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HARLAN CCR STUDY 1502500

GENE MUTATION ASSAY
IN CHINESE HAMSTER V79 CELLS
IN VITRO
(V79 / HPRT)
WITH
Bis(2-ethylhexyl)tetrabromophthalate

REPORT
(FIRST ORIGINAL OF TWO)

Study Completion Date:
February 26, 2013

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 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

Harlan Cytotest Cell Research GmbH
Harlan 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	**2** Toxicity studies
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	**3** Mutagenicity studies
6 Prüfungen zur Bestimmung von Rückständen	**6** Residues
8 Analytische Prüfungen an biologischen Materialien	**8** Analytical studies on biological materials

15.08. und 27. – 29.10.2008
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 **30. März 2009**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, LÄNDLICHEN RAUM UND VERBRAUCHERSCHUTZ 2

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

2 CONTENTS

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3 PREFACE

3.1 General

Title:	Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with Bis(2-ethylhexyl)tetrabromophthalate
Sponsor:	Lanxess Deutschland GmbH 51369 Leverkusen Germany
Study Monitor:	Dr. Gabriele Schmuck Bayer HealthCare Pharmaceuticals Bayer Pharma AG Global Drug Discovery-GED-PSICO-AHGLP Aprather Weg 42096 Wuppertal Germany
Test Facility:	Harlan Cytotest Cell Research GmbH (Harlan CCR) In den Leppsteinswiesen 19 64380 Rossdorf Germany

3.2 Responsibilities

Study Director:	Dr. Hans-Eric Wollny
Deputy Study Director:	Dipl. Biol. Andrea Sokolowski
Management:	Dr. Wolfgang Völkner
Head of Quality Assurance Unit:	Frauke Hermann

3.3 Schedule

Experimental Starting Date:	January 08, 2013
Experimental Completion Date:	February 06, 2013

3.4 Project Staff Signature

Study Director Dr. Hans-Eric Wollny

Wollny

Date: February 26, 2013

3.5 Good Laboratory Practice

The study was performed in compliance with:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version.

"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 Regulation (EC) No. 440/2008 B.17 "Mutagenicity – *In vitro* Mammalian Cell Gene Mutation Test", dated May 30, 2008.

3.7 Archiving

Harlan CCR will archive:

Raw data, study plan, report, and specimens (if any) for at least 3 years at the test facility's archive. Thereafter, the material will be transferred to the GLP archive of Harlan Laboratories Ltd. in Füllinsdorf, Switzerland for archiving the remaining time up to a total archiving period of 15 years. No data will be discarded without the sponsor's prior written consent.

A sample of the test item will be archived two years after the expiration date provided by the Sponsor. If no expiration date is given, the archiving period will be the required 15 years. Thereafter, the samples will be discarded without further notice.

3.8 Deviations to the study plan

There were no deviations to the study plan.

4 STATEMENT OF COMPLIANCE

Harlan CCR Study:	1502500
Test Item:	Bis(2-ethylhexyl)tetrabromophthalate
Study Director:	Dr. Hans-Eric Wollny
Title:	Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with Bis(2-ethylhexyl)tetrabromophthalate

This study performed in the test facility of Harlan CCR was conducted in compliance with Good Laboratory Practice Regulations:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version.

"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

Harlan CCR
Dr. Hans-Eric Wollny

Wollny
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Date: February 26, 2013

5 STATEMENT OF QUALITY ASSURANCE UNIT

Harlan CCR Study: 1502500

Test Item: Bis(2-ethylhexyl)tetrabromophthalate

Study Director: Dr. Hans-Eric Wollny

Title: Gene Mutation Assay
in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)
with Bis(2-ethylhexyl)tetrabromophthalate

The general facilities and activities of Harlan CCR 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:	August 10, 2012	August 10, 2012
1st Amendment to Study Plan:	November 06, 2012	November 06, 2012
Study Inspection Preparation for Application and Application:	January 15, 2013	January 15, 2013
Report:	February 25, 2013	February 25, 2013

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

Head of Quality Assurance Unit Frauke Hermann

F. Hermann

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Date: February 26, 2013

6 SUMMARY

The study was performed to investigate the potential of Bis(2-ethylhexyl)tetrabromophthalate 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 maximum test item concentration of 5800 µg/mL used in the pre-experiment with and without metabolic activation was equal to approximately 5000 µg/mL of the pure substance. The test item was dissolved in acetone. The dose range of the main experiments was limited by phase separation of the test item.

The tested concentrations are described in chapter 8.6 (page 14). The evaluated experimental points and the results are summarised in Table 1 (page 22).

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.

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, Bis(2-ethylhexyl)tetrabromophthalate is considered to be non-mutagenic in this HPRT assay.

7 OBJECTIVE

7.1 Aims of the Study

This *in vitro* assay was performed to assess the potential of the test item to induce gene mutations by means of two independent HPRT experiments using the Chinese hamster cell line V79. The treatment time was 4 hours in the first experiment with and without metabolic activation. In the second experiment the cells were exposed to the test item for 24 hours without metabolic activation and 4 hours with 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.

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 6-TG (6-thioguanine) to its toxic ribophosphorylated derivative. Therefore, cells deficient in HPRT due to a forward mutation are resistant to 6-TG. These cells are able to proliferate in the presence of 6-TG 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 6-TG to the culture medium.

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 Test Item Number: S1388311

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

Identity:	Bis(2-ethylhexyl)tetrabromophthalate
Batch No.:	12128E71
Purity:	90.6%; dose calculation adjusted to purity
Molecular Weight:	706.1 g/mol
Storage:	At room temperature
Expiration Date:	May 09, 2015

Results of the analyses for stability of Bis(2-ethylhexyl)tetrabromophthalate:

Nominal value mg/mL	Theoretical value mg/mL	Content of Test Item mg/mL			Recovery as % of theoretical value		
		Start	After 2 hours	After 24 hours	Start	After 2 hours	After 24 hours
0.05	0.0506	0.0520	0.0524	0.0525	103	104	104
500	547.9	572.1	577.2	585.7	104	105	107

The 0.05 mg/mL and 500 mg/mL formulations of the test item in acetone are homogeneous and stable at room temperature for at least 24 hours.

On the day of the experiment (immediately before treatment), the test item was dissolved in acetone (purity 99.9%). The final concentration of acetone 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	Test Item 5800 µg/mL
Osmolarity mOsm	368	329
pH-value	7.49	7.48

8.2 Controls

8.2.1 Solvent Controls

Concurrent solvent controls (acetone) were performed.

Name:	Acetone
Supplier:	Merck, 64293 Darmstadt, Germany
Purity:	99.9%
Lot. No.:	K42445614127 (pre-experiment and experiment I) 1299231 (experiment II)
Expiry Date:	June 2016 (pre-experiment and experiment I) August 2017 (experiment II)

8.2.2 Positive Control Substances

Without metabolic activation

Name:	EMS; ethylmethane sulfonate
Supplier:	Acros Organics, 2440 Geel, Belgium
Lot No.:	A 0315915
Expiry date:	May 2013
Dissolved in:	Nutrient medium
Final concentration:	0.15 mg/mL = 1.2 mM

With metabolic activation

Name:	DMBA; 7,12-dimethylbenz(a)anthracene
Supplier:	Sigma Chemie GmbH, 82041 Deisenhofen, Germany
Lot No.:	100M1178V (experiment I)
	061M1197V (experiment II)
Expiry date:	September 2016 (experiment I)
	May 2017 (experiment II)
Dissolved in:	DMSO; dimethylsulfoxide
Supplier:	Merck, 64293 Darmstadt, Germany,
	final concentration in nutrient medium 0.5%
Final concentration:	1.1 µg/mL = 4.3 µM

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, 64287 Darmstadt, Germany) are stored in liquid nitrogen in the cell bank of Harlan 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 (4). Each batch is screened for mycoplasm 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. About 5×10^5 cells were seeded into each flask with 15 mL of MEM (minimal essential medium) containing Hank's salts supplemented with 10% foetal bovine serum (FBS), neomycin (5 µg/mL) and amphotericin B (1%). The cells were sub-cultured twice weekly. The cell cultures were incubated at 37°C in a 1.5% carbon dioxide atmosphere (98.5% air).

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 Harlan CCR)

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 were prepared from 8 – 12 weeks old male Wistar rats (Hsd Cpb: WU, weight approx. 220 – 320 g, Harlan Laboratories B.V., 5960 AD Horst, The Netherlands) induced by intraperitoneal applications of 80 mg/kg b.w. phenobarbital (Desitin, 22335 Hamburg, Germany) and by peroral administrations of 80 mg/kg b.w. β-naphthoflavone (Sigma-Aldrich Chemie GmbH, 82024 Taufkirchen, Germany) 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 parts) 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. Each batch of S9 mix was routinely tested with 2-aminoanthracene as well as benzo(a)pyrene.

The protein concentration of the S9 preparation was 27.9 mg/mL (Lot. No.: 191012) in the pre-experiment and in the main experiments.

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-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.

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 current OECD Guideline for Cell Gene Mutation Tests at least four analysable concentrations should be used in two parallel cultures. The pre-experiment was performed using eight concentrations. For freely-soluble and non-cytotoxic test items the maximum concentration should be 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest. For cytotoxic test items the maximum concentration should result in approximately 10 to 20% relative survival or cell density at sub-cultivation and the analysed concentrations should cover a range from the maximum to little or no cytotoxicity. Relatively insoluble test items should be tested up to the highest concentration that can be formulated in an appropriate solvent as solution or homogenous suspension. These test items should be tested up or beyond their limit of solubility. Precipitation should be evaluated at the end of treatment by the unaided eye. Furthermore, test item induced changes in osmolarity are considered.

The highest test item concentration used in the pre-test was 5800 µg/mL equal to approximately 5000 µg/mL of the pure substance. The test item was dissolved in acetone. Test item concentrations between 45.3 µg/mL and 5800 µg/mL were used to evaluate toxicity in the presence (4 hours treatment) and absence (4 hours and 24 hours treatment) of metabolic activation. No relevant toxic effect occurred up to the maximum concentration with and without metabolic activation following 4 and 24 hours treatment.

The test medium was checked for precipitation or phase separation at the end of each treatment period (4 or 24 hours) prior to removal to the test item. Phase separation occurred at the lowest to the highest concentration at the end of 4 and 24 hours treatment with and without metabolic activation.

There was no relevant shift of pH and osmolarity of the medium even at the maximum concentration of the test item.

Based on the results of the pre-experiment, the maximum concentration of the main experiments was selected. In both main experiments the individual concentrations were spaced by a factor of 3 to cover a range of soluble as well as insoluble concentrations.

Doses applied in the gene mutation assay with Bis(2-ethylhexyl)tetrabromophthalate (concentrations given in bold characters were chosen for the mutation rate analysis):

exposure period	S9 mix	concentrations in µg/mL					
		Experiment I					
4 hours	-	**3.0**	**9.0**	**18.0**	**54.0** **PS**	**162.0** **PS**	486.0 **PS**
4 hours	+	**3.0**	**9.0**	**18.0**	**54.0** **PS**	**162.0** **PS**	486.0 **PS**
		Experiment II					
24 hours	-	**3.0**	**9.0**	**18.0**	**54.0** **PS**	**162.0** **PS**	486.0 **PS**
4 hours	+	**3.0**	**9.0**	**18.0**	**54.0** **PS**	**162.0** **PS**	486.0 **PS**

PS = Phase Separation

In experiment I and II the cultures at the maximum concentration with and without metabolic activation were not continued to avoid analysis of too many concentrations showing phase separation.

8.7 Experimental Performance

8.7.1 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).

8.7.2 Seeding

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.

The PBS is composed as follows (per litre):

NaCl	8000 mg
KCl	200 mg
KH_2PO_4	200 mg
Na_2HPO_4	150 mg

Prior to the trypsin treatment the cells were rinsed with Ca-Mg-free salt solution (PBS) 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.

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

NaCl	8000 mg
KCl	400 mg
Glucose	1100 mg
$Na_2HPO_4\times2H_2O$	192 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.

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.

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

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 (see Annex II).
- the positive control substances should produce a significant increase in mutant colony frequencies.
- 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 cloning efficiency 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 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.

8.11 Statistical Analysis

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. The number 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 were considered together.

experimental group	p-value
experiment I, culture I without S9 mix	0.503
experiment I, culture II without S9 mix	0.125
experiment I, culture I with S9 mix	0.416
experiment I, culture II with S9 mix	0.608
experiment II, culture I without S9 mix	0.802
experiment II, culture II without S9 mix	0.168
experiment II, culture I with S9 mix	0.389
experiment II, culture II with S9 mix	0.416

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 Bis(2-ethylhexyl)tetrabromophthalate was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster.

The study was performed in two independent experiments, using identical experimental procedures. In the first experiment the treatment period was 4 hours with and without metabolic activation. The second experiment was performed with a treatment time of 4 hours with and 24 hours without metabolic activation.

Doses applied in the gene mutation assay with Bis(2-ethylhexyl)tetrabromophthalate:

exposure period	S9 mix	concentrations in µg/mL				
				Experiment I		
4 hours	-	3.0	9.0	18.0	54.0 PS	162.0 PS
4 hours	+	3.0	9.0	18.0	54.0 PS	162.0 PS
				Experiment II		
24 hours	-	3.0	9.0	18.0	54.0 PS	162.0 PS
4 hours	+	3.0	9.0	18.0	54.0 PS	162.0 PS

PS = Phase Separation

Phase separation of the test item at the end of treatment was noted at 54.0 µg/mL and above in experiment I and II in the presence and absence of metabolic activation.

No relevant toxic effects occurred up to the maximum evaluated concentration of 162.0 µg/mL.

No relevant and reproducible increase in mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration with and without metabolic activation. The mutation frequency did not exceed the range of solvent controls.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. No significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in any of the experimental groups.

In both experiments of this study (with and without S9 mix) the range of the solvent controls was from 10.0 up to 23.4 mutants per 10^6 cells; the range of the groups treated with the test item was from 5.5 up to 26.7 mutants per 10^6 cells.

EMS (150 µg/mL) and DMBA (1.1 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, Bis(2-ethylhexyl)tetrabromophthalate is considered to be non-mutagenic in this HPRT assay.

11 REFERENCES

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4. M.O. Bradley, B. Bhuyan, M.C. Francis, R. Langenbach, A. Peterson, and E. Huberman
   Mutagenesis by chemical agents in V79 Chinese hamster cells: a review and analysis of the literature. A report of the gene-tox program

12 DISTRIBUTION OF THE REPORT

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13 ANNEX I: TABLES OF RESULTS

Table 1: Summary of results

				relative	relative	relative	mutant		relative	relative	relative	mutant	
	conc.	PS	S9	cloning	cell	cloning	colonies/	induction	cloning	cell	cloning	colonies/	induction
	µg/mL		mix	efficiency I	density	efficiency II	10^6 cells	factor	efficiency I	density	efficiency II	10^6 cells	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 acetone			-	100.0	100.0	100.0	12.6	1.0	100.0	100.0	100.0	13.0	1.0
Positive control (EMS)	150.0		-	86.3	76.3	121.5	305.0	24.2	78.7	115.8	103.0	210.3	16.2
Test item	3.0		-	105.1	95.2	144.0	9.8	0.8	87.3	66.8	101.7	11.9	0.9
Test item	9.0		-	99.0	95.5	127.4	8.8	0.7	85.6	88.1	86.8	14.4	1.1
Test item	18.0		-	95.4	85.4	121.5	5.5	0.4	91.1	61.8	96.1	15.0	1.2
Test item	54.0	PS	-	109.5	80.1	105.1	12.9	1.0	86.5	81.1	95.7	10.2	0.8
Test item	162.0	PS	-	105.5	89.9	127.6	11.9	0.9	85.9	81.0	108.6	19.3	1.5
Test item	486.0	PS	-	94.7	0.0	culture was not continued[#]			82.0	0.0	culture was not continued[#]		
Experiment I / 4 h treatment				culture I					culture II				
Solvent control with acetone			+	100.0	100.0	100.0	14.3	1.0	100.0	100.0	100.0	13.6	1.0
Positive control (DMBA)	1.1		+	85.6	93.1	98.5	345.9	24.3	87.6	100.4	93.5	251.4	18.5
Test item	3.0		+	95.2	98.8	123.0	10.8	0.8	97.0	90.0	106.8	18.6	1.4
Test item	9.0		+	95.6	95.0	108.3	13.8	1.0	96.2	118.0	100.1	14.4	1.1
Test item	18.0		+	105.6	108.4	124.5	12.9	0.9	104.0	91.5	94.9	16.3	1.2
Test item	54.0	PS	+	90.8	106.8	115.5	16.2	1.1	97.4	96.4	94.7	20.2	1.5
Test item	162.0	PS	+	98.8	124.0	129.6	10.2	0.7	94.7	97.9	95.9	13.2	1.0
Test item	486.0	PS	+	91.0	0.0	culture was not continued[#]			91.1	0.0	culture was not continued[#]		
Experiment II / 24 h treatment				culture I					culture II				
Solvent control with acetone			-	100.0	100.0	100.0	10.0	1.0	100.0	100.0	100.0	15.5	1.0
Positive control (EMS)	150.0		-	85.8	121.2	59.6	747.2	74.5	98.4	138.3	56.6	649.8	41.9
Test item	3.0		-	80.8	64.6	107.5	8.2	0.8	104.8	165.7	104.3	7.9	0.5
Test item	9.0		-	89.9	91.4	110.4	13.3	1.3	107.6	138.9	94.2	12.4	0.8
Test item	18.0		-	94.6	101.6	105.7	9.5	0.9	78.9	163.3	90.0	26.6	1.7
Test item	54.0	PS	-	83.5	130.9	103.2	14.8	1.5	101.9	166.1	97.3	22.8	1.5
Test item	162.0	PS	-	91.0	114.7	99.6	10.5	1.1	123.5	145.5	87.2	26.7	1.7
Test item	486.0	PS	-	100.3	0.0	culture was not continued[#]			110.0	0.0	culture was not continued[#]		
Experiment II / 4 h treatment													
Solvent control with acetone			+	100.0	100.0	100.0	14.8	1.0	100.0	100.0	100.0	23.4	1.0
Positive control (DMBA)	1.1		+	92.7	65.6	50.0	764.0	51.6	106.8	80.7	76.1	882.2	37.8
Test item	3.0		+	90.4	89.6	84.9	15.2	1.0	120.1	99.8	122.7	10.3	0.4
Test item	9.0		+	102.0	88.9	88.0	18.4	1.2	94.1	99.9	126.2	10.9	0.5
Test item	18.0		+	86.0	88.5	85.9	9.6	0.6	96.2	106.3	107.9	11.0	0.5
Test item	54.0	PS	+	71.5	93.0	88.1	11.7	0.8	91.1	104.4	101.5	16.9	0.7
Test item	162.0	PS	+	104.1	90.5	88.9	19.5	1.3	123.1	107.4	114.7	8.3	0.4
Test item	486.0	PS	+	58.2	0.0	culture was not continued[#]			103.8	0.0	culture was not continued[#]		

PS = Phase separation

culture was not continued to avoid analysis of too many phase separating concentrations

13.1 Pre-test

Table 2: Toxicity data

	conc.	S9	Duration of	cells	number of colonies per flask			CE%	CE%	
	µg/mL	mix	treatment	seeded	found			absolute	relative	phase separation
Test group			(h)	I/II	I	II	mean			
Column	1	2	3	4	5	6	7	8	9	10
Solvent control with acetone		-	4	505	311	307	309	61.2	100.0	
Test item	45.3	-	4	505	302	321	312	61.7	100.8	phase separation
Test item	90.6	-	4	505	319	297	308	61.0	99.7	phase separation
Test item	181.3	-	4	505	296	301	299	59.1	96.6	phase separation
Test item	362.5	-	4	505	315	316	316	62.5	102.1	phase separation
Test item	725.0	-	4	505	304	324	314	62.2	101.6	phase separation
Test item	1450.0	-	4	505	297	283	290	57.4	93.9	phase separation
Test item	2900.0	-	4	505	299	290	295	58.3	95.3	phase separation
Test item	5800.0	-	4	505	271	260	266	52.6	85.9	phase separation
Solvent control with acetone		+	4	505	281	271	276	54.7	100.0	
Test item	45.3	+	4	505	293	280	287	56.7	103.8	phase separation
Test item	90.6	+	4	505	291	288	290	57.3	104.9	phase separation
Test item	181.3	+	4	505	275	283	279	55.2	101.1	phase separation
Test item	362.5	+	4	505	290	282	286	56.6	103.6	phase separation
Test item	725.0	+	4	505	286	275	281	55.5	101.6	phase separation
Test item	1450.0	+	4	505	259	264	262	51.8	94.7	phase separation
Test item	2900.0	+	4	505	266	271	269	53.2	97.3	phase separation
Test item	5800.0	+	4	505	281	273	277	54.9	100.4	phase separation
Solvent control with acetone		-	24	505	342	329	336	66.4	100.0	
Test item	45.3	-	24	505	336	340	338	66.9	100.7	phase separation
Test item	90.6	-	24	505	348	327	338	66.8	100.6	phase separation
Test item	181.3	-	24	505	313	308	311	61.5	92.5	phase separation
Test item	362.5	-	24	505	336	319	328	64.9	97.6	phase separation
Test item	725.0	-	24	505	351	338	345	68.2	102.7	phase separation
Test item	1450.0	-	24	505	317	329	323	64.0	96.3	phase separation
Test item	2900.0	-	24	505	346	351	349	69.0	103.9	phase separation
Test item	5800.0	-	24	505	312	317	315	62.3	93.7	phase separation

13.2 Main Experiment

13.2.1 Experiment I Culture I

Table 3: Cloning Efficiency I (Survival)

	conc.	PS	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	11
Solvent control with acetone			-	508	393	375	384.0	0.8	100.0	1424000	100.0
Positive control with EMS	150.0		-	508	328	335	331.5	0.7	86.3	1086000	76.3
Test item	3.0		-	508	410	397	403.5	0.8	105.1	1356000	95.2
Test item	9.0		-	508	386	374	380.0	0.7	99.0	1360000	95.5
Test item	18.0		-	508	362	371	366.5	0.7	95.4	1216000	85.4
Test item	54.0	PS	-	508	415	426	420.5	0.8	109.5	1140000	80.1
Test item	162.0	PS	-	508	401	409	405.0	0.8	105.5	1280000	89.9
Test item	486.0	PS	-	508	368	359	363.5	0.7	94.7	culture was not continued[#]	
Solvent control with acetone			+	508	421	397	409.0	0.8	100.0	1632000	100.0
Positive control with DMBA	1.1		+	508	356	344	350.0	0.7	85.6	1519000	93.1
Test item	3.0		+	508	398	381	389.5	0.8	95.2	1612000	98.8
Test item	9.0		+	508	370	412	391.0	0.8	95.6	1551000	95.0
Test item	18.0		+	508	428	436	432.0	0.9	105.6	1769000	108.4
Test item	54.0	PS	+	508	372	371	371.5	0.7	90.8	1743000	106.8
Test item	162.0	PS	+	508	395	413	404.0	0.8	98.8	2023000	124.0
Test item	486.0	PS	+	508	368	376	372.0	0.7	91.0	culture was not continued[#]	

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

Table 4: Cloning Efficiency II (Viability), Experiment I, culture I

	conc.	PS	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	11
Solvent control with acetone			-	522	361	327	344.0	0.7	100.0	481350	317211.5
Positive control with EMS	150.0		-	527	428	416	422.0	0.8	121.5	398850	319382.7
Test item	3.0		-	539	549	474	511.5	0.9	144.0	406950	386187.2
Test item	9.0		-	520	476	397	436.5	0.8	127.4	433200	363638.1
Test item	18.0		-	512	445	375	410.0	0.8	121.5	501750	401792.0
Test item	54.0	PS	-	533	363	375	369.0	0.7	105.1	449100	310915.4
Test item	162.0	PS	-	531	481	412	446.5	0.8	127.6	481050	404498.7
Test item	486.0	PS	-	culture was not continued[#]							
Solvent control with acetone			+	540	345	296	320.5	0.6	100.0	543600	322636.7
Positive control with DMBA	1.1		+	543	365	270	317.5	0.6	98.5	501300	293117.4
Test item	3.0		+	541	354	436	395.0	0.7	123.0	404700	295483.4
Test item	9.0		+	539	297	396	346.5	0.6	108.3	427050	274532.1
Test item	18.0		+	534	432	357	394.5	0.7	124.5	418950	309505.2
Test item	54.0	PS	+	525	355	365	360.0	0.7	115.5	395550	271234.3
Test item	162.0	PS	+	526	378	431	404.5	0.8	129.6	407250	313179.9
Test item	486.0	PS	+	culture was not continued[#]							

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

Table 5: Mutagenicity data (Mutation rates), Experiment I, culture I

	conc.	PS	S9	number of mutant colonies per flask							mutant	
Test group	µg/mL		mix	found after plating in TG medium						standard	colonies	induction
				I	II	III	IV	V	mean	deviation	per 10^6 cells	factor
Column	1	2	3	4	5	6	7	8	9	10	11	12
Solvent control with acetone			-	3	5	2	4	6	4.0	1.6	12.6	1.0
Positive control with EMS	150.0		-	95	87	63	125	117	97.4	24.7	305.0	24.2
Test item	3.0		-	5	3	4	3	4	3.8	0.8	9.8	0.8
Test item	9.0		-	3	4	3	2	4	3.2	0.8	8.8	0.7
Test item	18.0		-	3	3	1	2	2	2.2	0.8	5.5	0.4
Test item	54.0	PS	-	3	4	3	5	5	4.0	1.0	12.9	1.0
Test item	162.0	PS	-	5	4	7	6	2	4.8	1.9	11.9	0.9
Test item	486.0	PS	-	culture was not continued[#]								
Solvent control with acetone			+	5	4	3	6	5	4.6	1.1	14.3	1.0
Positive control with DMBA	1.1		+	109	98	112	87	101	101.4	9.9	345.9	24.3
Test item	3.0		+	1	3	6	4	2	3.2	1.9	10.8	0.8
Test item	9.0		+	5	2	2	5	5	3.8	1.6	13.8	1.0
Test item	18.0		+	6	4	4	2	4	4.0	1.4	12.9	0.9
Test item	54.0	PS	+	6	3	5	5	3	4.4	1.3	16.2	1.1
Test item	162.0	PS	+	6	2	1	3	4	3.2	1.9	10.2	0.7
Test item	486.0	PS	+	culture was not continued[#]								

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

13.2.2 Experiment I Culture II

Table 6: Cloning Efficiency I (Survival), culture II

	conc.	PS	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	11
Solvent control with acetone			-	508	465	430	447.5	0.9	100.0	1600000	100.0
Positive control with EMS	150.0		-	508	376	328	352.0	0.7	78.7	1852000	115.8
Test item	3.0		-	508	398	383	390.5	0.8	87.3	1068000	66.8
Test item	9.0		-	508	375	391	383.0	0.8	85.6	1410000	88.1
Test item	18.0		-	508	412	403	407.5	0.8	91.1	988000	61.8
Test item	54.0	PS	-	508	376	398	387.0	0.8	86.5	1298000	81.1
Test item	162.0	PS	-	508	387	382	384.5	0.8	85.9	1296000	81.0
Test item	486.0	PS	-	508	373	361	367.0	0.7	82.0	culture was not continued[#]	
Solvent control with acetone			+	508	394	415	404.5	0.8	100.0	1869000	100.0
Positive control with DMBA	1.1		+	508	355	354	354.5	0.7	87.6	1876000	100.4
Test item	3.0		+	508	397	388	392.5	0.8	97.0	1683000	90.0
Test item	9.0		+	508	381	397	389.0	0.8	96.2	2206000	118.0
Test item	18.0		+	508	426	415	420.5	0.8	104.0	1711000	91.5
Test item	54.0	PS	+	508	401	387	394.0	0.8	97.4	1802000	96.4
Test item	162.0	PS	+	508	377	389	383.0	0.8	94.7	1830000	97.9
Test item	486.0	PS	+	508	372	365	368.5	0.7	91.1	culture was not continued[#]	

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

Table 7: Cloning Efficiency II (Viability), experiment I, culture II

	conc.	PS	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	11
Solvent control with acetone			-	534	498	412	455.0	0.9	100.0	398700	339716.3
Positive control with EMS	150.0		-	510	452	443	447.5	0.9	103.0	392400	344311.8
Test item	3.0		-	548	457	493	475.0	0.9	101.7	386400	334927.0
Test item	9.0		-	541	414	386	400.0	0.7	86.8	395400	292347.5
Test item	18.0		-	518	450	398	424.0	0.8	96.1	392100	320946.7
Test item	54.0	PS	-	523	457	396	426.5	0.8	95.7	386400	315104.4
Test item	162.0	PS	-	548	531	483	507.0	0.9	108.6	413400	382470.4
Test item	486.0	PS	-	culture was not continued[#]							
Solvent control with acetone			+	533	365	373	369.0	0.7	100.0	424200	293676.9
Positive control with DMBA	1.1		+	513	339	325	332.0	0.6	93.5	431400	279190.6
Test item	3.0		+	529	392	390	391.0	0.7	106.8	392400	290034.8
Test item	9.0		+	521	363	359	361.0	0.7	100.1	421200	291848.8
Test item	18.0		+	536	352	352	352.0	0.7	94.9	411600	270304.5
Test item	54.0	PS	+	555	371	357	364.0	0.7	94.7	377400	247520.0
Test item	162.0	PS	+	548	359	369	364.0	0.7	95.9	387000	257058.4
Test item	486.0	PS	+	culture was not continued[#]							

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

Table 8: Mutagenicity data (Mutation rates), experiment I, culture II

	conc.	PS	S9	number of mutant colonies per flask							mutant	
Test group	µg/mL		mix	found after plating in TG medium						standard	colonies	induction
				I	II	III	IV	V	mean	deviation	per 10^6 cells	factor
Column	1	2	3	4	5	6	7	8	9	10	11	12
Solvent control with acetone			-	4	3	2	5	8	4.4	2.3	13.0	1.0
Positive control with EMS	150.0		-	56	64	66	85	91	72.4	14.9	210.3	16.2
Test item	3.0		-	5	3	6	2	4	4.0	1.6	11.9	0.9
Test item	9.0		-	2	5	6	3	5	4.2	1.6	14.4	1.1
Test item	18.0		-	3	4	6	5	6	4.8	1.3	15.0	1.2
Test item	54.0	PS	-	2	2	5	6	1	3.2	2.2	10.2	0.8
Test item	162.0	PS	-	8	6	10	7	6	7.4	1.7	19.3	1.5
Test item	486.0	PS	-	culture was not continued[#]								
Solvent control with acetone			+	5	2	4	6	3	4.0	1.6	13.6	1.0
Positive control with DMBA	1.1		+	78	64	91	65	53	70.2	14.6	251.4	18.5
Test item	3.0		+	4	5	7	5	6	5.4	1.1	18.6	1.4
Test item	9.0		+	5	3	7	5	1	4.2	2.3	14.4	1.1
Test item	18.0		+	5	4	5	6	2	4.4	1.5	16.3	1.2
Test item	54.0	PS	+	6	6	7	2	4	5.0	2.0	20.2	1.5
Test item	162.0	PS	+	2	3	4	3	5	3.4	1.1	13.2	1.0
Test item	486.0	PS	+	culture was not continued[#]								

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

13.2.3 Experiment II, Culture I

Table 9: Cloning Efficiency I (Survival)

	conc.	PS	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	11
Solvent control with acetone			-	536	371	339	355.0	0.7	100.0	3126000	100.0
Positive control with EMS	150.0		-	536	292	317	304.5	0.6	85.8	3788000	121.2
Test item	3.0		-	536	284	290	287.0	0.5	80.8	2020000	64.6
Test item	9.0		-	536	321	317	319.0	0.6	89.9	2856000	91.4
Test item	18.0		-	536	342	330	336.0	0.6	94.6	3176000	101.6
Test item	54.0	PS	-	536	306	287	296.5	0.6	83.5	4092000	130.9
Test item	162.0	PS	-	536	347	299	323.0	0.6	91.0	3584000	114.7
Test item	486.0	PS	-	536	352	360	356.0	0.7	100.3	culture was not continued[#]	
Solvent control with acetone			+	536	338	317	327.5	0.6	100.0	2494000	100.0
Positive control with DMBA	1.1		+	536	298	309	303.5	0.6	92.7	1636000	65.6
Test item	3.0		+	536	291	301	296.0	0.6	90.4	2234000	89.6
Test item	9.0		+	536	293	375	334.0	0.6	102.0	2216000	88.9
Test item	18.0		+	536	246	317	281.5	0.5	86.0	2206000	88.5
Test item	54.0	PS	+	536	243	225	234.0	0.4	71.5	2320000	93.0
Test item	162.0	PS	+	536	301	381	341.0	0.6	104.1	2258000	90.5
Test item	486.0	PS	+	536	298	83	190.5	0.4	58.2	culture was not continued[#]	

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

Table 10: Cloning Efficiency II (Viability), experiment II, culture I

	conc.	PS	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	11
Solvent control with acetone			-	555	364	374	369.0	0.7	100.0	450000	299189.2
Positive control with EMS	150.0		-	550	215	221	218.0	0.4	59.6	360600	142928.7
Test item	3.0		-	558	386	412	399.0	0.7	107.5	375000	268145.2
Test item	9.0		-	500	333	401	367.0	0.7	110.4	472200	346594.8
Test item	18.0		-	537	371	384	377.5	0.7	105.7	480000	337430.2
Test item	54.0	PS	-	529	367	359	363.0	0.7	103.2	472200	324023.8
Test item	162.0	PS	-	523	358	335	346.5	0.7	99.6	372000	246458.9
Test item	486.0	PS	-	culture was not continued[#]							
Solvent control with acetone			+	500	401	396	398.5	0.8	100.0	457200	364388.4
Positive control with DMBA	1.1		+	519	215	199	207.0	0.4	50.0	447000	178283.2
Test item	3.0		+	610	425	401	413.0	0.7	84.9	524100	354841.5
Test item	9.0		+	578	399	412	405.5	0.7	88.0	419100	294022.6
Test item	18.0		+	590	408	400	404.0	0.7	85.9	456300	312449.5
Test item	54.0	PS	+	557	397	385	391.0	0.7	88.1	511200	358849.6
Test item	162.0	PS	+	504	361	353	357.0	0.7	88.9	522300	369962.5
Test item	486.0	PS	+	culture was not continued[#]							

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

Table 11: Mutagenicity data (Mutation rates), experiment II, culture I

	conc.	PS	S9	number of mutant colonies per flask							mutant	
Test group	µg/mL		mix	found after plating in TG medium						standard	colonies	induction
				I	II	III	IV	V	mean	deviation	per 10^6 cells	factor
Column	1	2	3	4	5	6	7	8	9	10	11	12
Solvent control with acetone			-	2	3	5	2	3	3.0	1.2	10.0	1.0
Positive control with EMS	150.0		-	113	121	98	117	85	106.8	15.0	747.2	74.5
Test item	3.0		-	1	1	2	1	6	2.2	2.2	8.2	0.8
Test item	9.0		-	3	5	7	4	4	4.6	1.5	13.3	1.3
Test item	18.0		-	5	2	2	4	3	3.2	1.3	9.5	0.9
Test item	54.0	PS	-	5	5	5	7	2	4.8	1.8	14.8	1.5
Test item	162.0	PS	-	2	3	1	3	4	2.6	1.1	10.5	1.1
Test item	486.0	PS	-	culture was not continued[#]								
Solvent control with acetone			+	6	5	6	5	5	5.4	0.5	14.8	1.0
Positive control with DMBA	1.1		+	134	151	113	145	138	136.2	14.5	764.0	51.6
Test item	3.0		+	5	5	5	7	5	5.4	0.9	15.2	1.0
Test item	9.0		+	6	4	7	7	3	5.4	1.8	18.4	1.2
Test item	18.0		+	1	1	6	2	5	3.0	2.3	9.6	0.6
Test item	54.0	PS	+	5	3	2	7	4	4.2	1.9	11.7	0.8
Test item	162.0	PS	+	8	9	7	9	3	7.2	2.5	19.5	1.3
Test item	486.0	PS	+	culture was not continued[#]								

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

13.2.4 Experiment II, Culture II

Table 12: Cloning Efficiency I (Survival)

	conc.	PS	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	11
Solvent control with acetone			-	536	279	299	289.0	0.5	100.0	2088000	100.0
Positive control with EMS	150.0		-	536	308	261	284.5	0.5	98.4	2888000	138.3
Test item	3.0		-	536	317	289	303.0	0.6	104.8	3460000	165.7
Test item	9.0		-	536	305	317	311.0	0.6	107.6	2900000	138.9
Test item	18.0		-	536	258	198	228.0	0.4	78.9	3410000	163.3
Test item	54.0	PS	-	536	313	276	294.5	0.5	101.9	3468000	166.1
Test item	162.0	PS	-	536	334	380	357.0	0.7	123.5	3038000	145.5
Test item	486.0	PS	-	536	272	364	318.0	0.6	110.0	culture was not continued[#]	
Solvent control with acetone			+	536	294	312	303.0	0.6	100.0	2202000	100.0
Positive control with DMBA	1.1		+	536	364	283	323.5	0.6	106.8	1778000	80.7
Test item	3.0		+	536	381	347	364.0	0.7	120.1	2198000	99.8
Test item	9.0		+	536	278	292	285.0	0.5	94.1	2200000	99.9
Test item	18.0		+	536	308	275	291.5	0.5	96.2	2340000	106.3
Test item	54.0	PS	+	536	237	315	276.0	0.5	91.1	2298000	104.4
Test item	162.0	PS	+	536	382	364	373.0	0.7	123.1	2366000	107.4
Test item	486.0	PS	+	536	321	308	314.5	0.6	103.8	culture was not continued[#]	

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

Table 13: Cloning Efficiency II (Viability), experiment II, culture II

	conc.	PS	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	11
Solvent control with acetone			-	522	384	343	363.5	0.7	100.0	462600	322136.2
Positive control with EMS	150.0		-	534	213	208	210.5	0.4	56.6	365400	144038.8
Test item	3.0		-	506	358	377	367.5	0.7	104.3	450600	327263.8
Test item	9.0		-	547	401	317	359.0	0.7	94.2	391800	257141.1
Test item	18.0		-	525	343	315	329.0	0.6	90.0	420000	263200.0
Test item	54.0	PS	-	544	368	369	368.5	0.7	97.3	453000	306857.5
Test item	162.0	PS	-	573	325	371	348.0	0.6	87.2	407400	247426.2
Test item	486.0	PS	-	culture was not continued[#]							
Solvent control with acetone			+	575	333	394	363.5	0.6	100.0	419850	265418.2
Positive control with DMBA	1.1		+	520	258	242	250.0	0.5	76.1	462600	222403.8
Test item	3.0		+	528	398	421	409.5	0.8	122.7	450600	349471.0
Test item	9.0		+	515	415	407	411.0	0.8	126.2	481200	384025.6
Test item	18.0		+	563	373	395	384.0	0.7	107.9	426450	290864.7
Test item	54.0	PS	+	565	360	365	362.5	0.6	101.5	404850	259748.9
Test item	162.0	PS	+	513	361	383	372.0	0.7	114.7	398250	288789.5
Test item	486.0	PS	+	culture was not continued[#]							

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

Table 14: Mutagenicity data (Mutation rates), experiment II, culture II

	conc.	PS	S9	number of mutant colonies per flask							mutant	
Test group	µg/mL		mix	found after plating in TG medium						standard	colonies	induction
				I	II	III	IV	V	mean	deviation	per 10^6 cells	factor
Column	1	2	3	4	5	6	7	8	9	10	11	12
Solvent control with acetone			-	2	6	5	6	6	5.0	1.7	15.5	1.0
Positive control with EMS	150.0		-	78	67	109	121	93	93.6	22.0	649.8	41.9
Test item	3.0		-	3	3	1	1	5	2.6	1.7	7.9	0.5
Test item	9.0		-	4	4	4	1	3	3.2	1.3	12.4	0.8
Test item	18.0		-	7	7	8	8	5	7.0	1.2	26.6	1.7
Test item	54.0	PS	-	6	9	6	8	6	7.0	1.4	22.8	1.5
Test item	162.0	PS	-	5	8	7	9	4	6.6	2.1	26.7	1.7
Test item	486.0	PS	-	culture was not continued[#]								
Solvent control with acetone			+	5	5	6	7	8	6.2	1.3	23.4	1.0
Positive control with DMBA	1.1		+	230	199	187	201	164	196.2	24.0	882.2	37.8
Test item	3.0		+	4	4	5	2	3	3.6	1.1	10.3	0.4
Test item	9.0		+	4	3	5	4	5	4.2	0.8	10.9	0.5
Test item	18.0		+	5	2	4	2	3	3.2	1.3	11.0	0.5
Test item	54.0	PS	+	5	5	2	6	4	4.4	1.5	16.9	0.7
Test item	162.0	PS	+	1	2	2	2	5	2.4	1.5	8.3	0.4
Test item	486.0	PS	+	culture was not continued[#]								

culture not continued to avoid evaluation of too many insoluble concentrations

PS = Phase Separation

14 ANNEX II: HISTORICAL DATA

These values represent the historical control data from 2010 – 2011

Number of mutant colonies per 10^6 cells		
without metabolic activation (4 hours treatment time)		
	Positive control EMS 150 µg/mL	Solvent control (medium, acetone, water, DMSO, ethanol, THF)
Range:	63.3 - 1386.4	2.8 - 43.5
Mean value:	161.1	18.1
Standard deviation:	146.6	8.8
Number of studies:	76	76
with metabolic activation (4 hours treatment time)		
	Positive control DMBA 1.1 - 2.0 µg/mL	Solvent control (medium, acetone, water, DMSO, ethanol, THF)
Range:	91.4 - 2666.3	3.4 - 36.6
Mean value:	896.3	16.4
Standard deviation:	386.8	7.0
Number of studies:	75	75
without metabolic activation (24 hours treatment time)		
	Positive control EMS 75 – 300 µg/mL	Solvent control (medium, acetone, water, DMSO, ethanol, THF)
Range:	108.4 - 2746.9	2.6 - 40.3
Mean value:	436.1	17.8
Standard deviation:	325.4	8.5
Number of studies:	56	56