RCC-CCR STUDY NUMBER 1165200

GENE MUTATION ASSAY
IN CHINESE HAMSTER V79 CELLS
IN VITRO
(V79 / HPRT)
WITH
C6SFA MONOMER

REPORT

Study Completion Date:

June 12, 2008

1 COPY OF GLP CERTIFICATE

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 88/320/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 88/320/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

RCC – Cytotest Cell Research GmbH
RCC – Cytotest Cell Research GmbH
In den Leppsteinswiesen 19
64380 Rossdorf

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according chemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)
6 Prüfungen zur Bestimmung von Rückständen
8 Analytische Prüfungen an biologischen Materialien
9 Virussicherheitsprüfungen

2 Toxicity studies
3 Mutagenicity studies
6 Residues
8 Analytical studies on biological materials
9 Virus validation studies

02.09.2006
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Th. Zimmermann, Referent, Wiesbaden, den **19. Januar 2007**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, LÄNDLICHEN RAUM UND VERBRAUCHERSCHUTZ

Hess. Ministerium für Umwelt, ländlichen Raum und Verbraucherschutz,
Mainzer Straße 80 D65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority

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3 PREFACE

3.1 General

Title:	Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with C6SFA MONOMER
Sponsor:	Daikin Industries, LTD. 1-1 Nishi Hitotsuya Settsu-Shi Osaka 566-8585 Japan
Study Monitor:	
Test Facility:	RCC Cytotest Cell Research GmbH (RCC-CCR) In den Leppsteinswiesen 19 64380 Rossdorf Germany
Contracting Institute:	RCC Ltd 4452 Itingen, Switzerland
Reference Number:	B87647

3.2 Responsibilities

Study Director:

Deputy Study Director:

Management:

Head of Quality Assurance Unit:

3.3 Schedule

Experimental Starting Date:	March 26, 2008
Experimental Completion Date:	May 29, 2008

3.4 Project Staff Signatures

Study Director

.....

Date: June 12, 2008

Management

.....

Date: June 12, 2008

3.5 Good Laboratory Practice

The study was performed in compliance with:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anlage 1" (Annexe 1) dated July 25, 1994 ("BGBl. I 1994", pp. 1703), last revision dated June 27, 2002.

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final].

3.6 Guidelines

This study was conducted according to the procedures indicated by the following internationally accepted guidelines and recommendations:

Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, February 1998, adopted July 21, 1997, Guideline No. 476 "In vitro Mammalian Cell Gene Mutation Test".

Commission Directive 2000/32/EC, L1362000, Annex 4E, dated May 19, 2000.

3.7 Archiving

RCC Cytotest Cell Research GmbH will archive the following data for 15 years:

Raw data, study plan, report, and a sample of the test item.

No data will be discarded without the sponsor's consent.

3.8 Deviations to the study plan

There were no deviations to the study plan.

4 STATEMENT OF COMPLIANCE

Study Number:	1165200
Test Item:	C6SFA MONOMER
Study Director:	
Title:	Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with C6SFA MONOMER

This study performed in the test facility of RCC Cytotest Cell Research GmbH was conducted in compliance with Good Laboratory Practice Regulations:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), dated July 25, 1994 ("BGBl. I 1994", pp. 1703), last revision June 27, 2002.

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final].

There were no circumstances that may have affected the quality or integrity of the study.

Study Director RCC-CCR

.....

Date: June 12, 2008

5 STATEMENT OF QUALITY ASSURANCE UNIT

Study Number: 1165200

Test Item: C6SFA MONOMER

Study Director:

Title: Gene Mutation Assay
in Chinese Hamster V79 Cells
in vitro (V79/HPRT) with C6SFA MONOMER

The general facilities and activities of RCC Cytotest Cell Research GmbH are inspected periodically and the results are reported to the responsible person and the Management.

Study procedures were inspected periodically. The study plan and this report were audited by the Quality Assurance Unit. The dates are given below.

Phases and Dates of QAU Inspections/ Audits		Dates of Reports to the Study Director and to Management
Study Plan:	March 14, 2008	March 14, 2008
Study Inspection Preparation for Application:	March 26, 2008	March 26, 2008
Report:	June 10, 2008	June 10, 2008

This statement is to confirm that the present report reflects the raw data.

Head of Quality Assurance Unit

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Date: June 12, 2008

6 SUMMARY

The study was performed to investigate the potential of C6SFA MONOMER to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster.

The assay was performed in two independent experiments. The cells were exposed to the test item for 4 hours in the first experiment with and without metabolic activation. Experiment II was performed with a treatment period of 4 hours with and 24 hours without metabolic activation.

The maximum dose was 4200 µg/mL corresponding to a molar concentration of about 10 mM.

The tested concentrations are described in table 2 (page 16). The evaluated experimental points and the results are summarised in table I (page 10).

No substantial and reproducible dose dependent increase of the mutation frequency was observed in both main experiments.

Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies and thus showed the sensitivity of the test item and the activity of the S9 mix.

Conclusion

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, C6SFA MONOMER is considered to be non-mutagenic in this HPRT assay.

Table 1: Summary of results

			relative	relative	mutant		relative	relative	mutant	
	conc.	S9	cloning	cloning	colonies/	induction	cloning	cloning	colonies/	induction
	µg per mL	mix	efficiency 1	efficiency 2	10^6 cells	factor	efficiency 1	efficiency 2	10^6 cells	factor
			%	%			%	%		
column	1	2	3	4	5	6	7	8	9	10
Experiment I			culture I				culture II			
Solvent control THF		-	100.0	100.0	5.9	1.0	100.0	100.0	12.4	1.0
Pos. contr. with EMS	150.0	-	56.8	91.8	81.1	13.7	57.3	102.7	100.9	8.2
Test item	131.3	-		culture was not continued#			101.8	culture was not continued#		
Test item	262.5	-	94.0	127.9	4.4	0.7	95.2	131.2	7.8	0.6
Test item	525.0	-	101.5	106.4	6.4	1.1	99.2	93.3	16.0	1.3
Test item	1050.0	-	93.4	87.8	14.3	2.4	93.0	68.6	17.8	1.4
Test item	2100.0 (p)	-	96.9	77.9	12.8	2.2	98.2	78.5	15.9	1.3
Test item	4200.0 (p)	-	91.4	46.4	18.1	3.1	90.7	48.9	47.0	3.8
Solvent control THF		+	100.0	100.0	18.0	1.0	100.0	100.0	10.4	1.0
Pos. contr. with DMBA	1.3	+	28.4	48.9	1247.1	69.2	29.1	57.5	937.0	90.3
Test item	131.3	+	95.5	culture was not continued#			89.1	culture was not continued#		
Test item	262.5	+	95.8	92.2	17.9	1.0	95.6	115.8	8.0	0.8
Test item	525.0	+	94.3	86.0	10.9	0.6	97.8	118.9	13.2	1.3
Test item	1050.0	+	95.7	95.8	18.2	1.0	92.5	112.8	9.0	0.9
Test item	2100.0 (p)	+	86.2	90.1	9.0	0.5	100.3	115.0	5.1	0.5
Test item	4200.0 (p)	+	86.7	83.2	14.7	0.8	97.2	114.7	14.8	1.4
Experiment II			culture I				culture II			
Solvent control THF		-	100.0	100.0	13.1	1.0	100.0	100.0	20.5	1.0
Pos. contr. with EMS	75.0	-	84.5	72.4	202.4	15.4	75.2	97.0	188.7	9.2
Test item	131.3	-	culture was not continued#				culture was not continued#			
Test item	262.5	-	17.8	79.2	15.2	1.2	22.7	82.5	28.5	1.4
Test item	525.0	-	55.8	75.2	11.1	0.8	59.8	90.8	38.4	1.9
Test item	1050.0	-	69.8	73.3	26.9	2.1	45.1	97.6	24.3	1.2
Test item	2100.0 (p)	-	29.3	77.8	13.0	1.0	57.8	89.5	14.3	0.7
Test item	4200.0 (p)	-	75.7	63.6	28.9	2.2	49.3	120.6	11.3	0.5
Solvent control THF		+	100.0	100.0	17.1	1.0	100.0	100.0	15.6	1.0
Pos. contr. with DMBA	1.3	+	11.8	68.8	971.2	56.9	32.7	89.9	722.6	46.3
Test item	131.3	+	89.1	culture was not continued#			96.7	culture was not continued#		
Test item	262.5	+	90.9	111.7	19.3	1.1	87.0	101.6	15.2	1.0
Test item	525.0	+	95.1	100.1	28.9	1.7	98.4	99.2	13.5	0.9
Test item	1050.0	+	100.0	108.9	31.0	1.8	93.3	102.8	7.4	0.5
Test item	2100.0 (p)	+	90.9	91.6	15.9	0.9	84.1	103.5	18.0	1.2
Test item	4200.0 (p)	+	96.5	97.6	16.2	1.0	70.1	111.6	16.5	1.1

culture was not continued since a minimum of only four analysable concentrations is required
p precipitation, visible to the naked eye

7 OBJECTIVE

7.1 Aims of the Study

This *in vitro* experiment was performed to assess the potential of the test item to induce gene mutations using the Chinese hamster cell line V79. Two parallel cultures were used throughout the assay.

Experiment I was performed with a treatment duration of 4 hours in the presence and absence of metabolic activation. Experiment II was performed with a treatment period of 4 hours with and 24 hours without metabolic activation.

7.2 Relevance of the Test System

In vitro methods are valuable when it is desired to accurately control the concentration and exposure time of cells to the test item under study. However, the limited capacity of metabolic activation of potential mutagens requires an exogenous metabolic activation system.

This *in vitro* test is an assay for the detection of forward gene mutations in mammalian cells. Gene mutations are discussed as an initial step in the carcinogenic process (02).

The V79 cells are exposed to the test item both with and without exogenous metabolic activation. At a defined time interval after treatment the descendants of the treated original population are monitored for the loss of functional HPRT enzyme.

HPRT (hypoxanthine-guanine phosphoribosyl transferase) catalyzes the conversion of the nontoxic 6TG (6-thioguanine) to its toxic ribophosphorylated derivative. Therefore, cells deficient in HPRT due to a forward mutation are resistant to 6TG. These cells are able to proliferate in the presence of 6TG whereas the non-mutated cells die. However, the mutant phenotype requires a certain period of time before it is completely expressed. The phenotypic expression is achieved by allowing exponential growth of the cells for 7 - 9 days. The expression period is terminated by adding 6TG to the culture medium (3).

Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the surviving cells. After a suitable period the colonies are counted. Mutant frequencies are calculated from the number of mutant colonies corrected for cell survival.

In order to establish a concentration response effect of the test item at least four concentration levels are tested. These concentration levels should yield a concentration related toxic effect. The highest concentration level should induce a reduced level of survival.

To demonstrate the sensitivity of the test system reference mutagens are tested in parallel to the test item.

8 MATERIALS AND METHODS

8.1 Test Item

Internal RCC-CCR Test Item Number: S 857311

The test item and the information concerning the test item were provided by the sponsor.

Identity:	C6SFA MONOMER
Batch No.:	N2SFA6X001
Molecular Weight:	418 g/mol
Purity:	Approximately 100 %
Storage:	Will be stored at +15 - +25 °C
Stability in solvent:	Not indicated by the sponsor
Expiration Date:	January 31, 2009

On the day of the experiment (immediately before treatment), the test substance was dissolved in THF (Tetrahydrofurane, E. MERCK, 64293 Darmstadt; Germany, purity 99.5 %). The final concentration of THF in the culture medium was 0.5 % v/v.

The osmolarity and pH-value were determined in the solvent control and the maximum concentration of the pre-experiment without metabolic activation:

	solvent control	C6SFA MONOMER 4200 µg/mL
Osmolarity mOsm	363	342
pH-value	7.37	7.34

8.2 Controls

8.2.1 Solvent Controls

Concurrent solvent controls (THF) were performed.

8.2.2 Positive Control Substances

8.2.2.1 Without metabolic activation

Name:	EMS; ethylmethane sulfonate
Supplier:	ACROS ORGANICS, B-2440 Geel
Purity:	≥ 98 %
Lot no.:	Experiment I: A0236026, expiry date: April 2008
	Experiment II: A0246840, expiry date: April 2009
Dissolved in:	Nutrient medium
Final concentration:	0.075 mg/mL = 0.6 mM (experiment II)
	0.150 mg/mL = 1.2 mM (experiment I)

8.2.2.2 With metabolic activation

Name:	DMBA; 7,12-dimethylbenz(a)anthracene
Supplier:	SIGMA CHEMIE GMBH, D-82041 Deisenhofen
Lot no.:	78 H 1078
Expiry date:	October 2008 (experiment I and II)
Dissolved in	DMSO, Dimethylsulfoxide;
Supplier:	(E. MERCK, D-64293 Darmstadt; Purity 99.5 %)
	final concentration in nutrient medium 0,5 %
Final concentration:	1.3 µg/mL = 5.1 µM (Experiment I and II)

The dilutions of the stock solutions were prepared on the day of the experiment and used immediately.

The stability of both positive control substances in solution is unknown, but a mutagenic response in the expected mutation range is sufficient evidence of biological stability. The dilutions of the stock solutions were prepared on the day of the experiment and used immediately.

8.3 Test System

8.3.1 Reasons for the Choice of the Cell Line V79

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 (3).

8.3.2 Cell Cultures

Large stocks of the V79 cell line (supplied by Laboratory for Mutagenicity Testing; Technical University; D-64287 Darmstadt) are stored in liquid nitrogen in the cell bank of RCC-CCR allowing the repeated use of the same cell culture batch in experiments. Before freezing, the level of spontaneous mutants was depressed by treatment with HAT-medium as described in [3]. Each batch is screened for mycoplasma contamination and checked for karyotype stability and spontaneous mutant frequency. Consequently, the parameters of the experiments remain similar because of the reproducible characteristics of the cells.

Thawed stock cultures are propagated at 37 °C in 80 cm^2 plastic flasks (GREINER, D-72632 Frickenhausen). About 5×10^5 cells are seeded into each flask with 15 mL of MEM (minimal essential medium; SEROMED, D-12247 Berlin) supplemented with 10 % fetal calf serum (FCS; PAA Laboratories GmbH, D-35091 Cölbe). The cells are subcultured twice weekly. The cell cultures are incubated at 37 °C in a 4.5 % carbon dioxide atmosphere (95.5% air).

For the selection of mutant cells the medium is supplemented with 11 µg/mL thioguanine (6TG, SIGMA GmbH, D-82041 Deisenhofen).

8.4 Mammalian Microsomal Fraction S9 Mix

Lacking metabolic activities of cells under *in vitro* conditions are a disadvantage of assays with cell cultures as many chemicals only develop a mutagenic potential when they are metabolized by the mammalian organism. However, metabolic activation of chemicals can be achieved at least partially by supplementing the cell cultures with mammalian liver microsome preparations (S9 mix).

8.4.1 S9 (Preparation by RCC-CCR)

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 HanIbm rats, weight approx. 220 - 320 g induced by applications of 80 mg/kg b.w. Phenobarbital i.p. (Desitin; D-22335 Hamburg) and β-Naphthoflavone p.o. (Aldrich, D-89555 Steinheim) 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. Aliquots of the supernatant were frozen and stored in ampoules at -80° C. Small numbers of the ampoules were kept at -20°C for up to one week.

The protein concentration in the S9 preparation was 31.0 mg/mL (Lot No.: 150208) in the pre-experiment, in experiment I, and in experiment II.

8.4.2 S9 Mix

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:

8 mM	$MgCl_2$
33 mM	KCl
5 mM	glucose-6-phosphate
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. The S9 mix preparation was performed according to Ames et al. (1).

8.5 Pre-Test on Toxicity

A pre-test was performed in order to determine the concentration range for the mutagenicity experiments. The general culture conditions and experimental conditions in this pre-test were the same as described for the mutagenicity experiment below. In this pre-test the colony forming ability of approximately 500 single cells (duplicate cultures per concentration level) after treatment with the test item was observed and compared to the controls. Toxicity of the test item is indicated by a reduction of the cloning efficiency (CE).

8.6 Dose Selection

According to the recommendations of the guidelines (see page 6), several concentrations (usually at least four) of the test item should be used. These should yield a concentration-related toxic effect. The highest concentration should produce a low level of survival and the survival in the lowest concentration should approximate the solvent control. Relatively insoluble substances should be tested up to their limit of solubility under culture conditions. For freely-soluble nontoxic substances the maximum concentration should be 5 mg/mL or 10 mM. If the maximal concentration is based on cytotoxicity the cloning efficiency should be reduced to less than 50 % and/or culture growth at subcultivation should be at least 20 % of the corresponding solvent control.

In the range finding pre-experiment test item concentrations between 32.8 µg/mL and 4200 µg/mL were used to evaluate toxicity in the presence (4 h treatment) and absence (4 h and 24 h treatment) of metabolic activation. The highest concentration was chosen with regard to the purity (approximately 100 %) and the molecular weight (418 g/mol) of the test item.

No relevant toxic effect was observed in any of the experimental parts up to the maximum concentration. The test medium was checked for precipitation at the end of each treatment period (4 or 24 hours) prior to removal to the test item. No precipitation of the test item was noted up to the highest concentration with and without metabolic activation.

There was no relevant shift of pH and osmolarity of the medium even in the stock solution of the test item.

Based on the results of the pre-experiment the concentration range of the main experiments was selected. The maximum concentration was 4200 µg/mL corresponding to approximately 10mM. The lower concentrations were spaced by a factor of 2.

To overcome problems with possible deviations in toxicity both main experiments were started with more than four concentrations. The individual concentrations were spaced by a factor of 2.0.

Table 2: Doses applied in the gene mutation assay with C6SFA MONOMER:

	concentrations in µg/mL					
	Experiment I					
without S9 mix*	**131.3**	262.5	525	1050	2100	4200
with S9 mix*	**131.3**	262.5	525	1050	2100	4200
	Experiment II					
without S9 mix**	**131.3**	262.5	525	1050	2100	4200
with S9 mix*	**131.3**	262.5	525	1050	2100	4200

* 4 hour treatment ** 24 hour treatment

In experiment I and II the cultures at the lowest concentration with and without metabolic activation (131.3 µg/mL) were not continued since a minimum of only four analysable concentrations is required by the guidelines.

8.7 Experimental Performance

8.7.1 Seeding

Three days old exponentially growing stock cultures (more than 50 % confluent) were trypsinized at 37 °C for 5 minutes. Then the enzymatic digestion was stopped by adding complete culture medium and a single cell suspension was prepared. The trypsin concentration for all subculturing steps was 0.2 % in Ca-Mg-free salt solution (Trypsin: Difco Laboratories, Detroit, USA).

The Ca-Mg-free salt solution had the following constituents (per litre):

NaCl	8000 mg
KCl	400 mg
Glucose	1000 mg
$NaHCO_3$	350 mg

Prior to the trypsin treatment the cells were rinsed with Ca-Mg-free salt solution containing 200 mg/l EDTA (ethylene diamine tetraacetic acid).

The cell suspension was seeded into plastic culture flasks (Greiner, 72632 Frickenhausen, Germany). Approximately 1.5×10^6 (single culture) and 5×10^2 cells (in duplicate) were seeded in MEM with 10 % FCS (complete medium) for the determination of mutation rate and toxicity, respectively.

8.7.2 Treatment

After 24 h 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 h 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 h in complete medium in the absence of metabolic activation.

The "saline G" solution had the following constituents (per litre):

NaCl	8000 mg
KCl	400 mg
Glucose	1100 mg
$Na_2HPO_4\times7H_2O$	290 mg
KH_2PO_4	150 mg

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 as described below.

The cultures were incubated at 37 °C in a humidified atmosphere with 4.5 % CO_2 for about 8 days. The colonies were stained with 10 % methylene blue in 0.01 % KOH solution (E. MERCK, 64293 Darmstadt, Germany).

The stained colonies with more than 50 cells were counted. In doubt the colony size was checked with a preparation microscope (Nikon, 40407 Düsseldorf, Germany).

8.8 Data Recording

The data generated were recorded in the raw data. The results are presented in tabular form, including experimental groups with the test item, solvent, and positive controls.

8.9 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 fall within the laboratory historical control data range of 2001 – 2006 (see Annex II).
- the positive control substances must produce a significant increase in mutant colony frequencies (see Annex II, Historical data).
- the cloning efficiency II (absolute value) of the solvent controls must exceed 50 %.

The data of this study comply with the above mentioned criteria [see Annex I (Tables of Results, mutation rate and factor calculated referring to the C.E. of the untreated cultures) and Annex II (Historical data)].

8.10 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 in the range normally found (0.5 – 31.8 mutants per 10^6 cells) 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.

8.11 Statistical Analysis

A linear regression was performed to assess a possible dose dependent increase of mutant frequencies using SYSTAT® statistics software. The number of mutant colonies obtained in the groups treated with the test item was 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 should be considered together.

experimental group	p-value
first experiment, culture 1 without S9	0.020
first experiment, culture 2 without S9	0.008
first experiment, culture 1 with S9	0.536
first experiment, culture 2 with S9	0.518
second experiment, culture 1 without S9	0.161
second experiment, culture 2 without S9	0.112
second experiment, culture 1 with S9	0.466
second experiment, culture 2 with S9	0.553

9 CALCULATION AND PROCESSING OF THE DATA

The data listed in the tables (Annex I) are calculated and processed as described below.

Pre-test

cloning efficiency, absolute	mean number of colonies per flask divided by the number of cells seeded × 100
cloning efficiency, relative	(mean number of colonies per flask divided by the mean number of colonies per flask of the corresponding control) × 100

Main test

Cloning efficiency I (survival):	cloning efficiency determined immediately after treatment to measure toxicity.
Cloning efficiency II (viability):	cloning efficiency determined after the expression period to measure viability of the cells without selective agent.
cloning efficiency I (survival, absolute):	mean number of colonies per flask divided by the number of cells seeded
cloning efficiency I (survival, relative):	(mean number of colonies per flask divided by the mean number of colonies per flask of the corresponding control) × 100
cell density % of control	(cell density at 1st subcultivation divided by the cell density at 1st subcultivation of the corresponding control) × 100
cloning efficiency II (viability, absolute):	mean number of colonies per flask divided by the number of cells seeded
cloning efficiency II (viability, relative):	cloning efficiency II absolute divided by the cloning efficiency II absolute of the corresponding control × 100
cells survived (after plating in TG containing medium)	number of cells seeded × cloning efficiency II absolute
mutant colonies / 10^6 cells:	mean number of mutant colonies per flask found after plating in TG medium × 10^6 divided by the number of cells survived
induction factor:	mutant colonies per 10^6 cells / mutant colonies per 10^6 cells of the corresponding solvent control

10 RESULTS AND DISCUSSION

The test item C6SFA MONOMER 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 with identical experimental procedures, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 h. The second experiment was performed with a treatment period of 24 hours in the absence of metabolic activation and 4 hours in the presence of metabolic activation.

The cell cultures were evaluated at the following concentrations:

Experiment I:

without S9 mix:	262.5; 525; 1050; 2100; and 4200 µg/mL
with S9 mix:	262.5; 525; 1050; 2100; and 4200 µg/mL

Experiment II:

without S9 mix:	262.5; 525; 1050; 2100; and 4200 µg/mL
with S9 mix:	262.5; 525; 1050; 2100; and 4200 µg/mL

The maximum concentration of the test item in the main experiments equals a molar concentration of approximately 10 mM.

Precipitation of the test item was observed in all experimental parts at 2100 and 4200 µg/mL.

No relevant toxic effect as indicated by a relative cloning efficiency I of less than 50 % in both parallel cultures occurred after 4h of treatment up to the maximum concentration with and without metabolic activation. Following 24h of treatment moderate toxic effects were noted in both parallel cultures at 262.5 µg/mL, in culture II at 1050.0 µg/mL, and in culture I at 2100 µg/mL. However, these cytotoxic effects were not dose dependent and except at the lowest evaluated concentration, not reproduced in the parallel culture under identical conditions. Therefore, the occasional cytotoxic effects are not judged to be biologically relevant.

No relevant and reproducible increase in mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration. The induction factor exceeded the threshold of three times the corresponding solvent control in both cultures of the first experiment without metabolic activation at 4200 µg/mL. This effect however, was judged to be based upon the rather low solvent controls of 5.9 and 11.4 mutant colonies/10^6 cells, respectively. In culture I at 4200 µg/mL the number of mutant colonies/10^6 cells (18.1) remained well within the historical range of solvent controls. Furthermore, the increase occurred at a precipitating concentration and was not reproduced in the second experiment without metabolic activation. Precipitation often is a reason for the generation of artefacts due to unspecific cell damages caused by mechanical shear forces.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using SYSTAT® statistics software. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in both cultures of experiment I without metabolic activation. However, this trend is based on an increase of the mutation frequency at the maximum con-

centration in the presence of precipitation and, as discussed above, is not reproduced in the second experiment without metabolic activation. Therefore, the statistical significance is considered biologically irrelevant.

In both experiments of this study (with and without S9 mix) the range of the solvent controls was from 5.9 up to 20.5 mutants per 10^6 cells; the range of the groups treated with the test item was from 4.4 up to 47.0 mutants per 10^6 cells.

EMS (150 µg/mL in experiment I and 75 µg/mL in experiment II) and DMBA (1.3 µg/mL) were used as positive controls and showed a distinct increase in induced mutant colonies.

Conclusion

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, C6SFA MONOMER is considered to be non-mutagenic in this HPRT assay.

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12 DISTRIBUTION OF THE REPORT

Study Monitor	2x copy
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13 ANNEX I: TABLES OF RESULTS

13.1 Pre-test

Table 3: Toxicity data

	conc.	S9	Duration of	cells	number of colonies per flask			CE%	CE%
	µg/mL	mix	treatment	seeded	found			absolute	relative
Test group			(h)	I/II	I	II	mean		
Column	1	3	2	4	5	6	7	8	9
Solvent control		-	4	503	478	469	474	94.1	100.0
Test item	32.8	-	4	503	498	457	478	94.9	100.8
Test item	65.6	-	4	503	471	472	472	93.7	99.6
Test item	131.3	-	4	503	463	496	480	95.3	101.3
Test item	262.5	-	4	503	485	468	477	94.7	100.6
Test item	525.0	-	4	503	499	512	506	100.5	106.8
Test item	1050.0	-	4	503	452	478	465	92.4	98.2
Test item	2100.0	-	4	503	467	507	487	96.8	102.9
Test item	4200.0	-	4	503	477	488	483	95.9	101.9
Solvent control		+	4	503	472	451	462	91.7	100.0
Test item	32.8	+	4	503	490	468	479	95.2	103.8
Test item	65.6	+	4	503	479	462	471	93.5	102.0
Test item	131.3	+	4	503	481	494	488	96.9	105.6
Test item	262.5	+	4	503	468	473	471	93.5	102.0
Test item	525.0	+	4	503	530	491	511	101.5	110.6
Test item	1050.0	+	4	503	452	479	466	92.5	100.9
Test item	2100.0	+	4	503	491	470	481	95.5	104.1
Test item	4200.0	+	4	503	498	499	499	99.1	108.0
Solvent control		-	24	503	471	492	482	95.7	100.0
Test item	32.8	-	24	503	501	464	483	95.9	100.2
Test item	65.6	-	24	503	458	493	476	94.5	98.8
Test item	131.3	-	24	503	481	468	475	94.3	98.5
Test item	262.5	-	24	503	493	482	488	96.9	101.2
Test item	525.0	-	24	503	474	475	475	94.3	98.5
Test item	1050.0	-	24	503	461	487	474	94.2	98.4
Test item	2100.0	-	24	503	509	470	490	97.3	101.7
Test item	4200.0	-	24	503	461	471	466	92.6	96.8

13.2 Main Experiment

13.2.1 Experiment I Culture I

Table 4: Cloning Efficiency I (Survival)

	conc.	S9	cells	number of colonies per flask			CE I	CE I	cells/mL	cell density
Test group	µg/mL	mix	seeded	found			absolute	relative	at 1st	%
			I/II	I	II	mean		%	subcultivation	of control
Column	1	2	3	4	5	6	7	8	9	10
Solvent control with THF		-	513	408	398	403	0.79	100.0	1591000	100.0
Positive control with EMS	150.0	-	513	233	225	229	0.45	56.8	1558000	97.9
Test item	131.3	-	513	406	388	397	0.77	98.5	culture was not continued#	
Test item	262.5	-	513	397	361	379	0.74	94.0	1522000	95.7
Test item	525.0	-	513	415	403	409	0.80	101.5	1632000	102.6
Test item	1050.0	-	513	375	378	377	0.73	93.4	1630000	102.5
Test item	2100.0	-	513	385	396	391	0.76	96.9	1529000	96.1
Test item	4200.0	-	513	366	371	369	0.72	91.4	1216000	76.4
Solvent control with THF		+	513	544	474	509	0.99	100.0	1620000	100.0
Positive control with DMBA	1.3	+	513	151	138	145	0.28	28.4	1329000	82.0
Test item	131.3	+	513	471	501	486	0.95	95.5	culture was not continued#	
Test item	262.5	+	513	482	493	488	0.95	95.8	1699000	104.9
Test item	525.0	+	513	489	471	480	0.94	94.3	1235000	76.2
Test item	1050.0	+	513	525	449	487	0.95	95.7	1413000	87.2
Test item	2100.0	+	513	451	427	439	0.86	86.2	1459000	90.1
Test item	4200.0	+	513	502	381	442	0.86	86.7	1605000	99.1

culture was not continued since a minimum of only four analysable concentrations is required

Table 5: Cloning Efficiency II (Viability), experiment I, culture I

	conc.	S9	cells	number of colonies per flask			CE II	CE II	cells	cells
Test group	µg/mL	mix	seeded	found			absolute	relative	seeded	survived
			I/II	I	II	mean		%		
Column	1	2	3	4	5	6	7	8	9	10
Solvent control THF		-	545	404	428	416.0	0.76	100.0	444300	339135
Positive control with EMS	150.0	-	545	385	379	382.0	0.70	91.8	482100	337912
Test item	131.3	-	culture was not continued[#]							
Test item	262.5	-	503	489	493	491.0	0.98	127.9	420300	410273
Test item	525.0	-	504	422	397	409.5	0.81	106.4	458100	372206
Test item	1050.0	-	505	362	315	338.5	0.67	87.8	416100	278911
Test item	2100.0	-	501	307	289	298.0	0.59	77.9	471000	280156
Test item	4200.0	-	504	172	185	178.5	0.35	46.4	435900	154381
Solvent control with THF		+	530	418	398	408.0	0.77	100.0	403650	310734
Positive control with DMBA	1.3	+	560	209	213	211.0	0.38	48.9	406050	152994
Test item	131.3	+	culture was not continued[#]							
Test item	262.5	+	558	389	403	396.0	0.71	92.2	439650	312010
Test item	525.0	+	609	412	394	403.0	0.66	86.0	359550	237929
Test item	1050.0	+	537	409	383	396.0	0.74	95.8	461400	340250
Test item	2100.0	+	568	378	410	394.0	0.69	90.1	416700	289049
Test item	4200.0	+	612	398	386	392.0	0.64	83.2	362250	232029

culture was not continued since a minimum of only four analysable concentrations is required

Table 6: Mutagenicity data (Mutation rates), experiment I, culture I

	conc.	S9	number of mutant colonies per flask							mutant
Test group	µg/mL	mix	found after plating in TG medium						standard	colonies
			I	II	III	IV	V	mean	deviation	per 10^6 cells
Column	1	2	3	4	5	6	7	8	9	10
Solvent control with THF		-	2	1	1	4	2	2.0	1.2	5.9
Positive control with EMS	150.0	-	29	27	30	23	28	27.4	2.7	81.1
Test item	131.3	-	culture was not continued#							
Test item	262.5	-	1	1	1	2	4	1.8	1.3	4.4
Test item	525.0	-	3	2	2	3	2	2.4	0.5	6.4
Test item	1050.0	-	5	4	2	7	2	4.0	2.1	14.3
Test item	2100.0	-	2	6	3	4	3	3.6	1.5	12.8
Test item	4200.0	-	2	2	4	4	2	2.8	1.1	18.1
Solvent control with THF		+	7	6	5	6	4	5.6	1.1	18.0
Positive control with DMBA	1.3	+	184	198	207	172	193	190.8	13.4	1247.1
Test item	131.3	+	culture was not continued#							
Test item	262.5	+	6	7	6	3	6	5.6	1.5	17.9
Test item	525.0	+	3	2	1	4	3	2.6	1.1	10.9
Test item	1050.0	+	5	9	8	8	1	6.2	3.3	18.2
Test item	2100.0	+	2	3	1	2	5	2.6	1.5	9.0
Test item	4200.0	+	4	4	5	1	3	3.4	1.5	14.7

culture was not continued since a minimum of only four analysable concentrations is required

13.2.2 Experiment I Culture II

Table 7: Cloning Efficiency I (Survival), culture II

	conc.	S9	cells	number of colonies per flask			CE I	CE I	cells/mL	cell density
Test group	µg/mL	mix	seeded	found			absolute	relative	at 1st	%
			I/II	I	II	mean		%	subcultivation	of control
Column	1	2	3	4	5	6	7	8	9	10
Solvent control with THF		-	513	381	395	388	0.76	100.0	1420000	100.0
Positive control with EMS	150.0	-	513	219	226	223	0.43	57.3	1576000	111.0
Test item	131.3	-	513	393	397	395	0.77	101.8	culture was not continued#	
Test item	262.5	-	513	374	365	370	0.72	95.2	1464000	103.1
Test item	525.0	-	513	382	388	385	0.75	99.2	1567000	110.4
Test item	1050.0	-	513	365	357	361	0.70	93.0	1770000	124.6
Test item	2100.0	-	513	389	373	381	0.74	98.2	1482000	104.4
Test item	4200.0	-	513	358	346	352	0.69	90.7	1189000	83.7
Solvent control with THF		+	513	522	496	509	0.99	100.0	1277000	100.0
Positive control with DMBA	1.3	+	513	145	151	148	0.29	29.1	871000	68.2
Test item	131.3	+	513	434	473	454	0.88	89.1	culture was not continued#	
Test item	262.5	+	513	512	461	487	0.95	95.6	1380000	108.1
Test item	525.0	+	513	485	511	498	0.97	97.8	1140000	89.3
Test item	1050.0	+	513	472	470	471	0.92	92.5	1460000	114.3
Test item	2100.0	+	513	527	494	511	1.00	100.3	900000	70.5
Test item	4200.0	+	513	503	487	495	0.96	97.2	935000	73.2

culture was not continued since a minimum of only four analysable concentrations is required

Table 8: Cloning Efficiency II (Viability), experiment I, culture II

	conc.	S9	cells	number of colonies per flask			CE II	CE II	cells	cells
Test group	µg/mL	mix	seeded	found			absolute	relative	seeded	survived
			I/II	I	II	mean		%		
Column	1	2	3	4	5	6	7	8	9	10
Solvent control THF		-	573	415	432	423.5	0.74	100.0	503700	372281
Positive control with EMS	150.0	-	506	389	379	384.0	0.76	102.7	399600	303254
Test item	131.3	-		culture was not continued[#]						
Test item	262.5	-	500	492	478	485.0	0.97	131.2	395100	383247
Test item	525.0	-	580	412	388	400.0	0.69	93.3	526500	363103
Test item	1050.0	-	636	337	308	322.5	0.51	68.6	509300	258254
Test item	2100.0	-	507	312	276	294.0	0.58	78.5	456300	264600
Test item	4200.0	-	501	164	198	181.0	0.36	48.9	400200	144583
Solvent control with THF		+	604	410	423	416.5	0.69	100.0	447300	308444
Positive control with DMBA	1.3	+	516	211	198	204.5	0.40	57.5	596200	236285
Test item	131.3	+		culture was not continued[#]						
Test item	262.5	+	501	398	402	400.0	0.80	115.8	500400	399521
Test item	525.0	+	500	412	408	410.0	0.82	118.9	424800	348336
Test item	1050.0	+	506	386	401	393.5	0.78	112.8	430800	335019
Test item	2100.0	+	508	408	398	403.0	0.79	115.0	499200	396019
Test item	4200.0	+	504	385	412	398.5	0.79	114.7	444600	351534

culture was not continued since a minimum of only four analysable concentrations is required

Table 9: Mutagenicity data (Mutation rates), experiment I, culture II

	conc.	S9	number of mutant colonies per flask							mutant
Test group	µg/mL	mix	found after plating in TG medium						standard	colonies
			I	II	III	IV	V	mean	deviation	per 10^6 cells
Column	1	2	3	4	5	6	7	8	9	10
Solvent control with THF		-	5	6	8	1	3	4.6	2.7	12.4
Positive control with EMS	150.0	-	32	29	30	33	29	30.6	1.8	100.9
Test item	131.3	-	culture was not continued[#]							
Test item	262.5	-	3	2	2	3	5	3.0	1.2	7.8
Test item	525.0	-	4	10	4	2	9	5.8	3.5	16.0
Test item	1050.0	-	5	3	5	6	4	4.6	1.1	17.8
Test item	2100.0	-	4	3	5	7	2	4.2	1.9	15.9
Test item	4200.0	-	7	8	8	6	5	6.8	1.3	47.0
Solvent control with THF		+	5	1	3	5	2	3.2	1.8	10.4
Positive control with DMBA	1.3	+	195	245	223	201	243	221.4	23.1	937.0
Test item	131.3	+	culture was not continued[#]							
Test item	262.5	+	2	3	6	1	4	3.2	1.9	8.0
Test item	525.0	+	3	2	5	10	3	4.6	3.2	13.2
Test item	1050.0	+	2	4	2	6	1	3.0	2.0	9.0
Test item	2100.0	+	2	3	3	1	1	2.0	1.0	5.1
Test item	4200.0	+	9	2	6	6	3	5.2	2.8	14.8

culture was not continued since a minimum of only four analysable concentrations is required

13.2.3 Experiment II, Culture I

Table 10: Cloning Efficiency I (Survival)

	conc.	S9	cells	number of colonies per flask			CE I	CE I	cells/mL	cell density
Test group	µg/mL	mix	seeded	found			absolute	relative	at 1st	%
			I/II	I	II	mean		%	subcultivation	of control
Column	1	2	3	4	5	6	7	8	9	10
Solvent control with THF		-	514	425	416	421	0.82	100.0	1997000	100.0
Positive control with EMS	75.0	-	514	350	361	356	0.69	84.5	2373000	118.8
Test item	131.3	-	514	culture was not continued#						
Test item	262.5	-	514	63	87	75	0.15	17.8	678900	34.0
Test item	525.0	-	514	221	248	235	0.46	55.8	1478000	74.0
Test item	1050.0	-	514	309	278	294	0.57	69.8	1575000	78.9
Test item	2100.0	-	514	178	68	123	0.24	29.3	1492000	74.7
Test item	4200.0	-	514	284	353	319	0.62	75.7	1009000	50.5
Solvent control with THF		+	514	326	354	340	0.66	100.0	1728000	100.0
Positive control with DMBA	1.3	+	514	35	45	40	0.08	11.8	396900	23.0
Test item	131.3	+	514	295	311	303	0.59	89.1	culture was not continued#	
Test item	262.5	+	514	304	314	309	0.60	90.9	2048000	118.5
Test item	525.0	+	514	340	307	324	0.63	95.1	3024000	175.0
Test item	1050.0	+	514	332	348	340	0.66	100.0	1821000	105.4
Test item	2100.0	+	514	310	308	309	0.60	90.9	1729000	100.1
Test item	4200.0	+	514	331	325	328	0.64	96.5	1781000	103.1

culture was not continued since a minimum of only four analysable concentrations is required

Table 11: Cloning Efficiency II (Viability), experiment II, culture I

	conc.	S9	cells	number of colonies per flask			CE II	CE II	cells	cells
Test group	µg/mL	mix	seeded	found			absolute	relative	seeded	survived
			I/II	I	II	mean		%		
Column	1	2	3	4	5	6	7	8	9	10
Solvent control THF		-	502	573	491	532.0	1.06	100.0	489500	518753
Positive control with EMS	75.0	-	506	399	378	388.5	0.77	72.4	440100	337903
Test item	131.3	-	culture was not continued[#]							
Test item	262.5	-	608	518	503	510.5	0.84	79.2	440200	369609
Test item	525.0	-	526	415	423	419.0	0.80	75.2	522300	416053
Test item	1050.0	-	559	430	438	434.0	0.78	73.3	526400	408690
Test item	2100.0	-	558	456	464	460.0	0.82	77.8	505100	416391
Test item	4200.0	-	575	384	391	387.5	0.67	63.6	523800	352996
Solvent control with THF		+	502	383	371	377.0	0.75	100.0	405450	304491
Positive control with DMBA	1.3	+	602	307	315	311.0	0.52	68.8	342000	176681
Test item	131.3	+	culture was not continued[#]							
Test item	262.5	+	500	431	408	419.5	0.84	111.7	358050	300404
Test item	525.0	+	500	367	385	376.0	0.75	100.1	367500	276360
Test item	1050.0	+	508	430	401	415.5	0.82	108.9	347400	284143
Test item	2100.0	+	502	330	361	345.5	0.69	91.6	346350	238374
Test item	4200.0	+	504	364	375	369.5	0.73	97.6	336000	246333

culture was not continued since a minimum of only four analysable concentrations is required

Table 12: Mutagenicity data (Mutation rates), experiment II, culture I

	conc.	S9	number of mutant colonies per flask							mutant
Test group	µg/mL	mix	found after plating in TG medium						standard	colonies
			I	II	III	IV	V	mean	deviation	per 10^6 cells
Column	1	2	3	4	5	6	7	8	9	10
Solvent control with THF		-	4	11	7	5	7	6.8	2.7	13.1
Positive control with EMS	75.0	-	80	73	62	66	61	68.4	8.0	202.4
Test item	131.3	-	culture was not continued#							
Test item	262.5	-	2	5	8	6	7	5.6	2.3	15.2
Test item	525.0	-	4	4	5	4	6	4.6	0.9	11.1
Test item	1050.0	-	16	8	11	9	11	11.0	3.1	26.9
Test item	2100.0	-	7	8	6	4	2	5.4	2.4	13.0
Test item	4200.0	-	10	15	6	13	7	10.2	3.8	28.9
Solvent control with THF		+	5	3	4	6	8	5.2	1.9	17.1
Positive control with DMBA	1.3	+	158	183	168	177	172	171.6	9.4	971.2
Test item	131.3	+	culture was not continued#							
Test item	262.5	+	7	8	4	4	6	5.8	1.8	19.3
Test item	525.0	+	6	8	10	9	7	8.0	1.6	28.9
Test item	1050.0	+	5	11	7	18	3	8.8	5.9	31.0
Test item	2100.0	+	1	6	3	7	2	3.8	2.6	15.9
Test item	4200.0	+	2	4	4	4	6	4.0	1.4	16.2

culture was not continued since a minimum of only four analysable concentrations is required

13.2.4 Experiment II, Culture II

Table 13: Cloning Efficiency I (Survival)

	conc.	S9	cells	number of colonies per flask			CE I	CE I	cells/mL	cell density
Test group	µg/mL	mix	seeded	found			absolute	relative	at 1st	%
			I/II	I	II	mean		%	subcultivation	of control
Column	1	2	3	4	5	6	7	8	9	10
Solvent control with THF		-	514	350	364	357	0.69	100.0	2581000	100.0
Positive control with EMS	75.0	-	514	259	278	269	0.52	75.2	1316000	51.0
Test item	131.3	-	514	culture was not continued[#]						
Test item	262.5	-	514	83	79	81	0.16	22.7	399500	15.5
Test item	525.0	-	514	220	207	214	0.42	59.8	992200	38.4
Test item	1050.0	-	514	118	204	161	0.31	45.1	1435000	55.6
Test item	2100.0	-	514	155	258	207	0.40	57.8	1199000	46.5
Test item	4200.0	-	514	184	168	176	0.34	49.3	533500	20.7
Solvent control with THF		+	514	368	379	374	0.73	100.0	2421000	100.0
Positive control with DMBA	1.3	+	514	121	123	122	0.24	32.7	260200	10.7
Test item	131.3	+	514	357	365	361	0.70	96.7	culture was not continued[#]	
Test item	262.5	+	514	316	334	325	0.63	87.0	1688000	69.7
Test item	525.0	+	514	371	364	368	0.71	98.4	1406000	58.1
Test item	1050.0	+	514	344	353	349	0.68	93.3	1484000	61.3
Test item	2100.0	+	514	300	328	314	0.61	84.1	1104000	45.6
Test item	4200.0	+	514	251	273	262	0.51	70.1	1255000	51.8

culture was not continued since a minimum of only four analysable concentrations is required

Table 14: Cloning Efficiency II (Viability), experiment II, culture II

	conc.	S9	cells	number of colonies per flask			CE II	CE II	cells	cells
Test group	µg/mL	mix	seeded	found			absolute	relative	seeded	survived
			I/II	I	II	mean		%		
Column	1	2	3	4	5	6	7	8	9	10
Solvent control THF		-	500	315	341	328.0	0.66	100.0	445200	292051
Positive control with EMS	75.0	-	562	363	352	357.5	0.64	97.0	543300	345605
Test item	131.3	-	culture was not continued[#]							
Test item	262.5	-	500	245	296	270.5	0.54	82.5	466200	252214
Test item	525.0	-	553	325	334	329.5	0.60	90.8	507300	302270
Test item	1050.0	-	555	367	344	355.5	0.64	97.6	538800	345123
Test item	2100.0	-	501	276	312	294.0	0.59	89.5	477300	280092
Test item	4200.0	-	500	416	375	395.5	0.79	120.6	425400	336491
Solvent control with THF		+	500	315	327	321.0	0.64	100.0	479100	307582
Positive control with DMBA	1.3	+	578	346	321	333.5	0.58	89.9	486900	280936
Test item	131.3	+	culture was not continued[#]							
Test item	262.5	+	506	327	333	330.0	0.65	101.6	483000	315000
Test item	525.0	+	504	317	325	321.0	0.64	99.2	442500	281830
Test item	1050.0	+	504	345	320	332.5	0.66	102.8	411000	271146
Test item	2100.0	+	504	329	341	335.0	0.66	103.5	485400	322637
Test item	4200.0	+	538	393	378	385.5	0.72	111.6	438750	314383

culture was not continued since a minimum of only four analysable concentrations is required

Table 15: Mutagenicity data (Mutation rates), experiment II, culture II

	conc.	S9	number of mutant colonies per flask							mutant
Test group	µg/mL	mix	found after plating in TG medium						standard	colonies
			I	II	III	IV	V	mean	deviation	per 10^6 cells
Column	1	2	3	4	5	6	7	8	9	10
Solvent control with THF		-	6	6	3	5	10	6.0	2.5	20.5
Positive control with EMS	75.0	-	56	63	68	68	71	65.2	5.9	188.7
Test item	131.3	-	culture was not continued#							
Test item	262.5	-	8	9	4	11	4	7.2	3.1	28.5
Test item	525.0	-	10	13	13	8	14	11.6	2.5	38.4
Test item	1050.0	-	13	4	9	10	6	8.4	3.5	24.3
Test item	2100.0	-	6	2	3	2	7	4.0	2.3	14.3
Test item	4200.0	-	3	3	4	3	6	3.8	1.3	11.3
Solvent control with THF		+	9	6	3	4	2	4.8	2.8	15.6
Positive control with DMBA	1.3	+	190	194	205	199	227	203.0	14.5	722.6
Test item	131.3	+	culture was not continued#							
Test item	262.5	+	6	3	2	8	5	4.8	2.4	15.2
Test item	525.0	+	3	3	2	5	6	3.8	1.6	13.5
Test item	1050.0	+	3	0	4	2	1	2.0	1.6	7.4
Test item	2100.0	+	8	1	8	3	9	5.8	3.6	18.0
Test item	4200.0	+	9	1	6	8	2	5.2	3.6	16.5

culture was not continued since a minimum of only four analysable concentrations is required

14 ANNEX II: HISTORICAL DATA

These values represent the historical control data from 2001 – 2007

Number of mutant colonies per 106 cells						
4 h treatment						
	Negative control*		Positive control		Solvent control **	
	without S9 mix	with S9 mix	without S9 mix EMS	with S9 mix DMBA	without S9 mix	with S9 mix
range:	0.6 – 24.0	0.8 – 30.2	58.3 – 721.7	94.9 – 2873.9	1.7 – 31.1	1.1 – 29.1
Mean value:	8.8	9.2	163.2	813.2	9.6	9.4
Standard deviation:	5.1	5.6	90.3	428.2	5.7	5.1
24 h treatment						
	Negative control*		Positive control		Solvent control **	
	without S9 mix		without S9 mix EMS		without S9 mix	
range:	0.5 – 28.0		61.8 – 1528.2		1.0 – 31.8	
Mean value:	9.5		435.3		10.9	
Standard deviation:	5.9		343.6		6.9	

* medium ** deionised water, DMSO, acetone, ethanol, DMF, THF