Gene mutation assays

Two independent gene mutation assays were performed as follows:

Seeding:

Three days after sub-cultivation stock cultures were trypsinised at 37 °C for approx. 5 to 10 minutes. Then the enzymatic digestion was stopped by adding complete culture medium with 10% FBS and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.2% in saline.

Prior to the trypsin treatment the cells were rinsed with PBS. Approx. 0.7 to 1.2 × 10^7 cells were seeded in plastic flasks. The cells were grown for 24 hours prior to treatment.

Treatment:

After 24 hours the medium was replaced with serum-free medium containing the test substance, 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 trypsinised as described above and sub-cultivated. At least 2.0 × 10^6 cells per experimental point (concentration series plus controls) were subcultured in 175 cm^2 flasks containing 30 mL medium.

Two additional 25 cm² flasks were seeded per experimental point with approx. 500 cells each to determine the relative survival (cloning efficiency I) as measure of test substance induced cytotoxicity. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

The colonies used to determine the cloning efficiency I were fixed and stained 6 to 8 days after treatment as described below.

Three or four days after first sub-cultivation approx. 2.0×10^6 cells per experimental point were sub-cultivated in 175 cm² flasks containing 30 mL medium.

Following the expression time of 7 days five 75 cm² cell culture flasks were seeded with about 4 to 5×10^5 cells each in medium containing 6-TG (11 µg/mL). Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability (cloning efficiency II).

The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2 for about 8 days. The colonies were stained with 10% methylene blue in 0.01% KOH solution.

The stained colonies with more than 50 cells were counted. In doubt the colony size was checked with a preparation microscope.

Calculations and processing of the data

The data listed in the tables of results are calculated as follows:

cloning efficiency I (survival)	cloning efficiency determined immediately after treatment to measure toxicity.
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 × 100
relative cell density % of control	(cell density at 1st subcultivation divided by the cell density at 1st subcultivation of the corresponding control) × 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 × 100
cells survived (after plating in TG containing medium)	number of cells seeded × cloning efficiency II absolute
mutant colonies / 10^6 cells	mean number of mutant colonies per flask found after plating in TG medium × 106 divided by the number of cells survived
relative adjusted cloning efficiency I	relative cloning efficiency I x relative cell density at first subcultivation / 100

4. Statistics

Linear regression (least squares, calculated using a validated excel spreadsheet) was performed to assess a possible dose dependent increase of mutant frequencies. The numbers of mutant colonies generated with the test substance were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05. However, both, biological and statistical significance were considered together.

A t-test was not performed since all mean mutant frequencies were well within the 95% confidence interval of our laboratory's historical negative control data.

5. Acceptance criteria

The gene mutation assay is considered acceptable if it meets the following criteria:

a) The mean values of the numbers of mutant colonies per 10^6 cells found in the solvent controls of both parallel cultures remain within the 95% confidence interval of the laboratory historical control data range.

b) Concurrent positive controls should induce responses that are compatible with those generated in the historical positive control data base and produce a statistically 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 substance concentrations) are analysable even for the cultures treated at concentrations that cause 90% cytotoxicity during treatment.

e) The criteria for the selection of the top concentration are fulfilled (see 0 dose selection).

7. Evaluation of results

A test substance is classified as clearly mutagenic if, in any of the experimental conditions examined, all of 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 test substance is classified as clearly non-mutagenic if, in all experimental conditions examined, all of the following criteria are met:

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

b) there is no concentration-related increase when evaluated with an appropriate trend test,

c) all results are inside the distribution of the historical negative control data (based 95% control limits).

In cases when the response is neither clearly negative nor clearly positive as described above, or in order to judge the biological relevance of a result, the data should be evaluated by expert judgement or further investigations.

II. Results and Discussion

A. Pre-test for cytotoxicity

In the pre-experiment no relevant cytotoxic effect, indicated by a relative cloning efficiency of 20% or below occurred up to the highest concentration with and without metabolic activation.

The test medium was checked for precipitation or phase separation at the beginning and at the end of treatment (4 hours) prior to removal to the test substance. No precipitation or phase separation was noted after 4 hours treatment with and without metabolic activation.

There was no relevant shift of pH and osmolality of the medium even at the maximum concentration of the test substance.

Table 5.8.1- 184: Osmolarity and pH

Parameter	Solvent control	M-15 (2146 µg/mL)
Osmolarity [mOsm]	405	378
pH-value	7.3	7.06

Table 5.8.1- 185: Cytotoxicity data, pre-experiment

Dose (µg/ml)	± S9	CE absolute (% control)	CE relative (% control)
0$	-	40.3	100.0
16.8		46.3	115.1
33.5		46.8	116.3
67.1		48.5	120.4
134.1		44.8	111.1
268.3		57.5	142.7
536.5		46.1	114.4
1073		44.9	111.5
2146		39.5	98.1
0$	+	53.2	100.0
16.8		54.3	102.2
33.5		55.3	103.9
67.1		52.7	99.1
134.1		53.3	100.1
268.3		51.0	96.0
536.5		50.6	95.0
1073		51.8	97.3
2146		48.9	92.0

$ solvent control

B. Gene mutation assays

No relevant cytotoxic effects indicated by an adjusted cloning efficiency I below 20% occurred up to the highest concentration in the presence and absence of metabolic activation.

No biologically relevant increase in mutant colony numbers was observed in the main experiment up to the maximum concentrations scored for gene mutations.

In the main experiment the mean mutant frequency of the solvent controls was 12.7 mutants per 10^6 cells in the absence of metabolic activation, and 27.5 mutants per 10^6 cells in the presence of metabolic activation. The mean mutant frequencies of the groups treated with the test item was in the range of 8.1 up to 22.1 mutants per 10^6 cells. The values were well within the 95% confidence interval of our laboratory's historical negative control data and, thus, fulfilled the requirements of the current OECD Guideline 476.

EMS (300 µg/mL) and DMBA (2.3 µg/mL) were used as positive controls and showed a distinct increase in induced mutant colonies and thus the sensitivity of this method.

Table 5.8.1- 186: Summary of results of the gene mutation assay

Dose (µg/mL)	Metabolic activation	relative cloning efficiency I (% control)	relative cell density (% control)	rel. adjusted cloning efficiency I (% control)	Mutant frequency (x 10^{-6})	95% confidence interval solvent control
0$	-	100.0	100.0	100.0	12.7	1.7-30.2
67.1		98.4	98.9	97.1	#	#
134.1		86.7	117.5	100.5	11.8	1.7-30.2
268.3		99.0	100.7	99.1	8.1	1.7-30.2
536.5		98.2	105.4	103.6	16.3	1.7-30.2
1073		97.8	107.2	104.9	8.1	1.7-30.2
2146		96.1	117.2	113.8	9.6	1.7-30.2
EMS 300		93.4	113.2	107.2	261.0	1.7-30.2
0$	+	100.0	100.0	100.0	27.5	2.0-29.4
67.1		99.2	122.2	120.3	#	#
134.1		97.4	109.1	106.1	12.9	2.0-29.4
268.3		105.7	113.8	119.9	14.9	2.0-29.4
536.5		85.1	99.9	86.2	22.1	2.0-29.4
1073		75.9	88.8	68.4	21.9	2.0-29.4
2146		93.0	88.5	81.6	19.7	2.0-29.4
DMBA 2.3		93.9	74.3	68.5	115.2	2.0-29.4

$ solvent control

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. No significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in any of the experimental groups.

Table 5.8.1- 187: Results of the linear regression analysis

Linear Regression / Main experiment	
S9 mix	**p-value (mean of both cultures)**
-	0.516
+	0.937

III. Conclusion

In conclusion it can be stated that under the experimental conditions reported the test substance did not induce gene mutations at the HPRT locus in V79 cells. Therefore, M-15 (AE 1413903) is considered to be non-mutagenic in this HPRT assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD 476 and is valid and acceptable to assess the potential of M-15 to induce gene mutations at the HPRT locus in Chines Hamster Lung V79 cells. M-15 is not mutagenic in mammalian cells under the conditions of this study.

Data Point:	KCA 5.8.1/51
Report Author:	[redacted]
Report Year:	2018
Report Title:	AE 1413903, pure: Micronucleus test in human lymphocytes in vitro
Report No:	1876302
Document No:	M-620774-01-1
Guideline(s) followed in study:	OECD 487 (2016)
Deviations from current test guideline:	Cells were harvested 40 hours after the beginning of the treatment which is slightly longer then the recommended 1.5-2 cell cycle length. However, a series of in-house non-GLP validation experiments using specified positive controls showed that the optimum in responses was found with an extended harvest time of 40 hours
Previous evaluation:	yes, evaluated and accepted by RMS Austria in 2018
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The fluopicolide metabolite M-15 (AE 1413903, batch no. SFS 13490-2-4, purity 93.2%) dissolved in DMSO was tested for mutagenic effects at the HGPRT locus (forward mutation assay) in human lymphocytes after *in vitro* treatment at concentrations up to 2146 µg/mL with and without S9 mix. The highest applied concentration in this study was chosen with regard to the purity (93.2%) of the test substance and with respect to the current OECD Guideline 487. The study was performed in two independent experiments, using two parallel cultures each. Experiment I was performed with a treatment period of 4 hours with and without metabolic activation and experiment II with a treatment period of 20 hours without metabolic activation only. Ethane methane sulfonate (EMS) was used as a positive control without S9 mix and dimethylbenzanthracene (DMBA) as a positive control with S9 mix.

In both cytogenetic experiments, in the absence and presence of S9 mix, no cytotoxicity was observed.

In the absence and presence of S9 mix, no biologically relevant increases in the numbers of micronucleated cells were observed after treatment with the test substance. In the presence of S9 mix, however, the value micronucleated cells after treatment with 1431 µg/mL was statistically significantly increased. Since the value of 1.03% is within the range of the 95% control limit of the laboratory control data (0.08 – 1.38%) and no dose dependency was observed, this finding is regarded as biologically irrelevant. Appropriate reference mutagens, used as positive controls, induced a distinct increase in mutant colonies and thus showed the sensitivity of the test system and the activity of the metabolic activation system.

In conclusion, the fluopicolide metabolite M-15 is considered to be non-clastogenic in this *in vitro* micronucleus test under the test conditions described, when tested up to the highest required concentration and there was no evidence of aneugenicity.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	M-15 (referred to as AE 1413903 in the report)
Purity:	93.2%
Batch no.:	SES 13490-2-4

2. Vehicle and/or positive control

Vehicle:	0.5% DMSO
Positive control:	<u>Without S9 mix:</u> Mitomycin C (MMC, 0.8 µg/ml; 4 h exposure) dissolved in deionized water Demecolcine (100 ng/ml; 20 h exposure) dissolved in deionized water <u>With S9 mix:</u> Cyclophosphamide (CPA, 15 µg/ml) dissolved in saline

3. Activation:

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as metabolic activation system. The S9 was prepared and stored according to the currently valid version of the lab's SOP for rat liver S9 preparation. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test.

The S9 mix contained the following components:

S9 fraction	40.0% (v/v)
$MgCl_2$	8 mM
KCl	33 mM
Glucose-6-phosphate	5 mM
NADP	4 mM
Sodium-ortho-phosphate-buffer	100 mM; pH 7.4

4. Blood cultures:

Human peripheral blood lymphocytes from healthy non-smoking donors not receiving medication: male donor (24 years old) for the 1st assay and from a male donor (25 years old) for the 2nd assay. The following culture conditions were used:

Culture medium:	Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1) already supplemented with 200 mM GlutaMAX™. Additionally, the medium was supplemented with penicillin/streptomycin (100 U/ml/100 µg/ml), the mitogen PHA (3 µg/ml), 10% foetal bovine serum (FBS), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/ml)
Culture conditions:	All cultures were incubated at 37 °C in a humidified atmosphere with 5.5% CO_2

5. Test substance concentrations used:

The following test substance concentrations were used:

1st micronucleus assay (4 h exposure): 0, 16.3, 28.5, 49.8, 87.2, 153, 267, 467, **818, 1431, 2146** µg/mL (+/- S9)

2nd micronucleus assay (20 h exposure): 0, 267, 467**, 818, 1431, 2146** µg/mL (-S9)

Concentrations in bold were used for mutation analysis.

B. Test performance

Experimental phase: 06 December 2017 to 30 January 2018

1. Test substance preparation

Stock formulations of the test substance and serial dilutions were made in DMSO (final concentration in culture medium 0.5%). The solvent was chosen due to its solubility properties and its relative non-toxicity to the cell cultures. All formulations were prepared freshly before treatment and used within two hours of preparation. The formulation is assumed to be stable for this period.

2. Dose selection

Dose selection was performed according to the current OECD Guideline 487 for the *in vitro* micronucleus test. The highest test substance concentration should be 10 mM, 2 mg/mL or, 2 µL/mL, whichever is the lowest. At least three test substance concentrations should be evaluated for cytogenetic damage.

In case of test substance induced cytotoxicity, measured by a reduced cytokinesis-block proliferation index (CBPI) and expressed as cytostasis, or precipitation (observed at the end of test substance exposure by the unaided eye) the dose selection should reflect these properties of the test substance. Where cytotoxicity occurs, the applied concentrations should cover a range from no to approx. 55 ± 5% cytostasis. For poorly soluble test substance, which are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration analysed should produce turbidity or visible precipitation (phase separation for liquid test substances).

With regard to the purity (93.2%) of the test substance, 2146 µg/mL were applied as top concentration for treatment of the cultures in the pre-test. Test substance concentrations ranging from 16.3 to 2146 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. In the pre-test for toxicity, no precipitation of the test substance was observed. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.

Using a reduced Cytokinesis-block proliferation index (CBPI) as an indicator for toxicity, no cytotoxic effects were observed in Experiment I after 4 hours treatment in the absence and presence of S9 mix. Therefore, 2146 µg/mL were chosen as top treatment concentration for experiment II.

3. Pre-experiment

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison with the controls (% cytostasis) by counting 500 cells per culture. The experimental conditions in this pre-experimental phase were identical to those required and described below for the mutagenicity assay.

The pre-test was performed with 10 concentrations of the test substance separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate.

Exposure time was 4 hours (with and without S9 mix). The preparation interval was 40 hours after start of the exposure.

This preliminary test was designated Experiment I, since the cultures fulfilled the criteria for cytogenetic evaluation and appropriate concentrations could be selected for evaluation.

4. Micronucleus test

The cultures were treated according to the following scheme:

	Without S9 mix		With S9 mix
	1st assay	2nd assay	1st assay
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

Pulse exposure:

About 48 hours after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test substance concentration. The culture medium was replaced with serum-free medium containing the test substance. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H_2O, 192 mg/L Na_2HPO_4 • 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10% FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approx. 20 hours until preparation.

Continuous exposure (without S9 mix):

About 48 hours after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test substance concentration. The culture medium was replaced with complete medium (with 10% FBS) containing the test substance. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10% FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured another approx. 20 hours until preparation.

Preparation of cells:

The cultures were harvested by centrifugation 40 hours after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded, and the cells were re-suspended in approx. 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

5. Data Evaluation

Evaluation of cytotoxicity and cytogenetic damage:

Evaluation of the slides was performed using microscopes with 40 x objectives. The micronuclei were counted in cells showing a clearly visible cytoplasm area. The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area of the main nucleus. 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 is 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

$$\text{Cytostasis \%} = 100 - 100 \times [(CBPI_T - 1) / (CBPI_C - 1)]$$

T	Test substance
C	Solvent control

6. Acceptance criteria

The micronucleus assay will be considered acceptable if it meets the following criteria:

- The concurrent solvent control will normally be within the laboratory historical solvent control data range.
- The concurrent positive controls should produce a statistically significant increase in the micronucleus frequency and should be within the laboratory historical positive control data range.
- Cell proliferation criteria in the solvent control are considered to be acceptable.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- The quality of the slides must allow the evaluation of an adequate number of cells and concentrations.
- The criteria for the selection of top concentration are consistent with those described in section 'Dose selection'.

7. Assessment criteria

Providing that all of the acceptability criteria are fulfilled, a test substance is considered to be clearly negative if, in all of the experimental conditions examined:

- None of the test substance concentrations exhibits a statistically significant increase compared with the concurrent solvent control.
- There is no concentration-related increase.
- The results in all evaluated test substance concentrations should be within the range of the laboratory historical solvent control data.

The test substance is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test substance is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test substance 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.

When all of the criteria are met, the test substance is then considered able to induce chromosome breaks and/or gain or loss in this test system.

There is no requirement for verification of a clear positive or negative response.

In case the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations.

8. Statistical analysis

Statistical significance was confirmed by the Chi square test ($\alpha < 0.05$), using a validated test script of "R", a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those values that indicated an increase in the number of cells with micronuclei compared to the concurrent solvent control.

II. Results and Discussion

A. Treatment conditions and cytotoxicity

In this study, no precipitation of the test substance in the culture medium was observed.

No relevant influence on osmolarity or pH was observed in the solvent control and the maximum concentration without metabolic activation:

Table 5.8.1- 188: Osmolality and pH

		Concentration [µg/mL]	Osmolarity [mOsm]	pH
Exp. I	Solvent control	-	448	7.5
	Test item	2146	392	7.5
Exp. II	Solvent control		383	n.d.
	Test item	2146	395	n.d.

n.d. not determined

In both cytogenetic experiments, in the absence and presence of S9 mix, no relevant cytotoxicity was observed.

B. Micronucleus Assay

In the absence and presence of S9 mix, no biologically relevant increases in the numbers of micronucleated cells were observed after treatment with the test substance at any concentration. In the presence of S9 mix, however, the value of 1.05% micronucleated cells after treatment with 1431 µg/mL was statistically significantly increased. Since the value is within the range of the 95% control limit of the laboratory control data (0.08 – 1.38%) and no dose dependency is observed, this finding can be regarded as biologically irrelevant (see also Assessment criteria above).

Demecolcine (100 ng/mL), MMC (0.8 µg/mL) and CPA (15.0 µg/mL) used as positive controls showed distinct increases in cells with micronuclei.

Table 5.8.1- 189: Summary of results

Exp.	Preparation interval	Test substance concentration in µg/mL	Proliferation index CBPI	Cytostasis (% control)	Micronucleated cells in %*	95% confidence interval solvent control
Exposure period 4 hrs without S9 mix						
I	40 hrs	Solvent control[1]	1.98		0.25	0.06-1.19
		Positive control[2]	1.85	13.0	**9.00**[S]	0.06-1.19
		818	1.92	5.8	0.05	0.06-1.19
		1431	1.95	2.6	0.25	0.06-1.19
		2146	1.98	n.c.	0.20	0.06-1.19
Exposure period 20 hrs without S9 mix						
II	40 hrs	Solvent control[1]	1.91		0.50	0.00-1.11
		Positive control[3]	1.55	39.9	**3.15**[S]	0.00-1.11
		818	1.83	8.6	0.40	0.00-1.11
		1431	1.82	9.5	0.50	0.00-1.11
		2146	1.76	16.2	0.45	0.00-1.11
Exposure period 4 hrs with S9 mix						
I	40 hrs	Solvent control[1]	2.10		0.50	0.08-1.38
		Positive control[4]	1.55	50.1	**3.00**[S]	0.08-1.38
		818	2.02	7.5	0.40	0.08-1.38
		1431	2.03	6.7	**1.05**[S]	0.08-1.38
		2146	2.00	9.0	0.40	0.08-1.38

* The number of micronucleated cells was determined in a sample of 2000 binucleated cells
S The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c. Not calculated as the CBPI is equal or higher than the solvent control value
1 DMSO 0.5% (v/v)
2 MMC 0.8 µg/mL
3 Demecolcine 100 ng/mL
4 CPA 15.0 µg/mL

III. Conclusions

In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes.

Therefore, M-15 (AE 1413903) is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 473 and is valid and acceptable to assess the potential of M-15 to induce chromosome aberrations in cultured human peripheral blood lymphocytes.

M-15 did not induce structural chromosome aberrations in cultured human peripheral blood lymphocytes under the conditions of this study when tested up to the highest required concentration.

Non-relevance of fluopicolide metabolites

Data Point:	KCA 5.8.1/40
Report Author:	[redacted]
Report Year:	2004
Report Title:	The non-relevance of the metabolites of AEC 638206 found in lysimeter leachate and field leaching studies - Position paper
Report No:	C039866
Document No:	M-227293-01-1
Guideline(s) followed in study:	not specified
Deviations from current test guideline:	Not applicable
Previous evaluation:	yes, evaluated and accepted in DAR (2005)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	No

In the previous submission (DAR, 2005), this statement was evaluated and accepted as valid to assess the non-relevance of the metabolites of fluopicolide found in lysimeter leachate and field leaching studies. However additional studies have been conducted and the groundwater exposure assessments have changed, thus the statement is no longer considered as valid. For procedural reasons it is included in the current dossier, but it has been superseded by Document N4 Relevance of Metabolites in Groundwater and hence a summary is not presented in this dossier.

Assessment and conclusion by applicant:
Superseded by Document N4 Relevance of Metabolites in Groundwater.

CA 5.8.2 Supplementary studies on the active substance

Data Point:	KCA 5.8.2/01
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE C638206 - 28-day explanatory toxicity study in the C57BL/6 female mouse
Report No:	C040806
Document No:	M-229594-01-1
Guideline(s) followed in study:	US-EPA OPPTS 870.4200
Deviations from current test guideline:	none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In order to find a mode of action for the liver tumours observed in the oncogenicity study in mice, fluopicolide was administered continuously via the diet to a group of 15 female C57BL/6 mice for at least 28 days at concentrations of 0 (control) and 3,200 ppm, equivalent to 575 mg/kg bw/day. Satellite subgroups of 20 female mice were added to each group for interim sacrifice after 7 days of treatment. Clinical signs were recorded daily, body weight and food consumption were measured weekly. A detailed physical examination was performed weekly.

Bromodeoxyuridine (BrdU) was administered in drinking water for 7 days before scheduled sacrifice for cell proliferation assessment. At both interim and final sacrifice times, liver was weighed and sampled. Hepatic cellular proliferation was assessed as well as morphological changes of the liver. In addition, at interim sacrifice, hepatic cytochrome P-450 isoenzymes were assessed.

At 3,200 ppm, there were no mortalities or clinical signs during the course of the study. There was a body weight loss (-2 kg) between Days 1-7. The mean body weight was thereafter lower than controls (-6 to -9%). Mean food consumption was lower than controls between Days 1-7 (-25%).

At interim sacrifice, mean terminal body weight was statistically significantly lower (-7%) when compared to controls. Mean absolute and relative liver weights were increased by 27 to 37% compared to controls, 9/20 livers appeared to be dark and 1/20 livers was enlarged. There was a diffuse, perilobular to panlobular hepatocellular hypertrophy in all treated animals and a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in 3/20 treated animals when compared to controls. An increased number of mitotic cells and some foci of single cell necrosis/apoptosis were seen in 5/20 treated animals. The mean BrdU labelling index was approx. 6.5-fold higher in treated animals, when compared to controls, indicative of a marked hepatocellular proliferation in the liver. Fluopicolide also induced a marked increase in total cytochrome P450 content (+97%) as well as in BROD (+1785%) and PROD (+1143%) activities.

At final sacrifice, mean terminal body weight was not affected. Mean absolute and relative liver weights were increased by 48 to 56% compared to controls, 11/15 livers appeared to be dark and 3/15 livers were enlarged. There was a diffuse, perilobular to panlobular hepatocellular hypertrophy in all treated animals together with a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in 3/15 treated animals, when compared to controls. Minimal single cell necrosis/apoptosis were seen in only 1/15 treated animals and an increased number of mitotic cells in 2/15 treated animals. At terminal sacrifice, there was no increased hepatocellular proliferation (mean BrdU labelling index) in treated animals when compared to controls.

In conclusion, treatment with fluopicolide at 3200 ppm in female C57BL/6 mice, caused a marked but transient hepatocellular proliferation, which returned to control levels after a total of 28 days treatment. Additionally, fluopicolide induces total cytochrome P450, BROD and PROD. As confirmed by a separate positive control study (see Langrand-Lerche, C.; 2004; M-232813-01-1), fluopicolide induced hepatic changes, both histopathological and in terms of enzyme induction activities with a phenobarbital-like profile.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	99.3% w/w
Batch no.:	R001737

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Female mice
Strain:	C57BL/6 J@Ico mice
Age:	10 weeks of age
Weight at start:	18.6 to 22.2 g
Source:	[redacted]
Acclimation period:	Yes
Diet:	Certified rodent powdered and irradiated diet A04C-10 PI from S.A.F.E. (Scientific Animal food and Engineering, Epinay-sur-Orge, France)
Water:	Water ad lib
Housing:	Individually in suspended stainless steel wire mesh cages
Temperature:	22 ± 2 °C
Humidity:	55 ± 15%
Air changes:	15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: October 29, 2003 to January 29, 2004

2. Animal assignment and treatment

The dose levels were set after taking into account the results of a carcinogenicity study (21557 TCS) where hepatocellular adenomas were observed in females at 3,200 ppm. Groups of 35 female mice (20 females designated for interim sacrifice on Day 8 and 15 females designated for final sacrifice on Day 29) were given control diet or the appropriate diet mixture. Table 5.8.2- 1 provides an overview of the study design.

Table 5.8.2- 1: Study design

Group no.	Dose (ppm)	Number of animals for interim sacrifice	Number of animals for final sacrifice
1	0	20	15
2	3,200	20	15

3. Diet preparation and analysis

Fluopicolide was incorporated into the diet to provide the required concentration. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing.

There was one preparation of the test formulation for the entire study. When not in use the diet formulation was stored at approx. -18 °C.

The stability of the test substance in the diet at 25 and 10,000 ppm has been demonstrated in the 90-day mouse study ([redacted] 2006; M-205579-02-1) after a freezing period of 7 weeks followed by 1 week at room temperature.

Before the start of the study, homogeneity and concentration were checked on the study mix at 3,200 ppm.

All results for homogeneity and concentration of fluopicolide in the diet were within a range of 97-99% of the nominal concentration. Hence all values were within the in-house target ranges of 85-115% of the nominal concentration. Regarding BrdU, results for concentration on the study solutions at 0.8 g/L were within the range of 104-105% of the nominal concentration.

4. Statistics

Variables analysed:

- Body weight parameters
- Body weight gain/day parameters calculated according to time intervals
- Average food and water consumption/day parameters calculated according to time intervals
- Total cytochrome P-450 content
- Cytochrome P-450 isoenzyme activities
- BrdU labeling indexes
- Organ weight parameters

Statistical analysis:

Means and standard deviations were calculated separately for each group at each time period.

Total cytochrome P-450 content:

The F test was performed to compare the homogeneity of group variances. If the F test was not significant (α=0.05), means were compared using the t-test (2-sided). If the F test was significant, data were transformed using the log transformation. If the F test on log transformed data was not significant (<x=0.05), means were compared using the t-test (2-sided) on log transformed data. If the F test was significant (<x=0.05) even after log transformation, group means were compared using the modified t-test (2-sided).

If one or more group variance(s) equaled 0, means were compared using non-parametric procedures.

Cytochrome P-450 isoenzyme activities:

For each substrate of enzymatic activity (ethoxyresorufin, pentoxyresorufin, benzoxyresorufin, lauric acid), group means were compared using the non-parametric Mann-Whitney exact test (2-sided).

BrdU labeling indices:

Because investigations were interested only in distinguishing between the hypothesis of no difference between means or the hypothesis of an increase of the mean in the treated group compared to the control mean, group means were compared using the non-parametric Mann-Whitney exact test (1-sided).

Statistical analyses were carried out using SAS programs (SAS Software Release 8.2, SAS Institute Inc., Cary, NC, USA).

Body weight gain/day parameters, organ weights:

Mean and standard deviation were calculated for each group and per time period for body weight gain/day parameters.

The F test was performed to compare the homogeneity of group variances. If the F test was not significant (a=0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided). If the F test was significant (a=0.05), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

Body weight and average food or water consumption/day parameters:

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters. The F test was performed to compare the homogeneity of group variances. If the F test was not significant (0=0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided). If the F test was significant (a=0.05), data were transformed using the log transformation. If the F test on log transformed data was not significant (a=0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data. If the F test was significant (a=0.05) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided). If one or more group variance(s) equaled 0, means were compared using the non-parametric Mann-Whitney test (2-sided).

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

Group means were compared at the 5% and 1% levels of significance.

C. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals.

Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body weight and food intake

All animals were weighed twice during the acclimatization phase. Each animal was weighed on the first day of test substance administration, then at approximately weekly intervals throughout the treatment period and before scheduled necropsy. Animals for interim sacrifice were weighed on Days 1 and 7 and before sacrifice only.

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

3. Cell proliferation assessment (in-life)

Preparation:

Solutions of 5-bromo-2'-deoxyuridine (BrdU) in drinking water were prepared twice at 0.8 g/L. When not in use, the solutions were stored at ambient temperature.

Analysis:

The stability of BrdU in drinking water was demonstrated in a previous study (SA 01416) over a 14-day period under storage and usage conditions similar to those of the current study.

Concentration of BrdU in drinking water was checked for each of the preparations used in the study.

BrdU delivery:

BrdU at 0.8 g/L in drinking water was delivered to selected animals in water bottles for seven days before scheduled sacrifice. Animals selected for interim sacrifice were given BrdU-containing water between Days 1-8 and animals selected for final sacrifice were given BrdU-containing water between Days 22-29.

Water consumption:

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration. Empty water bottles were weighed on the day before scheduled sacrifice. The mean water consumption in g/day was calculated at each scheduled sacrifice.

4. Post mortem examinations

On Days 8 (interim sacrifice) and 29 (final sacrifice), all designated animals from all groups were sacrificed. All sacrifices were performed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of approx. 60 mg/kg body weight). Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled, because they were considered to be incidental for this species at this age.

Duodenum and liver were sampled and fixed in 10% neutral buffered formalin for histological and immunohistochemical assessment. Histological sections of the liver (left and median lobes), including a sample of duodenum, were prepared for all animals in all groups for all scheduled sacrifice and stained with hematoxylin and eosin. Histopathological examination was performed on the liver of all animals from all groups.

5. Cell proliferation assessment

An immunohistochemical staining to visualize the incorporation of an analog of a nucleic acid (BrdU) was used to assess hepatocytic cell cycling. A section from a formalin-fixed paraffin-embedded block containing 2 liver samples and one duodenum sample was prepared (the duodenum has a high proliferation rate and serves as a positive staining control). The immunohistochemical reaction involved incubation with a monoclonal antibody raised against BrdU, an amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, a detection of the complex with the chromogen diamino-benzidine (DAB) and a Feulgen nuclear counterstaining. The immunohistochemical staining for BrdU and determination of the labeling index were performed on all animals. The labeling index, expressed as the number of BrdU-positive hepatocytes per thousand hepatocytes, was measured separately on random fields comprising approx. 1000 centrilobular and 1000 periportal cells using an automatic image analysis system. The mean labeling index with standard deviation was calculated for each zone, each liver and each group.

6. Hepatotoxicity testing

At interim sacrifice, the remaining portions of the liver pooled by five within each group were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile to check the hepatotoxic potential of the test substance. Microsomal preparations were not performed from animals sacrificed at the final sacrifice date. Each microsomal sample was identified by the animal number of the first animal from the pool of five.

Total cytochrome P-450 content:

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. One quantification was performed for each sample.

Enzyme activities:

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry using the following substrates

- benzoxyresonifin (BROD),
- ethoxyresorufin (EROD),
- pentoxyresorufin (PROD)

and by HPLC with fluorimetric detection following derivatization by 4-(bromomethyl)-7-methoxycoumarin of 12-hydroxy-lauric acid (lauric acid used as substrate).

Ethoxyresorufin is a highly selective substrate for the isoform IA, the isoform IIB metabolizes preferentially the O-dealkylation of pentoxyresorufin while the benzoxyresonifin O-debenzylation is mainly metabolized by the isoform IIA. Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37 °C.

Samples were prepared to follow the hydroxylation of lauric acid by the isoform IVA over a period of 10 minutes at 37 °C. Two replicates of each incubation mixture were collected. One replicate was analysed, the other one was stored frozen. 12-hydroxylauric acid was quantified in the incubation mixtures using the method N° ANL/046-94E.

Rat microsomes induced by well-known reference compounds (3-naphtoflavone, phenobarbital and clofibric acid) were measured at the same time as the study samples to have positive controls for each assay, but results were not reported in the present report.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities and no treatment-related clinical signs.

2. Body weight and food and water intake

At 3,200 ppm, there was a reduction of mean body weight gain (-0.35 g/day compared to 0.00 g/day in the control group, $p \leq 0.01$) and a body weight loss (-2.1 g) between days 1-7. The mean body weight gain was thereafter transiently higher and then again lower than controls between days 15-28 resulting in a mean body weight reduction throughout treatment (-6 to -9%).

Table 5.8.2- 2: Mean body weight and body weight gain measurements

	Dose level (ppm)		
	0	3,200	
Body weight (g) *(% difference to control)*			
Day 1	20.1	20.4	*(+2)*
Day 7	20.1	18.3**	*(-9)*
Day 15	20.5	19.3**	*(-6)*
Day 22	20.5	19.2**	*(-6)*
Day 28	21.0	19.3**	*(-8)*
Body weight gain (g) *(% difference to control)*			
Day 1-7	0.00	-0.35	-
Day 7-15	0.05	0.08	*(+60)*
Day 15-22	0.00	-0.02	-
Day 22-28	0.08	0.03*	*(-62)*

* $p \leq 0.05$; ** $p \leq 0.01$, significantly different from controls

At 3,200 ppm, mean food consumption was lower than controls between days 1-7 (-25 %, $p \leq 0.01$). Thereafter, food consumption was comparable between the treated and the control group. The mean achieved dosage intake of fluopicolide in mg/kg bw/day for Weeks 1 to 4 was 575 mg/kg bw/day.

Table 5.8.2- 3: Mean food consumption data (g/day) *(% difference to control)*

	Dose level (ppm)		
	0	3,200	
Day 7	3.6	2.7**	*(-25)*
Day 15	3.7	3.5	*(-5)*
Day 22	3.8	3.5	*(-8)*
Day 28	4.0	4.0	*(±0)*

* $p \leq 0.05$; ** $p \leq 0.01$, significantly different from controls

Mean water consumption was unaffected by treatment.

Table 5.8.2- 4: Mean water consumption data (g/day) *(% difference to control)*

	Dose level (ppm)		
	0	3,200	
Day 7	4.3	4.2	*(-2)*
Day 28	4.6	4.7	*(2)*

3. Post mortem examinations

Liver weight:

At interim sacrifice, mean terminal body weight was statistically significantly lower (-7%, $p < 0.01$) at 3,200 ppm, when compared to controls.

Mean absolute and relative liver weights were statistically significantly higher at 3,200 ppm, when compared to controls.

Mean brain to body weight ratio was statistically significantly higher at 3,200 ppm (+6%, $p < 0.01$), but was considered not toxicologically relevant since it was considered to be related to the lower terminal body weight at 3,200 ppm.

Table 5.8.2- 5 gives an overview.

Table 5.8.2- 5: Liver weight changes in % of control at interim sacrifice

Parameter	% change
Mean absolute liver weight	+27 ($p < 0.01$)
Mean relative liver weight	+37 ($p < 0.01$)
Mean liver to brain weight ratio	+29 ($p < 0.01$)

At terminal sacrifice, there was no change in mean terminal body weight at 3,200 ppm, when compared to controls. Mean absolute and relative liver weights were statistically significantly higher at 3,200 ppm, when compared to controls.

Table 5.8.2- 6: Liver weight changes in % of control at terminal sacrifice

Parameter	% change
Mean absolute liver weight	+48 ($p < 0.01$)
Mean relative liver weight	+56 ($p < 0.01$)
Mean liver to brain weight ratio	+56 ($p < 0.01$)

Mean absolute brain weight was statistically significantly lower at 3,200 ppm (-5%, $p < 0.01$) when compared to controls but the reduction was so slight that it is considered to be within normal biological variations.

Gross pathology:

At interim sacrifice, 9/20 livers appeared to be dark at 3,200 ppm, compared to controls, and 1/20 livers was enlarged. Other macroscopic changes were considered to be incidental or strain-related.

At terminal sacrifice, 11/15 livers appeared to be dark at 3,200 ppm, compared to controls and 3/15 livers were enlarged. Other macroscopic changes were considered to be incidental or strain-related.

Microscopic pathology:

At interim sacrifice, several treatment-related changes were seen in the liver:

- Diffuse, perilobular to panlobular hepatocellular hypertrophy was observed in all treated animals. This hepatocellular hypertrophy was associated with a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in treated animals.
- An increased number of mitotic cells was observed in treated animals, when compared to controls.
- Some foci of minimal single cell necrosis/apoptosis was observed in 5 animals at 3200 ppm.

Table 5.8.2- 7 gives an overview.

Table 5.8.2- 7: Incidence and severity of treatment-related changes in the liver at interim sacrifice

	Dose level (ppm)	
	0	**3,200**
Number of animals examined	20	20
Hepatocellular hypertrophy, perilobular to panlobular, diffuse		
Minimal	0	5
Slight	0	13
Moderate	0	2
Total	0	20
Hepatocellular vacuolation, mainly Centrilobular, diffuse		
Minimal	6	3
Slight	12	0
Moderate	2	0
Total	20	3
Increased number of mitoses		
Present	0	5
Single cell necrosis/apoptosis		
Minimal	0	5
Total	0	5

At terminal sacrifice, several treatment-related changes were seen in the liver:

- A diffuse, perilobular to panlobular hepatocellular hypertrophy was observed in all treated animals. This hepatocellular hypertrophy was associated with a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in treated animals.
- An increased number of mitotic cells was observed in two treated animals, when compared to controls. Minimal single cell necrosis/apoptosis was observed in 1/15 animals only.

Table 5.8.2- 8 gives an overview.

Table 5.8.2- 8: Incidence and severity of treatment-related changes in the liver at final sacrifice

	Dose level (ppm)	
	0	**3,200**
Number of animals examined	15	15
Hepatocellular hypertrophy, perilobular to panlobular, diffuse		
Slight	0	12
Moderate	0	3
Total	0	15
Hepatocellular vacuolation, mainly Centrilobular, diffuse		
Slight	10	2
Moderate	5	1
Total	15	3
Increased number of mitoses		
Present	0	2
Single cell necrosis/apoptosis		
Minimal	0	1
Total	0	1

The other changes seen were few and considered to be incidental in origin and within the range of expected changes for animals of this strain and age kept under laboratory conditions.

4. Cell cycling assessment

At interim sacrifice a marked hepatocellular proliferation was noted and considered to be treatment-related:

- Centrilobular, perilobular and total proliferation indexes were significantly and statistically higher in treated animals, when compared to controls.
- Mean BrdU labeling index was approximately 6.5-fold higher in treated animals, when compared to controls.

Table 5.8.2- 9: Mean BrdU labeling index (number of BrdU positive cells out of 1000 cells) at interim sacrifice

Interim sacrifice		**Centrilobular**	**Perilobular**	**Total**
Control	animal no.	20	20	20
	Mean	22.95	24.16	23.55
	STD	19.00	20.32	18.97
Fluopicolide	animal no.	20	20	20
	Mean	93.55**	215.88**	152.95**
	STD	35.40	57.91	36.64

**: significantly different from the control group ($p \leq 0.01$)

At terminal sacrifice, there was no increased hepatocellular proliferation in treated animals when compared to controls. The mean BrdU labeling index was even slightly lower in treated animals, when compared to controls (see Table 5.8.2- 10).

Table 5.8.2- 10: Mean BrdU labeling index (number of BrdU positive cells out of 1000 cells) at terminal sacrifice

Terminal sacrifice		Centrilobular	Perilobular	Total
Control	animal no.	15	15	15
	Mean	25.79	33.29	29.62
	STD	17.94	18.01	16.72
Fluopicolide	animal no.	15	15	15
	Mean	7.23	28.53	17.90
	STD	2.51	10.90	5.27

5. Total cytochrome P450 content

Fluopicolide administration at 3,200 ppm for 7 days induced a marked increase in total cytochrome P450 in female mice (+97% compared to control mean).

Enzymatic activities:

Fluopicolide markedly induced BROD (+1785%) and PROD (+1143%) activities, slightly induced cytochrome P450 (+97%) EROD activity (+79%) and decreased lauric acid hydroxylation compared to control meanafter 7 days of treatment. The magnitude of these changes are presented in Table 5.8.2- 11.

Table 5.8.2- 11: Total cytochrome P-450 content and enzymatic activities at interim sacrifice

Parameter	Fluopicolide at 3,200 ppm % change compared to control mean
P450	+ 97 %
BROD	+ 1785 %
EROD	+ 79 %
PROD	+ 1143%
Lauric acid	- 67 %

III. Conclusion

In conclusion, treatment with fluopicolide at 3,200 ppm in female C57BL/6 mice, caused a marked but transient hepatocellular proliferation, which returned to control levels after a total of 28 days treatment. Additionally, fluopicolide induces total cytochrome P450, BROD and PROD activities after 7 days of treatment. As confirmed by a separate positive control study (see [redacted] 2004; M-232813-01-1), fluopicolide induced hepatic changes, both histopathological and in terms of enzyme induction activities with a phenobarbital-like profile.

Assessment and conclusion by applicant:

This exploratory study was conducted in order to find a mode of action for the liver tumours observed in the oncogenicity study in mice and is valid and acceptable for this purpose. A marked increase in hepatocellular proliferation was seen along with inductions of P450, PROD and BROD enzyme activities.

Data Point:	KCA 5.8.2/02
Report Author:	[redacted]
Report Year:	2004
Report Title:	Phenobarbital and clofibric acid: Reference 28-day study for hepatotoxicity in the C57BL/6 mouse
Report No:	C042531
Document No:	M-232813-01-1
Guideline(s) followed in study:	OPPTS 870.4200
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The potential liver changes caused by two reference compounds, phenobarbital and clofibric acid were assessed after oral administration by gavage at dose levels of 80 or 300 mg/kg bw/day, respectively, for 28 days, to groups of 15 male and 15 female C57BL/6 mice. A similarly constituted group of 15 animals/sex received untreated diet and acted as a control. Satellite subgroups of 20 male and female mice were added to each group for interim sacrifice after 7 days of treatment. Clinical signs were recorded daily, with body weight and food consumption and a detailed physical examination performed weekly. Bromodeoxyuridine (BrdU) was administered in drinking water for 7 days before scheduled sacrifice for cell proliferation assessment. Water consumption was measured during the period of BrdU administration. At both interim and final sacrifice times, livers were weighed and sampled. Hepatic cellular proliferation was assessed as well as morphological changes. In addition, at interim sacrifice, hepatic cytochrome P450 isoenzymes were assessed.

Phenobarbital (80 mg/kg bw/day):

There was no mortality in phenobarbital-treated groups during the study. Reduced motor activity was observed during most of the treatment period for all animals just after administration of the test substance by gavage. In males, a slight reduction mean body weight on Day 7 (-4%) was caused by reductions in body weight gains over the same period. Mean body weight and body weight gain of females were unaffected by treatment. Mean food consumption was decreased by 7% between Days 1-7 in males only. Hepatic effects were recorded as increased liver weights (interim and final sacrifices), dark livers in 5/20 males and 10/20 females at interim sacrifice and in 8/15 males and 9/15 females at final sacrifice, and loss of centrilobular to diffuse hepatocellular vacuolation was seen in some males and females, corroborated with the persistence of a residual, diffuse periportal hepatocellular vacuolation in some males at bother sacrifices. At the interim sacrifice, marked hepatocellular proliferation was noted in males and females with increases in global proliferation indexes increased compared to controls. At the terminal sacrifice, this measurement was minimal compared to the change at the interim sacrifice and was seen in males only. At interim sacrifice, phenobarbital markedly induced total cytochrome P450 and BROD and PROD activities in both sexes compared to control means, with only a slight increase in males only for EROD activity.

Clofibric acid (300 mg/kg bw/day):

One male was found dead on Day 6. Two males and two females were killed for humane reasons during the first 12 days of treatment of which one male and one female showed clinical signs indicative of a gavage error that was confirmed at necropsy. Clinical signs observed on the other decedent animals were reduced motor activity, laboured respiration, soiled anogenital region, prostration, piloerection and/or coldness to touch. In the surviving animals, one male showed reduced motor activity and laboured respiration on Day 6 and one other male showed soiling around the mouth on the first day of treatment. Mean body weight, body weight gain and food consumption were unaffected by treatment. Hepatic effects were recorded as increased liver weights (interim and final sacrifices), dark livers observed in 3/19 males and 8/19 females at interim sacrifice and in 13/13 males and 12/14 females at final sacrifice and a diffuse, centrilobular to pan lobular hepatocellular hypertrophy in all males and females at both sacrifices. An increased number of mitotic hepatocytes was noted in 11/19 males and 9/19 females at interim sacrifice only. A loss of centrilobular to diffuse hepatocellular vacuolation was seen in the majority of males and females at both sacrifices, which was corroborated with the persistence of a residual, diffuse periportal hepatocellular vacuolation in some males and females. At the interim sacrifice, marked hepatocellular proliferation was noted in males and females with increases in global proliferation indexes compared to controls. The global proliferation index remained unchanged at the terminal sacrifice. At the interim sacrifice, clofibric acid statistically significantly induced lauric acid hydroxylation in both males and females, but not total cytochrome P450, BROD, PROD or EROD activities.

In conclusion, phenobarbital at 80 mg/kg bw/day induced a marked hepatocellular proliferation in male and female C57BL/6 mice after 7 days of treatment, which remained statistically significant but slight in males and returned to control levels in females after 28 days of treatment. In addition, phenobarbital was found to be a strong inducer of hepatocellular hypertrophy and of total cytochrome P450, BROD and PROD activities. Clofibric acid at 300 mg/kg bw/day induced a marked hepatocellular proliferation in male and female C57BL/6 mice after 7 days of treatment, which returned to control levels after 28 days of treatment. In addition, clofibric acid was found to be a strong inducer of hepatocellular hypertrophy and of lauric acid hydroxylation activities.

I. Materials and Methods

A. Materials

1. Test material

Test substance 1:	Phenobarbital (sodium salt)
Purity:	99% w/w
Batch no.:	088H0023
Test substance 2:	Clofibric acid
Purity:	97% w/w
Batch no.:	01220BT-121

2. Vehicle and/or positive control

Vehicle:	0.5% methylcellulose in sterilized water

3. Test animals

Species:	Mice
Strain:	C57BL/6J@lco mice
Age:	10 weeks of age
Weight at start:	21.8 to 25.7 g (males), 17.8 to 21.6 g (females)
Source:	[redacted]
Acclimation period:	Yes
Diet:	Certified rodent powdered and irradiated diet A04C-10 PI from S.A.F.E (Scientific Animal food and Engineering, Epinay-sur-Orge, France)
Water:	Softened tap water ad lib
Housing:	Individually in suspended stainless steel wire mesh cages
Temperature:	22 ± 2 °C
Humidity:	55 ± 15%
Air changes:	15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: November 05, 2003 to May 26, 2004

2. Animal assignment and treatment

Groups of 35 male and 35 female mice were given the vehicle (0.5% methylcellulose in sterilized water) or the test substance formulations (see Table 5.8.2- 12).

Table 5.8.2- 12: Study design

Group no.	Group	Dose (mg/kg bw/day)	Interim sacrifice, satellite group 1	Interim sacrifice, satellite group 2	Final sacrifice satellite group 1	Final sacrifice satellite group 2
Males						
1	Control	0	10	10	7	8
2	Phenobarbital	80	10	10	8	7
3	Clofibric acid	300	10	10	7	8
Females						
1	Control	0	10	10	8	7
2	Phenobarbital	80	10	10	7	8
3	Clofibric acid	300	10	10	8	7

3. Diet preparation and analysis

The dosing formulations were prepared by suspending each test substance in 0.5% methylcellulose in sterilized water to produce the required dosing concentrations (w/v). There was one preparation of each test formulation for the study. When not in use, the test formulations were stored at approx. 4 °C.

The stability of phenobarbital at 8 g/L and clofibric acid at 30 g/L in aqueous methylcellulose was studied during the course of the study under conditions of storage and usage similar to those used in the present study. Before the start of the study homogeneity and concentration of phenobarbital and clofibric acid were checked on the study suspensions at 8 and 30 g/L, respectively.

4. Statistics

Means and standard deviations were calculated separately for each group at each time period.

Total cytochrome P450 content:

The F test was performed to compare the homogeneity of group variances. If the F test was not significant ($p > 0.05$), means were compared using the t-test (2-sided). If the F test was significant, data were transformed using the log transformation. If the F test on log transformed data was not significant ($p > 0.05$), means were compared using the t-test (2-sided) on log transformed data. If the F test was significant ($p < 0.05$) even after log transformation, group means were compared using the modified t-test (2-sided).

If one or more group variance(s) equalled 0, means were compared using non-parametric procedures.

Cytochrome P450 isoenzyme activities:

For each substrate of enzymatic activity (ethoxyresorufin, pentoxyresorufin, benzoxyresorufin, lauric acid), group means were compared using the non-parametric Mann-Whitney exact test (2-sided).

BrdU labelling indexes:

Because investigations were interested only in distinguishing between the hypothesis of no difference between means or the hypothesis of an increase of the mean in the treated group compared to the control mean, group means were compared using the non-parametric Mann-Whitney exact test (1-sided). Consequently, no statistical comparison was performed when the mean of the treated group was lower than the mean of the control group.

Statistical analyses were carried out using SAS programs (SAS Software Release 8.2, SAS Institute Inc., Gary, NC, USA).

Body weight changes, organ weights:

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

The F test was performed to compare the homogeneity of group variances. If the F test was not significant ($p > 0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ($p < 0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

If one or more group variance(s) equalled 0, means were compared using the non-parametric Mann-Whitney test (2-sided).

Body weight and average food or water consumption/day parameters:

Mean and standard deviation were calculated for each group and per time period for average food or water consumption/day parameters. The F test was performed to compare the homogeneity of group variances. If the F test was not significant ($p > 0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ($p < 0.05$), data were transformed using the Jog transformation. If the F test on log transformed data was not significant ($p > 0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data. If the F test was significant ($p < 0.05$) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

If one or more group variance(s) equalled 0, means were compared using the non-parametric Mann-Whitney test (2-sided).

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). Group means were compared at the 5% and 1% levels of significance.

C. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose faeces.

2. Body weight and food intake

All animals were weighed three times during the acclimatization phase. Each animal was weighed on the first day of test substance administration, then at approximately weekly intervals throughout the treatment period. Animals for interim sacrifice were weighed on Days 1 and 7 only. Additionally, diet fasted animals were weighed before scheduled necropsy. The weight of food supplied and of that remaining at the end of the food consumption period was recorded approximately weekly for all animals during the treatment period. From these records the mean daily food consumption was calculated. Food spillage was also noted.

3. Cell proliferation (In-life)

Preparation:

Solutions of 5-bromo-2'-deoxyuridine (BrdU) in drinking water were prepared twice at 0.8 g/L. When not in use, the solutions were stored at ambient temperature.

Analysis:

The stability of BrdU in drinking water was demonstrated in a previous study over a 14-day period under storage and usage conditions similar to those of the current study. Concentration of BrdU in drinking water was checked for each of the preparations used in the study.

BrdU delivery:

BrdU at 0.8 g/L in drinking water was delivered to selected animals in water bottles for seven days before scheduled sacrifice. Animals selected for interim sacrifice were given water containing BrdU between Days 1-8 and animals selected for final sacrifice were given water containing BrdU between Days 22-29.

Water consumption:

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration. Empty water bottles were weighed on the day before scheduled sacrifice. The mean water consumption in g/day was calculated for each scheduled sacrifice.

Postmortem examinations:

On Days 8 (interim sacrifice) and 29 (final sacrifice), all designated surviving animals from all groups were sacrificed. All sacrifices were performed by exsanguination under deep anaesthesia (pentobarbital, intraperitoneal injection of approx. 60 mg/kg bw). Animals were diet fasted overnight prior to sacrifice. All animals, surviving, found dead or killed for humane reasons, were necropsied. The necropsy included the examination of all major organs, tissues, and body cavities. Macroscopic abnormalities were recorded, sampled, and examined microscopically. Brain and liver were weighed fresh. Duodenum and liver were sampled and fixed in 10% neutral buffered formalin for histological and immunohistochemical assessment. Histological sections of the liver (left and median lobes), including a sample of duodenum, were prepared for all animals in all groups for all scheduled sacrifice and stained with haematoxylin and eosin. Histopathological examination was performed on the livers of all animals from all groups.

Cell proliferation assessment:

An immunohistochemical staining to visualize the incorporation of an analogue of a nucleic acid (BrdU) was used to assess hepatocytic cell cycling. A section from a formalin-fixed paraffin-embedded block containing 2 liver samples and one duodenum sample was prepared (the duodenum has a high proliferation rate and serves as a positive staining control). The immunohistochemical reaction involved an incubation with a monoclonal antibody raised against BrdU, an amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, a detection of the complex with the chromogen diamino-benzidine (DAB) and a Feulgen nuclear counterstaining. The immunohistochemical staining for BrdU and determination of the labelling index were performed on all surviving animals. The labelling index expressed as the number of BrdU-positive hepatocytes per thousand hepatocytes, was measured separately on random fields comprising approx. 1000 centrilobular and 1000 periportal cells using an automatic image analysis system. The mean labelling index with standard deviation was calculated for each zone, each liver and each group.

Hepatotoxicity testing:

At interim sacrifice, the remaining portions of the liver pooled by five within each group were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile to check the hepatotoxic potential of the test substance. Microsomal preparations were not performed from animals sacrificed at the final sacrifice date. Each microsomal sample was identified by the animal number of the first animal from the pool of five.

Total cytochrome P-450 content:

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. One quantification was performed for each sample.

Enzymatic activities:

Specific cytochrome P450 enzymatic activities were evaluated by spectrofluorimetry using the following substrates:

- benzoxyresorufin (BROD)
- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)

and by HPLC with fluorimetric detection following derivatization by 4-(bromomethyl)-7-methoxycoumarin of 12-hydroxy-lauric acid (lauric acid used as substrate). Ethoxyresorufin is a highly selective substrate for the isoform 1A, the isoform IIB metabolizes preferentially the O-dealkylation of pentoxyresorufin, while the benzoxyresorufin O-debenzylation is mainly metabolized by the isoform IIIA. Cytochrome P450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37 °C.

Samples were prepared to follow the hydroxylation of lauric acid by the isoform IVA over a period of 10 minutes at 37 °C. Two replicates of each incubation mixture were collected. One replicate was analysed, the other one was stored frozen. 12-hydroxylauric acid was quantified in the incubation mixtures using the method N° ANL/046-94E. Rat microsomes induced by well-known reference compounds (3-naphtoflavone, phenobarbital and clofibric acid were measured at the same time as the study samples to have positive controls for each assay. Results are not reported in the present report.

II. Results and Discussion

A. Results

1. Clinical results

Phenobarbital:

At 80 mg/kg bw/day, reduced motor activity was observed during most of the treatment period in all treated males and females just after administration of the test substance. No mortality occurred.

Clofibric acid:

At 300 mg/kg bw/day, four females showed reduced motor activity on Day 15. Due to the isolated occurrence of this finding, this observation was considered not to be related to the treatment.

At 300 mg/kg bw/day, one male was found dead on Day 6 showing reduced motor activity and laboured respiration on the day of death. Two males were killed for humane reason on Days 2 and 4, respectively, showing piloerection, reduced motor activity, soiled anogenital region and cold to touch or reduced motor activity, hunched posture, and focal swelling in the thorax area on the day of sacrifice. Two females were killed for humane reason on Days 7 and 12, respectively. One showed reduced motor activity, prostration, and coldness to touching on Day 7 and the other showed reduced motor activity on Days 11 and 12, laboured respiration, soiling around the mouth and focal swelling on the left forelimb on Day 12.

2. Body weight and food intake

Phenobarbital (PB):

Mean body weight of males was 4% and 3% lower than controls on Day 7 ($p < 0.01$) and on Day 28 ($p < 0.05$), respectively. There was a mean cumulative body weight loss of 0.7 g in males between Days 1-7 compared to a mean cumulative body weight gain of 0.2 g in the control group ($p < 0.05$). Cumulative body weight gain between Days 1-28 was also reduced by 46% compared to controls ($p < 0.05$) due to the combined body weight loss observed on Week 1 and a slightly reduced body weight gain observed between Days 22-28 (not statistically significant). In females, mean body weight was 4% lower than controls on Day 7 ($p < 0.01$). There was a mean cumulative body weight loss of 0.5 g in females between Days 1-7 compared to an absence of body weight gain in the control group ($p < 0.01$). Thereafter, mean body weight and body weight gain of females were comparable to controls.

Table 5.8.2- 13: Body weight and body weight gains (g)

Day	Treatment	
	Control	Phenobarbital
Males		
1	23.9	23.8
7	24.1	23.1^{+}
15	24.5	24.1
22	24.8	24.4
28	25.3	24.6
BWG 1-7	0.2	-0.7^{+}
BWG 7-15	0.5	0.2
BWG 15-22	0.8	0.5
BWG 22-28	1.3	0.7
Females		
1	20.0	19.8
7	20.0	19.3^{+}
15	20.6	20.1
22	21.3	20.7
28	21.6	20.9
BWG 1-7	0.0	-0.5^{+}
BWG 7-15	0.4	0.5
BWG 15-22	1.1	1.1
BWG 22-28	1.4	1.3

* $p < 0.05$; $^{+}$ $p < 0.01$

Mean food consumption was decreased by 7% ($p < 0.01$) between Days 1-7 in males and was thereafter comparable to controls. Mean food consumption of females was unaffected by treatment.

Table 5.8.2- 14: Mean food consumption (g/day)

Day	Treatment	
	Control	Phenobarbital
Males		
1-7	4.3	4.0^{+}
7-15	4.5	4.5
15-22	4.4	4.6
22-28	4.4	4.5
Females		
1-7	4.0	3.9
7-15	4.3	4.1
15-22	4.4	4.3
22-28	4.3	4.3

* $p < 0.05$; $^{+}$ $p < 0.01$

Clofibric acid (CA):

Generally mean body weight, body weight gain and food consumption were unaffected by treatment. Mean body weight of males was marginally lower than controls on Day 28 ($p < 0.01$). There was a slight mean body weight loss of 0.03 g/day between Days 15-22 compared to a body weight gain of 0.04 g/day in the control group ($p < 0.05$).

Table 5.8.2- 15: Liver weight changes (interim sacrifice)

Day	Treatment	
	Control	Clofibric Acid
Males		
1	23.9	23.6
7	24.1	23.9
15	24.5	24.5
22	24.8	24.2
28	25.3	24.5+
BWG 1-7	0.2	0.3
BWG 7-15	0.5	1.1*
BWG 15-22	0.8	0.9
BWG 22-28	1.3	1.1
Females		
1	20.0	19.9
7	20.0	20.2
15	20.6	21.0
22	21.3	21.0
28	21.6	21.0
BWG 1-7	0.0	0.3
BWG 7-15	0.4	1.0
BWG 15-22	1.1	1.0
BWG 22-28	1.4	1.0

* $p < 0.05$; + $p < 0.01$

Mean food consumption was slightly decreased by 7% ($p < 0.05$) between Days 1-7 in males and was thereafter comparable to controls. Mean food consumption of females was unaffected by treatment, the statistically significant increase in food consumption observed between Days 1-7 being considered not to be related to the treatment, in light of the small magnitude of the increase.

Table 5.8.2- 16: Mean food consumption (g/day)

Day	Treatment	
	Control	Clofibric Acid
Males		
1-7	4.3	4.0*
7-15	4.5	4.5
15-22	4.4	4.4
22-28	4.4	4.7
Females		
1-7	4.0	4.1*
7-15	4.3	4.4
15-22	4.4	4.3
22-28	4.3	4.5

* $p < 0.05$; + $p < 0.01$

3. Post mortem examinations

Terminal body weight and organ weights:

Phenobarbital:

Interim sacrifice:

In males, mean terminal body weight was statistically significantly lower (-5%, $p < 0.01$), when compared to controls. In males, mean absolute liver weight and mean liver to brain weight ratio were slightly higher, when compared to controls but these changes were not statistically significant.

Mean liver to body weight ratio was statistically significantly higher, when compared to controls (see Table 5.8.2- 17). This statistically significant change was considered to be partially related to the lower terminal body weight. In females, mean absolute and relative liver weights were statistically significantly higher, when compared to controls ($p < 0.01$). These changes were considered to be treatment-related.

Table 5.8.2- 17: Liver weight changes (interim sacrifice)

Mean Liver weight changes (% change when compared to controls) Phenobarbital – Interim sacrifice		
	Male	**Female**
Mean absolute liver weight	+ 5%	+ 17%$^{+}$
Mean liver to body weight ratio	+ 10%$^{+}$	+ 19%
Mean liver to brain weight ratio	+ 4%	+ 16%$^{+}$

* $p < 0.05$; $^{+}$ $p < 0.01$

In males, mean brain to body weight ratio was statistically significantly higher (+7%, $p < 0.01$) when compared to controls; this statistically significant change was considered to be related to the lower terminal body weight in treated animals.

Terminal sacrifice:

There were no statistically significant differences for mean terminal body weights between treated and control males and females.

In both sexes, mean absolute and relative liver weights were statistically significantly higher in treated animals, when compared to controls (see Table 5.8.2- 18). These changes were considered to be treatment-related.

Table 5.8.2- 18: Liver weight changes (terminal sacrifice)

Mean Liver weight changes (% change when compared to controls) Phenobarbital – Terminal sacrifice		
	Male	**Female**
Mean absolute Liver weight	+ 12%$^{+}$	+ 16%$^{+}$
Mean Liver to body weight ratio	+ 16%$^{+}$	+ 14%$^{+}$
Mean Liver to brain weight ratio	+ 11%$^{+}$	+ 20%$^{+}$

* $p < 0.05$; $^{+}$ $p < 0.01$

In females, mean absolute brain weight was statistically significantly lower (-4%, $p < 0.05$) when compared to controls. With the small magnitude, the fact that this was considered to be within the normal range of biological variation and the potential for it being a secondary effect of reduced bodyweight, this change was considered to be not treatment-related.

Clofibric acid:

Interim sacrifice:

In males and females, there was no relevant change in mean terminal body weights, when compared to controls. In males and females, mean absolute and relative liver weights were statistically significantly higher, when compared to controls ($p < 0.01$) (see Table 5.8.2- 19). These changes were considered to be treatment-related.

Table 5.8.2- 19: Liver weight changes after clofibric acid (interim sacrifice)

Mean Liver weight changes (% change when compared to controls) Clofibric acid – Interim sacrifice		
	Male	**Female**
Mean absolute Liver weight	+ 19%$^{+}$	+ 22%$^{+}$
Mean Liver to body weight ratio	+ 20%$^{+}$	+ 20%$^{+}$
Mean Liver to brain weight ratio	+ 18%$^{+}$	+ 22%$^{+}$

* $p < 0.05$; $^{+}$ $p < 0.01$

Terminal sacrifice:

There were no statistically significant differences for mean terminal body weights between treated and control males and females. In males and females, mean absolute and relative liver weights were statistically significantly higher in treated animals, when compared to controls (see Table 5.8.2- 20). These changes were considered to be treatment-related.

Table 5.8.2- 20: Liver weight changes after clofibric acid (terminal sacrifice)

Mean Liver weight changes (% change when compared to controls) Clofibric acid – Terminal sacrifice		
	Male	**Female**
Mean absolute Liver weight	+ 26%$^{+}$	+ 29%$^{+}$
Mean Liver to body weight ratio	+ 27%$^{+}$	+ 25%$^{+}$
Mean Liver to brain weight ratio	+ 27%$^{+}$	+ 34%$^{+}$

* $p < 0.05$; $^{+}$ $p < 0.01$

In females, mean absolute brain weight was statistically significantly lower in treated animals (-4%, $p < 0.01$) when compared to controls: this statistically significant change was considered to be within normal ranges of biological variations, and not treatment-related. Mean brain to body weight ratio was statistically significantly lower (-8%, $p < 0.05$) when compared to controls: this statistically significant change was considered to be related to the slightly higher (+3%) terminal body weight at 300 mg/kg bw/day.

Gross pathology:

Unscheduled deaths:

All deaths were from the clofibric acid group. Two males were killed for humane reasons on Days 2 and 4, another male was found dead on Day 6. One animal which was in a moribund state with a focal thoracic swelling) had a mottled, brown lung probably associated with a gavage error (despite the absence of pulmonary lesions at histology). Macroscopically, no clear cause of death was determined for the two other two animals.

Two females were killed for humane reasons on Days 7 and 12. One, with laboured respiration) showed an esophageal perforation with a pleura Effusion which was considered to have resulted from a gavage error. No clear cause of death was determined for the other female with prostration, coldness to touching and reduced motor activity): a macroscopic dark liver was noted (correlated with a slight centrilobular to panlobular hepatocellular hypertrophy at histology).

Phenobarbital

Interim sacrifice:

Dark liver was observed in 5/20 males and 10/20 females at 80 mg/kg bw/day. Other macroscopic lesions were considered to be incidental or strain-related and not treatment-related.

Terminal sacrifice:

Dark liver was noted in 8/15 males and 9/15 females at 80 mg/kg bw/day. Other macroscopic lesions were considered to be incidental or strain-related and not treatment-related.

Clofibric acid

Interim sacrifice:

Dark liver was observed in 3/19 males and 8/19 females at 300 mg/kg bw/day. Other macroscopic lesions were considered to be incidental or strain-related and not treatment-related.

Terminal sacrifice:

Dark liver was observed in 13/13 males and 12/14 females at 300 mg/kg bw/day. Other macroscopic lesions were considered to be incidental or strain-related and not treatment-related.

Microscopic pathology:

Unscheduled deaths:

A diffuse congestion in lung and acute inflammation in the subcutis were observed in animal NT3M5636 and a diffuse congestion was found in the liver of animal NT3F5669. These findings had no specific correlation with the macroscopic observations of a gavage error.

A slight centrilobular to panlobular hepatocellular hypertrophy, correlated with a macroscopic dark liver was noted for the female NT3F5660 (as observed in some treated animals at scheduled sacrifice). No microscopic findings indicative of any cause of death were noted for animals NT3M5623, NT3M5629 and NT3F5660.

Phenobarbital:

Interim sacrifice (Table 5.8.2- 21):

Several treatment-related changes were seen in the liver in treated males:

- a diffuse centrilobular to midzonal hypertrophy was observed in all males and 18/20 females.
- a loss of centrilobular to diffuse hepatocellular vacuolation was seen in some males and females.
- corroborated with the loss of the centrilobular to diffuse vacuolation, persistence of a residual, diffuse periportal hepatocellular vacuolation was seen in some males.

Table 5.8.2- 21: Phenobarbital liver changes at interim sacrifice

Incidence and severity of treatment-related changes in the liver Interim sacrifice - Phenobarbital				
	Males		**Females**	
Dose (mg/kg/d)	**0**	**80**	**0**	**80**
Animals examined	**20**	**20**	**20**	**20**
Hepatocellular hypertrophy, centrilobular to midzonal diffuse				
Minimal	0	8	0	7
Slight	0	12	0	11
Total	**0**	**20**	**0**	**18**
Hepatocellular vacuolation, centrilobular to diffuse				
Minimal	13	3	3	5
Slight	6	1	12	7
Moderate	1	0	5	1
Total	**20**	**4**	**20**	**13**
Hepatocellular vacuolation, periportal, diffuse				
Minimal	0	5	0	0
Slight	0	1	0	0
Total	**0**	**6**	**0**	**0**

Terminal sacrifice (see Table 5.8.2- 22):

Several treatment-related changes were seen in the liver in treated males:

- a diffuse centrilobular to midzonal hepatocellular hypertrophy was observed in all males and females.
- A loss of centrilobular to diffuse hepatocellular vacuolation was seen in some males and females.
- corroborated with the loss of the centrilobular to diffuse vacuolation, persistence of a residual, diffuse periportal hepatocellular vacuolation was seen in some males.

Table 5.8.2- 22: Phenobarbital liver changes at terminal sacrifice

Incidence and severity of treatment-related changes in the liver Terminal sacrifice - Phenobarbital				
	Males		Females	
Dose (mg/kg/d)	**0**	**80**	**0**	**80**
Animals examined	**15**	**15**	**15**	**15**
Hepatocellular hypertrophy, centrilobular to midzonal diffuse				
Minimal	0	8	0	7
Slight	0	7	0	8
Total	**0**	**15**	**0**	**15**
Hepatocellular vacuolation, centrilobular to diffuse				
Minimal	9	1	6	5
Slight	5	0	8	2
Moderate	1	0	1	0
Total	**15**	**1**	**15**	**7**
Hepatocellular vacuolation, periportal, diffuse				
Minimal	0	2	0	0
Total	**0**	**2**	**0**	**0**

Clofibric acid

Interim sacrifice (see Table 5.8.2- 23*):*

Several treatment-related changes were seen in the liver in treated males and females:

- a diffuse, centrilobular to panlobular hepatocellular hypertrophy was observed in all males and females.
- an increased number of mitotic hepatocytes was noted in 11/19 males and 9/19 females.
- a loss of centrilobular to diffuse hepatocellular vacuolation was seen in the majority of the males and females.
- corroborated with the loss of the centrilobular to diffuse vacuolation, persistence of a residual, diffuse periportal hepatocellular vacuolation was seen in some males and females.

Table 5.8.2- 23: Clofibric acid liver changes at interim sacrifice

Incidence and severity of treatment-related changes in the liver Interim sacrifice – Clofibric acid				
	Males		Females	
Dose (mg/kg/d)	**0**	**300**	**0**	**300**
Animals examined	**20**	**19**	**20**	**19**
Hepatocellular hypertrophy, centrilobular to panlobular diffuse				
Minimal	0	7	0	11
Slight	0	12	0	8
Total	**0**	**19**	**0**	**19**
Increased number of mitoses				
Present	**0**	**11**	**0**	**9**
Hepatocellular vacuolation, centrilobular to diffuse				
Minimal	13	0	3	3
Slight	6	0	12	0
Moderate	1	0	5	0
Total	**20**	**0**	**20**	**3**
Hepatocellular vacuolation, periportal, diffuse				
Minimal	0	11	0	4
Slight	0	2	0	1
Total	**0**	**13**	**0**	**5**

Terminal sacrifice (see Table 5.8.2- 24):

Several treatment-related changes were seen in the liver in treated males and females:

- a diffuse centrilobular to panlobular hepatocellular hypertrophy was observed in all males and females.
- a loss of centrilobular to diffuse hepatocellular vacuolation was seen in all males and females.
- corroborated with the loss of the centrilobular to diffuse vacuolation, persistence of a residual diffuse periportal hepatocellular vacuolation was seen in some males and females.

Table 5.8.2- 24: Clofibric acid liver changes at terminal sacrifice

Incidence and severity of treatment-related changes in the liver Terminal sacrifice – Clofibric acid				
	Males		Females	
Dose (mg/kg/d)	**0**	**300**	**0**	**300**
Animals examined	**15**	**13**	**15**	**14**
Hepatocellular hypertrophy, centrilobular to panlobular diffuse				
Minimal	0	0	0	1
Slight	0	5	0	11
Moderate	0	8	0	2
Total	**0**	**13**	**0**	**14**
Hepatocellular vacuolation, centrilobular to diffuse				
Minimal	9	0	6	0
Slight	5	0	8	0
Moderate	1	0	1	0
Total	**15**	**0**	**15**	**0**
Hepatocellular vacuolation, periportal, diffuse				
Slight	0	3	0	3
Total	**0**	**3**	**0**	**3**

Cell cycle assessment

Interim sacrifice: phenobarbital and clofibric acid (see Table 5.8.2- 25):

At interim sacrifice, in males and females, a marked hepatocellular proliferation was noted and was considered to be treatment-related: centrilobular, perilobular and global proliferation indexes were 6.6 to 14.6 fold higher in phenobarbital treated animals and 7.1 to 12.6 fold higher in clofibric acid treated animals, when compared to controls).

Table 5.8.2- 25: Cell cycling phenobarbital and clofibric at interim sacrifice

Interim sacrifice Phenobarbital and Clofibric acid				
		Centrilobular	**Perilobular**	**Total**
Males				
Control	N	20	20	20
	Mean	8.0	10.2	9.1
	STD	7.36	7.79	7.00
Phenobarbital	N	20	20	20
	Mean	116.5**	71.9**	92.1**
	STD	47.06	28.61	31.04
Clofibric acid	N	19	19	19
	Mean	100.6**	76.1**	87.5**
	STD	55.76	62.34	57.75
Females				
Control	N	20	20	20
	Mean	12.2	20.7	16.4
	STD	9.65	14.21	9.70
Phenobarbital	N	20	20	20
	Mean	140.7**	137.1**	138.7**
	STD	58.01	37.87	44.16
Clofibric acid	N	19	19	19
	Mean	136.8**	147.8**	142.5**
	STD	76.91	76.58	73.16

** = significantly different from the control group ($p \leq 0.01$)

Terminal sacrifice: phenobarbital and clofibric acid (see Table 5.8.2-26):

Phenobarbital

In males, centrilobular and global proliferation indexes were statistically significantly higher, when compared to controls. There was no relevant change for females, when compared to controls. A centrilobular effect was still present in males but this change was minimal, compared to the effect observed at interim sacrifice.

Clofibric acid

In females, perilobular proliferation index was statistically significantly higher when compared to controls. Global index was similar and centrilobular index was lower, when compared to controls. There was no relevant change for males, when compared to controls. A perilobular effect was still present in females but this change was minimal, compared to the effect observed at interim sacrifice.

Table 5.8.2- 26: Cell cycling phenobarbital and clofibric at terminal sacrifice

Terminal sacrifice Phenobarbital and Clofibric acid				
		Centrilobular	Perilobular	Total
Males				
Control	N	15	15	15
	Mean	13.4	20.1	16.9
	STD	5.34	7.39	5.77
Phenobarbital	N	15	15	15
	Mean	34.4**	15.1	23.7*
	STD	17.50	7.05	10.29
Clofibric acid	N	13	13	13
	Mean	12.9	21.3	17.4
	STD	6.62	15.39	20.21
Females				
Control	N	15	15	15
	Mean	29.8	29.1	29.4
	STD	28.29	18.67	22.96
Phenobarbital	N	15	15	15
	Mean	25.8	31.2	28.7
	STD	23.69	20.50	21.31
Clofibric acid	N	14	14	14
	Mean	16.4	42.5*	30.0
	STD	10.21	19.16	13.83

* = significantly different from the control group ($p \leq 0.05$); ** = significantly different from the control group ($p \leq 0.01$)

Special testing

Total cytochrome P450 content

Phenobarbital

Phenobarbital administration for 7 days induced a marked statistically significant increase in total cytochrome P-450 in both sexes compared to control means (+95% and +77% for the males and the females, respectively).

Clofibric acid

Clofibric acid administration for 7 days induced a slight increase in total cytochrome P-450, but without reaching statistical significance (+28% and +24% for the males and the females, respectively).

Enzymatic activities

Phenobarbital (see Table 5.8.2- 27):

Phenobarbital markedly induced BROD and PROD activities in both sexes compared to control means. The magnitude of the changes are indicated in the table below. A slight increase was also observed in EROD activity for males only. No significant changes were observed in lauric acid hydroxylation.

Table 5.8.2- 27: Enzymatic activities of phenobarbital

Phenobarbital		
% change compared to control means		
	Males	Females
P450	+95%	+77%
BROD	+6326%	+1494%
EROD	+ 83%	NC
PROD	+1920%	+819%
Lauric acid	NC	NC

NC = no significant change

Clofibric acid (see Table 5.8.2- 28):

Clofibric acid significantly induced lauric acid hydroxylation in both males and females. The magnitude of the changes is indicated in the table below. The slight decreases observed in males for EROD activity and in females for BROD and PROD activities were considered not to be toxicologically meaningful.

Table 5.8.2- 28: Enzymatic activities of clofibric acid

Clofibric acid		
% change compared to control means		
	Males	Females
P450	+28%	+24%
BROD	NC	-34%
EROD	-27%	NC
PROD	NC	-44%
Lauric acid	+173%	+112%

NC = no significant change

III. Conclusion

Phenobarbital at 80 mg/kg/day induced a marked hepatocellular proliferation in male and female C57BL/6 mice after 7 days of treatment, which remained statistically significant but slight in males and returned to control levels in females after 28 days of treatment. In addition, phenobarbital was found to be a strong inducer of hepatocellular hypertrophy and of total cytochrome P-450 and BROD and PROD activities. Clofibric acid at 300 mg/kg/day induced a marked hepatocellular proliferation in male and female C57BL/6 mice after 7 days of treatment, which returned to control levels after 28 days of treatment. In addition, clofibric acid was found to be a strong inducer of hepatocellular hypertrophy and of lauric acid hydroxylation activities.

Assessment and conclusion by applicant:

The study was conducted as a reference study to investigate the hepatotoxicity of phenobarbital and clofibric acid and is valid and acceptable for this purpose. Marked hepatocellular proliferation was seen with both substances.

Data Point:	KCA 5.8.2/03
Report Author:	[redacted]
Report Year:	2004
Report Title:	fluopicolide (AE C638206) - Assessment of the non potential interactions between fluopicolide and fosetyl-aluminium or propamocarb-hydrochloride
Report No:	C042301
Document No:	M-232336-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	not applicable
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Assessment and conclusion by applicant:

This paper investigates the potential interactions of fluopicolide with fosetyl-aluminium and propamocarb hydrochloride. Fosetyl-aluminium is no longer an active substance in the representative products of fluopicolide; therefore, this paper is no longer relevant and has not been considered further for this renewal.

Data Point:	KCA 5.8.2/04
Report Author:	[redacted]
Report Year:	2017
Report Title:	Fluopicolide - Preliminary concentration range finding study in cultured male and female C57BL/6 mouse hepatocytes
Report No:	CXR1748
Document No:	M-600904-01-1
Guideline(s) followed in study:	US-EPA OCSPP 870.SUPP
Deviations from current test guideline:	none
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Data Point:	KCA 5.8.2/05
Report Author:	[redacted]
Report Year:	2017
Report Title:	Fluopicolide - Enzyme and DNA-synthesis induction in cultured male and female C57BL/6 mouse hepatocytes
Report No:	CXR1749
Document No:	M-603455-01-1
Guideline(s) followed in study:	US-EPA OCSPP 870.SUPP
Deviations from current test guideline:	none
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The aim of this study was to investigate the potential for fluopicolide to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and modulate cytochrome P450 (Cyp) enzyme activities in isolated male and female C57BL/6 mouse hepatocyte cultures. Cytotoxicity, as evaluated by adenosine 5'-triphosphate (ATP) depletion, was assessed in parallel.

Phenobarbital (PB) at 10, 100 and 1000 µM was tested in parallel as an assay control to confirm hepatocytes responded to the reference compound in the expected manner (induction of Cyp2b and Cyp3a- activities and increased cell proliferation). In addition, Epidermal Growth Factor (EGF, 25 ng/mL) was included as a positive control for hepatocyte proliferation.

In a preliminary range finding study, fluopicolide administration to C57BL/6 male and female mouse hepatocytes in culture resulted in cytotoxicity at concentrations > 3 µM and > 10 µM respectively ([redacted] 2017, M-600904-01-1).

Therefore, in this study, male and female hepatocytes were treated with fluopicolide up to and including 3 µM or 10 µM respectively, resulting in a 48% decrease in ATP levels in male hepatocytes at 3 µM and a 55% decrease in ATP levels in female hepatocytes at 10 µM.

Fluopicolide induced replicative DNA synthesis in a dose-dependent manner with maximal induction at 0.3 µM (1.7-fold in male hepatocytes and 2.3-fold in female hepatocytes). PB induced replicative DNA synthesis to a maximum of 1.8-fold and 2.2-fold in the male and female hepatocytes, respectively; the proliferative capability of these cells in culture was confirmed using EGF (25 ng/mL).

Hepatic pentoxyresorufin-O-depentylation (PROD), benzyloxyresorufin O-debenzylase (BROD) and benzoxyquinoline-O-debenzylation (BQ) rates are indicative of Cyp2b and Cyp3a induction. In male C57BL/6 mouse hepatocytes, Fluopicolide caused a dose dependant increase in PROD and BROD. BQ was also increased in these cells following administration of fluopicolide (1 and 2 µM only).

In female C57BL/6 mouse hepatocytes, fluopicolide induced a dose dependent increase in PROD, but not BROD or BQ activities.

PB (1000 µM) administration to both male and female mouse hepatocytes induced PROD, BROD and BQ activities. Treatment with the positive control items PB and EGF gave the expected set of responses, indicating the suitability of the test system.

In conclusion, fluopicolide induced both hepatocellular S-phase replicative DNA synthesis and Cyp2b enzyme activity in both male and female C57BL/6 mouse primary hepatocyte cultures.

Taken together, these data suggest that fluopicolide activated the nuclear hormone receptor constitutive androstane receptor (Car) in male and female C57BL/6 mouse hepatocytes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	Fluopicolide
Purity:	98.2% w/w
Batch no.:	2016-012208

2. Vehicle and/or positive control

Vehicle:	0.1% v/v DMSO
Positive controls:	Phenobarbital sodium salt (PB), catalogue no. P-5178
	Epidermal growth factor (EGF), catalogue no. E-9644)
Supplier:	Sigma-Aldrich Company Ltd, Poole, Dorset, UK

3. Test animals

Species:	Mice (male and female)
Strain:	C57BL/6 mice
Age:	10 weeks old at study start
Source:	Taconic Biosciences, 273 Hover Avenue, Germantown, NY 12526
Acclimation period:	yes
Diet:	RM1 pelleted diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK) (the specification of the diet is held by the Medical School Resource Unit (MSRU))
Water:	Not mentioned

Housing:	Housing in groups on saw-dust in solid-bottom, polypropylene cages.
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	14-15/hour
Photoperiod:	12 hours

B. Study conduct

1. Study Dates

Study initiation date:	05 May 2017
Experimental start date:	15 May 2017
Experimental finish date:	14 July 2017

2. Experimental procedures

2.1 Hepatocyte isolation

Mice were terminally anaesthetised using Euthatal™ and hepatocytes isolated by in situ perfusion according to Mitchell A.M. et al., (1984). Viabilities of the hepatocyte preparations as determined by Trypan Blue exclusion (Laboratory Method Sheet (LMS) Tic-002), were in excess of 81% for the males and 78% for the females. Hepatocytes used in this study were pooled from multiple independent perfusions of each sex.

2.2 Hepatocyte culture

Primary monolayers of hepatocytes were cultured in plastic tissue culture flasks/plates (25 cm^2 flasks at 2 x 10^6 cells/flask, 6-well plates at 0.8 x 10^6 cells/well, and 96-well plates at 0.02 x 10^6 cells/well). In all 96-well plate cultures, the outside wells of the culture plates were filled with sterile phosphate buffered saline to reduce culture media evaporation.

Following isolation, hepatocytes were cultured in Leibowitz CL15 medium (LMS Tic-001) for 4 hours to allow adherence. The medium was then changed and the hepatocytes were exposed to PB (10, 100 and 1000 µM) or to fluopicolide (male mouse hepatocytes: 0.03, 0.1, 0.3, 1, 2 and 3 µM; females mouse hepatocytes: 0.03, 0.1, 0.3, 1, 3 and 10 µM), to epidermal growth factor (EGF) (25 ng/mL) or a vehicle control [0.1% v/v DMSO]. All test substances were formulated in DMSO. The final concentration of DMSO in all hepatocyte cultures was 0.1% (v/v).

Fluopicolide concentrations were defined in a preliminary range finding study ([redacted] 2017; M-600904-01-1). Fluopicolide caused marked cytotoxicity in male and female C57BL/6 mouse primary hepatocytes at concentrations greater than 1 µM, with more pronounced cytotoxicity in male, than female C57BL/6 mouse primary hepatocytes. Therefore, the concentrations used in the main study were 0.03, 0.1, 0.3, 1, 2 and 3 mM for the male and 0.03, 0.1, 0.3, 1, 3 and 10 µM for the female mouse primary hepatocytes, respectively.

Hepatocytes were cultured for 96 hours and the culture medium, including test and control substances, was replaced at 24 h intervals. Hepatocytes used for enzyme assays (n=3 per test substance concentration) were cultured in 25 cm^2 flasks. To determine replicative DNA synthesis, hepatocytes (n=5 per test substance concentration) were cultured in 6-well plates.

To determine cytotoxicity, hepatocytes (n=6 per test substance concentration) were cultured in 96-well plates.

2.3 Hepatocyte culture harvest

After 96 hours in culture, hepatocytes were either fixed in methanol for assessment of the cell cycle S-phase labelling index or harvested into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4) for subsequent enzyme activity analysis. Hepatocytes harvested into SET were scraped into the buffer and subjected to sonication and stored at -70 °C until analysis. Protein was determined by the method of Lowry *et al.*[2].

2.4 Hepatocyte cytotoxicity

Hepatocyte toxicity, following 96 hours of culture, was assessed by measuring ATP depletion (LMS-Spec-009) using the CellTitre-Glo luminescent cell viability assay (Promega) according to manufacturer's instructions. Results were expressed relative to control cells.

2.5 Replicative DNA synthesis (cell cycle S-phase labelling index)

The number of cells undergoing replicative DNA synthesis (cell cycle S-phase labelling index) in any given cell population can be determined by measuring the incorporation of 5'-bromo-2'-deoxyuridine (BrdU) by immunocytochemistry (ICC). BrdU is a synthetic analog of the nucleoside thymidine that is incorporated into newly synthesised DNA; the incorporated BrdU is detected by ICC. Cell cycle S-phase labelling index was determined over the last 3 days of culture. At the end of the culture period (96 hours), ICC was performed on fixed cells; the number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes /total no. of hepatocytes) x 100].

2.6 Pentoxyresorufin-O-depentylation (PROD)

The activity of Cyp2b in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al.*[3].

2.7 Benzyloxyresorufin-0-debenzylation (BROD)

The activity of Cyp2b and Cyp3a in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin, as described by Burke *et al.*[3].

2.8 Benzyloxyquinoline-O-debenzylation (BQ)

The activity of Cyp3a in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline.

2.9 Statistics

Statistical comparisons between fluopicolide-treated mouse hepatocytes and their respective control groups were undertaken for all numerical data sets using a 2-tailed Student's t-test.

[2] Lowry, O.R., Rosebrough, N.J., Fair, A.L. and R.J. Randall (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275

[3] Burke *et al.* (1985) Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. Biochem. Pharmacol. 34: 3337-3345.

II. Results and Discussion

A. Results

1. Cytotoxicity determination by ATP depletion

Fluopicolide caused dose dependant decreases in ATP levels in the male and female mouse hepatocytes, falling to 48% and 55% of control values at 3 and 10 µM, respectively. These data are in agreement with the dose range finding study ([redacted] 2017; M-600904-01-1), where ATP levels of 53% and 62% relative to control were measured at 3 and 10 µM in the male and female C57BL/6 mouse hepatocytes, respectively.

Although some cytotoxicity was observed in male mouse hepatocytes after treatment with 100 µM PB this was not deemed to be biologically relevant as the ATP values remained above 85%. Neither 10 µM nor 1000 µM PB depleted ATP in male or female hepatocytes, as can be seen in Table 5.8.2- 29.

Table 5.8.2- 29: ATP assay following PB or fluopicolide administration

Test substance and concentration	ATP content (luminescence)	
	Male Mouse hepatocytes	Female Mouse hepatocytes
Vehicle control (0.1% [v/v] DMSO)	482328 ± 12663 (100 ± 2.6)	467685 ± 25761 (100 ± 5.5)
PB 10 µM	457427 ± 30294 (94.8 ± 6.3)	485305 ± 8335 (103.8 ± 17.8)
PB 100 µM	418519 ± 8489*** (86.8 ± 1.8)	476751 ± 36014 (101.9 ± 7.7)
PB 1000 µM	452015 ± 28540* (93.7 ± 5.9)	506639 ± 61550 (108.3 ± 13.2)
Fluopicolide 0.03 µM	465107 ± 28825 (96.4 ± 6.00)	452015 ± 9028 (96.6 ± 1.9)
Fluopicolide 0.1 µM	42884 ± 9398*** (88.1 ± 1.9)	470627 ± 39236 (100.6 ± 8.4)
Fluopicolide 0.3 µM	441382 ± 14490*** (91.5 ± 3.0)	419130 ± 16967** (89.6 ± 3.6)
Fluopicolide 1 µM	375500 ± 21877*** (77.9 ± 4.5)	425745 ± 5186** (91.1 ± 1.1)
Fluopicolide 2 µM	300665 ± 21692*** (62.3 ± 4.5)	------
Fluopicolide 3 µM	231918 ± 13432*** (48.1 ± 2.8)	380615 ± 13882*** (81.4 ± 3.0)
Fluopicolide 10 µM	------	256467 ± 7142*** (54.8 ± 1.5)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n = 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.

2. Biochemical assay results

In the male mouse hepatocytes, fluopicolide caused dose dependant statistically significant increases in PROD and BROD activities (indicative of Cyp2b and Cyp2b/3a, respectively). Fluopicolide only slightly induced BQ activities (indicative of Cyp3a) at 1 and 2 µM (1.4- and 1.5-fold respectively). PROD, BROD and BQ activities were reduced at the highest concentration of fluopicolide (3 µM); this was due to cytotoxicity observed at this concentration (see Table 5.8.2- 29).

PB (1 mM) caused significant increases in PROD (6.5-fold), BROD (4.9-fold) and BQ (7.7-fold) activities in the male mouse hepatocytes.

The pattern of PROD activity in female mouse hepatocytes exposed to fluopicolide was similar to that of males. Here, the test substance caused a dose dependant increase up to and including 1 µM fluopicolide, where PROD activity at 1 µM was induced 1.7-fold relative to control. PROD activity at

3 µM fluopicolide was 1.6-fold relative to control but was not statistically significant in this instance. At 10 µM fluopicolide, PROD activity was reduced to 13% of control values coinciding with the cytotoxicity observed at this concentration of fluopicolide. Although there was a slight increase in BROD activity (maximum of 1.2- fold at 3 µM) this was not statistically significant. BQ activity was slightly reduced after treatment with fluopicolide, however, this was only statistically significant at 10 µM. Again, all three enzyme activity measurements were decreased at the top concentration assessed.

As in the male hepatocytes, PB (1 mM) caused significant increases in PROD, BROD and BQ activities in the female mouse hepatocytes, increasing activities by 2.6-, 1.6- and 4.4-fold, respectively.

All results are summarized in Table 5.8.2- 30 and Table 5.8.2- 31.

Table 5.8.2- 30: Biochemical measurements following PB or fluopicolide administration (males)

Test substance and concentration	Males Mouse hepatocytes		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control (0.1% [v/v] DMSO)	5.89 ± 0.249 (100 ± 4.2)	24.74 ±5.15 (100 ± 20.8)	0.241 ± 0.022 (100 ± 9.3
PB 10 µM	5.00 ± 0.469* (84.9 ±8.0)	21.66 ± 1.71 (87.6 ± 6.9)	0.228 ± 0.026 (94.5 ± 10.8)
PB 100 µM	5.45 ± 0.078* (92.5 ± 1.3)	19.54 ± 0.60 (79.0 ± 2.4)	0.241 ± 0.037 (100.0 ± 15.2)
PB 1000 µM	38.07 ± 2.757*** (646.5 ± 46.8)	121.37 ± 16.67*** (490.6 ± 67.4)	1.858 ± 0.186*** (770.2 ± 77.1)
Fluopicolide 0.03 µM	6.34 ± 0.356 (107.6 ± 6.1)	22.53 ± 0.57 (91.1 ± 2.3)	0.255 ± 0.024 (93.1 ± 9.8)
Fluopicolide 0.1 µM	7.91 ± 0.393*** (134.3 ± 6.7)	29.84 ± 6.05 (120.6 ± 24.4)	0.266 ± 0.007 (110.2 ± 2.9)
Fluopicolide 0.3 µM	10.74 ± 0.583*** (182.4 ± 9.9)	34.01 ± 2.37* (137.5 ± 9.6)	0.247 ± 0.008 (102.2 ± 3.5)
Fluopicolide 1 µM	14.40 ± 0.272*** (244.6 ± 4.6)	51.51 ± 4.64** (208.3 ± 18.8)	0.340 ± 0.021** (140.9 ± 8.7)
Fluopicolide 2 µM	17.6 ± 1.955*** (298.9 ± 33.2)	62.96 ± 9.56** (254.5 ± 38.6)	0.354 ± 0.023** (146.8 ± 9.5)
Fluopicolide 3 µM	11.58 ± 3.411* (196.6 ± 57.9)	34.32 ± 6.14 (139.5 ± 24.8)	0.220 ± 0.28 (91.4 ± 11.8)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$

Table 5.8.2- 31: Biochemical measurements following PB or fluopicolide administration (females)

Test substance and concentration	Females Mouse hepatocytes		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control (0.1% [v/v] DMSO)	13.51 ± 2.19 (100 ± 16.2)	44.90 ± 11.17 (100 ± 24.9)	0.396 ± 19.4 (100 ± 19.4)
PB 10 µM	15.43 ± 0.24 (114.2 ± 1.7)	43.97 ± 12.50 (97.9 ± 27.8)	0.357 ± 0.041 (90.3 ±10.3)
PB 100 µM	22.58 ± 5.51 (167.1 ± 40.8)	54.55 ± 12.95 (121.5 ± 28.8)	0.358 ± 0.069 (90.4 ± 17.4)
PB 1000 µM	34.73 ± 2.53*** (257.0 ± 18.7)	69.99 ± 8.71* (155.9 ± 19.4)	0.938 ± 0.081*** (236.9 ± 20.3)

Test substance and concentration	Females		
	Mouse hepatocytes		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Fluopicolide 0.03 µM	13.44 ± 0.42 (99.5 ± 3.1)	34.51 ± 6.83 (76.9 ± 15.2)	0.303 ± 0.032 (76.5 ± 8.0)
Fluopicolide 0.1 µM	13.25 ± 3.02 (98.0 ± 22.3)	30.38 ± 8.69 (67.7 ± 19.4)	0.278 ± 0.044 (70.2 ± 11.0)
Fluopicolide 0.3 µM	20.49 ± 0.89** (151.6 ± 6.6)	51.04 ±10.90 (113.7 ± 24.3)	0.371 ± 0.027 (93.7 ± 6.9)
Fluopicolide 1 µM	23.05 ± 0.6** (170.6 ± 4.4)	50.86 ± 1.79 (113.6 ± 4.0)	0.327 ± 0.017 (82.6 ± 4.3)
Fluopicolide 3 µM	21.33 ± 5.04 (157.8 ± 37.3)	53.10 ± 11.20 (118.3 ± 24.9)	0.269 ± 0.047 (67.9 ± 11.9)
Fluopicolide 10 µM	1.76 ± 0.28*** (13.0 ± 2.1)	7.15 ± 0.80** (15.9 ± 1.8)	0.042 ± 0.002*** (10.6 ± 0.5)

Values are mean ± SD. Values in parenthesis are mean % control ± SD, n= 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control p<0.05; ** p<0.01; ***p<0.001.

3. Replicative DNA synthesis (S-phase)

In the male mouse hepatocytes, fluopicolide caused dose-dependent increases in replicative DNA synthesis; maximal induction was 1.7-fold at 0.3 µM. At 1 µM fluopicolide, replicative DNA synthesis was 1.6-fold relative to control. Replicative DNA synthesis (S-phase) was not analysed at 2 and 3 µM due to reduced cell numbers at these concentrations. PB (100 µM) and EGF (25 ng/mL) also caused significant increases in replicative DNA synthesis of 1.8-fold and 8.3-fold, respectively.

Similarly, in the female mouse hepatocytes, fluopicolide caused dose-dependent increases in replicative DNA synthesis, with the maximal response being seen at 0.3 µM (2.3-fold). The increases in replicative DNA synthesis at 1 and 3 µM were lower, at 1.5- and 1.4-fold, respectively, relative to control. Due to reduced cell numbers, S-phase was not analysed at 10 µM fluopicolide. PB caused significant increases in replicative DNA synthesis at all concentrations, the maximal response (2.2-fold) was observed at 100 µM. As expected, EGF (25 ng/mL) significantly induced replicative DNA synthesis (6.0-fold) in the female hepatocytes.

The results are summarized in Table 5.8.2- 32.

Table 5.8.2- 32: Replicative DNA synthesis (S-phase) assessment following PB, EGF or Fluopicolide administration

Test substance and concentration	S-Phase labelling index	
	Mouse hepatocytes	
	Males	Females
Vehicle control (0.1% [v/v] DMSO)	0.28 ± 0.06 (100 ± 21.2)	1.07 ± 0.16 (100 ± 14.5)
PB 10 µM	0.24 ± 0.04 (87 ± 14.0)	2.22 ± 0.19*** (206.5 ±18.1)
PB 100 µM	0.52 ±0.08*** (184.2 ± 28.6)	2.41 ± 0.24*** (224.5 ± 22.6)
PB 1000 µM	0.24 ± 0.04 (84.9 ± 13.7)	2.06 ± 0.22*** (191.9 ± 20.6)
Fluopicolide 0.03 µM	0.31 ± 0.03 (111.6 ± 11.6)	1.83 ± 0.11*** (170.7 ± 10.6)
Fluopicolide 0.1 µM	0.38 ± 0.10 (135.0 ± 35.6)	2.45 ± 0.40*** (227.8 ± 37.6)
Fluopicolide 0.3 µM	0.48 ± 0.05*** (171.9 ± 18.8)	2.50 ± 0.18*** (171.9 ± 18.8)

Test substance and concentration	S-Phase labelling index Mouse hepatocytes	
	Males	Females
Fluopicolide 1 µM	0.46 ± 0.07** (163.5 ± 24.0)	1.61 ± 0.45* (149.6 ± 41.7)
Fluopicolide 2 µM	#	------
Fluopicolide 3 µM	#	1.52 ± 0.26* (141.3 ± 24.3)
Fluopicolide 10 µM	-------	#
EGF 25 ng/mL	2.32 ± 0.68*** (830.4 ± 242.7)	6.45 ± 0.90*** (600.1 ± 83.6)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control p<0.05; ** p<0.01; ***p<0.001. # not counted due to abnormal morphology and cell density following treatment with Fluopicolide.

III. Conclusion

Fluopicolide induced replicative DNA synthesis in a dose-dependent manner. In both sets of hepatocytes maximal induction occurred at 0.3 µM: 1.7-fold in male hepatocytes and 2.3-fold in female hepatocytes. Similar increases in replicative DNA synthesis were observed in cells exposed to PB, where fold increases of 1.8 and 2.2 were seen in male and female hepatocytes, respectively. As expected, the positive control EGF caused a robust proliferative response in both male and female mouse hepatocytes (8.3-fold in males and 6.0- fold in females).

Fluopicolide caused dose dependant increases in the activity of Cyp2b as determined by PROD and BROD activity in both sexes. However, BQ activities were slightly increased in the male mouse hepatocytes but not in the female hepatocytes, suggesting that Cyp3a was less affected by fluopicolide administration than was Cyp2b. In line with expectations, PB caused induction of both Cyp2b and Cyp3a enzyme activities in both sexes of mouse hepatocytes.

Treatment with the positive control items PB and EGF gave the expected set of responses, indicating the suitability of the test system.

In conclusion, fluopicolide induced hepatocellular S-phase (replicative DNA synthesis) and Cyp2b enzyme activity in both male and female C57BL/6 mouse primary hepatocyte cultures. Taken together, these data suggest that Fluopicolide activated the nuclear hormone receptor constitutive androstane receptor (Car) in male and female C57BL/6 mouse hepatocytes.

Assessment and conclusion by applicant:

The study (and accompanying range-finding study) is valid and acceptable to investigate the potential for fluopicolide to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and modulate cytochrome P450 (Cyp) enzyme activities in isolated male and female C57BL/6 mouse hepatocyte cultures. Fluopicolide induced hepatocellular S-phase (replicative DNA synthesis) and Cyp2b enzyme activity in both male and female C57BL/6 mouse primary hepatocyte cultures.

Data Point:	KCA 5.8.2/06
Report Author:	[redacted]
Report Year:	2017
Report Title:	Fluopicolide - Preliminary concentration range finding study in cultured male and female CarKO/PxrKO mouse hepatocytes
Report No:	CXR1750
Document No:	M-600908-01-1
Guideline(s) followed in study:	US-EPA OCSPP 870.SUPP
Deviations from current test guideline:	none
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Data Point:	KCA 5.8.2/07
Report Author:	[redacted]
Report Year:	2017
Report Title:	Fluopicolide - Enzyme and DNA-synthesis induction in cultured male and female CarKO/PxrKO mouse hepatocytes
Report No:	CXR1751
Document No:	M-604080-01-1
Guideline(s) followed in study:	US-EPA OCSPP 870.SUPP
Deviations from current test guideline:	none
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The aim of this study was to investigate the potential for fluopicolide to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and modulate cytochrome P450 (Cyp) enzyme activities in isolated male and female constitutive androstane receptor knockout/pregnane x-receptor knockout (CarKO/PxrKO) mouse hepatocyte cultures. Cytotoxicity, as evaluated by adenosine 5'-triphosphate (ATP) depletion, was assessed in parallel.

Phenobarbital (PB) at 10, 100 and 1000 µM was tested in parallel as an assay control to confirm CarKO/PxrKO hepatocytes responded to the reference compound in the expected manner (i.e. poor induction of Cyp2b- and Cyp3a- activities and no cell proliferation). In addition, Epidermal Growth Factor (EGF, 25 ng/mL) was included as a positive control for hepatocyte proliferation.

Fluopicolide administration to CarKO/PxrKO male and female mouse hepatocytes in culture resulted in severe cytotoxicity at concentrations > 3 and > 10 µM respectively ([redacted] 2017; M-600908-01-1). Therefore, in this study, male and female hepatocytes were treated with fluopicolide up to and including 3 or 10 µM respectively. This resulted in a decrease of 65% in ATP levels in male hepatocytes at 3 µM and a 61% decrease in ATP levels in female hepatocytes at 10 µM.

Treatment with fluopicolide or PB did not induce replicative DNA synthesis in male or female CarKO/PxrKO hepatocytes at any concentration but the proliferative capability of these cells in culture was confirmed using EGF (25 ng/mL).

Hepatic pentoxyresorufin-O-depentylation (PROD), benzyloxyresorufin 0-debenzylase (BROD) and benzoxyquinoline-O-debenzylation (BQ) rates are indicative of Cyp2b and Cyp3a induction. Fluopicolide did not cause any increases in PROD, BROD or BQ in male or female CarKO/PxrKO mouse hepatocytes.

PB administration (1000 µM only) to male mouse hepatocytes only slightly induced PROD, BROD and BQ to 3.5-, 1.5- and 1.7-fold respectively. 1000 µM PB also caused only slight induction in female mouse hepatocytes in PROD and BROD of 1.6- and 1.8-fold respectively, with no induction observed in BQ.

In conclusion, fluopicolide did not induce either hepatocellular S-phase replicative DNA synthesis, Cyp2b or Cyp3a enzyme activity in male or female CarKO/PxrKO mouse primary hepatocyte cultures. Taken together, these data suggest that fluopicolide requires the presence of the nuclear hormone receptors Car and/or Pxr to induce replicative DNA synthesis and enzyme activity in male and female mouse hepatocytes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	Fluopicolide
Purity:	98.2% w/w
Batch no.:	2016-0122-08

2. Vehicle and/or positive control

Vehicle:	0.1% v/v DMSO
Positive controls:	Phenobarbital sodium salt (PB), catalogue no. P-5178 Epidermal growth factor (EGF), catalogue no. E-9644)
Supplier:	Sigma-Aldrich Company Ltd, Poole, Dorset, UK

3. Test animals

Species:	Mice (male and female)
Strain:	Male and female CarKO/PxrKO mice (C57BL/6-Nrli2tm3Arle/Nrli3tmUArte, product number, 8222-M or 8222-F, respectively)
Age:	11-12 weeks old at study start
Source:	Taconic Biosciences, 273 Hover Avenue, Germantown, NY 12526
Acclimation period:	yes
Diet:	RMH pelleted diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK) (the specification of the diet is held by the Medical School Resource Unit (MSRU))
Water:	Not mentioned
Housing:	Housing in groups on saw-dust in solid-bottom, polypropylene cages.
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	14-15/hour
Photoperiod:	12 hours

B. Study conduct

1. Study Dates

Study initiation date: 02 June 2017

Experimental start date: 05 June 2017

Experimental finish date: 12 June 2017

2. Experimental procedures

2.1 Hepatocyte isolation

Mice were terminally anaesthetised using Euthatal™ and hepatocytes isolated by in situ perfusion according to Mitchell A.M, Bridges, J.W. & CR. Elcombe (1984)[4]. Viabilities of the hepatocyte preparations, as determined by Trypan Blue exclusion, were in excess of 81% for the males and 78% for the females. Hepatocytes used in this study were pooled from multiple independent perfusions of each sex.

2.2 Hepatocyte culture

Primary monolayers of hepatocytes were cultured in plastic tissue culture flasks/plates (25 cm^2 flasks at 2 x 10^6 cells/flask; 6-well plates at 0.8 x 10^6 cells/well; and 96-well plates at 0.02 x 10^6 cells/well). In all 96-well plate cultures, the outside wells of the culture plates were filled with sterile phosphate buffered saline to reduce culture media evaporation.

Following isolation, hepatocytes were cultured in Leibovitz CL15 medium for 4 hours to allow adherence. The medium was then changed and the hepatocytes were exposed to PB (10, 100 and 1000 µM) or to fluopicolide (male mouse hepatocytes: 0.03, 0.1, 0.3, 1, 2 and 3 µM; females mouse hepatocytes: 0.03, 0.1, 0.3, 1, 3 and 10 µM), to epidermal growth factor (EGF) (25 ng/mL) or a vehicle control [0.1% v/v DMSO]. All test substances were formulated in DMSO. The final concentration of DMSO in all hepatocyte cultures was 0.1% (v/v).

Fluopicolide concentrations were defined in a preliminary range finding study ([redacted] 2017; M-600908-01-1). In this study, fluopicolide caused pronounced cytotoxicity in CarKO/PxrKO mouse primary hepatocytes at concentrations greater than 1 and 3 µM in male and female, respectively. Therefore, the concentrations used in the main study were 0.03, 0.1, 0.3, 1, 2 and 3 µM for the male and 0.03, 0.1, 0.3, 1, 3 and 10 µM for the female CarKO/PxrKO mouse primary hepatocytes, respectively.

Hepatocytes were cultured for 96 hours and the culture medium, including test and control substances, was replaced at 24 h intervals. Hepatocytes used for enzyme assays (n=3 per test substance concentration) were cultured in 25 cm^2 flasks. To determine replicative DNA synthesis, hepatocytes (n=5 per test item concentration) were cultured in 6-well plates.

To determine cytotoxicity, hepatocytes (n=6 per test substance concentration) were cultured in 96-well plates.

[4] Mitchell A.M, Bridges, J.W. & CR. Elcombe (1984) Factors influencing peroxisome proliferation in cultured rat hepatocytes. Arch. Toxicol. 55: 239-246

2.3 Hepatocyte culture harvest

After 96 hours in culture, hepatocytes were either fixed in methanol for assessment of the cell cycle S-phase labelling index or harvested into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4) for subsequent enzyme activity analysis. Hepatocytes harvested into SET were scraped into the buffer and subjected to sonication and stored at -70 °C until analysis. Protein was determined by the method of Lowry *et al.*[2].

2.4 Hepatocyte cytotoxicity

Hepatocyte toxicity, following 96 hours of culture, was assessed by measuring ATP depletion (LMS-Spec-009) using the CellTitre-Glo luminescent cell viability assay (Promega) according to manufacturer's instructions.

Results were expressed relative to control cells.

2.5 Replicative DNA synthesis (cell cycle S-phase labelling index)

The number of cells undergoing replicative DNA synthesis (cell cycle S-phase labelling index) in any given cell population can be determined by measuring the incorporation of 5'-bromo-2'-deoxyuridine (BrdU) by immunocytochemistry (ICC). BrdU is a synthetic analog of the nucleoside thymidine that is incorporated into newly synthesised DNA; the incorporated BrdU is detected by ICC. Cell cycle S-phase labelling index was determined over the last 3 days of culture. At the end of the culture period (96 hours), ICC was performed on fixed cells; the number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes /total no. of hepatocytes) x 100].

2.6 Pentoxyresorufin-O-depentylation (PROD)

The activity of Cyp2b in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al.*[3] according to LMS Fluor-002.

2.7 Benzyloxyresorufin-O-debenzylation (BROD)

The activity of Cyp2b and Cyp3a in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin.

2.8 Benzyloxyquinoline-O-debenzylation (BQ)

The activity of Cyp3a in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline.

2.9 Statistics

Statistical comparisons between fluopicolide-treated mouse hepatocytes and their respective control groups were undertaken for all numerical data sets using a 2-tailed Student's t-test.

II. Results and Discussion

A. Results

1. Cytotoxicity determination by ATP depletion

Fluopicolide caused dose dependant decreases in ATP levels in the male and female mouse hepatocytes, falling to 35% and 39% of control values at 3 and 10 µM, respectively. These data are in agreement with the dose range finding study ([redacted] 2017; M-600908-01-1), where ATP levels of 36% and 46% relative to control were measured at 3 and 10 µM in the male and female CarKO/PxrKO mouse hepatocytes, respectively.

Although some cytotoxicity was observed in male mouse hepatocytes after treatment with PB (10, 100 and 1000 µM) and female hepatocytes (100 µM only) this was not deemed to be biologically relevant as the ATP values remained above 85%.

An overview is given in Table 5.8.2- 33.

Table 5.8.2- 33: ATP assay following PB or fluopicolide administration

Test substance and concentration	ATP content (luminescence units) Mouse hepatocytes	
	Males	Females
Vehicle control (0.1% [v/v] DMSO)	441352 ± 17781 (100 ± 4.0)	412962 ± 29443 (100.0 ± 7.1)
PB 10 µM	392636 ± 18325*** (89.0 ± 4.2)	415126 ± 19045 (100.5 ± 4.6)
PB 100 µM	406457 ± 16496** (92.1 ± 3.7)	376077 ± 13602* (90.3 ± 3.3)
PB 1000 µM	415126 ± 17249* (94.1 ± 3.9)	398332 ± 24209 (96.5 ± 5.9)
Fluopicolide 0.03 µM	413286 ± 16435* (93.6 ± 3.7)	407496 ± 17572 (98.6 ± 4.3)
Fluopicolide 0.1 µM	399551 ± 10509*** (90.5 ± 2.4)	387127 ± 21231 (93.7 ± 5.1)
Fluopicolide 0.3 µM	384198 ± 22427*** (87.1 ± 5.1)	363244 ± 44708* (88.0 ± 10.8)
Fluopicolide 1 µM	336433 ± 8650*** (77.9 ± 4.5)	369433 ± 27823* (89.5 ± 6.7)
Fluopicolide 2 µM	225484 ± 11574*** (51.1 ± 2.6)	Not analysed
Fluopicolide 3 µM	153540 ± 10158*** (34.8 ± 2.3)	309758 ± 25430*** (75.0 ± 6.2)
Fluopicolide 10 µM	Not analysed.	162451 ± 5137*** (39.3 ± 1.2)

Values are mean ± SD. Values in parenthesis are mean % control ± SD, n= 6 per group.
A student's t-test (2-sided) was performed on the results, * statistically different from control
$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

2. Biochemical assay results

In the male CarKO/PxrKO mouse hepatocytes, fluopicolide caused decreases in PROD activity (indicative of Cyp2b), with no activity at all observed after treatment with 3 µM fluopicolide.

This lack of activity was due to cytotoxicity observed at this concentration. Fluopicolide reduced BROD and BQ activities (indicative Cyp2b/3a and Cyp3a respectively) in a dose dependant manner.

PB administration (1000 µM only) to male mouse hepatocytes caused small but significant increases in PROD, BROD and BQ by 3.5-, 1.5- and 1.7-fold respectively.

In female CarKO/PxrKO mouse hepatocytes, treatment with fluopicolide caused decreases in PROD, BROD and BQ activities. Enzyme assays for 3 and 10 µM fluopicolide were n=l and n=2 respectively, therefore, statistics have not been carried out on these concentrations.

1000 µM PB also caused small, but significant inductions in PROD and BROD by 1.6- and 1.8-fold, respectively. BQ activity was decreased compared to control after treatment with 1000 µM PB.

All results are summarized in Table 5.8.2- 34.

Table 5.8.2- 34: Biochemical measurements following PB or fluopicolide administration

Test substance and concentration	Males		
	Mouse KO hepatocytes		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control (0.1% [v/v] DMSO)	0.14 ± 0.103 (100 ± 73.7)	4.18 ± 0.73 (100 ± 17.3	0.081 ± 0.007 (100 ± 8.7)
PB 10 µM	0.16 ± 0.064 (116.5 ± 45.6)	3.09 ± 0.47 (73.8 ± 11.3)	0.074 ± 0.004 (91.1 ± 4.7)
PB 100 µM	0.15 ± 0.070 (104.2 ± 50.4)	2.64 ± 0.74 (63.1 ± 17.8)	0.082 ± 0.007 (100.9 ± 8.0)
PB 1000 µM	0.49 ± 0.084** (354.7 ± 60.3)	6.07 ± 0.27* (145.1 ± 6.6)	0.142 ± 0.011** (174.1 ± 13.9)
Fluopicolide 0.03 µM	0.17 ± 0.038 (122.6 ± 27.2)	3.23 ± 0.36 (77.1 ± 8.7)	0.084 ± 0.005 (103.0 ± 6.3)
Fluopicolide 0.1 µM	0.16 ± 0.032 (118.2 ± 23.0)	2.69 ± 0.04* (64.2 ± 1.0)	0.082 ± 0.001 (100.4 ± 1.7)
Fluopicolide 0.3 µM	0.18 ± 0.059 (132.4 ± 9.9)	3.24 ± 0.23 (77.5 ± 5.5)	0.076 ± 0.006 (93.1 ± 7.4)
Fluopicolide 1 µM	0.08 ± 0.039 (60.6 ± 28.3)	2.26 ± 0.04* (54.0 ± 1.1)	0.072 ± 0.006 (89.0 ± 8.0)
Fluopicolide 2 µM	0.02 ± 0.012 (11.0 ± 8.5)	1.27 ± 0.08** (30.4 ± 1.8)	0.043 ± 0.0002*** (52.8 ± 0.2)
Fluopicolide 3 µM	#	0.51 ± 0.13*** (12.1 ± 3.0)	0.027 ± 0.003*** (33.0 ± 3.7)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 3 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$. # no activity was determined in that sample set.

Table 5.8.2- 34 (continued): Biochemical measurements following PB or fluopicolide administration

Test substance and concentration	Females		
	Mouse KO hepatocytes		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control (0.1% [v/v] DMSO)	2.08 ± 0.103 (100 ± 73.7)	11.74 ± 1.53 (100 ± 13.0)	0.509 ± 0.049 (100 ± 9.7)
PB 10 µM	1.83 ± 0.17 (88.1 ± 8.1)	12.56 ± 0.09 (107.0 ± 0.8)	0.502 ± 0.046 (98.6 ± 8.9)
PB 100 µM	1.61 ± 0.45 (77.7 ± 21.9)	12.9 ± 2.01 (103 ± 17.1)	0.432 ± 0.090 (84.8 ± 17.7)
PB 1000 µM	3.30 ± 0.14*** (257.0 ± 18.7)	21.69 ± 0.45*** (184.7 ± 3.8)	0.384 ± 0.025* (75.5 ± 4.9)

Test substance and concentration	Females		
	Mouse KO hepatocytes		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Fluopicolide 0.03 µM	1.70 ± 0.08* (81.8 ± 4.0)	12.33 ± 2.30 (105.0 ± 19.6)	0.591 ± 0.069 (116.2 ± 13.5)
Fluopicolide 0.1 µM	1.75 ± 0.25 (84.3 ± 11.8)	10.51 ± 0.41 (89.5 ± 19.4)	0.463 ± 0.056 (91.0 ± 11.1)
Fluopicolide 0.3 µM	2.01 ± 0.19 (97.0 ± 7.7)	12.41 ± 0.69 (105.7 ± 5.9)	0.480 ± 0.030 (94.4 ± 5.8)
Fluopicolide 1 µM	1.41 ± 0.21** (68.0 ± 10.2)	8.81 ± 0.72* (75.0 ± 6.2)	0.417 ± 0.062 (81.9 ± 12.2)
Fluopicolide 3 µM	0.57[a] (27.3)	4.74[a] (40.3)	165[a] (32.5)
Fluopicolide 10 µM	#[b]	0.36 ± 0.07[b] (3.1 ± 0.6)	0.005 ± 0.000[b] (1.1 ± 0.1)

Values are mean ± SD. Values in parenthesis are mean % control ± SD, n= 3 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
p < 0.05; ** p < 0.01; ***p < 0.001. # no activity was determined in that sample set.
[a] one samples analysed at the dose group; [b] two samples analysed at the dose group

3. Replicative DNA synthesis (S-phase)

In the male mouse hepatocytes, fluopicolide did not cause any significant increases in replicative DNA synthesis at any concentration analysed. S-phase labelling index was not analysed at 2 and 3 µM due to reduced cell numbers at these concentrations. EGF (25 ng/mL) caused a significant increase in replicative DNA synthesis of 5.8-fold, whereas PB did not cause any induction in replicative DNA synthesis.

Similarly, in the female mouse hepatocytes, fluopicolide did not cause any increases in replicative DNA synthesis at any concentration analysed. Due to reduced cell numbers, S-phase labelling index was not analysed at 3 or 10 µM fluopicolide. PB did not cause any significant increases in replicative DNA synthesis at any concentration analysed. As expected, EGF (25 ng/mL) significantly induced replicative DNA synthesis (3.2-fold) in the female hepatocytes.

The results are summarized in the following table.

Table 5.8.2- 35: Replicative DNA synthesis (S-phase) assessment following PB, EGF or fluopicolide administration

Test substance and concentration	S-Phase labelling index	
	Mouse hepatocytes	
	Males	Females
Vehicle control (0.1% [v/v] DMSO)	0.52 ± 0.13 (100 ± 24.9)	1.76 ± 0.35 (100 ± 20.1)
PB 10 µM	0.60 ± 0.09 (116.3 ± 16.8)	1.53 ± 0.18 (86.7 ± 10.4)
PB 100 µM	0.43 ± 0.11 (83.1 ± 21.8)	1.85 ± 0.36 (104.7 ± 20.4)
PB 1000 µM	0.42 ± 0.09 (81.0 ± 17.2)	1.68 ± 0.49 (95.3 ± 28.0)
Fluopicolide 0.03 µM	0.43 ± 0.07 (83.9 ± 14.4)	1.55 ± 0.24 (87.6 ± 13.8)
Fluopicolide 0.1 µM	0.48 ± 0.05 (92.1 ± 9.1)	1.63 ± 0.15 (92.6 ± 8.6)
Fluopicolide 0.3 µM	0.48 ± 0.18 (93.8 ± 33.5)	1.35 ± 0.21 (76.7 ± 12.2)
Fluopicolide 1 µM	0.40 ± 0.06 (78.1 ± 12.0)	1.30 ± 0.14 (73.9 ± 7.9)

Test substance and concentration	S-Phase labelling index	
	Mouse hepatocytes	
	Males	Females
Fluopicolide 2 µM	#	------
Fluopicolide 3 µM	#	#
Fluopicolide 10 µM	-------	#
EGF 25 ng/mL	3.01 ± 0.93*** (582.4 ± 180.7)	5.65 ± 1.05*** (320.2 ± 59.4)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 5 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.
not counted due to abnormal morphology and cell density following treatment fluopicolide.

III. Conclusion

Based on the toxicity observed in the preliminary ATP assay ([redacted], 2017; M-600908-01-1), the highest concentration of fluopicolide used to treat male and female mouse hepatocytes was 3 and 10 µM respectively. At these concentrations, ATP levels were depleted to 36% (male) and 46% (female) of control values.

Neither fluopicolide nor PB induced replicative DNA synthesis at any concentrations evaluated. As expected, the positive control, EGF, caused a robust proliferative response in both male and female mouse hepatocytes (5.8-fold in males and 3.2- fold in females).

Fluopicolide did not cause any increases in the activity of Cyp2b or Cyp3a as determined by PROD, BROD and BQ activity in both sexes. In line with expectations, PB did not cause robust induction of Cyp2b or Cyp3a enzyme activities in either sex of CarKO/PxrKO mouse hepatocytes.

Treatment with the positive control for replicative DNA synthesis, EGF, gave the expected set of responses, indicating the suitability of the test system.

In conclusion, fluopicolide did not induce either hepatocellular S-phase replicative DNA synthesis, Cyp2b or Cyp3a enzyme activity in male or female CarKO/PxrKO mouse primary hepatocyte cultures. Taken together, these data suggest that fluopicolide requires the presence of the nuclear hormone receptors Car and/or Pxr to induce replicative DNA synthesis and enzyme activity in male and female mouse hepatocytes.

Assessment and conclusion by applicant:

The study (and accompanying range-finding study) is valid and acceptable to investigate the potential for fluopicolide to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and modulate cytochrome P450 (Cyp) enzyme activities in isolated male and female constitutive androstane receptor knockout/pregnane x receptor knockout (CarKO/PxrKO) mouse hepatocyte cultures. Fluopicolide did not induce DNA synthesis or Cyp2b/Cyp3a enzyme activity.

Data Point:	KCA 5.8.2/08
Report Author:	[redacted]
Report Year:	2017
Report Title:	Fluopicolide - Preliminary concentration range finding study in cultured human hepatocytes from three individual donors
Report No:	CXR1752
Document No:	M-600911-01-1
Guideline(s) followed in study:	US-EPA OCSPP 870.SUPP
Deviations from current test guideline:	none
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Data Point:	KCA 5.8.2/09
Report Author:	[redacted]
Report Year:	2017
Report Title:	Fluopicolide - Enzyme and DNA-synthesis induction in cultured human hepatocytes from three individual donors
Report No:	CXR1753
Document No:	M-604094-01-1
Guideline(s) followed in study:	US-EPA OCSPP 870.SUPP
Deviations from current test guideline:	none
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The aim of this study was to investigate the potential for fluopicolide to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and modulate cytochrome P450 (CYP) enzyme activities in cryopreserved male and female human hepatocyte cultures from three independent donors. Cytotoxicity, as evaluated by adenosine 5'-triphosphate (ATP) depletion, was assessed in parallel.

Phenobarbital (PB) at 10, 100 and 1000 µM was tested in parallel as an assay control to confirm hepatocytes responded to the reference compound in the expected manner (induction of CYP2B- and CYP3A-activities). In addition, Epidermal Growth Factor (EGF, 25 ng/mL) was included as a positive control for hepatocyte proliferation.

In a preliminary study, fluopicolide administration to male and female human hepatocytes in culture resulted in cytotoxicity at concentrations > 3 and > 10 µM respectively ([redacted] 2017; M-600911-01-1). In this study, male and female human hepatocytes were treated with fluopicolide up to and including 10 or 100 µM, respectively, resulting in an estimated 38% decrease in ATP levels in male hepatocytes at 10 µM and estimated 69% or 57% decreases in ATP levels in female hepatocytes at 100 µM (donor 8239 and 1765, respectively).

Neither administration with fluopicolide nor PB induced replicative DNA synthesis in cultured male or female human hepatocytes. However, the proliferative capability of these cells in culture was confirmed using EGF (25 ng/mL).

Hepatic pentoxyresorufin O-dependentylation (PROD), benzyloxyresorufin O-debenzylase (BROD) and benzoxyquinoline-O-debenzylation (BQ) rates are indicative of CYP2B and CYP3A induction. In male human hepatocytes, Fluopicolide caused a dose-dependent increase in BQ. BROD was also slightly increased in these cells following administration of fluopicolide. PB administration to male human hepatocytes induced BROD and BQ in a dose-dependent manner. PROD activity could not be analysed as levels were below the level of quantification.

Treatment with fluopicolide resulted in dose-dependent increases in BQ activity in female hepatocytes from both donors. Fluopicolide caused no increased PROD or BROD activity in female hepatocytes, however, slight, but significant, decreases in BROD activity were observed at the top concentrations. In female human hepatocytes, PB induced a dose-dependent increase in BROD and BQ in both donors, however, only donor 1765 responded in a dose-dependent manner after treatment with PB. Treatment with the positive control item EGF gave the expected set of responses, indicating the suitability of the test system.

In summary, treatment of cultured male or female human hepatocytes with fluopicolide resulted in weak induction of CYP3A enzyme activity (BROD (male only) and BQ activities (male and females)). There was no evidence of fluopicolide or PB-stimulated proliferation in cultured male or female human hepatocytes.

In conclusion, these data suggest that fluopicolide is a weak activator of human PXR (as shown by the effects on CYP3A enzyme activity levels), with no effect on DNA-synthesis in male or female human hepatocytes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	Fluopicolide
Purity:	98.2% w/w
Batch no.:	2016-012208

2. Vehicle and/or positive control

Vehicle:	0.1% v/v DMSO
Positive controls:	Phenobarbital sodium salt (PB), catalogue no. P-5178 Epidermal growth factor (EGF), catalogue no. E-9644)
Supplier:	Sigma-Aldrich Company Ltd, Poole, Dorset, UK

3. Human Hepatocytes

Primary male and female human hepatocytes (cryopreserved and plateable) were sourced from Life Technologies, 7 Kingsland Grange, Warrington, Cheshire, WA1 4SR. Hepatocytes were sourced from a single male donor with reference number 8210 (Caucasian, 51 yrs.) and two female donors with reference numbers 8239 (Caucasian, 52 yrs.) and 1765 (Caucasian, 37 yrs.). Data sheets are provided in the report.

B. Study conduct

1. Study Dates

Study initiation date: 27 June 2017

Experimental start date: 03 July 2017

Experimental finish date: 21 July 2017

2. Experimental procedures

2.1 Hepatocyte culture

Viabilities of the hepatocyte preparations, as determined by Trypan Blue exclusion (Laboratory Method Sheet (LMS) Tic-002), were in excess of 80%.

Primary monolayers of hepatocytes were cultured in collagen-coated plastic tissue culture flasks/plates (25 cm^2 flasks at 3.2 x 10^6 cells/flask; 6-well plates at 1.6 x 10^6 cells/well; and 96-well plates at 4 x 10^4 cells/well). In all 96-well plate cultures, the outside wells of the culture plates were filled with sterile phosphate buffered saline to reduce culture media evaporation.

Hepatocytes were resuscitated in Cryopreserved Hepatocyte Thaw Medium, then cultured in Cryopreserved Hepatocyte Plating Medium at 37 °C in a humidified incubator under an atmosphere of 95% air/5% CO_2 for 6 hours to allow adherence. The medium was then changed to Leibowitz human complete L15 (HCL15) medium and the hepatocytes exposed to PB at 3 concentrations (10, 100 and 1000 µM) as an assay control, the test substance at 6 concentrations (the male human donor was treated with 0.03, 0.1, 0.3, 1, 3 and 10 µM, the female human donors were treated with 0.3, 1, 3, 10, 30 and 100 µM fluopicolide) and a vehicle control [0.1% v/v DMSO].

The concentrations of fluopicolide used were selected with reference to the preliminary range finding study ([redacted] 2017; M-600911-01-1). In this range finder, fluopicolide caused pronounced cytotoxicity in primary cultures of hepatocytes from one male donor at concentrations greater than 3 µM and two female donors at concentrations greater than 10 or 100 µM, with more pronounced cytotoxicity being observed in the male primary hepatocytes. Therefore, following discussions with the sponsor, the concentrations to be taken to the main study were 0.03, 0.1, 0.3, 1, 3 and 10 µM for the male donor, and 0.3, 1, 3, 10, 30 and 100 µM for the two female donors.

Hepatocytes were cultured for 96 hours and the culture medium, including test and control substances, was replaced at 24 h intervals. Hepatocytes used for enzyme assays (n=3 per test substance concentration) were cultured in 25 cm^2 flasks. To determine replicative DNA synthesis, hepatocytes (n=5 per test substance concentration) were cultured in 6-well plates. To determine cytotoxicity, hepatocytes (n=6 per test substance concentration) were cultured in 96-well plates.

2.2 Hepatocyte culture harvest

After 96 hours in culture, hepatocytes were either fixed in methanol for assessment of the cell cycle S-phase labelling index or harvested into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4) for subsequent enzyme activity analysis. Hepatocytes harvested into SET were scraped into the buffer, subjected to sonication, and stored at -70 °C until analysis. Protein was determined by the method of Lowry *et al.*[5]

[5] Lowry, O. H., Rosebrough, N. J., Farr, A. L., Ransall, R. J., 1951, Protein measurement with the Folin phenol reagent, Journal of Biological Chemistry, 193(1): 265-75

2.3 Hepatocyte cytotoxicity

Hepatocyte toxicity, following 96 hours of culture, was assessed by measuring ATP depletion (LMS-Spec-009) using the CellTitre-Glo luminescent cell viability assay (Promega) according to manufacturer's instructions.

Results were expressed relative to control cells.

2.4 Replicative DNA synthesis (cell cycle S-phase labelling index)

The number of cells undergoing replicative DNA synthesis (cell cycle S-phase labelling index) in any given cell population can be determined by measuring the incorporation of 5'-bromo-2'-deoxyuridine (BrdU) by immunocytochemistry (ICC). BrdU is a synthetic analogue of the nucleoside thymidine that is incorporated into newly-synthesised DNA; the incorporated BrdU is detected by ICC. Cell cycle S-phase labelling index was determined over the last 3 days of the culture. At the end of the culture period (96 hrs), ICC was performed on fixed cells; the number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes/total no. of hepatocytes) x 100].

2.5 Pentoxyresorufin-O-depentylation (PROD)

The activity of CYP2B in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al.*[3] according to LMS Fluor-002.

2.6 Benzyloxyresorufin-0-debenzylation (BROD)

The activity of CYP2B and CYP3A in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin, as described by Burke *et al.*[3], according to LMS Fluor-002.

2.7 Benzyloxyquinoline-O-debenzylation (BQ)

The activity of CYP3A in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline, as described by the Corning Gentest HTS technical bulletin and according to LMS Fluor-005.

2.8 Statistics

Statistical comparisons between fluopicolide-treated human hepatocytes and their respective control groups were undertaken for all numerical data sets using a 2-tailed Student's t-test.

II. Results and Discussion

A. Results

1. Cytotoxicity determination by ATP depletion

Fluopicolide caused dose-dependent decreases in ATP levels in male and female human hepatocytes, falling to 62%, 31% and 43% of control values at 10 µM (donor 8210) and 100 µM (donors 8239 and 1765), respectively. These data are in broad agreement with the dose range finding study ([redacted] 2017; M-600911-01-1), where declines in ATP levels of 58% and 37%, relative to control, were measured at 10 µM (donor 8210) and 100 µM (donor 8239), respectively. However, Donor 1765 did not suffer as great a decline in ATP levels at 100 µM, compared to control, in this study compared with the previous dose range finding study (43% vs. 70%, respectively).

No cytotoxicity was observed in either male or female human hepatocytes after treatment with PB.

An overview is given in Table 5.8.2- 36.

Table 5.8.2- 36: ATP assay following PB or fluopicolide administration

Test substance and concentration	ATP content (luminescence units)		
	Male human hepatocytes (Donor 8210)	Female human hepatocytes (Donor (8239)	Female human hepatocytes (Donor 1765)
Vehicle control (0.1% [v/v] DMSO)	301240 ± 15625 (100 ± 5.2)	82474 ± 6272 (100.0 ± 7.6)	72652 ± 4952 (100.0 ± 6.8)
PB 10 µM	375108 ± 26769*** (124.5 ± 8.9)	103164 ± 9620** (125.1 ± 11.7)	74811 ± 4250 (103.0 ± 5.8)
PB 100 µM	334775 ± 17404** (111.1 ± 5.8)	102030 ± 10472** (123.7 ± 12.7)	81209 ± 6504* (111.8 ± 9.0)
PB 1000 µM	308482 ± 19913 (102.4 ± 6.6)	85064 ± 7689 (103.1 ± 9.3)	76352 ± 6198 (105.1 ± 8.5)
Fluopicolide 0.03 µM	319470 ± 16891 (106.1 ± 5.6)	-------	-------
Fluopicolide 0.1 µM	335622 ± 17799** (111.4 ± 5.9)	-------	-------
Fluopicolide 0.3 µM	334441 ± 29050* (111.0 ± 9.6)	87427 ± 10547 (106.0 ± 12.8)	74283 ± 4862 (102.2 ± 6.7)
Fluopicolide 1 µM	315283 ± 27899 (104.7 ± 9.0)	95004 ± 8775* (115.2 ± 10.6)	67440 ± 6446 (92.8 ± 8.9)
Fluopicolide 3 µM	283059 ± 8089* (94.0 ± 2.7)	73538 ± 6146* (89.2 ± 7.5)	62939 ± 6116* (86.6 ± 8.4)
Fluopicolide 10 µM	186593 ± 18036*** (61.9 ± 6.0)	56856 ± 5868*** (68.9 ± 7.1)	63557 ± 5527* (87.5 ± 7.6)
Fluopicolide 30 µM	------	41382 ± 3699*** (50.2 ± 4.5)	50481 ± 3742*** (69.5 ± 5.2)
Fluopicolide 100 µM	-------	25465 ± 2523*** (30.9 ± 1.2)	31253 ± 23131 (43.0 ± 5.1)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control. ; * statistically different from control $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2. Biochemical assay results

In the male human hepatocytes, PROD activities could not be determined as the values were below the limit of quantification, therefore, no results are presented for this assay in the male Donor 8210. Fluopicolide caused slight increases in BROD and BQ activities to a maximum of 1.5 - and 2.6-fold respectively. All levels of PB resulted in statistically significant increases in BROD and BQ activities in the male human hepatocytes, with maximum increases observed at 1 mM (2.0- and 5.3-fold respectively).

PROD activity was not altered after exposure to fluopicolide in hepatocytes from female human Donor 8239. However, BROD activity was significantly reduced at 30 and 100 µM fluopicolide. BQ activity increased in a dose-dependent manner, to a maximum of 1.7- fold induction compared to control, this increase was observed at 3 µM.

Fluopicolide induced small, but statistically significant, increases in PROD and BQ activities in female human Donor 1765, to a maximum of 1.5- and 2.8-fold respectively, with a dose-dependent increase observed in BQ activities. No increases were observed in BROD activity after administration with fluopicolide and a small but statistically significant decrease was observed after treatment with 100 µM fluopicolide.

Treatment with PB resulted in increased PROD, BROD and BQ activities in the female human hepatocytes from Donor 8239 to a maximum of 1.1-, 1.7- and 3.6-fold, respectively. PB also caused significant increases in PROD, BROD and BQ activities in the female human hepatocytes from Donor 1765, to a maximum of 2.3-, 3.9- and 5.4-fold, respectively.

The results are summarized in Table 5.8.2- 37 to Table 5.8.2- 39.

Table 5.8.2- 37: Biochemical measurements following PB fluopicolide administration in Donor 8210 (male)

Test substance and concentration	Human hepatocytes (Donor 8210)	
	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
	Male	
Vehicle control (0.1% [v/v] DMSO)	0.53 ± 0.006 (100 ± 1.1)	0.110 ± 0.004 (100 ± 3.5)
PB 10 µM	0.71 ± 0.04** (135.5 ± 7.2)	0.141 ± 0.007** (127.6 ± 6.3)
PB 100 µM	1.06 ± 0.10** (200.8 ± 19.6)	0.243 ± 0.0016*** (220.6 ± 14.7)
PB 1000 µM	2.88 ± 0.34*** (547 ± 65.2)	0.579 ± 0.023*** (525.6 ± 20.52)
Fluopicolide 0.0 3 µM	0.71 ± 0.05* (135.6 ± 9.4)	0.146 ± 0.008** (132.3 ± 7.1)
Fluopicolide 0.1 µM	0.59 ± 0.09 (112.9 ± 16.6)	0.122 ± 0.006* (110.7 ± 5.4)
Fluopicolide 0.3 µM	0.65 ± 0.05* (124.3 ± 9.2)	0.164 ± 0.007*** (149.0 ± 5.9)
Fluopicolide 1 µM	0.77 ± 0.07** (145.7 ± 12.9)	0.193 ± 0.009*** (174.8 ± 8.1)
Fluopicolide 3 µM	0.78 ± 0.03** (147.6 ± 4.8)	0.285 ± 0.013*** (258.4 ± 11.9)
Fluopicolide 10 µM	0.40 ± 0.04 (76.6 ± 6.8)	0.227 ± 0.024 (206.1 ± 22.1)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 3 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 5.8.2- 38: Biochemical measurements following PB or fluopicolide administration in Donor 8239 (female)

Test substance and concentration	Female Donor 8239		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control 0.1% [v/v] DMSO)	0.21 ± 0.08 (100 ± 38.8)	0.81 ± 0.14 (100 ± 17.7)	0.17 ± 0.08 (100 ± 44.98)
PB 10 µM	0.20 ± 0.04 (97.3 ± 18.7)	0.78 ± 0.07 (97.1 ± 8.9)	0.19 ± 0.08 (108.63 ± 48.71)
PB 100 µM	0.23 ± 0.08 (111.1 ± 38.8)	108 ± 0.02* (134.4 ± 2.4)	0.28 ± 0.11 (161.3 ± 62.4)
PB 1000 µM	0.20 ± 0.07 (96.2 ± 32.0)	1.39 ± 0.14** (171.9 ± 17.9)	0.57 ± 0.27 (333.84 ± 156.54)
Fluopicolide 0.3 µM	0.20 ± 0.01 (97.1 ± 6.1)	0.78 ± 0.03 (96.4 ± 3.4)	0.19 ± 0.08 (109.09 ± 45.68)
Fluopicolide 1 µM	0.21 ± 0.09 (102.1 ± 43.6)	0.88 ± 0.05 (108.4 ± 6.6)	0.22 ± 0.09 (130.49 ± 50.03)
Fluopicolide 3 µM	0.14 ± 0.01 (69.4 ± 5.2)	0.64 ± 0.08 (78.8 ± 9.9)	0.29 ± 0.12 (169.49 ± 68.44)
Fluopicolide 10 µM	0.21 ± 0.03 (101.4 ± 15.4)	0.55 ± 0.13 (68.4 ± 16.4)	0.16 ± 0.08 (95.05 ± 45.44)
Fluopicolide 30 µM	0.19 ± 0.02 (92.2 ± 10.2)	0.54 ± 0.03 (66.6 ± 3.3)	0.07 ± 0.03 (38.90 ± 15.82)
Fluopicolide 100 µM	#	0.34 ± 0.14 (42.0 ± 17.2)	#

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 3 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.
no activity observed at this concentration.

Table 5.8.2- 39: Biochemical measurements following PB or fluopicolide administration in Donor 1765 (female)

Test substance and concentration	Female Donor 1765		
	PROD (pmol resorufin/min/ mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control (0.1% [v/v] DMSO)	0.12 ± 0.01 (100 ± 12.31)	0.87 ± 0.01 (100 ± 79)	0.08 ± 0.01 (100 ± 15.04
PB 10 µM	0.12 ± 0.07 (101.0 ± 6.4)	0.96 ± 0.12 (110.29 ± 13.87)	0.09 ± 0.01 (114.14 ± 14.45)
PB 100 µM	0.15 ± 0.018 (128.7 ± 15.4)	1.63 ± 0.25** (184.67 ± 28.93)	0.18 ± 0.01 (224.72 ± 18.66)
PB 1000 µM	0.28 ± 0.04 (230.3 ± 35.1)	3.42 ± 0.024*** 392.82 ± 28)	0.42 ± 0.03 (542.27 ± 39.73)
Fluopicolide 0.3 µM	0.11 ± 0.019 (95.6 ± 16.4)	0.86 ± 0.11 (98.91 ± 12.26)	0.08 ± 0.02 (100.67 ± 21.77)
Fluopicolide 1 µM	0.09 ± 0.01 (75.0 ± 10.6)	0.95 ± 0.06 (109.36 ± 7.39)	0.12 ± 0.01 (152.30 ± 17.4)
Fluopicolide 3 µM	0.10 ± 0.008 (80.2 ± 6.5)	1.01 ± 0.12 (116.5 ± 13.22)	0.20 ± 0.03 (254.20 ± 32.06)

Test substance and concentration	Female		
	Donor 1765		
	PROD (pmol resorufin/min/ mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Fluopicolide 10 µM	0.103 ± 0.008 (85.3 ± 6.3)	0.92 ± 0.07 (105.39 ± 8.43)	0.22 ± 0.01 (286 ± 16.39)
Fluopicolide 30 µM	0.175 ± 0.036* (145.6 ± 30.2)	0.71 ± 0.11 (81.3 ± 12.83)	0.06 ± 001 (72.93 ± 1.87)
Fluopicolide 100 µM	0.132 ± 26.5 (109.8 ± 26.5)	0.72 ± 0.086* (82.52 ± 9.90)	0.03 ± 0.001 (34.26 ± 0.09)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 3 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.
no activity observed at this concentration.

3. Replicative DNA synthesis (S-phase)

In the male human hepatocytes, Donor 8210, treatment with either fluopicolide or PB did not result in any increases in replicative DNA synthesis. S-phase was not analysed at 10 µM fluopicolide due to reduced cell numbers at these concentrations. EGF (25 ng/mL) caused significant increases in replicative DNA synthesis of 7.4-fold.

Similarly, in the female human hepatocytes, treatment with either fluopicolide or PB did not result in any increases in replicative DNA synthesis. Due to reduced cell numbers, S-phase was not analysed at 30 µM (Donor 8239) or 100 µM (Donors 8239 and 1765) fluopicolide. As expected, EGF (25 ng/mL) significantly induced replicative DNA synthesis (9.7-fold for Donor 8239 and 3.9-fold for Donor 1765) in the female human hepatocytes.

The results are summarized in Table 5.8.2- 40.

Table 5.8.2- 40: Replicative DNA synthesis (S-phase) assessment following PB, EGF or fluopicolide administration

Test substance and concentration	S-phase labelling index		
	Male human hepatocytes (Donor 8210)	Female human hepatocytes (Donor 8239)	Female human hepatocytes (Donor 1765)
Vehicle control (0.1% [v/v] DMSO)	0.28 ± 0.04 (100 ± 15.8)	0.22 ± 0.07 (100 ± 30.0)	0.16 ± 0.06 (100 ± 38.9)
PB 10 µM	0.30 ± 0.06 (107.5 ± 22.8)	0.25 ± 0.07 (115.1 ± 29.9)	0.13 ± 0.03 (82.1 ± 19.7)
PB 100 µM	0.32 ± 0.08 (113.0 ± 28.7)	0.27 ± 0.04 (121.8 ± 18.8)	0.17 ± 0.08) (110.2 ± 52.2)
PB 1000 µM	0.30 ± 0.07 (105.8 ± 23.4)	0.30 ± 0.07 (137.5 ± 29.8)	0.19 ± 0.04 (121.2 ± 24.7
Fluopicolide 0.03 µM	0.33 ± 0.07 (118.7 ± 24.2)	-------	-------
Fluopicolide 0.1 µM	0.34 ± 0.07 (120.3 ± 23.6)	-------	-------
Fluopicolide 0.3 µM	0.30 ± 0.07 (107.7 ± 24.0)	0.24 ± 0.06 (108.4 ± 25.8)	0.17 ± 0.04 (111.5 ± 26.1)
Fluopicolide 1 µM	0.29 ± 0.07 (103.85 ± 26.10	0.22 ± 0.10 (101.2 ± 46.8)	0.17 ± 0.08 (112.7 ± 53.1)
Fluopicolide 3 µM	0.24 ± 0.06 (86.9 ± 21.0)	0.29 ± 0.09 (131.9 ± 42.0)	0.19 ± 0.06 (120.3 ± 39.6)

Test substance and concentration	S-phase labelling index		
	Male human hepatocytes (Donor 8210)	Female human hepatocytes (Donor (8239)	Female human hepatocytes (Donor 1765)
Fluopicolide 10 µM	#	0.16 ± 0.04 (73.82 ± 42.0)	0.12 ± 0.04 (77.3 ± 26.74)
Fluopicolide 30 µM	-------	#	0.09 ± 0.03 (58.63 ± 21.19)
Fluopicolide 100 µM	-------	#	#
EGF 25 ng/mL	2.07 ± 0.2*** (736.01 ± 57.3)	2.12 ± 0.16*** (968.16 ± 73.89)	0.91 ± 0.11 (589.42 ± 71.19)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n = 5per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$. # Concentrations not counted due to abnormal morphology and cell density following treatment with fluopicolide.

III. Conclusion

Neither fluopicolide nor PB induced replicative DNA synthesis in male or female human hepatocytes. As expected, the positive control EGF caused a robust proliferative response in both male and female human hepatocytes (7.4 -fold for male Donor 8210, 9.7-fold for female Donor 8239 and 5.9-fold for female Donor 1765).

Fluopicolide caused dose-dependent increases in the activity of CYP3A as determined by BQ activity in both sexes. PROD activities were not increased in the female human hepatocytes and levels could not be detected in male human hepatocytes, suggesting that CYP2B was not affected by fluopicolide administration.

In conclusion, treatment of cultured male or female human hepatocytes with fluopicolide resulted in weak induction of CYP3A enzyme activity (BROD (male only) and BQ activities (male and female)). There was no evidence of fluopicolide or PB-stimulated proliferation in cultured male or female human hepatocytes.

Treatment with the positive control item EGF gave the expected set of responses, indicating the suitability of the test system.

These data suggest that fluopicolide is a weak activator of human PXR (as shown by the effects on CYP3A enzyme activity levels) with no effect on DNA synthesis in male or female human hepatocytes.

Assessment and conclusion by applicant:

The study (and accompanying range-finding study) is valid and acceptable to investigate the potential for fluopicolide to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and modulate cytochrome P450 (CYP) enzyme activities in cryopreserved male and female human hepatocyte cultures. The data suggest that fluopicolide is a weak activator of human PXR with no corresponding effect on DNA synthesis in human hepatocytes.

Data Point:	KCA 5.8.2/10
Report Author:	[redacted]
Report Year:	2008
Report Title:	Fluopicolide (AE C638206): UDP-glucuronosyl transferase and cytochrome P-450 related activities measurements after 7 days dietary administration in the rat
Report No:	SA 06139
Document No:	M-306998-02-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	not applicable
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 objective of this study was to investigate the total cytochrome P-450 levels and the enzymatic activities of EROD, PROD, BROD, lauric acid hydroxylation and UDPGT in liver microsomes obtained from male and female rats treated with fluopicolide. In addition, phenobarbital as known inducer of enzymes whose activity is mediated by the nuclear receptors CAR (constitutive androstane receptor) and PXR (pregnane X receptor) was used as a positive control. Rats were exposed to the appropriate compound for 7 days either in the diet (2500 ppm fluopicolide) or by gavage (80 mg/kg Phenobarbital).

No treatment-related clinical signs were reported in rats treated with fluopicolide or phenobarbital. Body weights and food consumption were unaffected by treatment with the test compound. The mean achieved dose levels of fluopicolide, received by the animals during the study were calculated as being 211 mg/kg/day for the males and 209 mg/kg/day for the females. No significant changes were noted in terminal body weights. Absolute and relative liver weights to body weights were significantly increased in both males and females treated with fluopicolide or phenobarbital.

Total cytochrome P-450 content was significantly increased in both male and female rats treated with fluopicolide. A similar increase but of higher magnitude was observed in male and female rats treated with Phenobarbital, when compared to concurrent controls. The CAR/PXR associated PROD (CYP2B) and BROD (CYP3A) activities were markedly and significantly increased in males and females treated with fluopicolide and with a similar profile compared to phenobarbital exposure. EROD (CYP1A) activity was also statistically significantly but less markedly induced by both compounds except in females following treatment with phenobarbital. This is not surprising since CYP1A activity is preferentially mediated by the AhR (aryl hydrocarbon receptor).

A slight decrease in lauric acid hydroxylation (mediated by CYP4A, known target gene of PPARα) was observed in rats treated with fluopicolide or phenobarbital for both sexes, when compared to control values. In addition, the glucuronidation of 4-nitrophenol was significantly increased following treatment with fluopicolide indicating induction of UGT (UDP-Glucuronosyltransferase) expression which is also known to be mediated by CAR/PXR. The profile and magnitude of response was similar to that induced by the positive control phenobarbital.

In conclusion, these data show that fluopicolide is a phenobarbital-like compound for liver enzyme induction in rats.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	Fluopicolide (AE C638206)
Purity:	97.6% w/w
Batch no.:	PFV052K012

2. Vehicle and/or positive control

Vehicle:	admixture in the diet
Positive control:	Phenobarbital (sodium salt) from Cooper, Coopération Pharmaceutique Française, Melun, France (purity 99%)

3. Test animals

Species:	rats
Strain:	Sprague Dawley Crl:CD(SD)
Age:	7 weeks of age
Weight at start:	219 to 277 g for the males and 176 to 219 g for the females
Source:	[redacted]
Acclimation period:	at least 5 days prior to the treatment
Diet:	Certified rodent powdered and irradiated diet A04C-10 PI from S.A.F.E. (Scientific Animal food and Engineering, Epinay-sur-Orge, France)
Water:	Water ad lib
Housing:	Individually in suspended stainless steel wire mesh cages
Temperature:	22 ± 2 °C
Humidity:	55 ± 15%
Air changes:	10-15 per hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: May 30, 2006 to June 09, 2006

2. Animal assignment and treatment

Eighty rats (40 males, 40 females) were selected for the study. An automatic randomization procedure was used to select animals for the study from the middle of the weight range of the available animals that ensured a similar body weight distribution among groups for each sex.

Groups of 10 male and 10 female rats were given the control diet or the diet mixture with the test substance at 2500 ppm. This dose level was set after evaluation of the results from the carcinogenicity study [redacted], 2003, M-225016-01-1). Additional groups of 10 male and 10 female rats were given (by gavage) the control vehicle or the suspension mixture with phenobarbital at 80 mg/kg.

Table 5.8.2- 41: Study design

Group no.	Dose	Males	Females
1	0 ppm	10	10
2	2,500 ppm	10	10
3	0 mg/kg bw/day	10	10
4	80 mg/kg bw/day	10	10

3. Diet preparation and analysis

The test substance was incorporated into the diet to provide the required dietary concentration of 2500 ppm. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. There was one preparation for the study. When not in use, the diet formulation was stored at room temperature

An appropriate amount of phenobarbital (positive control) was suspended (w/v) in aqueous solution of methylcellulose 400 at 0.5% to provide the required concentration: 8 g/L. There was one preparation for the study. When not in use, the suspension was stored at approximately +5°C (+/- 3°C).

The homogeneity of test substance in diet and phenobarbital in methylcellulose were verified before the study to demonstrate adequate formulation procedures. The concentration was determined by the mean results obtained for each compound.

The stability of the dietary formulation with fluopicolide was demonstrated in a previous study (Wason, S. M.; 2006; M-205579-02-1) where the diet preparations were found to be stable over 56 days at room temperature or under frozen storage. This time period covers the period of storage and usage for the current study.

The stability of the phenobarbital suspension (8g/l) has been demonstrated in a previous study (Langrand-Lerche, C.; 2004; M-232813-01-1). At this concentration, the suspension was found to be stable over a 29-day period after storage at +5 ± 3°C and mixing at room temperature before and during the time of administration. This time period covers the period of storage and usage for the current study.

4. Statistics

Variables analysed:

- Body weight parameters
- Body weight gain/day parameters calculated according to time intervals
- Average food and water consumption/day parameters calculated according to time intervals
- Terminal body weight, absolute and relative liver weight parameters
- Total cytochrome P-450 content
- Cytochrome P-450 isoenzyme activities
- UDP-glucuronosyltransferase activity

Fluopicolide (group 2) was compared to the diet control group 1 and phenobarbital (group 4) was compared to the gavage control group 3 using the following procedures.

Mean and standard deviation (STD) were calculated for each group. All statistical analyses were carried out separately for males and females. The F test was performed to compare the homogeneity of group variances. If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

Body weight change parameters, Terminal body weight, absolute and relative organ weight (liver) parameters

If the F test was significant ($p \leq 0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

Body weight and average food consumption/day parameters, *total cytochrome P-450 content*

If the F test was significant ($p \leq 0.05$), data were transformed using the log transformation. If the F test on log transformed data was not significant ($p > 0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2- sided) on log transformed data. If the F test was significant ($p \leq 0.05$) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided). If one or more group variance(s) equaled 0, means were compared using the non-parametric Mann-Whitney test (2-sided).

Cytochrome P-450 isoenzyme activities:

For each substrate of enzymatic activity (ethoxyresorufin, pentoxyresorufin, benzoxyresorufin, lauric acid), group means were compared using the non-parametric Mann-Whitney exact test (2-sided). Group means were compared at the 5% and 1% level of significance. For these parameters, statistical analysis was carried out using SAS programs (version 8.2).

C. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose faeces.

2. Body weight and food intake

All animals were weighed twice during the acclimatization phase. Each animal was weighed on the first day of test substance administration, then at day 5 and 7 and before necropsy at day 8.

The weight of food supplied at day 1 and of that remaining at the end of the treatment period was recorded for all animals. From these records the weekly mean achieved dosage intake was calculated. Food spillage was also noted.

3. Post mortem examinations

At the end of the treatment period, the animals were deeply anesthetized by inhalation of Isoflurane, and then exsanguinated before necropsy. Only the liver was removed, weighed fresh and prepared for microsomal preparations. All animals were diet fasted prior to scheduled sacrifice. Animals found dead or moribund during the study were not submitted to necropsy.

4. Hepatotoxicity testing

At necropsy, the livers were homogenized for microsomal preparations in order to determine cytochrome P-450 specific isoenzyme profile and UDP-glucuronosyltransferase activity.

Cytochrome P-450 isoenzyme activities:

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. One quantification was performed for each sample.

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry using the following substrates

- benzoxyresonifin (BROD)
- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)

and by HPLC with fluorimetric detection following derivatization by 4-(bromomethyl)-7-methoxycoumarin of 12-hydroxy-lauric acid (lauric acid used as substrate).

Ethoxyresorufin is a highly selective substrate for the CYP 1A, CYP 2B metabolizes preferentially the O-dealkylation of pentoxyresorufin, while the benzoxyresorufin O-debenzylation is mainly metabolized by the CYP 3A. Three replicates were performed for each sample and for each of the three enzymatic activities.

Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 2,5 or 7 minutes at 37°C.

Individual samples were prepared to follow the hydroxylation of lauric acid by the CYP 4A over a period of 10 minutes at 37°C. Two replicates of each incubation mixture were collected. One replicate was analysed, the other one was stored frozen.

Total microsomal protein was measured using the Bio-Rad protein assay based on the method of Bradford.

UDP-glucuronosyltransferase activity:

The glucuronidation towards the substrate 4-nitrophenol was assayed using a spectrophotometry method. The enzymatic kinetic (disappearance of the colored 4-nitrophenol) was followed at 405 nm during 2-3 minutes. Three replicates from each sample were assayed.

II. Results and Discussion

A. Results

1. Mortality & Clinical results

One female treated with phenobarbital at 80 mg/kg was found dead on Day 5. No other mortalities occurred in any group. No treatment-related clinical signs were reported in rats treated with fluopicolide or with phenobarbital.

2. Body weight and food intake

Body weights were unaffected by treatment with fluopicolide in both sexes. Body weight gain was slightly reduced during the first 5 days in males and increased in females treated with phenobarbital, when compared to controls.

Table 5.8.2- 42: Mean body weight and body weight gain measurements (*% of control*) - Fluopicolide

	Dose level (ppm)					
	Males			Females		
	0	2,500		0	2,500	
Body weight (g)						
Day 1	242	242	*(-0)*	193	191	*(-1)*
Day 5	272	267	*(-2)*	204	200	*(-2)*
Day 7	284	280	*(-1)*	209	206	*(-1)*
Body weight gain (g)						
Day 1-7	42	38	*(-10)*	16	15	*(-6)*

* p < 0.05 ; ** p < 0.01, significantly different from controls
Statistical analysis was not performed for body weight gain day 1-3

Table 5.8.2- 43: Mean body weight and body weight gain measurements (*% of control*) - Phenobarbital

	Dose level (mg/kg bw/day)					
	Males			Females		
	0	80		0	80	
Body weight (g)						
Day 1	262	260	*(-1)*	196	197	*(+1)*
Day 5	290	282	*(-3)*	205	211	*(+3)*
Day 7	302	295	*(-2)*	203	211	*(+4)*
Body weight gain (g)						
Day 1-7	40	35	*(-12)*	7	14	*(+100)*

* p < 0.05 ; ** p < 0.01, significantly different from controls
Statistical analysis was not performed for body weight gain day 1-7

No significant changes were noted in food consumption for both males and females treated with fluopicolide. Food intake was slightly increased (by +17%, p≤0.05) in females treated with phenobarbital, when compared to controls. The mean achieved dose levels of fluopicolide, received by the animals during the study were calculated as being 211 mg/kg/day for the males and 209 mg/kg/day for the females.

Table 5.8.2- 44: Mean food consumption *(% of control)* - Fluopicolide

	Dose level (ppm)					
	Males			Females		
	0	2,500		0	2,500	
Food consumption/day (g)						
Day 1-7	25.0	23.6	*(-6)*	17.8	17.2	*(-3)*

* p ≤ 0.05 ; ** p ≤ 0.01, significantly different from controls

Table 5.8.2- 45: Mean food consumption *(% of control)* - Phenobarbital

	Dose level (mg/kg bw/day)					
	Males			Females		
	0	80		0	80	
Food consumption/day (g)						
Day 1-7	25.1	24.4	*(-3)*	16.7	19.6*	*(+17)*

* p < 0.05 ; ** p < 0.01, significantly different from controls

3. Post mortem examinations

No significant changes were noted in terminal body weights for both male and female rats treated with fluopicolide or phenobarbital.

Absolute and/or relative liver weights were statistically significantly increased in male and female rats treated with fluopicolide (by 16% and 9%, respectively) and phenobarbital (by 31% and 27%, respectively).

Table 5.8.2- 46: Mean liver weight *(% of control)* - Fluopicolide

		Dose level (ppm)					
		Males			Females		
		0	2,500		0	2,500	
Terminal body weight (g)		256.8	250.7	*(-2)*	188.8	180.8	*(-4)*
Liver weight	abs. (g)	7.06	7.96**	*(+13)*	5.40	5.64	*(+4)*
	rel. (%)	2.747	3.173**	*(+16)*	2.854	3.114*	*(+9)*

* p < 0.05 ; ** p < 0.01, significantly different from controls

Table 5.8.2- 47: Mean liver weight *(% of control)* – Phenobarbital

		Dose level (mg/kg bw/day)					
		Males			Females		
		0	80		0	80	
Terminal body weight (g)		272.2	265.8	*(-2)*	186.0	191.9	*(+3)*
Liver weight	abs. (g)	7.40	9.44**	*(+28)*	5.16	6.71**	*(+30)*
	rel. (%)	2.718	3.551**	*(+31)*	2.768	3.502**	*(+27)*

* p < 0.05 ; ** p < 0.01, significantly different from controls

4. Hepatotoxicity testing

Total cytochrome P-450 content was significantly increased in male rats treated with fluopicolide (by around 35%). A similar increase but of higher magnitude was observed in male and female rats treated with Phenobarbital, when compared to concurrent controls (by around 128% and 80%, respectively).

The associated enzyme activities PROD, BROD (both indicative for CAR/PXR activation) and EROD (indicative for AhR activtaion) were increased in males and females treated with fluopicolide with a similar profile as after exposure to phenobarbital.

The PROD activities were markedly and significantly increased in male and female rats treated with fluopicolide (by around 781% and 126%, respectively) and to a higher extent with phenobarbital (by around 1141% and 1156%, respectively). The BROD activities were markedly and significantly increased in male and female rats treated with fluopicolide (by around 3442% and 543%, respectively) and with phenobarbital (by around 2478% and 3395%, respectively).

The EROD activities were statistically significantly but less markedly increased in male and female rats treated with fluopicolide (by around 73% and 169%, respectively) and with phenobarbital in male rats by 98%. No induction was observed in females treated with phenobarbital. This is not surprising since the cytochrome P450 isoform related to this enzymatic activity is mainly mediated by the AhR and not preferentially induced by PB.

A slight decrease in lauric acid hydroxylation (mediated by CYP4A, known target gene of PPARα) was observed in rats treated with fluopicolide (by 44% and 21%, respectively) or phenobarbital (by around 37% and 21%, respectively) for both sexes, when compared to control values.

In addition, the glucuronidation of 4-nitrophenol (also mediated by CAR/PXR) was significantly increased following treatment with fluopicolide (by 108% and 99%, respectively). The profile and magnitude of response was similar to that induced by the positive control phenobarbital (increase by around 42% and 111%, respectively).

As shown in Table 5.8.2- 48 the liver enzyme activity was generally higher in male animals and also the PROD and BROD induction was about 6-times higher in fluopicolide treated males compared to female animals.

Table 5.8.2- 48: Cytochrome P450 content and liver enzyme activities *(% of control)* - Fluopicolide

	Dose level (ppm)					
	Males			Females		
	0	2,500		0	2,500	
Animal No.	10	10		10	10	
CYP450 content [nmol/mg protein]	1.7	2.3**	*(+35)*	1.5	1.6	*(+7)*
PROD activity [pmol/min/mg protein]	13.4	118.1**	*(+781)*	5.0	11.3**	*(+126)*
BROD activity [pmol/min/mg protein]	13.2	467.6**	*(+3442)*	4.6	29.6**	*(+543)*
EROD activity [pmol/min/mg protein]	62.6	108.4**	*(+73)*	52.2	140.5**	*(+169)*
LAH activity [nmol/min/mg protein]	2.5	1.4**	*(-44)*	2.4	1.9	*(-21)*
UDPGT activity [nmol/min/mg protein]	14.35	29.80**	*(+108)*	7.76	15.47**	*(+99)*

* p < 0.05 ; ** p < 0.01 significantly different from controls

Table 5.8.2- 49: Cytochrome P450 content and liver enzyme activities *(% of control)* – Phenobarbital

	Dose level (mg/kg bw/day)			
	Males		Females	
	0	80	0	80
Animal No.	10	9[$]	10	9
CYP450 content [nmol/mg protein]	1.8	4.1** *(+128)*	1.5	2.7** *(+80)*
PROD activity [pmol/min/mg protein]	15.7	194.8** *(+1141)*	5.5	69.1** *(+1156)*
BROD activity [pmol/min/mg protein]	36.6	943.5** *(+2478)*	12.6	431.5** *(+3325)*
EROD activity [pmol/min/mg protein]	48.4	95.9** *(+98)*	58.9	60.1 *(+2)*
LAH activity [nmol/min/mg protein]	3.5	2.2 *(-37)*	2.9	2.3 *(-21)*
UDPGT activity [nmol/min/mg protein]	16.89	23.93** *(+42)*	6.99	14.74** *(+111)*

[$] During the microsomes preparation, the sample corresponding to one male phenobarbital treated animal was lost and could not be recovered.

* p < 0.05 ; ** p < 0.01, significantly different from controls

III. Conclusion

The dietary administration of 2,500 ppm fluopicolide to male and female rats for 7 days induced hepatic total cytochrome P-450 expression and associated PROD, BROD and to a lesser extent EROD activity as well as UDPGT activity. The profile and the magnitude of induction were similar to those observed with phenobarbital a well-known CAR/PXR-inducer used as a positive control in this study.

In conclusion, these data show that fluopicolide is a phenobarbital-like compound for liver enzyme induction in rats.

Assessment and conclusion by applicant:

This non-guideline mechanistic study is valid and acceptable to investigate the total cytochrome P-450 levels and the enzymatic activities of EROD, PROD, BROD, lauric acid hydroxylation and UDPGT in liver microsomes obtained from male and female rats treated with fluopicolide, comparing the results to the positive control substance phenobarbital. The data show that fluopicolide is a phenobarbital like enzyme inducer in rats.

CA 5.8.3 Endocrine disrupting properties

Fluopicolide has been sufficiently investigated for endocrine related adverse effects in level 4 and 5 short-term and chronic studies in rats, mice, and dogs, and in reproductive and developmental toxicity studies in rats and rabbits. In addition, endocrine activity was investigated in level 2 and 3 studies. The table below summarises the data considered for the ED assessment of fluopicolide; several studies are currently ongoing and the final reports and a detailed WoE assessment (in line with the respective guidance) will be submitted in November 2020.

Overall, based on the current data, ED criteria are not met for fluopicolide.

Table 5.8.3- 1: Data considered for the ED assessment of fluopicolide.

Study, reference	EATS modality	EATS relevant findings	Comments
Level 5 assays			
Two-generation reproduction study [redacted] 2003; M-232532-01-1	EAS	No findings	All endocrine parameters foreseen in the OECD 416 (2001) guidance were investigated and no endocrine mediated adversity was observed.
Level 4 assays			
Short-term toxicity studies [redacted] 2000; M-199377-01-1 [redacted] 2000; M-197343-01-1 [redacted] 2000; M-197380-01-1 [redacted] 2000; M-197622-01-1 [redacted] 2006; M-205879-02-1 [redacted] 2000; M-197623-01-1 [redacted] 2000; M-199397-01-1 [redacted] 2002; M-216694-01-1 [redacted] 2003; M-220782-01-1	EATS	No findings	Endocrine mediated parameters were investigated in rats, mice, and dogs from 28-days to 1-year and no EATS mediated adversity was observed.
Developmental toxicity studies [redacted] 2004; M-202155-02-1 [redacted] 2004; M-202513-02-1	EATS	No findings	Endocrine mediated parameters were investigated in rats and rabbits and no endocrine mediated adversity was observed.

Study, reference	EATS modality	EATS relevant findings	Comments
Chronic toxicity/carcinogenicity study in rats [redacted] 2003; M-225616-01-1	EATS	Prostate: ↑ incidence of acinar atrophy (associated with reduced colloid)	Acinar cell atrophy & reduced colloid only occurred at high dose above MTD
		Testes: ↑ incidence of Leydig cell hyperplasia/adenoma	Testes: Neither finding attained statistical significance, slightly increased adenoma incidence considered incidental as within HCD range and without clear dose-response relationship. Androgen-related endocrine activity ruled out with Hershberger assay
		Thyroid: ↑ weight, ↑ follicular cell hyperplasia & benign adenoma	Concomitant ↑ in liver weight and hepatocellular hypertrophy Thyroid effects are considered secondary to increased hepatic metabolism resulting in increased elimination of T4 by UDPGT conjugation which is supported by several MoA studies.
Oncogenicity study in mice [redacted] 2003; M-225595-01-1	EAS	Epididymides: enlargement, ↑ spermatic granuloma Testes: ↑ incidence of degenerated seminiferous tubules Ovary: ↑ incidence of ovaries without corpora lutea Uterus: ↑ weight	All effects occurred only above the MTD and are therefore considered secondary to excessive systemic toxicity and thus should not be considered as indicative of endocrine disruption.
Level 3 assays			
Hershberger assay*	EAS	No findings	
Level 2 assays			
Steroidogenesis *in vitro**	S	Treatment-related statistically significant inhibition of testosterone (-43% and -81%) and progesterone (-38% & -74%) secretion at the highest tested concentrations (10 & 100 µM)	The S-modality is partially covered by the Hershberger assay. Endocrine MoA is therefore considered unlikely given the available negative Hershberger assay.

Study, reference	EATS modality	EATS relevant findings	Comments
ToxCast/tox 21*	EATS	S	Inactive in 3/18 ER assays & not tested in 15/18 In active in 2/11 AR assays & not tested in 9/11 Inactive in 7/12 H295R steroidogenesis assay & active in 5/12 Negative in 1/6 thyroid receptor assays and not tested in 5/6 Active in 2 PXR activation assays and 1 PPARγ assay
NIS assay*	T	Negative	Alternative direct MoA for thyroid findings ruled out by this negative result
DIO assay*	T	Negative	Alternative direct MoA for thyroid findings ruled out by this negative result

* Summaries and reports of Level 2 and level 3 studies will be provided together with Appendix E and Appendix I for the November 2020 submission.

The following studies are currently ongoing and will be submitted on the specified date.

Dossier node	Draft title	Study ID	Planned submission
KCA 5.8.3	Evaluation of fluopicolide in the H295R steroidogenesis assay	TXAC0096	November 2020
KCA 5.8.3	Hersberger Bioassay of Fluopicolide Technical by Oral (Gavage) Administration in Castrated Male Rats	TXAC0091	November 2020
KCA 5.8.3	Fluopicolide: In-vitro inhibition of iodide uptake by sodium/iodide symporter in the rat thyroid-derived cell line FRTL-5	TXAC0087	November 2020
KCA 5.8.3	Fluopicolide: Evaluate the *in vitro* potential to inhibit recombinant rat deiodinases	TXAC0102	November 2020
KCA 5.8.3	ToxCast/tox 21 report	N/A	November 2020
KCA 5.8.3	Appendix E	N/A	November 2020
KCA 5.8.3	Appendix I	N/A	November 2020

CA 5.9 Medical data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

There were no reported incidences involving fluopicolide during its manufacture at any of the monitored sites. Monitoring of workers involved in the production of fluopicolide revealed no unusual findings and no ill effects were reported to medical professionals. The summary of the data can be found in the confidential Document JCA.

CA 5.9.2 Data collected on humans

There are no cases of human poisoning available in the literature relating to fluopicolide.

CA 5.9.3 Direct observations

Amongst 11 incidents reported to Bayer since 2012, none involved fluopicolide lone but in combinations with other more toxic active ingredients. In a few cases there were unspecified symptoms comprising nausea and stomach-ache, unrelated to fluopicolide.

CA 5.9.4 Epidemiological studies

No epidemiological studies have been published.

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

There are no reported poisoning cases in humans. Fluopicolide is of low acute toxicity (LD_{50} >2000 mg/kg bw in rats) and no specific symptoms of acute poisoning are therefore known.

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

First aid:

- Remove patient from exposure/terminate exposure
- Thorough skin decontamination with copious amounts of water and soap and if available polyethylenglykol 300, followed by water: note. Most formulations with this active ingredient can be decontaminated with water and soap and therefore polyethylenglykol 300 will not be required.
- Flushing of the eyes with luke-warm water for 15-minutes
- Induction of vomiting does not seem to be required in regard of the low toxicity. It should only be considered if a large amount has been swallowed, if the ingestion was less than an hour ago and if the patient is fully conscious (induced vomiting can remove a maximum of 50% of the ingested substance. Note. Induction of vomiting is forbidden if a formulation containing organic solvents has been ingested.

Treatment:

- Gastric lavage does not seem to be required in regard of the low toxicity
- The application of activated charcoal and sodium sulphate (or other cathartic) may be considered in significant ingestions.
- As there is no antidote, treatment should be symptomatic and supportive

CA 5.9.7 Expected effects of poisoning

No delayed or persistent effects are expected.