Bayer CropScience

Document Title

Summary of the toxicological and metabolism studies for Mesosulfuron-methyl

Data Requirements

EU Regulation 1107/2009 & EU Regulation 283/2013

Document MCA

Section 5: Toxicological and metabolism studies

According to the guidance document, SANCO 10181/2013, for preparing dossiers for the approval of a chemical active substance

Date

2015-02-26

2015-09-12

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Bayer CropScience

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Version history

Date	Data points containing amendments or additions[1] and brief description	Document identifier and version number
February 2015	MCA 5.1.1	
	MCA 5.8.1	

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

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CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

Mesosulfuron-methyl is a herbicidal active substance and was included into Annex I of Directive 91/414 in 2003 (Directive 2003/84/EC, dated 25th of September 2003, Entry into Force 1st of January 2004.

This Supplemental Dossier contains only summaries of studies, which were not available at the time of the first Annex I inclusion of mesosulfuron-methyl and were, therefore, not evaluated during the first EU review of this compound. All studies, which were already submitted by Bayer CropScience for the first Annex I inclusion, are contained in the Monograph, its Addenda and are included in the Baseline dossier provided by Bayer CropScience. These old studies are not summarized in detail again. For all new studies detailed summaries are provided with this Supplemental Dossier. As a new study a phototoxicity study in-vitro in BALB/c 3T3 cells is summarized in chapter CA 5.2.7.
For the soil metabolites AE F147447, AE F160460 and BCS-CV14885 new genotoxicity studies were conducted to support e-fate risk assessment.

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

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

Report:	[redacted]2; [redacted];1997;M-193715-01
Title:	Rat preliminary toxicokinetics: Absorption, distribution and elimination - oral low dose (10 mg/kg body weight) and oral high dose (1000 mg/kg body weight) Code: (2-pyrimidyl-14C) AE F130060
Report No:	C006347
Document No:	M-193715-01-1
Guidelines:	**OECD: 417; USEPA (=EPA): OPPTS 870.7485;not specified**
GLP/GEP:	**yes**

Report:	[redacted]6; [redacted]; [redacted];2000;M-197417-01
Title:	Rat preliminary toxicokinetics: Metabolism - oral low dose (10 mg/kg body weight) and oral high dose (1000 mg/kg body weight) Code:(2-14C-pyrimidyl)-AE F130060
Report No:	C008054
Document No:	M-197417-01-1
Guidelines:	**EU (=EEC): 91/414/EEC; OECD: 417; USEPA (=EPA): FS 85-1;not specified**
GLP/GEP:	**yes**

Report:	[redacted]h; [redacted];1997;M-193718-01
Title:	Rat - absorption, distribution and elimination - single oral low dose (10 mg/kg body weight) Code: (phenyl-U-14C) AE F130060
Report No:	C006348
Document No:	M-193718-01-1
Guidelines:	**OECD: 417; USEPA (=EPA): OPPTS 870.7485;not specified**
GLP/GEP:	**yes**

Report:	[redacted]k;[redacted];1999;M-193724-01
Title:	Rat - Excretion via the bile - single oral low dose (10 mg/kg body weight) Code: (phenyl-U-14C) AE F130060
Report No:	C006349
Document No:	M-193724-01-1
Guidelines:	**OECD: 417; USEPA (=EPA): OPPTS 870.7485;not specified**
GLP/GEP:	**yes**

Report:	[redacted]4;[redacted];[redacted];[redacted];2000;M-197419-01
Title:	Rat metabolism - single oral low dose (10 mg/kg body weight) (U-14C-phenyl)-AE F130060
Report No:	C008356
Document No:	M-197419-01-1
Guidelines:	**EU (=EEC): 94/79/EC; OECD: 417; USEPA (=EPA): F § 85-1;not specified**
GLP/GEP:	**yes**

Report:	[redacted];[redacted];[redacted];1998;M-147473-01
Title:	(Phenyl-U-14C) AE F130060: Rat absorption, distribution and elimination; oral high dose (1000 mg/kg body weight)
Report No:	A67074
Document No:	M-147473-01-1
Guidelines:	**EU (=EEC): 94/79/EC; OECD: 417; USEPA (=EPA): F § 85-1;not specified**
GLP/GEP:	**yes**

Report:	[redacted];[redacted];[redacted];[redacted];2000;M-197418-01
Title:	Rat metabolism - single oral high dose (1000 mg/kg body weight) Code: (U-14C-phenyl)-AE F130060
Report No:	C008395
Document No:	M-197418-01-1
Guidelines:	**EU (=EEC): 94/79/EC; OECD: 417; USEPA (=EPA): F § 85-1;not specified**
GLP/GEP:	**yes**

Report:	[redacted]h;[redacted];1999;M-193730-01
Title:	Rat - Absorption, distribution and elimination - repeated oral dose (7 x 250 mg/kg body weight) Code: (phenyl-U-14C) AE F130060
Report No:	C006350
Document No:	M-193730-01-1
Guidelines:	**OECD: 417; USEPA (=EPA): OPPTS 870.7485;not specified**
GLP/GEP:	**yes**

Report:	[redacted]8;[redacted];[redacted];[redacted];2000;M-197420-01
Title:	Rat metabolism - repeated oral dose (7 x 250 mg/kg body weight) (U-14C-phenyl)-AE F130060
Report No:	C008360
Document No:	M-197420-01-1
Guidelines:	**EU (=EEC): 94/79/EC; OECD: 417; USEPA (=EPA): F § 85-1;not specified**
GLP/GEP:	**yes**

The toxicological profile of mesosulfuron-methyl was already investigated and evaluated.

The absorption, distribution, metabolism and excretion, including plasma and blood pharmacokinetics, of mesosulfuron-methyl (AE F130060) was investigated in the Wistar rat following a nominal single oral gavage dose of 10 or 1 000 mg/kg body weight and following up to 7 daily oral doses of 250 mg/kg body weight. There were no significant differences in the excretion profile of the phenyl- or pyrimidyl- labeled mesosulfuron-methyl and a very low level of metabolism of the compound was found. In view of these results [U-^{14}C-phenyl]-mesosulfuron-methyl was used for the majority of the studies, though [2-^{14}C-pyrimidyl]-mesosulfuron-methyl was also dosed as part of the determination of the metabolism.

In the rat, mesosulfuron-methyl appeared to be moderately to poorly absorbed and rapidly excreted following a single oral dose of 10 or 1 000 mg/kg body weight, with a mean of 95.1% of the dose present in the 0-24 hour excreta at both dose levels. Faecal excretion was predominant, whilst only 12.8% and 1.3% of the low and high dose, respectively, were found in the urine. There was no significant sex difference in the route of excretion and no exhalation of radiolabelled carbon dioxide during the first 24 hours after administration of a single high dose. Repeated dosing at 250 mg/kg bw/day for 7 days had no significant effect on the excretion profile. More than 93% of the totally administered radioactivity were found in rat faeces.
The pharmacokinetic parameters of mesosulfuron-methyl showed that the maximum concentration of radioactivity in whole blood (C_{max}) occurred at 4 and 2 hours after dosing for males and females respectively in the 10 mg/kg bw dose group, and 4 hours after dosing for the 1 000 mg/kg bw dose group. A monophasic elimination occurred with half-lives between 8 and 12 hours depending on test conditions. An examination of the biliary excretion of mesosulfuron-methyl confirmed the generally moderate absorption of mesosulfuron-methyl after low-dose oral exposure. Following a single oral dose of 10 mg/kg body weight, the total amount absorbed, as determined by the radioactivity present in urine (measured in a separate study) and bile, was only between 20.2 and 22.8% of the dose.
The concentration of mesosulfuron-methyl residues in the tissues of rats 72 hours after dosing was generally low with several of the tissues containing residue levels that were below the limit of quantification at both dose levels. Following a single oral dose of 10 mg/kg body weight, only traces of radioactivity were detected in the organs and tissues. In the males, only liver (mean 0.17 µg equivalents/g) and plasma (mean 0.30 µg equivalents/g) showed radioactivity. In the females, the mean values in organs and tissues were below the limit of quantification. At the high-dose level (1 000 mg/kg body weight) all tissue residue levels were below 0.04 µg equivalents/g tissue.

The metabolism of mesosulfuron-methyl was determined in the rat following dosing at 10 or 1000 mg/kg body weight. The predominant excretion product was unchanged mesosulfuron-methyl (>68 % in studies with single application) excreted mainly in the faeces. A main metabolic pathway was identified, *i.e.* breakdown of the sulfonylurea-bridge leading to AE F092944 and AE F140584 which cyclised to AE F147447. Minor metabolic reactions observed were *O*-demethylation at the pyrimidine moiety leading to AE F160459, and cleavage of the methanesulfonamidomethyl side chain leading to AE F151015 and AE 0195141. A breakdown of the sulfonylurea-bridge of AE F160459 led to AE F119094, which was further metabolised by *O*-demethylation to AE F118772. The formation of the benzoic acid metabolite AE F154851 due to hydrolysis of the methyl ester by esterases was detected as a minor metabolic reaction in rats.

The metabolic profile of mesosulfuron-methyl in the rat is shown in Figure 5.1.1-1.

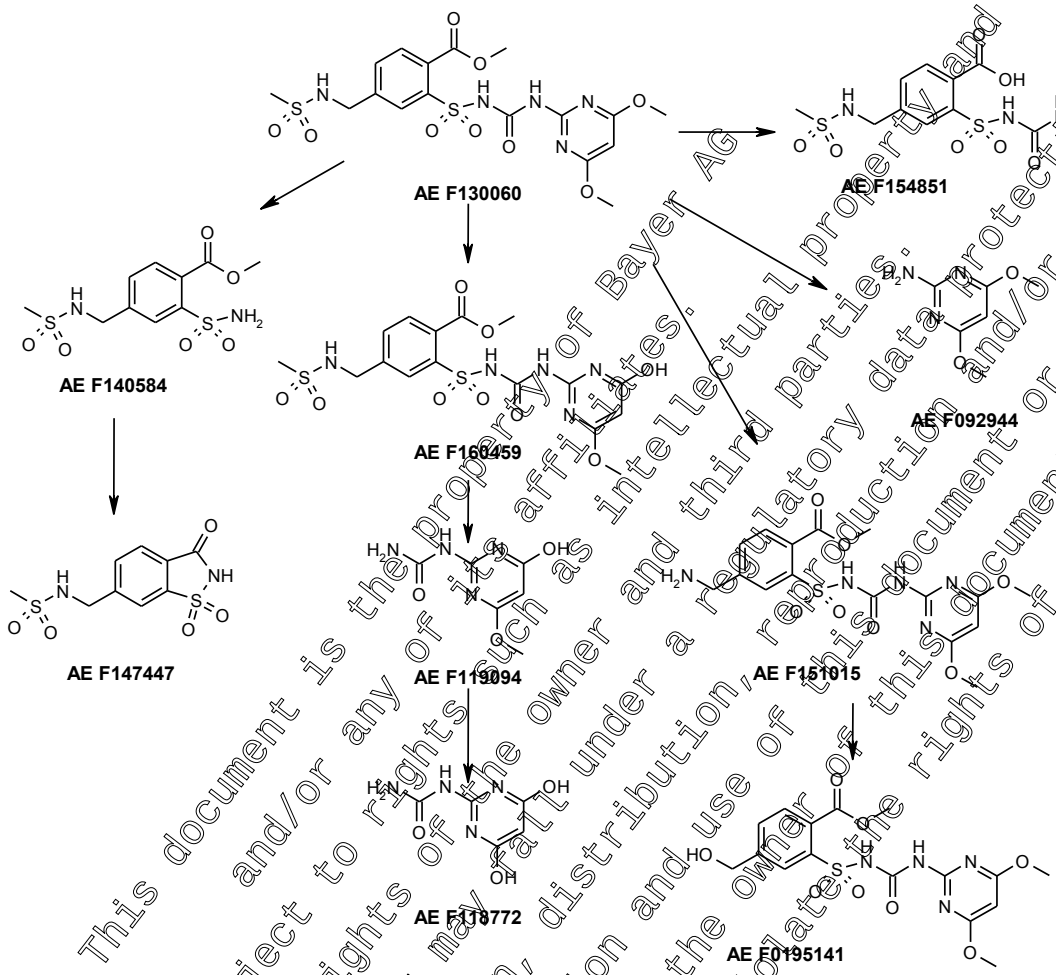

Figure 5.1.1-1: Metabolic profile of mesosulfuron-methyl in rats

Report:	[redacted]; [redacted];2013;M-470477-01
Title:	[Pyrimidyl-2-14C]mesosulfuron-methyl: Metabolic stability and profiling in liver microsomes from rats and humans for Inter-Species Comparison
Report No:	EnSa-13-0829
Document No:	M-470477-01-1
Guidelines:	**Regulation (EC) No 1107/2009 (Europe) amended by the Commission Regulation (EU) No. 283/2013 (Europe)** **US EPA OCSPP 870.SUPP;not specified**
GLP/GEP:	**yes**

According to the new data requirements (Commission regulation (EU) No 283/2013) an *in vitro* metabolism study was performed and is summarised below.

Materials and Methods

The comparative metabolism of [pyrimidine-2-^{14}C]-mesosulfuron (^{14}C-mesosulfuron) was investigated in animal in-vitro systems by incubating the test item with liver microsomes from male Wistar rats (RLM) and humans (HLM) in the presence of NADPH cofactor. The 15 µM test item concentration was chosen in order to have enough sample material for possible identification of metabolites by chromatographic or spectroscopic methods. The sampling times were 0 and 1 hour after test start. The metabolic activity of the microsomes was demonstrated by determining 6β-hydroxytestosterone that was formed from testosterone by testosterone 6β-hydroxylase. This biochemical reaction is well known for the CYP3A microsomal enzyme.

The test duration of 1 hour for the test item was considered as reasonable because positive results were obtained from the enzymatic reaction of Testosterone to hydroxy-testosterone already after 10 minutes. Samples were analyzed following protein precipitation by reversed phase HPLC with radiochemical detection (HPLC-RAD).

Results

The recovery of radioactivity was measured in the microsome incubations and amounted to 107.1% (RLM) and 112.8% (HLM) for the 1 hour samples.

The results of the tests with ^{14}C-mesosulfuron-methyl demonstrated that the in-vitro metabolism was only very slightly different between rats and humans.

While no metabolism was found in RLM, an unresolved peak region (M-2) was detected in the 1 h HPLC-chromatogram of the HLM incubation that accounted for only 8.1% of the total relative percentage (calculated from peak area values). Because of the approximately similar HPLC chromatograms of the incubations with rat and human microsomes (only a.i.), no further investigations were deemed necessary regarding peak region M-2. With regard to the peak region M-2 with the incubations of human microsomes our experts stated that this was not a clearly recognizable "Peak" (= metabolite), but a peak region which was not isolated, or a direct elution of a.i. from the column. Our experts decided that chances of success for a further isolation of this peak region which anyway was <10% would be minimal if at all.

Conclusion

Overall, the results suggest that phase I metabolism is not significantly involved in the biotransformation of mesosulfuron-methyl in rat and human liver microsomes.

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

Report:	[redacted]i;[redacted];2000;M-198123-01
Title:	In vivo dermal absorption in the rat using an oil suspension formulation (14C)-AE F130060 Code: AE F130060 01 1K12 A7
Report No:	C009130
Document No(s):	M-198123-01-1
Guidelines:	**EU (=EEC): 87/302 EEC; OECD: Draft June 1996; not specified**
GLP/GEP:	**yes**

Following 6-h dermal exposure to ^{14}C-mesosulfuron-methyl in an oil flowable formulation to rats only limited systemic absorption was observed, *i.e.,* max. 14 % after 24 h using a 10-fold diluted spray solution, and of max. 9% after 72 hours using the concentrated product.

CA 5.2 Acute toxicity

Mesosulfuron-methyl exhibited a low acute toxicity to mammals irrespective of the route of exposure (oral, dermal administration, inhalation exposure). The rat acute dermal LD_{50} was >5 000 mg/kg body weight. The rat acute inhalation LC_{50} (4-hour) was > 1.33 mg/L air, which was the highest achievable concentration and did not cause mortality.
No new studies were conducted since the first submission.

CA 5.2.1 Oral

Report:	[redacted]4;[redacted];1996;M-140405-01
Title:	Hoe 130060; Substance, technical; (Code: Hoe 130060 00 ZC96 0001) - Testing for acute oral toxicity in the male and female Wistar rat
Report No:	A56612
Document No(s):	M-140405-01-1
Guidelines:	**EU (=EEC): 92/69 B.1; JMAF: ; OECD: 401; USEPA (=EPA): 81-1;Deviation not specified**
GLP/GEP:	**yes**

CA 5.2.2 Dermal

Report:	[redacted]3;[redacted];1996;M-140406-01
Title:	Hoe 130060; Substance, technical; (Code: Hoe 130060 00 ZC96 0001) - Testing for acute dermal toxicity in the male and female Wistar rat
Report No:	A56613
Document No(s):	M-140406-01-1
Guidelines:	**EU (=EEC): 92/69 B.3; JMAF: ; OECD: 402; USEPA (=EPA): 81-2;Deviation not specified**
GLP/GEP:	**yes**

CA 5.2.3 Inhalation

Report:	[redacted]§;[redacted];1999;M-186735-02; Amended: 2001-03-22
Title:	Testing for acute dust inhalation toxicity in male and female Sprague Dawley rats 4-hour LC50 AE F130060 substance technical Code: AE F130060 00 1C95 0001
Report No:	C003755
Document No(s):	M-186735-02-1
Guidelines:	**EU (=EEC): 67/548 B.2.; OECD: 403; USEPA (=EPA): §81-3;Deviation not specified**
GLP/GEP:	**yes**

CA 5.2.4 Skin irritation

AE F130060 was not irritating to rabbit skin and only slightly irritating to rabbit eyes, but no classification is needed. No evidence of skin sensitisation was seen in a guinea pig maximisation test.
No new studies were conducted since the first submission.

Report:	[redacted]k[redacted];1996;M-140524-01
Title:	Hoe 130060; Substance, technical; (Code: Hoe 130060 00 ZC96 0001) - Testing for primary dermal irritation in the rabbit
Report No:	A56736
Document No(s):	M-140524-01-1
Guidelines:	**EU (=EEC): 92/69 B.4; JMAF: ; OECD: 404; USEPA (=EPA): 81-5;Deviation not specified**
GLP/GEP:	**yes**

CA 5.2.5 Eye irritation

Report:	[redacted]$;[redacted];1996;M-140517-01
Title:	Hoe 130060; Substance, technical; (Code: Hoe 130060 00 ZC96 0001) - Testing for primary eye irritation in the rabbit
Report No:	A56729
Document No(s):	M-140517-01-1
Guidelines:	**EU (=EEC): 92/69 B.5; JMAF: ; OECD: 405; USEPA (=EPA): 81-4;Deviation not specified**
GLP/GEP:	**yes**

CA 5.2.6 Skin sensitization

Report:	[redacted]6;[redacted];1998;M-148033-01
Title:	Sensitizing properties in the Pirbright-White guinea pig in a maximization test AE F130060 substance, technical Code: AE F130060 00 1C95 0001
Report No:	A61665
Document No(s):	M-148033-01-1
Guidelines:	**EU (=EEC): B.6., 96/54/EEC; 67/548/EEC; JMAF: 1985; OECD: 406, 1981 / update 1992; USEPA (=EPA): §81-6, Subdiv. F;Deviation not specified**
GLP/GEP:	**yes**

CA 5.2.7 Phototoxicity

According to the new data requirements (Commission Regulation (EU) No. 283/2013 of 1 March 2013; Official Journal of the European Union, L 93/1, 3.4.2013), the conduct of a phototoxicity study is required under certain conditions.

The circumstances in which a phototoxicity study, according to the new data requirements, is required are "where the active substance absorbs electromagnetic radiation in the range 290-700 nm and is liable to reach the eyes or light-exposed areas of the skin, either by direct contact or through systemic distribution. If the Ultraviolet / visible molar extinction / absorption coefficient of the active substance is less than 10 L x mol^{-1} x cm^{-1}, no toxicity testing is required.

As the Ultraviolet / visible molar extinction / absorption coefficient of the active substance exceeds the trigger of 10 L x mol^{-1} x cm^{-1}, a cytotoxicity study has been performed in vitro using BALB/c 3T3 cells.

Report:	[redacted]6[redacted];2014;M-476222-01
Title:	Mesosulfuron-methyl (AE F130060) technical: cytotoxicity assay in vitro with BALB/c3T3 c31 cells: Neutral Red (NR) test during simultaneous irradiation with artificial sunlight
Report No:	1592100
Document No:	M-476222-01-1
Guidelines:	Commission regulation (EC) No. 440/2008 B.41, dated May 30, 2008; Committee for Proprietary Medicinal Products (CPMP) Note for Guidance on Photosafety testing, EMEA, CPMP/SWP/398/01, adopted 27 June 2002, into operation in Dec 2002 ; OECD Guideline for Testing of Chemicals: Guideline: 432; In vitro 3T3 NRU phototoxicity test (Revised and approved by the National Co-ordinators in May 2002, approved by Council April 2004);not specified
GLP/GEP:	**yes**

Executive summary:
The phototoxic potential of Mesosulfuron-methyl (AE F130060) technical, was tested in this assay using BALB/c 3T3 cells. The experiment was performed twice. The first experiment served as a range finding experiment (RFE), the second one was the main experiment (ME).
Cytotoxic effects did not occur after exposure of the test item to the cells, neither in the presence nor in the absence of irradiation with artificial sunlight in the RFE as well as in the ME. Therefore, ED50-values or a PIF could not be calculated. The resulting MPE value was 0.048 and 0.000, respectively.

Therefore, the results show that the test item is not phototoxic.

I. Materials and methods

A. Materials

1. Test material:

Identification:	Mesosulfuron-methyl (AE F130060) technical
Batch Code:	AE F130060-01-02
Origin Batch No.:	EFME000144

Purity:	97.4% w/w (dose calculation was adjusted to purity)
Expiry Date:	25 January 2015
Storage Conditions:	At room temperature, protected from light
Stability in Solvent:	Generally stable for more than 24 hours in aqueous solution

2. Vehicle: DMSO (further diluted in EBSS, final concentration of DMSO in EBSS was 1% (v/v)

3. Positive control: Chlorpromazine dissolved in EBSS

4. Test system:

Cells: BALB/c 3T3 cells clone

Cell cultures: Thawed stock cultures (Master Cell Stock of the BALB/c 3T3 c31 cell line supplied by [redacted], Berlin) were propagated at 37 ± 1.5 °C in 75 cm² plastic flasks. Seeding was done with about 1 x 10^6 cells per flask in 15 mL of Dulbecco's Minimal essential Medium (DMEM), supplemented with 10 % NCS. The cells were sub-cultured twice weekly. The cell cultures were incubated at 37 ± 1.5 °C in a 7.5 ± 0.5% carbon dioxide atmosphere.

B. Study design and methods

1. Treatments: The experiment was performed twice. The first experiment served as a range finding experiment (RFE), the second one was the main experiment (ME).

Doses:

Test item	+/ - UV	Final concentrations in µg/mL
Mesosulfuron	+/-	7.41, 14.81, 29.64, 118.55, 237.10, 474.19, 948.06
Chlorpromazine	-	6.25, 12.5, 25, 37.5, 50, 75, 100, 200
	+	0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0
Solvent control	-	EBSS containing 1 % (v/v) DMSO

2. Experimental procedures:

Solar Simulator: Dr. Hönle Sol 500 solar simulator (filter H1 was used to keep the UVB irradiation as low as possible).

Seeding of Cultures: About 2 x 10^4 cells per well were seeded in 100 µL culture medium (two plates, one was exposed to artificial sunlight, one was kept in the dark).

Treatment: 24 hours after seeding the cultures were treated with the test item. The cultures were washed with EBSS. 8 dilutions of the solved test item were tested on two 96-well plates (100 µL/well), both plates were pre-incubated for 1 hour in the dark. After one hour one 96-well plate was irradiated through the lid at 1.65 mW/cm² (4.95 J/cm²), the other plate was stored in the dark (each 50 min, about 25-26 °C). After irradiation the test item was removed and both plates were washed twice with EBSS. Fresh culture medium was added and the cells were incubated about 22 - 23 hours at 37 ± 1.5 °C and 7.5 ± 0.5% CO2.

Cytotoxicity determination:
The medium was removed and 0.1 mL serum free medium containing 50 µg Neutral Red / mL added to each well. The plates were incubated for another 3 hours for the uptake of the vital dye into the lysosomes of viable cells. Thereafter, the medium was removed completely and the cells were washed with EBSS. For extraction of the dye 0.15 mL of a solution of 49% (v/v) deionised water, 50% (v/v) ethanol and 1% (v/v) acetic acid were added to each well. After approx. 10 minutes at room temperature and a brief agitation, the plates were transferred to a microplate reader (Versamax®, Molecular Devices) equipped with a 540 nm filter to determine the absorbance of the extracted dye. This absorbance showed a linear relationship with the number of surviving cells.

Number of measurements:
Mesosulfurone, positive control: 6 times
Solvent control: 12 times

Data Recording: Arithmetic means ± standard deviation were calculated for every test group. The ED50 values, the Photo-Irritancy-Factor (PIF), as well as the Mean Phototoxic Effect (MPE), were calculated using the software Phototox (Version 2.0) (distributed by [redacted], Germany, and recommended by the OECD guideline). The ED50 values (effective dose where only 50% of the cells survived) were determined by curve-fitting software.

Evaluation criteria: If **PIF < 2 or MPE < 0.1**: no phototoxic potential predicted.
If **PIF > 2 and < 5 or MPE >0.1 and <0.15** a probable phototoxic potential is predicted. If **PIF > 5 or MPE > 0.15** a phototoxic potential predicted.

II. Results and discussion

The first experiment served as a range finding experiment (RFE), the second one was the main experiment (ME). The following concentrations of the test item solved in DMSO (further diluted in EBSS, final concentration of DMSO in EBSS was 1% (v/v); dose calculation was adjusted to purity) were tested in the presence and in the absence of irradiation in both experiments: 7.41, 14.81, 29.64, 59.28, 118.55, 237.10, 474.19, 948.06. As solvent control EBSS containing 1% (v/v) DMSO was used. Chlorpromazine was used as positive control. One test group of cells treated with the test item was irradiated with artificial sunlight

for 50 minutes with 1.65 mW/cm² UVA, resulting in an irradiation dose of 4.95 J/cm² UVA. Another test group of test item treated cells were kept in the dark for 50 minutes.
Cytotoxic effects did not occur after exposure of the test item to the cells, neither in the presence nor in the absence of irradiation with artificial sunlight in the RFE as well as in the ME. Therefore, ED50-values or a PIF could not be calculated. The resulting MPE value was 0.048 and 0.000, respectively.
Therefore, the results show that the test item is not phototoxic.
The results are summarized in table CA 5.2.7-1.

Table CA 5.2.7-1 - Summary of Results

	Substance	ED_{50} (+UV) [µg/mL]	ED_{50} (-UV) [µg/mL]	PIF	MPE	% viability of solvent control of irradiated versus non-irradiated plate
RFE	Test Item	-	-	-	0.048	98.1
	Positive control	0.28	13.11	47.02	0.699	101.1
ME	Test Item	-	-	-	0.000	95.1
	Positive control	0.16	8.94	55.60	0.705	93.3

CA 5.3 Short-term toxicity

The short-term toxicity of mesosulfuron-methyl was assessed in an earlier EU review of the active substance, however these data are summarized here for reference in gray type. Further information is available in the Baseline Dossier provided by Bayer CropScience and in the Monograph.

TYPE OF STUDY	SPECIES / STRAIN	Dose levels (ppm)	NOAEL	Reference
Rat 90-d feeding study + 4 wk recovery	Wistar rat (Hoe:WISK)	0; 240; 1200; 6000; 12000	12000 ppm males: 908 mg/kg bw females: 976 mg/kg bw	[redacted],1999c; Doc. N° C004205 M-187497-01-1 KCA 5.3.2/01
Mouse 90-d feeding study	CD-1 mouse	0; 140; 1000; 7000	7000 ppm males: 1238mg/kg bw females: 1603 mg/kg bw	[redacted],1999b Doc. N° C006716 M-194489-01-1 KCA 5.3.2/02
Dog 28-d range finding	Beagle dog (Marshall)	0; 400; 2000; 10000; 20000	20000 ppm males: 775.9 mg/kg bw females: 804.5 mg/kg bw	[redacted],1997a Doc. N° A59274 M-142958-01-1 KCA 5.3.1/01
Dog 90-d feeding study	Beagle dog	0; 2000; 10000; 20000	20000 ppm males: 648 mg/kg bw females: 734 mg/kg bw	[redacted].,2000a Doc. N° C009014 M-198012-02-1 KCA 5.3.2/03
Dog 12 month feeding study	Beagle dog	0; 400; 4000; 16000	16000 ppm males: 574 mg/kg bw females: 646 mg/kg bw	[redacted].,2000a Doc. N° C009410 M-198511-01-1 KCA 5.3/01

No adverse effects were seen at any dose level of up to the highest tested dose of 1000 mg/kg bw/day of mesosulfuron-methyl in rats, mice and dogs. In all studies the NOAEL was the highest dose level given. This can be explained by the low toxicity of the test substance in combination with a non-linear absorption from the gastrointestinal tract, i.e., up to 23 % after oral low doses of 10 mg/kg bw and only 2 % or slightly above at the limit dose of 1,000 mg/kg bw after oral exposure.
Slight changes of some biochemical and hematological parameters which were seen in the subchronic study in rats did not show a clear dose-related pattern and were not consistently seen in both sexes and were therefore in the absence of any morphological consequence, regarded as findings due to variability.

No new short term toxicity study has been performed, and there are no new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance.

Report:	[redacted]§;[redacted];2000;M-198511-01
Title:	Dog 12 month dietary toxicity study Code: AE F130060 00 1C95 0001
Report No:	C009410
Document No(s):	M-198511-01-1
Guidelines:	**EU (=EEC): 88/302/EEC, V B; JMAF: 4200; OECD: 452; USEPA (=EPA): 83-1;not specified**
GLP/GEP:	**yes**

CA 5.3.1 Oral 28-day study

Report:	[redacted];[redacted];1997;M-142958-01
Title:	AE F130060 - substance technical; Code: AE F130060 00 1C94 0001 - 28 day repeated dose toxicity study in dogs (range finding study with dietary administration)
Report No:	A59274
Document No(s):	M-142958-01-1
Guidelines:	**JMAF: (1985); OECD: 409; USEPA (=EPA): 82-1;Deviation not specified**
GLP/GEP:	**yes**

CA 5.3.2 Oral 90-day study

Report:	[redacted];[redacted];1999;M-187497-01
Title:	Subchronic (90 days feeding) oral toxicity study in rats Hoe 130060 substance technical Code: Hoe 130060 00 ZC96 0002
Report No:	C004205
Document No(s):	M-187497-01-1
Guidelines:	**EU (=EEC): 88/302/EEC Annex V Part B; JMAF: 1985; OECD: 408; USEPA (=EPA): §82-1;Deviation not specified**
GLP/GEP:	**yes**

Report:	[redacted];[redacted];1999;M-194489-01
Title:	Subchronic (90 days feeding) oral toxicity study in mice Hoe 130060 substance technical Code: Hoe 130060 00 ZC96 0002
Report No:	C006716
Document No(s):	M-194489-01-1
Guidelines:	**EU (=EEC): 88/302/EEC Annex V Part B; JMAF: 1985; OECD: 408; USEPA (=EPA): F § 82-1;not specified**
GLP/GEP:	**yes**

Report:	[redacted];[redacted];2000;M-198012-02; Amended: 2001-03-27
Title:	Dog oral 90 day repeated dose toxicity study (dietary administration) AE F130060 substance technical Code: AE F130060 00 1C95 0001
Report No:	C009014
Document No(s):	M-198012-02-1
Guidelines:	**JMAF: 1985; OECD: 409; USEPA (=EPA): §82-1;not specified**
GLP/GEP:	**yes**

CA 5.3.3 Other routes

Studies with other routes are not generally required and were not conducted.

CA 5.4 Genotoxicity testing

Testing for possible genotoxic properties of mesosulfuron-methyl technical in several *in vitro* and *in vivo* test systems on different endpoints gave negative results. The *in vitro* testing battery comprised investigations for gene mutation in bacterial and mammalian cells, examination of chromosomal aberration in Chinese Hamster cells and testing for unscheduled DNA-synthesis in primary rat hepatocytes. Furthermore, a mouse micronucleus assay on chromosomal aberration *in vivo* was performed.

Since all 5 tests were negative and no evidence for carcinogenic properties was seen in life-time experiments in two species, further testing, e. g. tests using germ cells was not triggered. The following table 5.4-1 presents a summary of genotoxicity testing conditions and results that were evaluated during the first European evaluation.

Table CA 5.4-1 - Results of genotoxicity tests

Endpoint	Purity (%)	*Test system*	Concentration (dose) levels	Result	*Author*
Bacterial reverse mutation (Ames test)	95.6 %	*Salmonella typhimurium* TA 100, 1535, 1537, 98 *E. coli* WP2uvra	4 – 5000 µg/ plate +/- S9 mix	negative	[redacted] 1996i Doc. N° A56743 M-140530-01-1 KCA 5.4.1/01
Chromosomal aberration in vitro	94.6 %	Chinese Hamster V 79 lung fibroblasts	250 – 2500 µg/mL +/- S9 mix	negative	[redacted] 1998i Doc. N° A67555 M-147927-01-1 KCA 5.4.1/02
Mammalian cell gene mutation (HPRT test)	94.6 %	HPRT locus V79 Chinese Hamster lung fibroblasts	25 – 2500 µg/mL +/- S9 mix	negative	[redacted] 1998d Doc. N° A67081 M-147480-01-1 KCA 5.4.1/03
DNA damage and repair (UDS test)	94.6 %	Rat SD primary hepatocytes	1-2500 µg/mL +/- S9 mix	Negative	[redacted] 1998c Doc. N° A67689 M-148054-01-1 KCA 5.4.1/04
Chromosomal aberration (micronucleus test) in vivo	94.6 %	Hsd:Win:NMRI mice	2000/1000, 2500 mg/kg bw	Negative	[redacted] 1998e Doc. N° A67143 M-147538-01-1 KCA 5.4.2/01

CA 5.4.1 In vitro studies

Report:	[redacted]o;[redacted];1996;M-140530-01
Title:	Hoe 130060, Substance, technical, (Code: Hoe 130060 00 ZC96 0001) - Bacterial reverse mutation test
Report No:	A56743
Document No(s):	M-140530-01-1
Guidelines:	**EU (=EEC): 92/69 B 13, 92/69 B 14; OECD: 471, 472; USEPA (=EPA): 798.5100, 798.5265;not specified**
GLP/GEP:	**yes**

Report:	[redacted]4;[redacted];1998;M-147927-01
Title:	In vitro mammalian chromosome aberration test in V79 Chinese hamster lung cells AE F130060 substance, technical Code: AE F130060 00 1C95 0001
Report No:	A67555
Document No(s):	M-147927-01-1
Guidelines:	**EU (=EEC): 92/69, L383A Annex B.10; OECD: 473, May 1983; USEPA (=EPA): 798.5375, Fed.Reg.50, Subp. F;not specified**
GLP/GCP:	**yes**

Report:	[redacted]k; [redacted];1998;M-147480-01
Title:	AE F130060; substance, technical; Code: AE F130060 00 1C95 0001 - In vitro chinese hamster lung V79 cell HPRT mutation test
Report No:	A67081
Document No(s):	M-147480-01-1
Guidelines:	**EU (=EEC): 87/302, L133, 1987; OECD: 476, 1984; USEPA (=EPA): 798.5300, Part 700 to end, 1986;not specified**
GLP/GEP:	**yes**

Report:	[redacted]$; [redacted];1998;M-148054-01
Title:	Detection of DNA strand breaks in primary hepatocytes of male rats in vitro, UDS-test in primary rat hepatocytes AE F130060; substance, technical Code: AE F130060 00 1C95 0001
Report No:	A67689
Document No(s):	M-148054-01-1
Guidelines:	**EU (=EEC): 88/302, L 133; OECD: 482, 1986; USEPA (=EPA): 798.5500, 1985;not specified**
GLP/GEP:	**yes**

CA 5.4.2 In vivo studies in somatic cells

Report:	[redacted]*; [redacted];1998;M-147538-01
Title:	AE F130060; substance, technical; Code: AE F130060 00 1C95 0001 - Mouse micronucleus test
Report No:	A67143
Document No(s):	M-147538-01-1
Guidelines:	**EU (=EEC): 92/69, L383 A, Annex B.12; OECD: 474, 1983; USEPA (=EPA): 798.5395, Subpart F, 1985;not specified**
GLP/GEP:	**yes**

CA 5.4.3 In vivo studies in germ cells

Based on the toxicity and genotoxicity profile, in vivo studies in germ cells were not required.

CA 5.5 Long-term toxicity and carcinogenicity

Type of study	Dose levels (ppm)	NOAEL (mg/kg bw/d)	REFERENCES
Wistar Rat combined Chronic	0, 160, 1600, 16000	16000 ppm Males: 865 mg/kg bw/d Females: 1056 mg/kg bw/d	[redacted],2000a Doc. No.: C009379 M-198434-01-1 KCA 5.5/01
Oncogenicity	0, 160, 1600, 16000	Males: 764 mg/kg bw/d Females: 952 mg/kg bw/d	
Mouse oncogenicity	0, 80, 800 and 8000	800 ppm: Males:103 mg/kg bw/d Females:130 mg/kg bw/d	[redacted]2000b Doc. No.: C009460 M-198596-01-1 KCA 5.5/02

In a combined chronic toxicity and oncogenicity study in rats, continuous dietary treatment for 106 weeks with dose levels of up to 16 000 ppm, which were approximately equivalent to the limit dose of 1000 mg/kg bw/day, did not produce any evidence of toxicity or of oncogenicity during their natural lifespan. Also in mice, dietary treatment with up to 8 000 ppm (ca. 1000 mg/kg bw/d) for 80 consecutive weeks, with the exception of slight reductions in body weight gain in females, did not show any evidence of an oncogenic activity in this species.

Report:	[redacted]k; [redacted];2000;M-198434-01
Title:	Rat combined dietary chronic (12 and 24 months) and oncogenicity study AE F130060 technical substance Code: AE F130060 00 1C95 0001
Report No:	C009379
Document No(s):	M-198434-01-1
Guidelines:	**EU (=ECC): 88/302/EC; JMAF: 4200; OECD: 453; USEPA (=EPA): 83-5;not specified**
GLP/GEP:	**yes**

Report:	[redacted]; [redacted];2000;M-198596-01
Title:	Mouse dietary oncogenicity (18 months) study AE F130060 technical substance Code: AE F130060 00 1C95 0001
Report No:	C009460
Document No(s):	M-198596-01-1
Guidelines:	**EU (=ECC): 88/302/EEC; JMAF: 4200; OECD: 451; USEPA (=EPA): Series 83-2;not specified**
GLP/GEP:	**yes**

CA 5.6 Reproductive toxicity

Study type	Species	Dose levels	NOAEL	Reference
Multigeneration study	S.D. rats (Hsd:SD)	0, 160, 1600, 16 000 ppm	parents: 16000 ppm Offspring: 16000 ppm Males: 1175 mg/kg bw Females: 1388 mg/kg bw	[redacted] 2000a Doc N° C010081 M-198366-01-1 KCA 5.6.1/02
Developmental toxicity study	S.D. rats (Hsd:SD)	0, 100, 315, 1000 mg/kg bw/d	Maternal and foetal : 1000 mg/kg bw/d	[redacted] [redacted]., 1999b Doc. N° C003932 M-187036-01-1 KCA 5.6.2/02
Developmental toxicity study	Himalayan rabbits	0, 100, 315, 1000 mg/kg bw/d	Maternal and foetal : 1000 mg/kg bw/d	[redacted] 1998b Doc. N° C000843 M-181336-02-1 KCA 5.6.2/04

It was concluded that mesosulfuron-methyl did not have a reproduction or developmental toxicity potential. There was also no evidence of any teratogenic potential. No reproductive toxicity was seen, although due to the extremely high test substance intake during certain phases of dietary treatment the limit dose of 1 000 mg/kg bw/d was exceeded.
New studies were not conducted.

CA 5.6.1 Generational studies

In the multigeneration study administration of mesosulfuron-methyl at dietary concentrations of up to and including 16 000 ppm (equivalent to approximately 800 mg/kg bw/day up to 3000 mg/kg bw/day, depending on the food consumption in the different phases of the study) did not cause any substance-related adverse effects on reproduction, fertility, mating behaviour or malformations in the offspring in a multigeneration study in rats.

Report:	[redacted]§;[redacted];2000;M-198304-01
Title:	Range finding feeding-reproduction study for a rat two-generation reproduction toxicity study AE F130060 substance technical Code: AE F130060 00 1C95 0001
Report No:	C010056
Document No(s):	M-198304-01-1
Guidelines:	**JMAF: ;OECD: 416, 1981; USEPA (=EPA): §83-4, 1984;Deviation not specified**
GLP/GEP:	**yes**

Report:	[redacted]p;[redacted];2000;M-198366-01
Title:	Rat two-generation feeding-reproduction toxicity study with AE F130060 substance technical Code: AE F130060 00 1C95 0001
Report No:	C010081
Document No(s):	M-198366-01-1
Guidelines:	**JMAF: 1985; OECD: 416, 1981; USEPA (=EPA): OPPTS 870.3000, 1998;Deviation not specified**
GLP/GEP:	**yes**

CA 5.6.2 Developmental toxicity studies

Also the developmental toxicity studies in rats and rabbits, performed at the same laboratory like the reproduction toxicity study did not show substance-related adverse findings up to and including the limit dose level of 1000 mg/kg bw/d, which formed the NOAEL for dams and fetuses in both studies.

Report:	[redacted]v;[redacted];1997;M-147697-01
Title:	Range finding embryotoxicity study after oral administration in sprague dawley rats substance, technical Code: AE F130060 00 1C95 0001
Report No:	A67310
Document No(s):	M-147697-01-1
Guidelines:	**EU (=EEC): 88/302/EWG, 1987; JMAFF (1985); OECD: 414, 12-May-1981; USEPA (=EPA): F 83-3, 1984; Deviation not specified**
GLP/GEP:	**yes**

Report:	[redacted]6[redacted];1999;M-187036-01
Title:	Rat oral developmental toxicity (teratogenicity) study AE F130060 substance technical Code: AE F130060 00 1C95 0001
Report No:	C003932
Document No(s):	M-187036-01-1
Guidelines:	**EU (=EEC): 88/302; JMAFF: 1985; OECD: 414; USEPA (=EPA): 83-3;not specified**
GLP/GEP:	**yes**

Report:	[redacted]•;[redacted];1998;M-147696-01
Title:	Range finding embryotoxicity study after oral administration in rabbits substance, technical Code: AE F130060 00 1C95 0001
Report No:	A67309
Document No(s):	M-147696-01-1
Guidelines:	**Deviation not specified**
GLP/GEP:	**yes**

Report:	[redacted]4[redacted];1998;M-181336-02; Amended: 2001-03-16
Title:	Rabbit oral developmental toxicity (teratogenicity) study AE F130060 substance technical Code: AE F130060 00 1C95 0001
Report No:	C000843
Document No(s):	M-181336-02-1
Guidelines:	**EU (=EEC): 88/302; JMAFF: NohSan No. 4200 (1985) ; OECD: 414; USEPA (=EPA): F 83-3;not specified**
GLP/GEP:	**yes**

CA 5.7 Neurotoxicity studies

No special studies on a neurotoxic potential of mesosulfuron-methyl have been performed. The extensive toxicity data from all apical studies which are available do not provide evidence of any direct neurotoxic effects. Therefore, no additional testing was required and based on the available data, mesosulfuron-methyl does not present a neurotoxic hazard.

CA 5.7.1 Neurotoxicity studies in rodents

Based on the available information, mesosulfuron-methyl does not present a neurotoxic hazard. Therefore, neurotoxicity studies were not conducted.

CA 5.7.2 Delayed polyneuropathy studies

Mesosulfuron-methyl does not belong to the class of organophosphates from which some have an OPIDP potential. Therefore, an OPIDP study was not necessary and not performed.

CA 5.8 Other toxicological studies

Other toxicological studies with the active ingredient were not performed since the toxicological profile was sufficiently demonstrated.

The equivalence of the test material used in the key toxicological studies with the current specification was demonstrated in a position paper. Please refer to the confidential part of the dossier.

CA 5.8.1 Toxicity studies of metabolites

Genotoxicity studies were conducted with the metabolites AE F147447, AE F160460 and BCS-CV14885.

AE F147447

Report:	[redacted]p;[redacted];2012;M-428741-01
Title:	Salmonella typhimurium reverse mutation assay with AE F147447
Report No:	1462101
Document No:	M-428741-01-1
Guidelines:	**OECD No. 471, adopted July 21, 1997** **EC No. 440/2008 B13/14, dated May 30, 2008** **EPA OPPTS 870.5100, EPA 712-C-98-247, August 1998;not specified**
GLP/GEP:	**yes**

Executive summary:

This study the potential of AE F147447 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 item 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

The plates incubated with the test item 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 AE F147447 at any dose level, 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 item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.
Therefore, AE F147447 is considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

I. Material and methods

A Materials

1 Test Material:

Description:	White powder
Lot/Batch:	SES 10681-2-3
Purity:	98.9 % w/w; Dose calculation adjusted to purity
Stability of test compound:	not performed as part of this study.
Solvent used:	DMSO ([redacted]/Germany; purity > 99 %).

2 Control materials:

Negative:	Culture medium
Solvent:	DMSO
Positive: *non-activation (-S9 mix)*	
sodium azide:	10 µg/plate for TA1535 and TA100
4-nitro-o-phenylene-diamine:	50 µg/plate for TA1537 and 10 µg/plate for TA98
methyl-methane sulfonate:	3.0 µL/plate for TA 102
activation (+S9 mix) :	
2-aminoanthracene, 2-AA :	2.5 µg/plate for TA 1535, TA 1537, TA 98, TA 100, and 10 µg/plate for TA 102

3 Metabolic activation: S9 derived from Wistar rats (Hsd Cpb: WU)

Phenobarbital/β-naphthoflavone induced rat liver S9 were used as the metabolic activation system. The S9 is prepared from 8 - 12 weeks old male Wistar rats (Hsd Cpb: WU; weight approx. 220 – 320 g, [redacted], The Netherlands) induced by intraperitoneal applications of 80 mg/kg b.w. phenobarbital ([redacted], Germany) and by peroral administrations of β-naphthoflavone ([redacted], Germany) each, on three consecutive days. Each batch of S9 mix is routinely tested with 2-aminoanthracene as well as benzo[a]pyrene. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix: 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate and 4 mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

4 Test organisms: *Salmonella typhimurium*, strains TA1535, TA1537, TA98, TA100 and TA 102 (from [redacted], Germany). Regular checking of the properties of the strains regarding the membrane permeability, ampicillin- and tetracycline-resistance as well as spontaneous mutation rates are performed in the laboratory of [redacted]

5 Test 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

B Study Design and Methods

Test performance: The study was conducted at [redacted]/Germany) from January 19th to February 14th, 2012.

1 Preliminary cytotoxicity/mutation assay:

To evaluate the toxicity of the test item a pre-experiment was performed with all strains used. Eight concentrations were tested for toxicity and mutation induction with each 3 plates. For each strain and dose level, including the controls three plates were used.

Toxicity of the test item can be evident as a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn. In this 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 overlay agar (45 °C) was added to each tube. The mixture was poured on minimal agar plates and after solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark.

2 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 and after solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark.

3 Statistics:
According to OECD guideline 471, a statistical analysis of the data is not mandatory.

4 Acceptability of the assay:
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 a significant increase in mutant colony frequencies
- 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.

5 Evaluation criteria:
A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding 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 is observed.
A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration. An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.
A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II Results and Discussion

A Analytical determinations:
The stability of technical AE F147447 and the stability and homogeneity of technical AE F147447 in the solvent and analysis of achieved concentration were not performed as part of this study.

B Preliminary cytotoxicity assay:
The plates incubated with the test item 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.

C Mutation assays:
No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with AE F147447 at any concentration level, 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 in-crease in induced revertant colonies.

The results are summarized in the following tables:

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		15 ± 3	11 ± 2	27 ± 6	89 ± 2	330 ± 20
	Untreated		19 ± 4	9 ± 4	21 ± 6	87 ± 2	328 ± 9
	AE F147447	3 µg	18 ± 2	14 ± 6	24 ± 3	85 ± 6	301 ± 6
		10 µg	20 ± 3	11 ± 3	26 ± 3	82 ± 10	320 ± 8
		33 µg	18 ± 5	11 ± 2	22 ± 7	78 ± 7	306 ± 25
		100 µg	17 ± 2	10 ± 2	28 ± 6	89 ± 12	295 ± 11
		333 µg	16 ± 1	11 ± 1	24 ± 3	77 ± 2	305 ± 7
		1000 µg	17 ± 2	11 ± 2	28 ± 2	73 ± 5	309 ± 12
		2500 µg	23 ± 1	11 ± 2	29 ± 2	72 ± 6	308 ± 11
		5000 µg	19 ± 4	10 ± 2	29 ± 8	72 ± 2	328 ± 17
	NaN3	10 µg	1569 ± 54			1739 ± 53	
	4-NOPD	10 µg			345 ± 11		
	4-NOPD	50 µg		72 ± 2			
	MMS	3.0 µL					3518 ± 109
With Activation	DMSO		29 ± 2	19 ± 10	39 ± 4	110 ± 6	345 ± 3
	Untreated		31 ± 4	25 ± 3	45 ± 5	110 ± 8	424 ± 40
	AE F147447	3 µg	31 ± 5	16 ± 3	34 ± 8	105 ± 8	382 ± 56
		10 µg	31 ± 6	22 ± 9	36 ± 6	113 ± 18	360 ± 54
		33 µg	28 ± 4	20 ± 3	37 ± 9	96 ± 7	395 ± 41
		100 µg	27 ± 2	17 ± 3	39 ± 8	121 ± 8	368 ± 14
		333 µg	31 ± 8	22 ± 2	42 ± 6	97 ± 15	384 ± 27
		1000 µg	27 ± 5	19 ± 3	39 ± 4	106 ± 11	371 ± 6
		2500 µg	21 ± 8	20 ± 4	36 ± 8	109 ± 4	361 ± 10
		5000 µg	30 ± 1	19 ± 5	41 ± 11	100 ± 4	367 ± 22
	2-AA	2.5 µg	272 ± 14	289 ± 4	1665 ± 153	1790 ± 60	
	2-AA	10.0 µg					2342 ± 127

NaN3	sodium azide
2-AA	2-aminoanthracene
MMS	methyl methane sulfonate
4-NOPD	4-nitro-o-phenylene-diamine

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		19 ± 3	14 ± 3	23 ± 3	93 ± 16	311 ± 11
	Untreated		18 ± 4	15 ± 7	32 ± 5	118 ± 22	321 ± 4
	AE F147447	33 µg	19 ± 2	12 ± 4	25 ± 5	97 ± 15	304 ± 19
		100 µg	18 ± 2	12 ± 4	24 ± 3	92 ± 7	319 ± 8
		333 µg	16 ± 1	13 ± 3	27 ± 4	96 ± 9	301 ± 14
		1000 µg	17 ± 3	13 ± 4	19 ± 2	91 ± 5	272 ± 23
		2500 µg	12 ± 6	14 ± 4	22 ± 2	96 ± 3	308 ± 24
		5000 µg	17 ± 6	9 ± 1	24 ± 5	88 ± 8	339 ± 9
	NaN3	10 µg	1818 ± 50			2143 ± 114	
	4-NOPD	10 µg			343 ± 16		
	4-NOPD	50 µg		77 ± 19			
	MMS	3.0 µL					2173 ± 106
With Activation	DMSO		26 ± 5	21 ± 3	41 ± 3	110 ± 12	348 ± 27
	Untreated		27 ± 4	18 ± 2	36 ± 2	114 ± 4	421 ± 33
	AE F147447	33 µg	28 ± 1	24 ± 4	38 ± 1	112 ± 10	334 ± 42
		100 µg	26 ± 6	23 ± 3	43 ± 13	107 ± 13	350 ± 26
		333 µg	28 ± 9	21 ± 5	38 ± 10	118 ± 8	359 ± 31
		1000 µg	25 ± 3	21 ± 5	35 ± 2	100 ± 9	336 ± 16
		2500 µg	26 ± 4	20 ± 4	37 ± 5	102 ± 9	396 ± 19
		5000 µg	26 ± 12	23 ± 1	41 ± 6	114 ± 7	403 ± 32
	2-AA	2.5 µg	301 ± 11	163 ± 18	1297 ± 26	1553 ± 138	
	2-AA	10.0 µg					2326 ± 530

NaN3	sodium azide
2-AA	2-aminoanthracene
MMS	methyl methane sulfonate
4-NOPD	4-nitro-o-phenylene-diamine

III Conclusions

It can be concluded that in the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Report:	KCA 5.8.1 /02; [redacted] 2012;M-433931-02; Amended: 2015-01-30
Title:	In vitro chromosome aberration test in Chinese hamster V79 cells with AE F147447
Report No:	1462102
Document No:	M-433931-02-1
Guidelines:	Ninth Addendum to the OECD Guidelines for Testing of Chemicals, February 1998, adopted July 21, 1997, Guideline No. 473 In vitro Mammalian Chromosome Aberration Test; Commission Regulation (EC) No. 440/2008, B10: Mutagenicity - In vitro Mammalian Chromosome Aberration Test, dated May 30, 2008; United States Environmental Protection Agency Health Effects Test Guidelines, OPPTS 870.5375, In Vitro Mammalian Chromosome Aberration Test, EPA 712-C-98-223, August 1998;not specified
GLP/GEP:	**yes**

Executive summary

The test item AE F147447, dissolved in culture medium, was assessed for its potential to induce structural chromosome aberrations in V79 cells of the Chinese hamster in vitro in two independent experiments. The following study design was performed:

	Without S9 mix		With S9 mix	
	Exp. I	Exp. II	Exp. I	Exp. II
Exposure period	4 hours	18 hours	4 hours	4 hours
Recovery	14 hours	-	14 hours	14 hours
Preparation interval	18 hours	18 hours	18 hours	18 hours

In each experimental group two parallel cultures were set up. 100 metaphases per culture were evaluated for structural chromosome aberrations.

The highest applied concentration (2936.0 µg/mL; approx. 10 mM) was chosen with regard to the molecular weight and the purity (98.9 %) of the test item and with respect to the current OECD Guideline 473. Test item concentrations between 11.5 and 2936.0 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I. Since no cytotoxicity and test item precipitation was observed in the first experiment, concentrations between 11.5 and 2936.0 µg/mL in the absence of S9 mix and between 183.5 and 2936.0 µg/mL in the presence of S9 mix were chosen for the second experiment.

No clastogenicity was observed at the concentrations evaluated either with or without metabolic activation. In Experiment I in the absence of S9 mix statistically significant increases were observed after treatment with 1468.0 and 2936.0 µg/mL, however, the values are within the range of the historical solvent control data (0.0 - 4.0 % aberrant cells, excluding gaps) and therefore considered as being biologically irrelevant. No evidence of an increase in polyploid metaphases was found after treatment with the test item as compared to the frequencies of the control cultures.

Appropriate positive controls induced statistically significant increases in cells with structural chromosome aberrations.

Thus, it can be stated that under the experimental conditions reported, the test item did not induce structural chromosome aberrations in V79 cells (Chinese hamster cell line) *in vitro*. Therefore, AE F147447 is considered to be non-clastogenic in this chromosome aberration test up to the highest required concentration.

I. Material and methods

A Materials

1 Test Material: AE F147447

Description: White powder
Lot/Batch: SES 10681-2-3
Purity: 98.9 % w/w, Dose calculation adjusted to purity
Stability of test compound: not performed as part of this study
Solvent: MEM culture medium from [redacted], Germany (chosen due to its solubility properties and its relative non-toxicity to the cell cultures).

2 Control materials:

Negative: MEM Tissue Culture medium
Positive, w/o metabolic activation: EMS; ethylmethane sulfonate, dissolved in Nutrient medium, final Concentration: 1000.0 µg/mL (Experiment I) 600.0 µg/mL (Experiment II)
Positive, with metabolic activation: CPA; cyclophosphamide, dissolved in Saline (0.9 % [w/v]), Final Concentration: 1.4 µg/mL

The dilutions of the stock solutions were prepared on the day of the experiment. The stability of the positive control substances (EMS and CPA, respectively) in solution is unknown but a mutagenic response in the expected range was sufficient biological evidence of chemical stability.

3 Metabolic activation S9 mix: S9 from male Wistar rats (Hsd Cpb: WU)

The S9 was prepared from 8 - 12 weeks old male Wistar rats (Hsd Cpb: [redacted], The Netherlands) weight approx. 220 - 320 g induced by intraperitoneal applications of 80 mg/kg b.w. phenobarbital ([redacted], Germany) and by orally administrations of 80 mg/kg b.w. β-naphthoflavone ([redacted], Germany) each, on three consecutive days. Each batch of S9 mix was routinely tested with 2-aminoanthracene as well as benzo(a)pyrene. 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).

4 Test cells: The V79 cell line has been used successfully for many years in in vitro experiments with a high proliferation and a reasonable plating efficiency.

The cells have a stable karyotype with a modal chromosome number of 22 ± 1.

5 Test concentrations:

Preparation interval	Exposure period	Concentration in µg/mL								
		Without S9 mix								
18 hours	4 hours	11.5	22.9	45.9	91.8	183.5	367.0	**734.0**	**1468.0**	**2936.0**
18 hours	18 hours	11.5	22.9	45.9	91.8	183.5	367.0	**734.0**	**1468.0**	**2936.0**
		With S9 mix								
18 hours	4 hours	11.5	22.9	45.9	91.8	183.5	367.0	**734.0**	**1468.0**	**2936.0**
18 hours	4 hours					183.5	367.0	**734.0**	**1468.0**	**2936.0**

Evaluated experimental points are shown in bold characters

B Study Design and Methods:

1 Study performance: The study was conducted at [redacted] [redacted]/Germany). The experimental start and completion dates of the study were January 25th 2012 and March 26th 2012, respectively.

2 Culture Medium and Conditions:

For seeding and treatment of the cell cultures the culture medium was MEM (minimal essential medium) containing Hank's salts, glutamine and Hepes (25 mM). Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/ 100 µg/mL) and 10 % (v/v) fetal bovine serum (FBS). All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO2 (98.5 % air).

3 Seeding of the Cultures:

Exponentially growing stock cultures more than 50 % confluent were rinsed with Ca-Mg-free salt solution containing 8000 mg/L NaCl, 200 mg/L KCl, 200 mg/L KH2PO4 and 150 mg/L Na2HPO4. Afterwards the cells were treated with trypsin-EDTA-solution at 37 °C for approx. 5 minutes. Then, by adding complete culture medium including 10 % (v/v) FBS the enzymatic treatment were stopped and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.5 % (w/v) in Ca-Mg-free salt solution. The cells were seeded into Quadriperm dishes, which contained microscopic slides. Into each chamber $1 \times 10^4 - 6 \times 10^4$ cells were seeded with regard to the preparation time.

4 Treatment after the 4-hour exposure period:

The culture medium of exponentially growing cell cultures was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium were added.

After 4 hours the cultures were washed twice 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_2HPO4 • 2 H_2O and 150 mg/L KH_2PO_4. The cells were

then cultured in complete medium containing 10 % (v/v) FBS for the remaining culture time of 14 hours. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

5 Slides preparation:
Colcemid was added to the culture medium (0.2 µg/mL) 15.5 hours after the start of the treatment. The cells were treated, 2.5 hours later, on the slides in the chambers with hypotonic solution (0.4 % KCl) for 20 min at 37 °C. After incubation in the hypotonic solution the cells were fixed with a mixture of methanol and glacial acetic acid (3:1 parts, respectively). After preparation the cells were stained with Giemsa and labelled with a computer-generated random code to prevent scorer bias.

6 Evaluation of Cell Numbers:
The evaluation of cytotoxicity indicated by reduced cell numbers was made after the preparation of the cultures on spread slides. The cell numbers were determined microscopically by counting 10 defined fields per coded slide. The cell number of the treatment groups is given in percentage compared to the respective solvent control.

7 Analysis of Metaphase Cells:
Evaluation of the cultures was performed according to the OECD guideline using NIKON microscopes with 100x objectives. Breaks, fragments, deletions, exchanges, and chromosome disintegrations were recorded as structural chromosome aberrations. Gaps were also recorded but not included in the calculation of the aberration rates. 100 well spread metaphases per culture were evaluated for cytogenetic damage on coded slides.
Only metaphases with characteristic chromosome numbers of 22 ± 1 were included in the analysis. To describe a cytotoxic effect the mitotic index (% cells in mitosis) was determined.

8 Acceptability of the Test:
The chromosome aberration test is considered acceptable, if it meets the following criteria:

- The number of structural aberrations found in the solvent controls falls within the range of the laboratory historical control data.
- The positive control substances produce significant increases in the number of cells with structural chromosome aberrations, which are within the range of the laboratory historical control data.

9 Evaluation of Results:
A test item is classified as non-clastogenic if the number of induced structural chromosome aberrations in all evaluated dose groups is in the range of the laboratory historical control data and no significant increase of the number of structural chromosome aberrations is observed.
A test item is classified as clastogenic if the number of induced structural chromosome aberrations is not in the range of the laboratory historical control data and either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.
Both biological and statistical significance should be considered together. If the criteria mentioned above for the test item are not clearly met, the classification with regard to the historical data and the biological relevance is discussed and/or a confirmatory experiment is performed.

In this study together with chromosome aberration also polyploids and endoreduplications were considered. A test item can be classified as aneugenic if the number of induced numerical aberrations is not in the range of the laboratory historical control data.

II. Results and discussion

Two independent experiments were performed. In Experiment I the exposure period was 4 hours with and without S9 mix. In Experiment II the exposure period was 4 hours with S9 mix and 18 hours without S9 mix. The chromosomes were prepared 18 hours (Exp. I & II) after start of treatment with the test item.
In each experimental group two parallel cultures were set up. 100 metaphases per culture were evaluated for structural chromosome aberrations.
No relevant influence on osmolarity was observed. In Experiment I as well as in Experiment II, the pH of the stock solutions (2.94 mg/mL without S9 mix and 3.09 mg/mL with S9 mix) was adjusted prior to the preparation of the dilution series by using small amounts of 2N NaOH.
No cytotoxicity, indicated by reduced mitotic indices or reduced cell numbers could be observed in both experiments up to the highest applied concentration.
In both experiments, in the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying structural chromosome aberrations was observed. The aberration rates of the cells after treatment with the test item (0.5 – 2.5 % aberrant cells, excluding gaps) were within the range of the solvent control values (0.0 – 3.5 % aberrant cells, excluding gaps) and within the range of the laboratory historical solvent control data.
Two statistically significant increases were observed in Experiment I after treatment with 1468.0 and 2936.0 µg/mL. Since these increases of 2.0 % aberrant cells, excluding gaps were within the laboratory historical solvent control data range (0.0 – 4.0 % aberrant cells, excluding gaps), this has to be regarded as being biologically irrelevant.
No evidence of an increase in polyploid metaphases was found after treatment with the test item as compared to the frequencies of the control cultures.
In both experiments, either EMS (1000.0 or 600.0 µg/mL) or CPA (1.4 µg/mL) were used as positive controls which showed distinct increases in cells with structural chromosome aberrations.

The results are summarized in the following table:

Exp.	Preparation interval	Test item concentration in µg/mL	Cell numbers in % of control	Mitotic indices in % of control	Aberrant cells in %		
					incl. gaps*	excl. gaps*	with exchange*
Exposure period 4 hrs without S9 mix							
I	18 hrs	Solvent control[1]	100.0	100.0	0.0	0.0	0.0
		Positive control[2]	n.d.	119.8	21.0	**20.5**[S]	14.5
		734.0	98.1	115.8	1.5	1.5	0.5
		1468.0	90.2	111.1	2.0	**3.0**[S]	0.5
		2936.0	96.9	115.0	2.0	**2.0**[S]	0.0
Exposure period 18 hrs without S9 mix							
II	18 hrs	Solvent control[1]	100.0	100.0	2.5	2.5	0.5
		Positive control[3]	n.d.	84.5	13.0	**10.5**[S]	2.5
		734.0	91.6	85.9	1.5	1.0	0.0
		1468.0	73.0	107.5	0.5	0.5	0.0
		2936.0	90.3	104.4	2.5	2.5	0.0
Exposure period 4 hrs with S9 mix							
I	18 hrs	Solvent control[1]	100.0	100.0	3.0	2.0	0.0
		Positive control[4#]	n.d.	59.9	44.0	**42.0**[S]	13.0
		734.0	101.9	88.0	3.0	2.0	1.0
		1468.0	102.3	104.2	0.5	0.5	0.5
		2936.0	102.8	113.6	2.5	2.0	0.0
II	18 hrs	Solvent control[1]	100.0	100	4.0	3.5	1.5
		Positive control[4]	n.d.	57.2	24.5	**24.0**[S]	6.0
		734.0	94.6	93.0	2.5	2.5	0.0
		1468.0	96.5	100.4	2.5	2.5	0.0
		2936.0	93.3	105.6	1.5	1.0	0.0

III. Conclusions

Technical AE F147447 showed no evidence of clastogenic activity in this *in vitro* cytogenetic test system.

Report:	[redacted]ヨ; [redacted];2012;M-433935-01
Title:	Gene mutation assay in Chinese hamster V79 cells in vitro (V79 / HPRT) - AE F147447
Report No:	1462103
Document No:	M-433935-01-1
Guidelines:	Ninth Addendum to the OECD Guidelines for Testing of Chemicals, Section 4, No. 476: In vitro Mammalian Cell Gene Mutation Test, adopted July 21, 1997; Commission Regulation (EC) No. 440/2008, B17, dated May 30, 2008; United States Environmental Protection Agency Health Effects Test Guidelines, OPPTS 870.5300, In Vitro Mammalian Cell Gene Mutation Test, EPA 712-C-98-221, August 1998;not specified
GLP/GEP:	**yes**

Executive summary:
The study was performed to investigate the potential of AE F147447 to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster.
The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with a treatment time of 4 hours with and 24 hours without metabolic activation.
No substantial and reproducible dose dependent increase of the mutation frequency exceeding the historical range of solvent controls was observed in both main experiments.
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, AE F147447 is considered to be non-mutagenic in this HPRT assay.

I. Material and methods

A Materials:

1 Test Material:
Description: White powder
Lot/Batch: SES 10681-2-3
Purity: 98.9 % w/w, Dose calculation adjusted to purity
Stability of test compound: not performed as part of this study.
Solvent used: DMSO

2 Control materials:
Negative: Tissue Culture medium
Positive: *non- activation (-S9 mix)*:
Ethyl methanesulphonate (EMS) dissolved in nutrient medium.
Final concentration: 0.15 mg/mL (1.2 mM).
Positive: *with activation (+S9 mix):*
DMBA; 7,12-dimethylbenz(a)anthracene dissolved in DMSO.
Final concentration 1.1 µg/mL (= 4.3 µM).

3 Metabolic activation S9 mix: S9 from male Wistar rats

Phenobarbital/β-Naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from 8 - 12 weeks old male Wistar rats (Hsd Cpb: WU, weight approx. 220 – 320 g, [redacted], The Netherlands) induced by i.p. applications of 80 mg/kg b.w. phenobarbital and by peroral administrations of β-naphthoflavone each, on three consecutive days. The livers were prepared 24 hours after the last treatment. The S9 fractions were produced by dilution of the liver homogenate with a KCl solution (1+3) followed by centrifugation at 9000 g. Cofactors were added to the S9 supernatant to reach following concentrations in the S9 mix were: 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate and 4 mMNADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

4 Test cells:

The V79 cell line has been used successfully in in vitro experiments for many years. Especially the high proliferation rate (doubling time 12 -16 h in stock cultures) and a good cloning efficiency of untreated cells (as a rule more than 50 %) recommend the use of this cell line. The cells have a stable karyotype with a modal chromosome number of 22.

5 Test concentrations:

Exposure period	S9 mix	Concentrations in µg/mL					
		Experiment I					
4 hours	-	156.3	**312.5**	**625.0**	**1250**	**2500**	**3000**
4 hours	+	156.3	**312.5**	**625.0**	**1250**	**2500**	**3000**
		Experiment II					
24 hours	-	156.3	**312.5**	**625.0**	**1250**	**2500**	**3000**
4 hours	+	156.3	**312.5**	**625.0**	**1250**	**2500**	**3000**

Concentrations given in bold letters were chosen for the mutation rate analysis

B Study design and methods:

1 Study performance: The study was conducted at [redacted] [redacted] /Germany). The experimental start and completion dates of the study were January 26th 2012 and March 29th 2012, respectively.

2 Culture medium and conditions:

For seeding and treatment of the cell cultures the complete culture medium was MEM (minimal essential medium) containing Hanks's salts, neomycin (5 µg/mL) and amphotericin B (1 %). For the selection of mutant cells the complete medium was supplemented with 11 µg/mL 6-thioguanine. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

3 Seeding of the cultures:

Two to three days after sub-cultivation stock cultures were trypsinized at 37 °C for 5 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 PBS. Prior to the trypsin treatment the cells were rinsed with Ca-Mg-free salt solution containing 200 mg/l EDTA (ethylene diamine tetraacetic acid). Approximately 1.5 x 10^6 (single culture) and 5 x 10^2 cells (in duplicate) were seeded in plastic culture flasks. The cells were grown for 24 hours prior to treatment.

4 Treatment:

After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps with "saline G". In the second experiment the cells were exposed to the test item for 24 hours in complete medium, supplemented with 10 % FBS, in the absence of metabolic activation. The pH was adjusted to 7.2. The colonies used to determine the cloning efficiency (survival) were fixed and stained approx. 7 days after treatment. Three or four days after treatment 1.5x 10^6 cells per experimental point were subcultivated in 175 cm² flasks containing 30 mL medium. Following the expression time of 7 days five 80 cm² cell culture flasks were seeded with about 3 – 5 x10^5 cells each in medium containing 6-TG. Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability. 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.

5 Evaluation of cell numbers:

The stained colonies with more than 50 cells were counted. In doubt the colony size was checked with a preparation microscope.

6 Acceptability of the Assay:

The gene mutation assay is considered acceptable if it meets the following criteria:

- the numbers of mutant colonies per 106 cells found in the solvent controls falls within the laboratory historical control data (see Annex).
- the positive control substances should produce a significant increase in mutant colony frequencies.
- the cloning efficiency II (absolute value) of the solvent controls should exceed 50 %.

7 Evaluation of Results:

A test item is classified as positive if it induces either a concentration-related increase of the mutant frequency or a reproducible and positive response at one of the test points.
A test item producing neither a concentration-related increase of the mutant frequency nor a reproducible positive response at any of the test points is considered non-mutagenic in this system.
A positive response is described as follows:
A test item is classified as mutagenic if it reproducibly induces a mutation frequency that is three times above the spontaneous mutation frequency at least at one of the concentrations in the experiment.
The test item is classified as mutagenic if there is a reproducible concentration-related increase of the mutation frequency. Such evaluation may be considered also in the case that a threefold increase of the mutant frequency is not observed.

However, in a case by case evaluation this decision depends on the level of the corresponding solvent control data. If there is by chance a low spontaneous mutation rate within the laboratory´s historical control data range, a concentration-related increase of the mutations within this range has to be discussed. The variability of the mutation rates of solvent controls within all experiments of this study was also taken into consideration.

8 Statistical Analysis:
A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. The numbers of mutant colonies 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. However, both, biological and statistical significance was considered together.

II. Results and discussion

No relevant toxic effects occurred up to the maximum concentration of 3000 µg/mL equal to approximately 10 mM.
No relevant and reproducible increase of mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration. The mutant frequency remained well within the historical range of solvent controls. An increase of the induction in the second experiment without metabolic activation at 2500 µg/mL (culture II) and 3000 µg/mL (both cultures), and in the second culture of the second experiment with metabolic activation at 312.5, 1250, 2500, and 3000 µg/mL were judged as biologically irrelevant fluctuations since they were based on a rather low mutation frequency of the solvent controls (4.9, 4.4, and 3.6). The absolute values of the mutation frequency even remained within the actual range of the solvent controls of this study (3.6 to 20.4 colonies per 10^6 cells).
A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequency. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in the second experiment at both cultures without metabolic activation. However, the trend was judged as biologically irrelevant since the mutation frequency did not exceed the historical solvent control range discussed above.
EMS (150 µg/mL) and DMBA (1.1 µg/mL) were used as positive controls and showed a distinct increase in induced mutant colonies.

The results of experiments I and II are summarized in the following table:

	concentration µ/mL	S9 mix	relative cloning efficiency I %	relative cell density %	relative cloning efficiency II %	mutant colonies 10^6 cells	induction factor	relative cloning efficiency I %	relative cell density %	relative cloning efficiency II %	mutant colonies 10^6 cells	induction factor
Column	1	2	3	4	5	6	7	8	9	10	11	12
Experiment I/4 h treatment			culture I					culture II				
Solvent		-	100.0	100.0	100.0	9.6	1.0	100.0	100.0	100.0	9.2	1.0

control with DMSO												
Positive control (EMS)	150.0	-	88.7	122.8	85.8	81.6	8.5	80.1	82.1	62.8	105.4	11.5
Test item	156.3	-	95.2	culture was not continued*				96.8	culture was not continued*			
Test item	312.5	-	92.3	110.9	106.3	14.1	1.5	94.2	105.3	111.2	3.4	0.4
Test item	625.0	-	88.7	111.5	99.6	7.5	0.8	86.5	107.5	85.8	9.3	1.0
Test item	1250.0	-	13.9	102.6	92.3	13.5	1.4	20.1	99.6	116.3	8.1	1.0
Test item	2500.0	-	87.9	108.7	99.7	9.7	1.0	93.7	97.5	91.3	20.7	2.3
Test item	3000.0	-	92.5	124.1	89.7	16.3	1.7	99.4	84.5	93.1	7.6	0.8
Solvent control with DMSO		+	100.0	100.0	100.0	14.9	1.0	100.0	100.0	100.0	8.3	1.0
Positive control (DMBA)	1.1	+	45.5	63.3	91.7	481. 1	32.3	44.0	83.0	93.9	465.0	56.0
Test item	156.3	+	105.4	culture was not continued*				98.7	culture was not continued*			
Test item	312.5	+	99.2	90.3	93.6	9.4	0.6	95.5	117.4	96. 7	13.2	1.6
Test item	625.0	+	107.9	102.9	96.4	12.9	0.9	94.8	105.6	94. 2	14.7	1.8
Test item	1250.0	+	103.9	101.3	102.8	5.1	0.3	102.9	106.6	94. 3	11.5	1.4
Test item	2500.0	+	96.5	92.3	95.3	9.9	0.7	97.9	110.1	94. 6	11.4	1.4
Test item	3000.0	+	97.3	89.4	83.7	10.3	0.7	101.9	90.4	92. 6	7.9	1.0
Experiment II, 24 h treatment				culture I						culture II		
Solvent control with DMSO		-	100.0	100.0	100.0	4.9	1.0	100.0	100.0	100 .0	4.4	1.0
Positive control (EMS)	150.0	-	95.1	84.6	87.6	535. 5	108.1	102.0	92.9	62. 9	615.2	138.5
Test item	156.3	-	103.8	culture was not continued*				99.7	culture was not continued*			
Test item	312.5	-	99.5	96.7	100.0	5.6	1.1	101.2	112.2	97. 2	8.5	1.9

Test item	625.0	-	103.8	96.5	96.7	8.7	1.8	99.7	112.8	81.0	8.6	1.9
Test item	1250.0	-	101.2	90.7	104.3	12.1	2.5	102.4	93.3	100.1	10.0	2.2
Test item	2500.0	-	101.6	98.9	86.0	11.1	2.2	100.1	104.8	74.8	13.8	3.1
Test item	3000.0	-	101.7	71.0	84.0	19.4	3.9	100.5	103.6	71.4	19.6	4.4
Experiment II/ 4 h treatment												
Solvent control with DMSO		+	100.0	100.0	100.0	20.4	4.0	100.0	100.0	100.0	3.6	1.0
Positive control (DMBA)	1.1	+	49.6	56.6	89.4	988.8	48.5	58.6	84.4	95.6	1186.2	327.0
Test item	156.3	+	96.1	culture was not continued*				95.8	culture was not continued*			
Test item	312.5	+	99.1	106.9	94.0	9.7	0.5	92.7	102.1	95.6	13.9	3.8
Test item	625.0	+	92.3	98.1	92.5	13.7	0.7	96.8	124.0	95.9	3.3	0.9
Test item	1250.0	+	93.3	106.0	90.4	14.7	0.7	96.4	97.9	96.2	13.6	3.8
Test item	2500.0	+	95.3	101.1	91.4	15.7	0.8	93.5	96.7	95.6	16.9	4.7
Test item	3000.0	+	92.8	93.4	93.3	15.9	0.8	94.9	97.5	91.2	14.0	3.9
* culture was not continued since a minimum of only four analysable concentrations is required												

III. Conclusions

Technical AE F147447 did not demonstrate mutagenic potential in this in vitro HPRT cell mutation assay, under the experimental conditions described.

AE F160460

Report:	[redacted]6;[redacted];2012;M-428745-01
Title:	Salmonella typhimurium reverse mutation assay with AE F160460
Report No:	1462301
Document No:	M-428745-01-1
Guidelines:	**OECD No. 471, adopted July 21, 1997** **EC No. 440/2008 B13/14, dated May 30, 2008** **EPA OPPTS 870.5100, EPA 712-C-98-247, August 1998;not specified**
GLP/GEP:	**yes**

Executive summary:
This study was performed to investigate the potential of AE F160460 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.
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 item 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
The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without metabolic activation in both independent experiments.
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 AE F160460 at any dose level, 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 as positive controls 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 item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.
Therefore, AE F160460 is considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

I. Material and methods

1 Test Material: **AE F160460**

Description:	Off white powder
Lot/Batch:	SES 11562-12-4
Purity:	96.7 % w/w, Dose calculation adjusted to purity
Stability of test compound:	not performed as part of this study.
Solvent used:	DMSO

2 Control materials:

Negative:		Culture medium
Solvent:		DMSO
Positive:	*non- activation (-S9 mix):*	
		sodium azide: 10 µg/plate for TA1535 and TA100
		4-nitro-o-phenylene-diamine, 4-NOPD: 50 µg/plate for TA1537 and 10 µg/plate for TA98
		methyl methane sulfonate, MMS: 3.0 µL/plate for TA 102
	activation (+ S9 mix) :	
		2-aminoanthracene, 2-AA : 2.5 µg/plate for TA 1535, TA 1537, TA 98, TA 100, and 10 µg/plate for TA 102

3 Metabolic activation: S9 mix derived from male Wistar rats

Phenobarbital/β-naphthoflavone induced rat liver S9 were used as the metabolic activation system. The S9 is prepared from 8 – 12 weeks old male Wistar rats (Hsd Cpb: WU; weight approx. 220 – 320 g, [redacted] The Netherlands) induced by intraperitoneal applications of 80 mg/kg b.w. phenobarbital ([redacted], Germany) and by oral administrations of β-naphthoflavone ([redacted], Germany). Each batch of S9 mix is routinely tested with 2-aminoanthracene as well as benzo[a]pyrene. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix: 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate and 4mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. During the experiment the S9 mix was stored in an ice bath.

4 Test organisms:

Salmonella typhimurium, strains TA1535, TA1537, TA98, TA100 and TA 102 obtained from [redacted], Germany). Regular checking of the properties of the strains regarding the membrane permeability, ampicillin- and tetracycline-resistance as well as spontaneous mutation rates are performed in the laboratory of [redacted].

5 Test 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

B Study Design and Methods:

1 Test performance: The study was conducted at [redacted] ([redacted]/Germany) from January 19th to March 2nd 2012.

2 Assay procedure:

Preliminary cytotoxicity/mutation assay:
To evaluate the toxicity of the test item a pre-experiment was performed with all strains used. Eight concentrations were tested for toxicity and mutation induction with each 3 plates. Toxicity of the test item can be evident as a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn. In this 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 overlay agar (45 °C) was added to each tube. The mixture was poured on minimal agar plates and after solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark.

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 and after solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark.

3 Statistics:

According to OECD 471 a statistical analysis is not mandatory.

4 Acceptability of the assay:

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 a significant increase in mutant colony frequencies
- 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.

5 Evaluation criteria:

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding 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 is observed .
A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration. An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.
A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and Discussion

1 Analytical determinations:

The stability of AE F160460 and the stability and homogeneity of AE F160460 in the solvent and analysis of achieved concentration were not determined as part of this study.

2 Preliminary cytotoxicity assay:

The plates incubated with the test item 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.

3 Mutation assays:

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with AE F160460 at any dose level, 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.

The results are summarized in the following tables:

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		21 ± 1	10 ± 2	26 ± 7	91 ± 4	316 ± 19
	Untreated		23 ± 1	10 ± 2	23 ± 4	96 ± 6	345 ± 39
	AE F160460	3 µg	23 ± 4	9 ± 2	25 ± 3	81 ± 3	280 ± 11
		10 µg	17 ± 4	10 ± 3	26 ± 5	78 ± 13	288 ± 5
		33 µg	22 ± 8	10 ± 0	23 ± 5	87 ± 8	279 ± 11
		100 µg	19 ± 2	9 ± 4	27 ± 7	82 ± 11	306 ± 24
		333 µg	17 ± 5	12 ± 4	25 ± 3	69 ± 4	286 ± 20
		1000 µg	16 ± 5	12 ± 3	18 ± 1	75 ± 4	284 ± 16
		2500 µg	16 ± 5	9 ± 3	23 ± 8	71 ± 5	270 ± 23
		5000 µg	20 ± 4	11 ± 3	25 ± 5	62 ± 10	272 ± 23
	NaN3	10 µg	1386 ± 14			1691 ± 154	
	4-NOPD	10 µg			365 ± 15		
	4-NOPD	50 µg		63 ± 1			
	MMS	3.0 µL					3422 ± 59
With Activation	DMSO		26 ± 3	19 ± 3	35 ± 5	106 ± 2	339 ± 3
	Untreated		31 ± 2	25 ± 5	40 ± 11	122 ± 6	378 ± 40
	AE F160460	3 µg	29 ± 7	20 ± 6	35 ± 4	94 ± 12	321 ± 61
		10 µg	26 ± 3	22 ± 7	38 ± 2	93 ± 8	291 ± 15
		33 µg	30 ± 5	23 ± 4	33 ± 4	104 ± 4	346 ± 49
		100 µg	24 ± 3	24 ± 4	38 ± 4	94 ± 11	368 ± 25
		333 µg	30 ± 4	15 ± 4	34 ± 4	103 ± 9	277 ± 5
		1000 µg	31 ± 5	21 ± 5	39 ± 5	108 ± 3	294 ± 30
		2500 µg	31 ± 4	21 ± 7	33 ± 2	102 ± 4	272 ± 17
		5000 µg	26 ±	23 ± 4	36 ± 4	102 ± 9	290 ± 33
	2-AA	2.5 µg	242 ± 22	148 ± 47	1731 ± 48	1770 ± 110	
	2-AA	10.0 µg					2237 ± 32

NaN3	sodium azide
2-AA	2-aminoanthracene
MMS	methyl methane sulfonate
4-NOPD	4-nitro-o-phenylene-diamine

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		18 ± 3	16 ± 6	25 ± 5	104 ± 6	391 ± 25
	Untreated		14 ± 2	23 ± 8	21 ± 4	124 ± 14	387 ± 15
	AE F160460	33 µg	17 ± 3	17 ± 4	25 ± 4	104 ± 15	391 ± 27
		100 µg	16 ± 2	15 ± 2	25 ± 7	108 ± 11	392 ± 17
		333 µg	18 ± 7	18 ± 2	18 ± 3	109 ± 26	394 ± 14
		1000 µg	16 ± 3	19 ± 6	31 ± 5	87 ± 9	382 ± 19
		2500 µg	12 ± 4	17 ± 2	29 ± 8	96 ± 13	368 ± 23
		5000 µg	15 ± 2	13 ± 3	29 ± 5	101 ± 15	349 ± 9
	NaN3	10 µg	1889 ± 33			2077 ± 83	
	4-NOPD	10 µg			372 ± 24		
	4-NOPD	50 µg		87 ± 20			
	MMS	3.0 µL					3583 ± 505
With Activation	DMSO		21 ± 2	18 ± 5	38 ± 6	131 ± 12	483 ± 70
	Untreated		25 ± 4	23 ± 3	42 ± 5	137 ± 7	570 ± 81
	AE F160460	33 µg	20 ± 4	22 ± 2	32 ± 5	136 ± 15	464 ± 58
		100 µg	18 ± 4	18 ± 8	33 ± 3	143 ± 29	439 ± 32
		333 µg	26 ± 3	20 ± 1	34 ± 1	122 ± 7	449 ± 63
		1000 µg	22 ± 6	26 ± 3	34 ± 6	130 ± 10	515 ± 35
		2500 µg	19 ± 4	18 ± 2	36 ± 2	124 ± 21	516 ± 74
		5000 µg	24 ± 8	19 ± 4	38 ± 9	128 ± 21	523 ± 47
	2-AA	2.5 µg	326 ± 27	246 ± 22	1930 ± 52	1929 ± 41	
	2-AA	10.0 µg					2967 ± 656

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, AE F160460 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Report:	[redacted]6; [redacted];2012;M-433910-02; Amended: 2015-01-30
Title:	In vitro chromosome aberration test in Chinese hamster V79 cells with AE F160460
Report No:	1462302
Document No:	M-433910-02-1
Guidelines:	Ninth Addendum to the OECD Guidelines for Testing of Chemicals, February 1998, adopted July 21, 1997, Guideline No. 473 In vitro Mammalian Chromosome Aberration Test; Commission Regulation (EC) No. 440/2008, B10: Mutagenicity - In vitro Mammalian Chromosome Aberration Test, dated May 30, 2008; United States Environmental Protection Agency Health Effects Test Guidelines, OPPTS 870.5375, In Vitro Mammalian Chromosome Aberration Test, EPA 712-C-98-223, August 1998;not specified
GLP/GEP:	**yes**

Executive summary:
The test item AE F1604605, dissolved in DMSO, was assessed for its potential to induce structural chromosome aberrations in V79 cells of the Chinese hamster in vitro in two independent experiments. The following study design was performed:

	Without S9 mix		With S9 mix	
	Exp. I	Exp. II	Exp. I	Exp. II
Exposure period	4 hrs	18 hrs	4 hrs	4 hrs
Recovery	14 hrs	-	14 hrs	14 hrs
Preparation interval	18 hrs	18 hrs	18 hrs	18 hrs

In each experimental group two parallel cultures were set up. 100 metaphases per culture were evaluated for structural chromosome aberrations, except for the positive control in Experiment II without S9 mix, where only 50 metaphases were evaluated. The highest applied concentration (2400.0 µg/mL) was chosen with regard to the solubility properties of the test item and with respect to the current OECD Guideline 473. Dose selection was performed considering the toxicity data and the occurrence of precipitation. In the absence and presence of S9 mix no cytotoxicity was observed up to the highest applied concentration.
No clastogenicity was observed at the concentrations evaluated either with or without metabolic activation. No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.
Appropriate positive controls induced statistically significant increases in cells with structural chromosome aberrations.
It can be stated that under the experimental conditions reported, the test item did not induce structural chromosome aberrations in V79 cells (Chinese hamster cell line) in vitro. Therefore, AE F160460 is considered to be non-clastogenic in this chromosome aberration test, when tested up to the highest applied concentration.

I. Material and methods

A Materials

1 Test Material: **AE F160460**
Description: Off white powder
Lot/Batch: SES 11562-12-4
Purity: 96.7 % w/w, Dose calculation adjusted to purity
Stability of test compound: not performed as part of this study.
Solvent used: DMSO

2 Control materials:
Negative: MEM Tissue Culture medium
Positive - Without metabolic activation:

EMS; ethylmethane sulfonate, dissolved in Nutrient medium
Final Concentration: 1000.0 µg/mL (Experiment I) 600.0 µg/mL (Experiment II)

Positive - With metabolic activation:

CPA; cyclophosphamide, dissolved in Saline (0.9 % [w/v])
Final Concentration: 1.4 µg/mL

The dilutions of the stock solutions were prepared on the day of the experiment. The stability of the positive control substances (EMS and CPA, respectively) in solution is unknown but a mutagenic response in the expected range was sufficient biological evidence of chemical stability.

3 Metabolic activation S9 mix: S9 mix from male Wistar rats

The S9 was prepared from 8 - 12 weeks old male Wistar rats ([redacted] The Netherlands) weight approx. 220 - 320 g induced by intraperitoneal applications of 80 mg/kg b.w. phenobarbital ([redacted] Germany) and by orally administrations of 80 mg/kg b.w. β-naphthoflavone ([redacted] Germany) each, on three consecutive days. Each batch of S9 mix was routinely tested with 2-aminoanthracene as well as benzo(a)pyrene. For the experiments, an appropriate quantity of S9 supernatant 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).

5 Test cells: The V79 cell line has been used successfully for many years in in vitro experiments. The high proliferation rate and a reasonable plating efficiency of untreated cells (as a rule more than 70 %), both necessary for the appropriate performance of the study, support the use of this cell line. The cells have a stable karyotype with a modal chromosome number of 22 ± 1.

6 Test concentrations:

Preparation interval	Exposure period	Exp.	Concentration in µg/mL								
			Without S9 mix								
18 hours	4 hours	I	9.4	18.8	37.5	75.0	150.0	300.0	**600.0**	**1200.0**	**2400.0**[P]
18 hours	18 hours	II	9.4	18.8	37.5	75.0	150.0	300.0	**600.0**	**1200.0**	**2400.0**
			With S9 mix								
18 hours	4 hours	I	9.4	18.8	37.5	75.0	150.0	300.0	**600.0**	**1200.0**	**2400.0**[P]
18 hours	4 hours	II					150.0	300.0	**600.0**	**1200.0**	**2400.0**

Evaluated experimental points are shown in bold characters

[P] Precipitation occurred at the end of treatment

B Study Design and Methods:

1 Study performance:

The study was conducted at [redacted] ([redacted]/Germany). The experimental start and completion dates of the study were January 25th 2012 and February 25th 2012, respectively.

2 Culture Medium and Conditions:

For seeding and treatment of the cell cultures the culture medium was MEM (minimal essential medium) containing Hank's salts, glutamine and Hepes (25 mM). Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/ 100 µg/mL) and 10 % (v/v) fetal bovine serum (FBS). All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO2 (98.5 % air).

3 Seeding of the Cultures:

Exponentially growing stock cultures more than 50 % confluent were rinsed with Ca-Mg-free salt solution containing 8000 mg/L NaCl, 200 mg/L KCl, 200 mg/L KH_2PO_4 and 150 mg/L Na_2HPO_4. Afterwards the cells were treated with trypsin-EDTA-solution at 37 °C for approx. 5 minutes. Then, by adding complete culture medium including 10 % (v/v) FBS the enzymatic treatment were stopped and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.5 % (w/v) in Ca-Mg-free salt solution. The cells were seeded into Quadriperm dishes, which contained microscopic slides. Into each chamber 1 x 10^4 – 6 x 10^4 cells were seeded with regard to the preparation time.

4 Treatment after the 4-hour Exposure period:

The culture medium of exponentially growing cell cultures was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium were added. After 4 hours the cultures were washed twice 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_2PO4. The cells were then cultured in complete medium containing 10 % (v/v) FBS for the remaining culture time of 14 hours. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO2 (98.5 % air).

5 Slides preparation:

Colcémid was added to the culture medium (0.2 µg/mL) 15.5 hours after the start of the treatment. The cells were treated, 2.5 hours later, on the slides in the chambers with hypotonic solution (0.4 % KCl) for 20 min at 37 °C. After incubation in the hypotonic solution the cells were fixed with a mixture of methanol and glacial acetic acid (3:1 parts, respectively). After preparation the cells were stained with Giemsa and labelled with a computer-generated random code to prevent scorer bias.

6 Evaluation of Cell Numbers:

The evaluation of cytotoxicity indicated by reduced cell numbers was made after the preparation of the cultures on spread slides. The cell numbers were determined microscopically by counting 10 defined

fields per coded slide. The cell number of the treatment groups is given in percentage compared to the respective solvent control.

7 Analysis of Metaphase Cells:
Evaluation of the cultures was performed according to the OECD guideline using NIKON microscopes with 100x objectives. Breaks, fragments, deletions, exchanges, and chromosome disintegrations were recorded as structural chromosome aberrations. Gaps were recorded as well but not included in the calculation of the aberration rates. 100 well spread metaphases per culture were evaluated for cytogenetic damage on coded slides.
Only metaphases with characteristic chromosome numbers of 22 ± 1 were included in the analysis. To describe a cytotoxic effect the mitotic index (% cells in mitosis) was determined.

8 Acceptability of the Test:
The chromosome aberration test is considered acceptable, if it meets the following criteria:

- The number of structural aberrations found in the solvent controls falls within the range of the laboratory historical control data
- The positive control substances produce significant increases in the number of cells with structural chromosome aberrations, which are within the range of the laboratory historical control data.

9 Evaluation of Results:
A test item is classified as non-clastogenic if:

- the number of induced structural chromosome aberrations in all evaluated dose groups is in the range of the laboratory historical control data and
- no significant increase of the number of structural chromosome aberrations is observed.

A test item is classified as clastogenic if:

- the number of induced structural chromosome aberrations is not in the range of the laboratory historical control data and
- either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.

Both biological and statistical significance should be considered together. If the criteria mentioned above for the test item are not clearly met, the classification with regard to the historical data and the biological relevance is discussed and/or a confirmatory experiment is performed.
In this study also polyploids and endoreduplications were considered together with chromosome aberration. A test item can be classified as aneugenic if the number of induced numerical aberrations is not in the range of the laboratory historical control data

II. Results and discussion
Two independent experiments were performed. In Experiment I the exposure period was 4 hours with and without S9 mix. In Experiment II the exposure period was 4 hours with S9 mix and 18 hours without S9 mix. The chromosomes were prepared 18 hours after start of treatment with the test item.
In each experimental group two parallel cultures were set up. 100 metaphases per culture were evaluated for structural chromosome aberrations, except for the positive control in Experiment II without S9 mix, where only 50 metaphases were evaluated. The highest treatment concentration in this study, 2400.0

µg/mL was chosen with regard to the solubility properties of the test item and with respect to the OECD Guideline for in vitro mammalian cytogenetic tests.
Visible precipitation of the test item in the culture medium was observed in Experiment I at 2400.0 µg/mL in the absence and presence of S9 mix at the end of treatment. In the absence and presence of S9 mix no cytotoxicity was observed up to the highest applied concentration.
In the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying structural chromosome aberrations was observed. The aberration rates of the cells after treatment with the test item (0.0 - 3.5 % aberrant cells, excluding gaps) were within the range of the solvent control values (2.0 -4.0 % aberrant cells, excluding gaps) and within the range of the laboratory historical solvent control data. No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.In both experiments, either EMS (600.0 or 1000.0 µg/mL) or CPA (1.4 µg/mL) were used as positive controls which showed distinct increases in cells with structural chromosome aberrations.

The results are summarized in the following table:

Exp.	Preparation interval	Test item concentration in µg/mL	Cell numbers in % of control	Mitotic indices in % of control	Aberrant cells in % incl. gaps*	Aberrant cells in % excl. gaps*	Aberrant cells in % with exchanges
		Exposure period 4 hrs without S9 mix					
I	18 hrs	Solvent control[1]	100.0	100.0	2.5	2.0	0.0
		Positive control[2]	n.d.	70.6	10.5	**9.5**[S]	5.0
		600.0	99.5	90.9	2.0	1.5	0.0
		1200.0	99.8	95.3	2.0	2.0	0.0
		2400.0[P]	85.6	110.6	2.5	2.0	0.5
		Exposure period 18 hrs without S9 mix					
II	18 hrs	Solvent control[1]	100.0	100.0	3.5	3.5	0.5
		Positive control[3#]	n.d.	73.2	42.0	**39.0**[S]	16.0
		600.0	104.1	107.7	1.0	0.0	0.0
		1200.0	117.1	119.5	3.5	3.5	0.5
		2400.0	109.5	95.6	3.5	3.5	0.5
		Exposure period 4 hrs with S9 mix					
I	18 hrs	Solvent control[1]	100.0	100.0	4.0	2.5	0.5
		Positive control[4]	n.d.	74.1	9.0	**9.0**[S]	5.5
		600.0	80.0	107.0	1.5	1.5	0.5
		1200.0	71.7	110.5	3.0	3.0	1.0
		2400.0[P]	87.7	104.2	2.0	2.0	0.5
II	18 hrs	Solvent control[1]	100.0	100.0	4.5	4.0	0.5

Positive control[4]	n.d.	76.1	18.5	**18.5[S]**	6.0
600.0	81.0	85.7	3.0	3.0	0.5
1200.0	77.2	93.8	2.5	2.5	0.5
2400.0	100.4	114.0	3.5	3.0	1.0

III. Conclusions

In conclusion, it can be stated that under the experimental conditions reported, the test item AE F160460 did not induce structural chromosomal aberrations in V79 cells of the Chinese hamster in vitro, when tested up to the highest applied concentration.

Report:	[redacted];2012;M-433923-02; Amended: 2015-01-19
Title:	Gene mutation assay in Chinese hamster V79 cells in vitro (V79 / HPRT) - AE F160460
Report No:	1462303
Document No:	M-433923-02-1
Guidelines:	Ninth Addendum to the OECD Guidelines for Testing of Chemicals, Section 4, No. 476: In vitro Mammalian Cell Gene Mutation Test, adopted July 21, 1997; Commission Regulation (EC) No. 440/2008, B17, dated May 30, 2008; United States Environmental Protection Agency Health Effects Test Guidelines, OPPTS 870.5300, In Vitro Mammalian Cell Gene Mutation Test, EPA 712-C-98-221, August 1998;not specified
GLP/GEP:	yes

Executive summary:
The study was performed to investigate the potential of AE F160460 to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster.
The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with a treatment time of 4 hours with and of 24 hours without metabolic activation.
The highest concentration of the pre-experiment (2400 µg/mL) was limited by the solubility properties of the test item in DMSO. The concentration range of the main experiments was limited by cytotoxic effects and the solubility of the test item.
No substantial and reproducible dose dependent increase of the mutation frequency was observed in both main experiments.
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, AE F160460 is considered to be non-mutagenic in this HPRT assay.

I. Material and methods

A Materials

1 Test Material: **AE F160460**

Description:	Off white powder
Lot/Batch:	SES 11562-12-4
Purity:	96.7 % w/w, Dose calculation adjusted to purity
Stability of test compound:	not performed as part of this study.
Solvent used:	dimethylsulfoxide (DMSO)

2 Control materials:

Negative:	Tissue Culture medium
Positive: *non- activation (-S9 mix)*	Ethyl methanesulphonate (EMS) dissolved in nutrient medium. Final concentration 0.15 mg/mL (1.2 mM)
Positive: *with activation (+S9 mix)*	DMBA; 7,12-dimethylbenz(a)anthracene dissolved in DMSO. Final concentration 1.1 µg/mL (4.3 µM)

3 Metabolic activation: S9 mix from male Wistar rats

Phenobarbital/β-Naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from 8 – 12 weeks old male Wistar rats (Hsd Cpb: WU, weight approx. 220 – 320 g, [redacted] The Netherlands) induced by intraperitoneal applications of 80 mg/kg b.w. phenobarbital and by peroral administrations of β-naphthoflavone each, on three consecutive days.

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 supernatant to reach following concentrations in the S9 mix were: 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate and 4 mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. During the experiment, the S9 mix was stored in an ice bath.

4 Test cells:

The V79 cell line has been used successfully in *in vitro* experiments for many years. Especially the high proliferation rate (doubling time 12 -16 h in stock cultures) and a good cloning efficiency of untreated cells (as a rule more than 50 %) both necessary for the appropriate performance of the study, recommend the use of this cell line. The cells have a stable karyotype with a modal chromosome number of 22.

5 Test compound concentrations used (µg/mL):

Exposure period	S9 mix	Concentrations in µg/mL					
		Experiment I					
4 hours	-	75	**150**	**300**	**600**	**1200**	**2400**
4 hours	+	75	**150**	**300**	**600**	**1200**	**2400**
		Experiment II					
24 hours	-	150	**300**	**600**	**1200**	**2400**	**3600**
4 hours	+	300	**600**	**1200**	**1800**	**2400** [P]	**3600** [P]

Concentrations given in bold letters were chosen for the mutation rate analysis
[P] Precipitation

B Study Design and Methods:

1 Test performance:
The study was conducted at [redacted]/Germany). The experimental start and completion dates of the study were February 2nd 2012 and March 27th 2012, respectively.

2 Culture medium and conditions:
For seeding and treatment of the cell cultures the complete culture medium was MEM (minimal essential medium) containing Hank's salts, neomycin (5 µg/mL) and amphotericin B (1 %). For the selection of mutant cells the complete medium was supplemented with 11 µg/mL 6-thioguanine. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

3 Seeding of the cultures:
Two to three days after sub-cultivation stock cultures were trypsinized at 37 °C for 5 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 PBS.
Prior to the trypsin treatment the cells were rinsed with Ca-Mg-free salt solution containing 200 mg/l EDTA (ethylene diamine tetraacetic acid). Approximately 1.5 x 10^6 (single culture) and 5 x 10^2 cells (in duplicate) were seeded in plastic culture flasks. The cells were grown for 24 hours prior to treatment.

4 Cell treatment:
After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps with "saline G". In the second experiment the cells were exposed to the test item for 24 hours in complete medium, supplemented with 10 % FBS, in the absence of metabolic activation. The pH was adjusted to 7.2
Three or four days after treatment 1.5×10^6 cells per experimental point were subcultivated in 175 cm² flasks containing 30 mL medium. Following the expression time of 7 days five 80 cm² cell culture flasks

were seeded with about 3 - 5×105 cells each in medium containing 6-TG. Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability.
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.

5 Evaluation of cell numbers:

The stained colonies with more than 50 cells were counted. In doubt the colony size was checked with a preparation microscope.

6 Acceptability of the Assay:

The gene mutation assay is considered acceptable if it meets the following criteria:

- the numbers of mutant colonies per 10^6 cells found in the solvent controls falls within the laboratory historical control data (see Annex).
- the positive control substances should produce a significant increase in mutant colony frequencies.
- the cloning efficiency II (absolute value) of the solvent controls should exceed 50 %.

7 Evaluation of Results:

A test item is classified as positive if it induces either a concentration-related increase of the mutant frequency or a reproducible and positive response at one of the test points.
A test item producing neither a concentration-related increase of the mutant frequency nor a reproducible positive response at any of the test points is considered non-mutagenic in this system.
A positive response is described as follows:
A test item is classified as mutagenic if it reproducibly induces a mutation frequency that is three times above the spontaneous mutation frequency at least at one of the concentrations in the experiment.
The test item is classified as mutagenic if there is a reproducible concentration-related increase of the mutation frequency. Such evaluation may be considered also in the case that a threefold increase of the mutant frequency is not observed.
However, in a case by case evaluation this decision depends on the level of the corresponding solvent control data. If there is by chance a low spontaneous mutation rate within the laboratory´s historical control data range, a concentration-related increase of the mutations within this range has to be discussed. The variability of the mutation rates of solvent controls within all experiments of this study was also taken into consideration.

8 Statistical Analysis:

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. The numbers of mutant colonies 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. However, both, biological and statistical significance was considered together.

II. Results and discussion

The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The following concentrations were tested: 150, 300, 600, 1200 and 2400 µg/mL.

The second experiment was performed with a treatment time of 4 hours with and 24 hours without metabolic activation. The following concentrations were tested: 300, 600, 1200, 2400 and 3600 µg/mL without S9 and 600, 1200, 1800, 2400 and 3600 µg/mL with S9. No relevant and reproducible increase in mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration.
The threshold was reached or exceeded at 2400 µg/mL in the second culture of the first experiment without metabolic activation. This increase however, was neither reproduced in the parallel culture under identical conditions nor in the second experiment without metabolic activation and consequently, judged as biologically irrelevant. A similar increase occurred in both cultures of the first experiment with metabolic activation at 2400 µg/mL. The historical range of solvent controls was exceeded in culture one but not in culture II. Again, this increase was not reproduced in the second experiment at 2400 µg/mL or at any other, even higher concentration and therefore, judged as irrelevant. In a linear regression analysis (least squares) a significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in both cultures of the first experiment with metabolic activations. This trend was judged as irrelevant since the values of the mutation frequency remained either within the historical range of solvent controls or the increase was not reproduced as discussed above.
EMS (150 µg/mL) and DMBA (1.1 µg/mL) were used as positive controls and showed a distinct increase in induced mutant colonies.

The results are summarized in the following table:

AE F160460 HPRT, 1462303													
	Conc. µg/mL	P	S9 - mix	Relative cloning efficiency I	Relative cell density	Relative cloning efficiency II	Mutant colonies/ 10^6 cells	Induction factor	Relative cloning efficiency I	Relative cell density	Relative cloning efficiency II	Mutant colonies/ 10^6 cells	Induction factor
				%	%	%			%	%	%		
Column	1	2	3	4	5	6	7	8	9	10	11	12	13
Experiment I / 4 h treatment				Culture I					Culture II				
Solvent control with DMSO			-	100.0	100.0	100.0	19.8	1.0	100.0	100.0	100.0	9.2	1.0
Positice control (EMS)	150.0		-	72.7	78.9	80.6	94.1	4.8	69.8	97.8	94.2	112.4	12.2
Test item	75.0		-	96.1	Culture was not continued#					Culture was not continued#			
Test item	150.0		-	87.0	83.7	87.8	6.1	0.3	90.3	100.9	100.5	21.7	2.3
Test item	300.0		-	85.2	78.3	79.7	10.7	0.5	82.7	104.8	115.6	11.8	1.3
Test item	600.0		-	84.4	76.1	82.0	10.3	0.5	78.8	102.3	103.7	12.3	1.3

Test item	1200.0		-	82.3	79.2	83.3	2.4	0.1	70.1	101.6	132.5	11.9	1.3
Test item	2400.0		-	0.0	48.1	71.6	12.6	0.6	0.0	61.0	96.2	31.3	3.4
Solvent control with DMSO			+	100.0	100.0	100.0	8.3	1.0	100.0	100.0	100.0	8.5	1.0
Positice control (DMBA)	1.1		+	70.4	112.2	84.4	403.2	48.7	60.3	108.7	58.0	394.2	72.3
Test item	75.0		+	97.0	Culture was not continued[#]				89.1	Culture was not continued[#]			
Test item	150.0		+	96.7	104.3	88.2	15.6	1.9	88.6	100.0	92.3	7.9	1.4
Test item	300.0		+	94.1	104.7	83.7	14.0	1.7	87.1	97.7	92.4	9.5	1.7
Test item	600.0		+	98.3	110.8	68.1	17.5	2.1	81.5	109.2	91.0	10.0	1.8
Test item	1200.0		+	84.1	107.3	87.6	10.3	1.2	71.1	89.5	87.1	10.6	1.9
Test item	2400.0		+	62.4	87.0	66.0	12.0	0.3	56.6	105.3	86.9	22.6	4.1
Experiment II / 24 h treatment				Culture I					Culture II				
Solvent control with DMSO				100.0	100.0	100.0	11.4	1.0	100.0	100.0	100.0	23.1	1.0
Positice control (EMS)	150.0		-	92.3	95.5	84.8	231.8	20.3	94.4	79.3	109.6	236.0	10.2
Test item	150.0		-	29.5	Culture was not continued[#]				96.3	Culture was not continued[#]			
Test item	300.0		-	102.4	132.8	109.5	7.3	0.6	106.5	97.8	110.7	13.8	0.6
Test item	600.0		-	99.7	106.8	80.4	19.4	1.7	108.2	105.4	116.9	10.5	0.5
Test item	1200.0		-	124.1	131.4	92.2	15.9	1.4	94.4	100.3	115.6	8.2	0.4
Test item	2400.0		-	48.9	139.5	108.2	9.4	0.8	40.4	96.3	111.0	4.6	0.2
Test item	3600.0		-	120.6	155.6	98.7	17.1	1.5	97.1	103.3	100.1	7.5	0.3
Experiment II / 4 h treatment													
Solvent control with			+	100.0	100.0	100.0	14.3	1.0	100.0	100.0	100.0	9.9	1.0

DMSO													
Positice control (EMS)	1.1		+	84.7	102.9	71.8	512.1	35.9	86.3	101.7	88.5	495.6	49.9
Test item	300.0		+	93.0	Culture was not continued#				91.8	Culture was not continued#			
Test item	600.0		+	93.5	102.4	90.2	13.7	1.0	92.0	94.5	72.5	9.6	1.0
Test item	1200.0		+	93.5	115.4	91.3	18.7	1.3	93.6	88.6	77.0	6.4	0.6
Test item	1800.0		+	94.2	121.4	83.6	5.1	0.4	91.0	92.7	86.9	15.7	1.6
Test item	2400.0	P	+	91.0	127.0	86.2	14.9	1.0	96.9	101.6	85.7	21.8	2.2
Test item	3600.0	P	+	99.2	121.3	74.6	14.6	1.0	91.4	99.6	97.7	16.7	1.7
# culture was not continued since a minimum of only four analysable concentrations is required													
P Precipitation													

III. Conclusions

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, AE F160460 is considered to be non-mutagenic in this HPRT assay.

BCS-CV14885

Report:	[redacted]9; [redacted];2012;M-448998-01
Title:	Salmonella typhimurium reverse mutation assay with BCS-CV14885
Report No:	1490201
Document No:	M-448998-01-1
Guidelines:	Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471: Bacterial Reverse Mutation Test, adopted July 21, 1997; Commission Regulation (EC) No. 440/2008 B13/14, dated May 30, 2008; EPA Health Effects Test Guidelines, OPPTS 870.5100, Bacterial Reverse Mutation Test; EPA 712-C-98-247, August, 1998;none
GLP/GEP:	**yes**

Executive summary:

This study was performed to investigate the potential of BCS-CV14885 to induce gene mutations in 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.

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 item 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

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without metabolic activation in both independent experiments.

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 BCS-CV14885 at any dose level, 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.

The test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, BCS-CV14885 is considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

I. Material and methods

A. Materials

1 Test material: **BCS-CV14885**

Description:	white powder
Lot/Batch:	BCS-CV14885-01-01
Purity:	97.8 % w/w, dose calculation will be adjusted to purity
Stability of test compound:	not performed as part of this study
Solvent used:	DMSO

2 Control materials:

Negative:	Culture medium
Solvent:	DMSO
Positive: *non- activation (-S9 mix):*	
	sodium azide: 10 µg/plate for TA1535 and TA100
	4-nitro-o-phenylene-diamine, 4-NOPD: 50 µg/plate for TA1537 and 10 µg/plate for TA98
	methyl methane sulfonate, MMS: 3.0 µL/plate for TA 102
activation (+ S9 mix):	
	2-aminoanthracene, 2-AA : 2.5 µg/plate for TA 1535, TA 1537, TA 98, TA 100, and 10 µg/plate for TA 102

3 Metabolic activation: S9 mix derived from male Wistar rats

Phenobarbital/β-naphthoflavone induced rat liver S9 were used as the metabolic activation system. The S9 is prepared from 8 – 12 weeks old male Wistar rats (Hsd Cpb: WU; weight approx. 220 – 320 g, [redacted] The Netherlands) induced by intraperitoneal applications of 80 mg/kg b.w. phenobarbital ([redacted], Germany) and by peroral administrations of β-naphthoflavone ([redacted], Germany). Each batch of S9 mix is routinely tested with 2-aminoanthracene as well as benzo[a]pyrene. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix: 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-

phosphate and 4mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. During the experiment the S9 mix was stored in an ice bath.

4 Test organisms:
Salmonella typhimurium, strains TA1535, TA1537, TA98, TA100 and TA 102 obtained from [redacted], Germany). Regular checking of the properties of the strains regarding the membrane permeability, ampicillin- and tetracycline resistance as well as spontaneous mutation rates are performed in the laboratory of Harlan CCR.

5 Test 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

B Study Design and Methods:
1 Test performance: The study was conducted at [redacted] (Germany) from July 16th to August 1st, 2012.

2 Assay procedure:
Preliminary cytotoxicity/mutation assay:
To evaluate the toxicity of the test item a pre-experiment was performed with all strains used. Eight concentrations were tested for toxicity and mutation induction with each 3 plates. Toxicity of the test item can be evident as a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn. The pre-experiment is reported as main experiment I, since the following criteria are met: evaluable plates (>0 colonies) at five concentrations or more in all strains used.

3 Statistics:
According to OECD 471 a statistical analysis is not mandatory.

4 Acceptability of the assay:
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 a significant increase in mutant colony frequencies
- 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.

5 Evaluation criteria:

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding 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 is observed.

A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration. An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and Discussion

1 Analytical determinations:

The stability of BCS-CV14885 and the stability and homogeneity of BCS-CV14885 in the solvent and analysis of achieved concentration were not determined as part of this study.

2 Preliminary cytotoxicity assay:

The plates incubated with the test item 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.

3 Mutation assay:

The test item BCS-CV14885 was assessed for its potential to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

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 item 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

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without metabolic activation in both independent experiments.

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 BCS-CV14885 at any dose level, 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 as positive controls showed a distinct increase of induced revertant colonies.

The results are summarized in the following tables:

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		16 ± 7	11 ± 4	29 ± 2	167 ± 10	392 ± 35
	Untreated		20 ± 7	10 ± 3	29 ± 2	165 ± 8	409 ± 31
	BCS-CV14885	3 µg	18 ± 3	12 ± 3	26 ± 8	174 ± 11	437 ± 28
		10 µg	17 ± 5	12 ± 2	23 ± 7	159 ± 12	398 ± 21
		33 µg	15 ± 4	11 ± 4	29 ± 4	169 ± 5	395 ± 17
		100 µg	14 ± 4	12 ± 2	21 ± 2	168 ± 16	406 ± 14
		333 µg	14 ± 2	10 ± 4	26 ± 3	159 ± 4	410 ± 25
		1000 µg	20 ± 6	10 ± 2	21 ± 1	163 ± 20	425 ± 21
		2500 µg	14 ± 2	10 ± 3	20 ± 4	157 ± 5	416 ± 5
		5000 µg	12 ± 5	9 ± 2	18 ± 2	170 ± 14	397 ± 29
	NaN3	10 µg	2006 ± 102			2068 ± 137	
	4-NOPD	10 µg			362 ± 31		
	4-NOPD	50 µg		77 ± 15			
	MMS	3.0 µL					3693 ± 784
With Activation	DMSO		17 ± 3	18 ± 6	25 ± 5	175 ± 10	313 ± 27
	Untreated		22 ± 7	19 ± 4	36 ± 6	193 ± 7	388 ± 67
	BCS-CV14885	3 µg	21 ± 4	18 ± 5	33 ± 6	166 ± 7	520 ± 126
		10 µg	20 ± 3	16 ± 4	28 ± 7	158 ± 17	448 ± 34
		33 µg	17 ± 4	15 ± 3	32 ± 7	166 ± 19	458 ± 22
		100 µg	24 ± 3	15 ± 2	29 ± 8	164 ± 16	436 ± 31
		333 µg	26 ± 5	20 ± 3	43 ± 7	161 ± 14	480 ± 23
		1000 µg	18 ± 4	17 ± 5	32 ± 4	164 ± 15	456 ± 74
		2500 µg	22 ± 6	18 ± 3	28 ± 2	160 ± 11	462 ± 24
		5000 µg	16 ± 1	16 ± 4	28 ± 8	179 ± 26	451 ± 109
	2-AA	2.5 µg	335 ± 25	216 ± 27	1338 ± 147	1982 ± 114	
	2-AA	10.0 µg					1758 ± 337

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		17 ± 4	19 ± 5	27 ± 4	134 ± 9	344 ± 24
	Untreated		16 ± 5	21 ± 6	28 ± 0	179 ± 4	377 ± 18
	BCS-CV14885	33 µg	16 ± 4	17 ± 4	29 ± 7	144 ± 15	378 ± 24
		100 µg	17 ± 4	19 ± 4	25 ± 2	146 ± 14	372 ± 20
		333 µg	15 ± 1	15 ± 1	22 ± 2	149 ± 11	365 ± 19
		1000 µg	17 ± 5	17 ± 3	25 ± 3	148 ± 3	371 ± 18
		2500 µg	13 ± 5	17 ± 2	26 ± 3	143 ± 8	364 ± 21
		5000 µg	13 ± 5	20 ± 2	27 ± 8	161 ± 10	387 ± 23
	NaN3	10 µg	1603 ± 27			1583 ± 28	
	4-NOPD	10 µg			618 ± 71		
	4-NOPD	50 µg		91 ± 12			
	MMS	3.0 µL					1488 ± 236
With Activation	DMSO		17 ± 3	24 ± 6	38 ± 4	140 ± 12	432 ± 35
	Untreated		21 ± 5	32 ± 2	48 ± 12	206 ± 24	521 ± 20
	BCS-CV14885	33 µg	18 ± 4	22 ± 4	37 ± 7	144 ± 7	413 ± 45
		100 µg	16 ± 3	24 ± 4	36 ± 11	149 ± 7	390 ± 14
		333 µg	20 ± 4	26 ± 3	35 ± 3	160 ± 16	386 ± 16
		1000 µg	23 ± 2	21 ± 7	39 ± 5	152 ± 9	390 ± 49
		2500 µg	18 ± 7	20 ± 3	35 ± 8	161 ± 12	416 ± 66
		5000 µg	18 ± 2	27 ± 0	33 ± 9	157 ± 17	359 ± 19
	2-AA	2.5 µg	265 ± 22	184 ± 21	1455 ± 222	1469 ± 140	
	2-AA	10.0 µg					1432 ± 150

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 item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Report:	[redacted] b; [redacted] ;2013;M-449003-02; Amended: 2015-01-30
Title:	In vitro chromosome aberration test in Chinese hamster V79 cells with BCS-CV14885
Report No:	1490202
Document No:	M-449003-02-1
Guidelines:	Ninth Addendum to the OECD Guidelines for Testing of Chemicals, February 1998, adopted July 21, 1997, Guideline No. 473 In vitro Mammalian Chromosome Aberration Test; Commission Regulation (EC) No. 440/2008 B10: Mutagenicity - In vitro Mammalian Chromosome Aberration Test, dated May 30, 2008; United States Environmental Protection Agency Health Effects Test Guidelines, OPPTS 870.5375, In Vitro Mammalian Chromosome Aberration Test, EPA 712-C-98-223, August 1998;none
GLP/GEP:	yes

Executive summary:

The test item BCS-CV14885, dissolved in culture medium (minimal essential medium), was assessed for

its potential to induce structural chromosome aberrations in V79 cells of the Chinese hamster in vitro in three independent experiments. The following study design was performed:

	Without S9 mix		With S9 mix	
	Exp. IA	Exp. II	Exp. IB	Exp. II
Exposure period	4 hrs	18 hrs	4 hrs	4 hrs
Recovery	14 hrs	-	14 hrs	14 hrs
Preparation interval	18 hrs	18 hrs	18 hrs	18 hrs

In each experimental group two parallel cultures were set up. At least 100 metaphases per culture were evaluated for structural chromosome aberrations, except for the positive control in Experiment IA in the absence of S9 mix, where only 50 metaphases were evaluated.
The highest applied concentration (4022.0 µg/mL, approx. 10 mM) was chosen with regard to the molecular weight and the purity (97.8 %) of the test item and with respect to the current OECD Guideline 473.
Dose selection for the cytogenetic experiments was performed considering the toxicity data. In the absence and presence of S9 mix concentrations no cytotoxicity was observed up to the highest applied concentration.
No clastogenicity was observed at the concentrations evaluated. However, a single statistically significant increase was observed in Experiment IA in the absence of S9 mix after treatment with 2011.0 µg/mL (4.0 % aberrant cells, excluding gaps). The value is in the laboratory historical solvent control data range (0.0 – 4.0 % aberrant cells, excluding gaps) and therefore biologically irrelevant.
No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.
Appropriate positive control mutagens induced statistically significant increases in cells with structural chromosome aberrations.
Under the experimental conditions reported, the test item did not induce structural chromosome aberrations in V79 cells (Chinese hamster cell line) in vitro. Therefore, BCS-CV14885 is considered to be non-clastogenic in this chromosome aberration test in the absence and presence of metabolic activation, when tested up to the highest required concentration.

I. Material and methods

A. Materials

1. Test material: **BCS-CV14885**

Description: white powder
Lot/Batch: BCS-CV14885-01-01
Purity: 97.8 % w/w, dose calculation will be adjusted to purity
Stability of test compound: not performed as part of this study
Solvent used: culture medium, (MEM)

2 Control materials:

Negative: MEM Tissue Culture medium

Positive - Without metabolic activation:
EMS; ethylmethane sulfonate, dissolved in Nutrient medium.
Final Concentration: 1000.0 µg/mL (Experiment I) 600.0 µg/mL (Experiment II)

Positive - With metabolic activation:
CPA; cyclophosphamide, dissolved in Saline (0.9 % [w/v]).
Final Concentration: 2.0 µg/mL

The dilutions of the stock solutions were prepared on the day of the experiment. The stability of the positive control substances (EMS and CPA, respectively) in solution is unknown but a mutagenic response in the expected range was sufficient biological evidence of chemical stability.

3 Metabolic activation S9 mix: S9 mix from male Wistar rats

The S9 was prepared from 8 - 12 weeks old male Wistar rats ([redacted] The Netherlands) weight approx. 220 - 320 g induced by intraperitoneal applications of 80 mg/kg b.w. phenobarbital ([redacted], Germany) and by orally administrations of 80 mg/kg b.w. β-naphthoflavone ([redacted], Germany) each, on three consecutive days. Each batch of S9 mix was routinely tested with 2-aminoanthracene as well as benzo(a)pyrene. For the experiments, an appropriate quantity of S9 supernatant 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).

4 Test cells:

The V79 cell line has been used successfully for many years in in vitro experiments. The high proliferation rate and a reasonable plating efficiency of untreated cells (as a rule more than 70 %) both necessary for the appropriate performance of the study, support the use of this cell line. The cells have a stable karyotype with a modal chromosome number of 22 ± 1.

5 Test concentrations:

Preparation interval	Exposure period	Exp.	Concentration in µg/mL								
			Without S9 mix								
18 hrs	4 hrs	IA	15.7	31.4	62.8	125.7	251.4	502.8	**1005.5**	**2011.0**	**4022.0**
18 hrs	18 hrs	II	15.7	31.4	62.8	125.7	251.4	502.8	**1005.5**	**2011.0**	**4022.0**
			With S9 mix								
18 hrs	4 hrs	IA*	15.7	31.4	62.8	125.7	251.4	502.8	1005.5	2011.0	4022.0

Preparation interval	Exposure period	Exp.	Concentration in µg/mL					
18 hrs	4 hrs	IB	125.7	251.4	502.8	**1005.5**	**2011.0**	**4022.0**
18 hrs	4 hrs	II		251.4	502.8	**1005.5**	**2011.0**	**4022.0**

Evaluated experimental points are shown in bold characters

* Was repeated due to invalid solvent control data

B. Study design and methods

1 Study performance: [redacted] and

The study was conducted at [redacted] ([redacted]/Germany). The experimental start and completion dates of the study were June 27th 2012 and September 4th, 2012, respectively.

2 Culture Medium and Conditions:

For seeding and treatment of the cell cultures the culture medium was MEM (minimal essential medium) containing Hank's salts, glutamine and Hepes (25 mM). Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/ 100 µg/mL) and 10 % (v/v) fetal bovine serum (FBS). All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

3 Seeding of the Cultures:

Exponentially growing stock cultures more than 50 % confluent were rinsed with Ca-Mg-free salt solution containing 8000 mg/L NaCl, 200 mg/L KCl, 200 mg/L KH_2PO4 and 150 mg/L Na_2HPO4. Afterwards the cells were treated with trypsin-EDTA-solution at 37 °C for approx. 5 minutes. Then, by adding complete culture medium including 10 % (v/v) FBS the enzymatic treatment were stopped and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.5 % (w/v) in Ca-Mg-free salt solution. The cells were seeded into Quadriperm dishes, which contained microscopic slides. Into each chamber $1 \times 10^4 - 6 \times 10^4$ cells were seeded with regard to the preparation time.

4 4-hour exposure period:

The culture medium of exponentially growing cell cultures was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium were added. After 4 hours the cultures were washed twice 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 cells were then cultured in complete medium containing 10 % (v/v) FBS for the remaining culture time of 14 hours.

5 18-hour exposure period:

The culture medium of exponentially growing cell cultures was replaced with complete medium containing 10 % (v/v) FBS including the test item without S9 mix. The medium was not changed until preparation of the cells. Concurrent solvent and positive controls were performed in the absence and presence of metabolic activation.

All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

5 Slides preparation:
Colcemid was added to the culture medium (0.2 µg/mL) 15.5 hours after the start of the treatment. The cells were treated, 2.5 hours later, on the slides in the chambers with hypotonic solution (0.4 % KCl) for 20 min at 37 °C. After incubation in the hypotonic solution the cells were fixed with a mixture of methanol and glacial acetic acid (3:1 parts, respectively). After preparation the cells were stained with Giemsa and labelled with a computer-generated random code to prevent scorer bias.

6 Evaluation of Cell Numbers:
The evaluation of cytotoxicity indicated by reduced cell numbers was made after the preparation of the cultures on spread slides. The cell numbers were determined microscopically by counting 10 defined fields per coded slide. The cell number of the treatment groups is given in percentage compared to the respective solvent control.

7 Analysis of Metaphase Cells:
Evaluation of the cultures was performed according to the OECD guideline using NIKON microscopes with 100x objectives. Breaks, fragments, deletions, exchanges, and chromosome disintegrations were recorded as structural chromosome aberrations. Gaps were recorded as well but not included in the calculation of the aberration rates. 100 well spread metaphases per culture were evaluated for cytogenetic damage on coded slides, except for the positive control without S9 mix in experiment IA where only 50 metaphases were scored.
Only metaphases with characteristic chromosome numbers of 22 ± 1 were included in the analysis. To describe a cytotoxic effect the mitotic index (% cells in mitosis) was determined.

8 Acceptability of the Test:
The chromosome aberration test is considered acceptable, if it meets the following criteria:

- The number of structural aberrations found in the solvent controls falls within the range of the laboratory historical control data.
- The positive control substances produce significant increases in the number of cells with structural chromosome aberrations, which are within the range of the laboratory historical control data.

9 Evaluation of Results:
A test item is classified as non-clastogenic if:

- the number of induced structural chromosome aberrations in all evaluated dose groups is in the range of the laboratory historical control data and
- no significant increase of the number of structural chromosome aberrations is observed.

A test item is classified as clastogenic if:

- the number of induced structural chromosome aberrations is not in the range of the laboratory historical control data and
- either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.

Statistical significance was confirmed by means of the Fisher's exact test (7) ($p < 0.05$). However, both biological and statistical significance should be considered together. If the criteria mentioned above for the

test item are not clearly met, the classification with regard to the historical data and the biological relevance is discussed and/or a confirmatory experiment is performed. Although the inclusion of the structural chromosome aberrations is the purpose of this study, it is important to include the polyploids and endoreduplications. The following criterion is valid:
A test item can be classified as aneugenic if the number of induced numerical aberrations is not in the range of the laboratory historical control data.

II. Results and discussion

The test item BCS-CV14885, dissolved in culture medium (minimal essential medium), was assessed for its potential to induce structural chromosome aberrations in *V79* cells of the Chinese hamster *in vitro* in the absence and presence of metabolic activation by S9 mix.
Three independent experiments were performed. In Experiment IA the exposure period was 4 hours without S9 mix. In Experiment IB the exposure period was 4 hours with S9 mix. In Experiment II the exposure period was 4 hours with S9 mix and 18 hours without S9 mix. The chromosomes were prepared 18 hours (Exp. IA, IB & II) after the start of treatment with the test item.
In each experimental group two parallel cultures were set up. At least 100 metaphases per culture were evaluated for structural chromosome aberrations, except for the positive control without S9 mix in Experiment IA, where only 50 metaphases were scored.
The highest treatment concentration in this study, 4022.0 µg/mL was chosen with regard to the molecular weight and the purity (97.8 %) of the test item and with respect to the OECD Guideline for *in vitro* mammalian cytogenetic tests.
Neither test item precipitation nor relevant influence on osmolarity or pH value was observed.
In the absence and presence of S9 mix no cytotoxicity was observed up to the highest applied concentration.
In all experimental parts no clastogenicity was observed at the concentrations evaluated. However, one single statistically significant increase was observed in Experiment IA in the absence of S9 mix after treatment with 2011.0 µg/mL (4.0 % aberrant cells, excluding gaps). The value is within the laboratory historical solvent control data range (0.0 – 4.0 % aberrant cells, excluding gaps) and therefore biologically irrelevant.
No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.
Either EMS (600 or 1000 µg/mL) or CPA (2.0 µg/mL) were used as positive controls which showed distinct increases in cells with structural chromosome aberrations.

The results are summarized in the following table:

Exp.	Preparation interval	Test item concentration in µg/mL	Cell numbers in % of control	Mitotic indices in % of control	Aberrant cells in % incl. gaps*	excl. gaps*	with exchanges
		Exposure period 4 hrs without S9 mix					
IA	18 hrs	Solvent control[1]	100.0	100.0	2.0	1.5	0.0
		Positive control[2#]	n.d.	92.0	47.0	**46.0[S]**	30.0
		1005.5	91.9	117.9	2.0	2.0	1.0
		2011.0[##]	104.5	96.0	4.0	**4.0[S]**	1.3
		4022.0	92.2	117.9	5.0	3.5	0.5

		Exposure period 18 hrs without S9 mix					
II	18 hrs	Solvent control[1]	100.0	100.0	1.5	1.5	0.0
		Positive control[3]	n.d.	88.3	10.0	**10.0**[S]	7.5
		1005.5	99.9	95.6	1.5	1.5	1.0
		2011.0	75.3	108.3	0.5	0.5	0.0
		4022.0	104.6	113.7	0.0	0.0	0.0
		Exposure period 4 hrs with S9 mix					
IB	18 hrs	Solvent control[1]	100.0	100.0	4.0	2.5	1.0
		Positive control[4]	n.d.	78.5	22.5	**21.0**[S]	6.5
		1005.5	77.7	106.5	2.0	2.0	0.5
		2011.0	80.2	119.1	1.5	1.0	1.0
		4022.0	97.3	113.8	3.0	1.5	0.5
II	18 hrs	Solvent control[1]	100.0	100.0	1.5	1.5	0.5
		Positive control[4]	n.d.	59.7	30.0	**30.0**[S]	9.0
		1005.5	80.1	104.4	1.0	1.0	0.0
		2011.0	97.1	106.9	1.0	1.0	0.0
		4022.0	67.0	98.1	1.5	1.5	0.5

* Including cells carrying exchanges
# Evaluation of 50 metaphases per culture
Evaluation of 200 metaphases per culture
n.d. Not determined
S Aberration frequency statistically significant higher than corresponding control values
1 Culture medium
2 EMS 1000.0 Mg/mL
3 EMS 6000 Mg/mL
4 CPA 2.0 Mg/mL

III. Conclusions

In conclusion, it can be stated that under the experimental conditions reported, the test item BCS-CV14885 did not induce structural chromosomal aberrations in *V79* cells of the Chinese hamster *in vitro*, when tested up to the highest required concentrations.

Report:	[redacted]ö; [redacted];2013;M-449010-02; Amended: 2015-01-19
Title:	Gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT) - BCS-CV14885
Report No:	1490203
Document No:	M-449010-02-1
Guidelines:	This study was conducted according to the procedures indicated by the following internationally accepted guidelines and recommendations: Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 476: In vitro Mammalian Cell Gene Mutation Test, adopted July 21, 1997; Commission Regulation (EC) No 440/2008 B17 dated May 30, 2008; United States Environmental Protection Agency Health Effects Test Guidelines, OPPTS 870.5300, In Vitro Mammalian Cell Gene Mutation Test, EPA 712-C-98-221, August 1998;not specified
GLP/GEP:	**yes**

Executive summary:
The study was performed to investigate the potential of BCS-CV 14885 to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster.
The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with a treatment time of 4 hours with and 24 hours without metabolic activation.
The highest concentration of 4020 µg/mL applied in the pre-experiment and in the main experiments was equal to a molar concentration of approximately 10mM.
No substantial and reproducible dose dependent increase of the mutation frequency exceeding the historical range of solvent controls was observed in both main experiments.
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, BCS-CV14885 is considered to be non-mutagenic in this HPRT assay.

I. Material and methods

A. Materials

1. Test material: BCS-CV14885

Description:	white powder
Lot/Batch:	BCS-CV14885-01-01
Purity:	97.8 % w/w, dose calculation will be adjusted to purity
Stability of test compound:	not performed as part of this study
Solvent used:	culture medium, (MEM)

2 Control materials:

Negative:	Tissue Culture medium
Positive: *non- activation (-S9 mix):*	Ethyl methanesulphonate (EMS) dissolved in nutrient medium. Final concentration 0.15 mg/mL (1.2 mM)

Positive: *with activation (+S9 mix):*

DMBA; 7,12-dimethylbenz(a)anthracene dissolved in DMSO,
Final concentration 1.1 µg/mL (4.3 µM)

3 Metabolic activation: S9 mix from male Wistar rats

Phenobarbital/β-Naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from 8 – 12 weeks old male Wistar rats (Hsd Cpb: WU, weight approx. 220 – 320 g, [redacted] The Netherlands) induced by intraperitoneal applications of 80 mg/kg b.w. phenobarbital and by peroral administrations of β-naphthoflavone each, on three consecutive days.
An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 supernatant to reach following concentrations in the S9 mix were: 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate and 4 mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. During the experiment, the S9 mix was stored in an ice bath.

4 Test cells:

The V79 cell line has been used successfully in *in vitro* experiments for many years. Especially the high proliferation rate (doubling time 12 -16 h in stock cultures) and a good cloning efficiency of untreated cells (as a rule more than 50 %) both necessary for the appropriate performance of the study, recommend the use of this cell line. The cells have a stable karyotype with a modal chromosome number of 22.

5 Test compound concentrations used (µg/mL):

exposure period	S9 mix	concentrations in µg/mL					
		Experiment I					
4 hours	-	125.6	**251.3**	**502.5**	**1005**	**2010**	**4020**
4 hours	+	125.6	**251.3**	**502.5**	**1005**	**2010**	**4020**
		Experiment II					
24 hours	-	125.6	**251.3**	**502.5**	**1005**	**2010**	**4020**
4 hours	+	125.6	**251.3**	**502.5**	**1005**	**2010**	**4020**

Concentrations given in bold letters were chosen for the mutation rate analysis

In experiment I and II the cultures at the lowest concentration of 125.6 µg/mL were not continued since a minimum of only four analysable concentrations is required by the guidelines.

B. Study design and methods

1 Test performance:

The study was conducted at [redacted]/Germany). The experimental start and completion dates of the study were November 22nd, 2012 and January 24th, 2013, respectively.

2 Culture Medium:

For seeding and treatment of the cell cultures the complete culture medium was MEM (minimal essential medium) containing Hank's salts, neomycin (5 µg/mL) and amphotericin B (1 %). For the selection of

mutant cells the complete medium was supplemented with 11 µg/mL 6-thioguanine. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

3 Seeding of the cultures:

Two to three days after sub-cultivation stock cultures were trypsinized at 37 °C for 5 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 PBS. Prior to the trypsin treatment the cells were rinsed with Ca-Mg-free salt solution containing 200 mg/l EDTA (ethylene diamine tetraacetic acid). Approximately 1.5×10^6 (single culture) and 5×10^2 cells (in duplicate) were seeded in plastic culture flasks. The cells were grown for 24 hours prior to treatment.

4 Treatment:

After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps with "saline G". In the second experiment the cells were exposed to the test item for 24 hours in complete medium, supplemented with 10 % FBS, in the absence of metabolic activation. The pH was adjusted to 7.2

The colonies used to determine the cloning efficiency (survival) were fixed and stained approx. 7 days after treatment.

Three or four days after treatment 1.5×10^6 cells per experimental point were sub-cultivated in 175 cm² flasks containing 30 mL medium. Following the expression time of 7 days five 80 cm² cell culture flasks were seeded with about 3 - 5×10^5 cells each in medium containing 6-TG. Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability.

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.

5 Evaluation of cell numbers:

The stained colonies with more than 50 cells were counted. In doubt the colony size was checked with a preparation microscope.

6 Acceptability of the Assay:

The gene mutation assay is considered acceptable if it meets the following criteria:

- the numbers of mutant colonies per 10^6 cells found in the solvent controls falls within the laboratory historical control data.
- the positive control substances should produce a significant increase in mutant colony frequencies.
- the cloning efficiency II (absolute value) of the solvent controls should exceed 50 %.

7 Statistical analysis:

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. The numbers of mutant colonies 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. However, both, biological and statistical significance was considered together.

8 Evaluation of Results:

A test item is classified as positive if it induces either a concentration-related increase of the mutant frequency or a reproducible and positive response at one of the test points.
A test item producing neither a concentration-related increase of the mutant frequency nor a reproducible positive response at any of the test points is considered non-mutagenic in this system.
A positive response is described as follows:
A test item is classified as mutagenic if it reproducibly induces a mutation frequency that is three times above the spontaneous mutation frequency at least at one of the concentrations in the experiment.
The test item is classified as mutagenic if there is a reproducible concentration-related increase of the mutation frequency. Such evaluation may be considered also in the case that a threefold increase of the mutant frequency is not observed.
However, in a case by case evaluation this decision depends on the level of the corresponding solvent control data. If there is by chance a low spontaneous mutation rate within the laboratory´s historical control data range, a concentration-related increase of the mutations within this range has to be discussed. The variability of the mutation rates of solvent controls within all experiments of this study was also taken into consideration.

II. Results and discussion

The test item BCS-CV14885 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster.
The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with a treatment time of 4 hours with and 24 hours without metabolic activation.
No precipitation of the test item was observed up to the maximum concentration in all experiments.
No cytotoxic effects indicated by a cloning relative efficiency I and/or relative cell density below 50 % in both parallel cultures occurred up to the maximum concentration of 4020 µg/mL in experiment I and II with and without metabolic activation following 4 and 24 hours treatment.
No relevant and reproducible increase of mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration with and without metabolic activation. The induction factor did not exceed the threshold of three times the corresponding solvent control at any experimental data point.
In experiment I the mutant colonies/10^6 cells exceeded the range of the historical solvent control data (3.4 – 36.6 mutant colonies/ 10^6 cells) in the presence of metabolic activation at 252.3 µg/mL (culture II) and at 2010 and 4020 µg/mL (culture I) (36.9, 40.9 and 38.3 mutant colonies/10^6 cells). However, the threshold of three times the mutation frequency of the corresponding solvent control was neither reached nor exceeded.
A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequency. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in both cultures of the second experiment without metabolic activation. However, the trend was judged as biologically irrelevant as the mutation frequency did not reach or exceed the threshold described above and all of the individual values of the mutation frequency remained within the historical range of solvent controls in the second culture.

The viability of the solvent control of the second culture of the first experiment with metabolic activation reached but did not exceed the lower limit of 50%. The data are acceptable however, as the lower limit was exceeded in the parallel culture under identical conditions.
EMS (150 µg/mL) and DMBA (1.1 µg/mL) were used as positive controls and showed a distinct increase in induced mutant colonies.

The results are summarized in the following table:

	Conc. µg/ml	S9-mix	Relative cloning efficiency I	Relative cell density	Relative cloning efficiency II	Mutant colonies / 10^6 cells	Induction factor	Relative cloning efficiency I	Relative cell density	Relative cloning efficiency II	Mutant colonies / 10^6 cells	Induction factor
			%	%	%			%	%	%		
Column	1	2	3	4	5	6	7	8	9	10	11	12
Experiment I / 4 h treatment			Culture I					Culture II				
Solvent control with medium		-	100.0	100.0	100.0	29.9	1.0	100.0	100.0	100.0	37.6	1.0
Positice control (EMS)	150.0	-	119.3	78.7	76.6	134.7	4.5	100.6	98.8	68.4	186.3	5.0
Test item	125.6	-	119.8	Culture was not continued[#]				111.8	Culture was not continued[#]			
Test item	251.3	-	96.4	100.8	89.9	9.0	0.3	102.9	110.5	82.0	23.3	0.6
Test item	502.5	-	113.4	117.4	82.7	31.9	1.1	101.2	141.4	85.4	33.3	0.9
Test item	1005.0	-	76.7	136.9	104.5	26.1	0.9	87.7	114.4	78.3	19.8	0.5
Test item	2010.0	-	96.1	101.6	92.6	20.9	0.7	90.2	139.0	85.3	37.3	1.0
Test item	4020.0	-	88.3	107.8	89.2	21.6	0.7	104.5	137.9	89.2	15.4	0.4
Experiment I / 4 h treatment			Culture I					Culture II				
Solvent control with medium		+	100.0	100.0	100.0	21.9	1.0	100.0	100.0	100.0	35.7	1.0
Positice control (DMBA)	1	+	126.8	92.9	79.2	556.4	25.4	92.0	89.5	100.7	718.9	20.1
Test item	125.6	+	135.3	Culture was not continued[#]				96.0	Culture was not continued[#]			
Test item	251.3	+	159.5	119.1	113.3	32.7	1.5	104.5	98.0	116.8	36.9	1.0
Test item	502.5	+	140.2	109.7	103.8	24.4	1.1	97.8	96.2	111.6	36.1	1.0
Test item	1005.0	+	160.4	115.0	97.8	24.2	1.1	117.0	95.0	107.2	33.6	0.9
Test item	2010.0	+	133.9	111.9	99.2	40.9	1.9	102.3	93.2	104.7	24.3	0.7
Test item	4020.0	+	130.0	104.6	108.1	38.3	1.7	115.7	74.0	104.0	27.7	0.8

Experiment II / 24 h treatment			Culture I					Culture II				
Solvent control with medium		-	100.0	100.0	100.0	27.2	1.0	100.0	100.0	100.0	12.9	1.0
Positice control (EMS)	150.0	-	92.0	105.5	85.8	450.6	16.6	105.3	97.1	88.5	391.5	30.4
Test item	125.6	-	101.5	Culture was not continued#				102.9	Culture was not continued#			
Test item	251.3	-	99.4	83.4	104.1	16.6	0.6	100.2	104.9	104.9	16.5	1.3
Test item	502.5	-	97.4	98.8	103.7	15.1	0.6	100.3	125.7	96.1	17.9	1.4
Test item	1005.0	-	101.7	86.1	98.7	19.0	0.7	102.5	118.6	96.1	19.9	1.5
Test item	2010.0	-	101.2	113.3	93.6	28.5	1.0	99.6	122.1	93.4	21.8	1.7
Test item	4020.0	-	99.5	122.1	88.4	43.1	1.6	100.6	134.1	88.0	26.7	2.1
Experiment II / 4 h treatment												
Solvent control with medium		+	100.0	100.0	100.0	15.3	1.0	100.0	100.0	100.0	18.6	1.0
Positice control (DMBA)	1.4	+	100.6	94.2	92.6	220.8	14.4	95.8	89.1	82.3	224.0	12.0
Test item	125.6	+	102.3	Culture was not continued#				98.3	Culture was not continued#			
Test item	251.3	+	102.1	106.6	95.7	20.7	1.3	99.7	93.4	95.4	22.1	1.2
Test item	502.5	+	102.0	108.9	121.3	21.4	0.7	97.4	109.1	98.8	17.0	0.9
Test item	1005.0	+	101.1	106.5	100.4	21.8	1.4	98.0	106.5	101.0	13.1	0.7
Test item	2010.0	+	102.9	102.2	99.8	11.7	0.8	98.0	105.1	91.7	22.4	1.2
Test item	4020.0	+	101.6	86.7	90.2	21.7	1.4	99.6	99.8	99.0	16.0	0.9
# culture was not continued since a minimum of only four analysabel concentrations is required												

III. Conclusions

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, BCS-CV14885 is considered to be non-mutagenic in this HPRT assay.

CA 5.8.2 Supplementary studies on the active substance

Since the toxicological profile was sufficiently demonstrated, no supplementary studies were necessary.

CA 5.8.3 Endocrine disrupting properties

It should be noted that to date, no clear criteria are available to define endocrine disrupting properties.

Mesosulfuron-methyl has no effects on reproductive indices nor fertility nor reproductive tissues and organs as shown in the multi-generation study. Mesosulfuron-methyl is not a developmental toxicant.
After a detailed analysis of all these apical toxicological studies under inclusion of scientific and regulatory hazard principles in discussion at present no evidence of endocrine disrupting properties are seen and mesosulfuron-methyl does not fall under the interim definition for endocrine disruption. Therefore, based on a complete toxicological data set, there is no evidence of endocrine disrupting properties of mesosulfuron-methyl.

RMS has considered the following reference as relevant for the dossier:

Report:	KCA 5.8.3 /01;[redacted];[redacted];[redacted];2013;M-476775-01
Title:	Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity
Report No:	M-476775-01-1
Document No:	M-476775-01-1
Guidelines:	**not applicable;not applicable**
GLP/GEP:	**n.a.**

As mentioned as footnotes of the tables of this publication, Mesosulfuron was negative in the tests

CA 5.9 Medical data

No poisoning cases have been reported in literature.

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Report:	[redacted] 1; [redacted] ;2000;M-199528-01
Title:	Medical surveillance of manufacturing plant personnel Medical data mesosulfuron-methyl Code: AE F130060
Report No:	C009926
Document No:	M-199528-01-1
Guidelines:	**Deviation not specified**
GLP/GEP:	**no**

The following information (CA 5.9.2 – 5.9.7) was provided by the global medical director of Bayer CropScience, [redacted], and gives the most current facts.

Medical assessment:
Occupational medical surveillance of workers exposed to Mesosulfuron-methyl performed since 2004 annually on a routine basis, not directly related to exposures, did not reveal any unwanted effects in the workers. The examinations included the following laboratory parameters and clinical and technical examinations:

No. of workers exposed:	6
Medical examinations:	History and full physical examination
Laboratory examinations:	FBC, liver enzymes, urine stick
Technical examinations:	Lung function testing, vision testing and audiometry as needed for specific job tasks

During the production period since 2004 no accidents with Mesosulfuron-methyl occurred in the workers. No further consultations of the Medical Department due to work or contact with Mesosulfuron-methyl were required.

CA 5.9.2 Data collected on humans

One suicide attempt has been reported from Iran. After drinking an unknown amount of a formulation containing mesosulfuron-methyl nausea, vomiting and stomach upset were seen. The ingestion was survived without sequelae.

CA 5.9.3 Direct observations

None.

CA 5.9.4 Epidemiological studies

No epidemiological studies are available in literature.

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

There are no reports on poisoning in humans.
Animal experiments with high doses showed unspecific symptoms like irregular breathing, weakness, salivation.
Though it is a sulfonyl urea compound, mesosulfuron-methyl does not influence glucose metabolism.

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

First Aid:

Remove patient from exposure/terminate exposure
Thorough skin decontamination with copious amounts of water and soap, if available with polyethylenglykol 300 followed by water.
Note: Most formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polyethyleneglykol 300 is not required.

Flushing of the eyes with lukewarm water for 15 minutes

Induction of vomiting does not seem to be required in regard of the low toxicity It should only be considered if a large amount has been swallowed, if the ingestion was less than one hour ago, and if the patient is fully conscious.
Induced vomiting can remove maximum 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 of the compound
The application of activated charcoal and sodium sulphate (or other carthartic) might be considered in significant ingestions.
As there is no antidote, treatment has to be symptomatic and supportive.

CA 5.9.7 Expected effects of poisoning

No persistent effects are expected after poisoning.