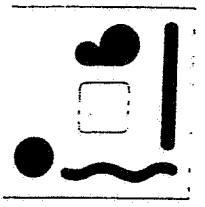
AR226-3029

[redacted]



CCR PROJECT 509800

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

WITH

[redacted]

REPORT

Study Completion Date: October 06, 1995

RCC

Company Sanitized. Does not contain TSCA CBI

COPY OF GLP CERTIFICATE

HESSISCHES MINISTERIUM FÜR
UMWELT, ENERGIE, JUGEND,
FAMILIE UND GESUNDHEIT

GLP-Bescheinigung

Bescheinigung

Hiermit wird bestätigt, daß die Prüfeinrichtung(en)
CCR Cytotest Cell Research GmbH & Co. KG
in 64380 Roßdorf, In den Leppsteinswiesen 19
(Ort, Anschrift)
der RCC/CCR Holding Verwaltungs GmbH
(Firma)
am 05./06./07. April 1995
(Datum)

von der für die Überwachung zuständigen Behörden über die Einhaltung der Grundsätze der Guten Laborpraxis inspiziert worden ist (sind).

Es wird hiermit bestätigt, daß folgende Prüfungen in dieser Prüfeinrichtung nach den Grundsätzen der Guten Laborpraxis durchgeführt werden.

Certificate

It is hereby certified that the test facility(ies)
CCR Cytotest Cell Research GmbH & Co. KG
in 64380 Roßdorf, In den Leppsteinswiesen 19
(location, address)
of RCC/CCR Holding Verwaltungs GmbH
(company name)
on 05./06./07. April 1995
(date)

was (were) inspected by the competent authority regarding compliance with the Principles of Good Laboratory Practice.

It is hereby certified that studies in this test facility are conducted in compliance with the Principles of Good Laboratory Practice.

Prüfkategorie nach § 19 d Abs. 3 Chemikaliengesetz in der Fassung vom 29.Juli 1994 (BGBl. I S. 1703), zuletzt geändert am 27. September 1994 (BGBl. I S. 2705) in Verbindung mit der Allgemeinen Verwaltungsvorschrift zum Verfahren der behördlichen Überwachung der Einhaltung der Grundsätze der Guten Laborpraxis vom 21. Oktober 1990 (BAnz. 204 a vom 31.10.1990):

Toxikologische Eigenschaften	Toxicological properties

Prüfkategorie gemäß OECD Panel on Good Laboratory Practice (January 1992)

Prüfungen auf toxikologische Eigenschaften	Toxicity studies
Prüfungen auf mutagene Eigenschaften (in vitro, in vivo)	Mutagenicity studies

Im Auftrag

Dr. Hecker

(Dr. Hecker) Wiesbaden, den 2. August 1995

HESSISCHES MINISTERIUM FÜR UMWELT, ENERGIE, JUGEND, FAMILIE UND GESUNDHEIT

Company Sanitized. Does not contain TSCA CBI

CONTENTS

PREFACE

General

Sponsor:	ISEGA Forschungs- und Untersuchungsgesellschaft mbH Zeppelinstraße 3 D-63741 Aschaffenburg
Study Monitor:	Dr. Derra
Testing Facility:	**C C R** CYTOTEST CELL RESEARCH GMBH & Co. KG In den Leppsteinswiesen 19 D-64380 Roßdorf, F.R.G.
CCR Project No.:	509800
Test Article:	[redacted]
Title:	Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT) with [redacted]

Project Staff

Study Director:	Dr. Hans-Eric Wollny
Management:	Markus Arenz
Quality Assurance Unit:	Frauke Hermann

Schedule

Date of Protocol	April 13, 1995
Start of Pre-Test:	July 27, 1995
End of Pre-Test:	July 31, 1995
Start of Experiments :	August 01, 1995
End of Experiments :	Sept. 11, 1995
Date of Draft:	Sept. 13, 1995
Date of Final Report:	Oct. 06, 1995

Project Staff Signatures

Study Director — Dr. Hans-Eric Wollny

Date: October 06, 1995

Management — Markus Arenz

ppa

Date: October 06, 1995

Good Laboratory Practice

The study was performed in compliance with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated July 25, 1994 (BGBL. I 1994 S. 1703)."

"The OECD Principles of Good Laboratory Practice", Paris 1981.

Guidelines

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

Second Addendum to the OECD Guideline for Testing of Chemicals, Section 4, No. 476, adopted April 4, 1984, "In vitro Mammalian Cell Gene Mutation Tests"

EEC Directive 87/302, L 133, p. 61 - 63

Archiving

C C-R, D-64380 Roßdorf/F.R.G. will archive the following data for 30 years:

Raw data, protocol and copy of report.

The following sample will be archived for at least 2 years following the date on which the report is audited by the Quality Assurance Unit:

sample of the test article

No raw data or material relating to the study will be discarded without the sponsor's prior consent.

STATEMENT OF COMPLIANCE

Project Number: 509800

Test Material : [redacted]

Study Director: Dr. Hans-Eric Wollny

Title: Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT) with [redacted]

This study performed in the testing facility of **C C R** was conducted in compliance with Good Laboratory Practice Regulations.

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated July 25, 1994 (BGBL. I 1994 S. 1703)."

"The OECD Principles of Good Laboratory Practice", Paris 1981."

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

Study Director

C C R
Dr. Hans-Eric Wollny

Wollny

Date: October 06, 1995

QUALITY ASSURANCE UNIT

C C R, Cytotest Cell Research GmbH & Co. KG,
In den Leppsteinswiesen 19,
D-64380 Roßdorf, F.R.G.

Statement

Project Number: 509800

Test Material : [redacted]

Study Director: Dr. Hans-Eric Wollny

Title: Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT) with [redacted]

This report was audited by the Quality Assurance Unit and the conduct of this study was inspected on the following dates.

Phases and Dates of QAU Inspections/ Audits		Dates of Reports to the Study Director and to Management
Protocol Audit:	April 18, 1995	April 18, 1995
Process Inspection	August 11, 1995	August 11, 1995
Draft Audit:	Sept. 22, 1995	Sept. 22, 1995

Head of Quality Assurance Unit Frauke Hermann

F. Hermann

Date: October 06, 1995

SUMMARY

The study was performed to investigate the potential o[redacted] to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster.

The assay was performed in two independent experiments, using identical procedures, both with and without liver microsomal activation.

The test article was tested with the following concentrations:

Experiment I:

without S9 mix: 1.0; 10.0*; 100.0; 200.0; 250.0* and 300.0 µg/ml
with S9 mix: 1.0; 10.0*; 100.0; 200.0; 250.0* and 300.0 µg/ml

Experiment II:

without S9 mix: 1.0; 100.0; 200.0; 250.0* and 300.0 µg/ml
with S9 mix: 1.0; 100.0; 200.0; 250.0* and 300.0 µg/ml

No relevant toxic effects occurred up to the limit of solubility and above. The highest concentration (300 µg/ml) resulted in a slight perturbation of the medium due to formation of small undisolved droplets. Survival at the lowest concentration was approximately in the range of the negative control.

Up to the highest investigated concentration no relevant increase in mutant colony numbers was observed in both independent experiments.

Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies.

Conclusion

In conclusion it can be stated that during the described mutagenicity test and under the experimental conditions reported the test article did not induce gene mutations at the HPRT locus in V79 cells.

Therefore [redacted]s considered to be non-mutagenic in this HPRT assay.

* not evaluated, culture not continued

OBJECTIVE

Aims of the Study

This in vitro assay is performed to assess the potential of the test article to induce gene mutations by means of two independent HPRT experiments using the Chinese hamster cell line V79.

Relevance of the Test System

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

This in vitro test is an assay for the detection of forward gene mutations in mammalian cells. Gene mutations are considered to be an initial step in the carcinogenic process (2).

The V79 cells are exposed to the test article 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 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 article 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 article.

MATERIALS AND METHODS

Test Article

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

Name:	[redacted]
Batch No.:	not indicated by the sponsor
Aggregate State at RT:	liquid
Colour:	yellow
Analysis:	not indicated by the sponsor
Purity:	35 % in Isopropanol and water
Stability	pure: not indicated by the sponsor
Stability	In solvent: not indicated by the sponsor
Storage:	4° C
Expiration Date:	not indicated by the sponsor

On the day of the experiment (immediately before treatment), the test article was dissolved in Ethanol (E. MERCK, D-64293 Darmstadt; purity 99.8 %). The solvent was chosen according to its solubility properties and its non-toxicity for the cells. The final concentration of DMSO in the culture medium did not exceed 1 % v/v.

Controls

Negative Controls

Concurrent negative and solvent controls were performed.

Positive Control Substances

Without metabolic activation

Name:	EMS; Ethylmethanesulfonate
Supplier:	Merck-Schuchardt, D-85662 München, F.R.G.
Catalogue no.:	820774; (Purity: > 98%)
Dissolved in:	nutrient medium
Final concentration:	0.6 mg/ml = 4.8 mM

Solution prepared on day of experiment.

With metabolic activation

Name:	DMBA; 7,12-dimethylbenz(a)anthracene
Supplier:	SIGMA CHEMIE GMBH, D-82041 Deisenhofen, F.R.G.
Catalogue no.:	D 3254; (Purity: approx. 95%)
Dissolved in	DMSO, Dimethylsulfoxide; final concentration in nutrient medium 1 %
Final concentration:	3.85 µg/ml = 15.0 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.

Test System

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 negative control 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).

Cell Cultures

Large stocks of the V79 cell line (supplied by Laboratory for Mutagenicity Testing; Technical University; D-64293 Darmstadt; F.R.G.) are stored in liquid nitrogen in the cell bank of **C C R** 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, F.R.G.). About 5×10^5 cells are seeded into each flask with 15 ml of MEM (minimal essential medium; SEROMED, D-12247 Berlin, F.R.G.) supplemented with 10 % foetal calf serum (FCS; Boehringer Mannheim, 68261-Mannheim, F.R.G.). 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 mutants the medium is supplemented with 11 µg/ml thioguanine (6TG, SIGMA GmbH, D-82041 Deisenhofen, F.R.G.).

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 after metabolisation 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).

S9 (Preparation by C C R)

The S9 liver microsomal fraction was obtained from the livers of 8 - 12 weeks old male Wistar rats, strain HanIbm (BRL, CH-4414 Füllinsdorf weight approx. 220 - 320 g) which received a single i.p. injection of 500 mg/kg b.w. Aroclor 1254 (Antechnika, D-76275 Ettlingen, F.R.G.) in olive oil 5 days previously.

After cervical dislocation the livers of the animals were re-moved, washed in 150 mM KCl and homogenised. The homogenate was diluted 1+3 in KCl and centrifuged at 9,000 g for 10 minutes at 4° C. A stock of the supernatant containing the microsomes was frozen in ampoules of 2, 3 or 5 ml and stored at -80° C. Small numbers of the ampoules are kept at -20° C for up to several weeks before use. The standardisation of the protein content was made using the analysis kit of Bio-Rad Laboratories, D-80939 München: Bio-Rad protein assay, Catalogue 500 000 6 (6).

The protein concentration in the S9 preparation was 33.2 mg/ml (lot 220595).

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 mix to reach following concentrations:

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).

Pre-Test on Toxicity

A pre-test was performed in order to determine the concentration range for the mutagenicity experiments. The general culturing and experimental conditions in this pre-test were the same as described below for the mutagenicity experiment.

The following method was used in the pre-test:

XTT-Assay:

The XTT-assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by hydrogenase activity in active mitochondria. 18 - 20 h after treatment with the test article the XTT-assay was initiated by adding a mixture of XTT-labelling reagent with an electron coupling reagent (PMS). After 4 h of incubation the absorption was determined at 450 nm (690 nm reference) using an ELISA reader (SLT, Labinstruments Austria, A-5082 Grödig). The viabilities of the cells are calculated as percentages of the solvent controls and reported as tables.

Dose Selection

According to the recommendations of the guidelines (see page 6), several concentrations (usually at least four) of the test article 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 negative 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 as recommended in the in vitro cytogenetic assay, namely 5 mg/ml or 10 mM. If the maximum 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 pre-test of toxicity (XTT-assay) the extinction (measured at 450/690 nm) was not significantly reduced after treatment with concentrations up to the limit of solubility at 300 µg/ml with and without metabolic activation (see table PRE-TEST OF TOXICITY).
Experiment I and II were performed with four concentrations ranging from 1.0 to 300.0 µg/ml with and without metabolic activation.

Therefore, the test article was tested with the following concentrations:

Experiment I:

without S9 mix: 1.0; 100.0; 200.0 and 300.0 µg/ml
with S9 mix: 1.0; 100.0; 200.0 and 300.0 µg/ml

Experiment II:

without S9 mix: 1.0; 100.0; 200.0 and 300.0 µg/ml
with S9 mix: 1.0; 100.0; 200.0 and 300.0 µg/ml

Experimental Performance

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 was composed as follows (per litre):

NaCl	8000 mg
KCl	400 mg
Glucose	1000 mg
$NaHC0_3$	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, D-72632 Frickenhausen). 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 (see experimental scheme).

Treatment

After 24 h the medium was replaced with serum-free medium containing the test article, either without S9 mix or with 50 µl/ml S9 mix. After 4 h this medium was replaced with complete medium after two washing steps with "saline G".

The "saline G" solution was composed as follows (per litre):

NaCl	8000 mg
KCl	400 mg
Glucose	1100 mg
$Na_2HP0_4\times7H_20$	290 mg
KH_2P0_4	150 mg

pH is adjusted to 7.2

Experimental Scheme:

Segment a): Procedure for determination of toxicity

Segment b): Procedure for determination of mutation rates

Day 1: Subculturing of a log-phase culture which showed an initial spontaneous mutation rate at the beginning of the experiment of 14.0 (experiment I) and 2.9 (experiment II) mutants per 10^6 cells.

a) About 500 cells in 5 ml medium/25 cm^2-plastic-flask for cloning efficiency; in duplicate per experimental point

b) 1×10^6 cells in 30 ml medium/175 cm^2-plastic-flask for the mutagenicity test, 1 flask per experimental point

Day 2: Treatment of a) and b)

experiment I

Day 5: Subculturing of b) in 175 cm^2-plastic-flasks 1.5×10^6 cells in 30 ml medium/175 cm^2-plastic-flasks

experiment II

Day 6: see day 5

Day 8: Fixation and staining of colonies in a)-flasks
determination of concentration-related cloning efficiency

Day 9: Subculturing of b) in five 80 cm^2-plastic-flasks containing selective medium: mutant selection (about $3\text{-}5 \times 10^5$ cells/flask);
subculturing of b) in two 25 cm^2-flasks for cloning efficiency (about 500 cells/flask)

Day 16: Fixation and staining of colonies in b) - derived flasks seeded on day 9 (cloning efficiency).

Day 18: Fixation and staining of colonies in b) - derived flasks seeded on day 9 (mutant selection).

The cultures were incubated at 37° C in a humidified atmosphere with 4.5 % CO_2. The colonies were stained with 10 % methylene blue in 0.01 % KOH solution (E. MERCK, D-64293 Darmstadt, F.R.G.).

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

Data Recording

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

Acceptability of the Assay

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

a) the numbers of mutant colonies per 10^6 cells found in the negative and/or solvent controls fall within the laboratory historical control data range: 0 - 45 mutants/10^6 cells.

b) the positive control substances must produce a significant increase in mutant colony frequencies.

c) the cloning efficiency (absolute value) of the negative and/or solvent controls must exceed 50 %.

The data of this study comply with the above mentioned criteria [a) and b) see mutation rate, tables III and VI, c) see tables II and V, factor calculated referring to the C.E. of the untreated cultures].

Evaluation of Results

A test article is classified as positive if it induces either a concentration-related increase of the mutant frequency or a reproducible and positive response for one of the test points.

A test article 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 significant response is described as follows:

The test article is classified as mutagenic if it induces a reproducible mutation frequency that is at least three times higher than the spontaneous mutation frequency in the experiment at one or more of the concentrations.

The test article 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 negative control data. If there is by chance a low spontaneous mutation rate in the range normally found (0 - 45 mutants per 10^6 cells) a concentration-related increase of the mutations within this range has to be discussed.

RESULTS

Pre-Test on Toxicity

Without S9 mix:

	concentration µg/ml	extinction (450/690 nm) mean	standard deviation	% of the corresponding control*
Blanc	/	0.18	0.00	0.00
Negative control	/	1.22	0.03	105.57
Solvent control	/	1.17	0.02	100.00
test article	0.3	1.19	0.04	102.35
test article	1.0	1.21	0.07	104.17
test article	3.0	1.16	0.03	99.44
test article	10.0	1.14	0.04	97.16
test article	30.0	1.08	0.05	91.36
test article	100.0	1.12	0.06	95.14
test article	200.0	1.10	0.06	93.33
test article	300.0	1.13	0.07	96.36

With S9 mix:

	concentration µg/ml	extinction (450/690 nm) mean	standard deviation	% of the corresponding control*
Blanc	/	0.178	0.007	0.000
Negative control	/	1.009	0.054	105.361
Solvent control	/	0.966	0.048	100.000
test article	0.300	0.926	0.074	94.893
test article	1.000	0.914	0.069	93.306
test article	3.000	0.872	0.038	87.977
test article	10.000	0.852	0.032	85.534
test article	30.000	0.829	0.030	82.568
test article	100.000	0.775	0.024	75.763
test article	200.000	0.716	0.032	68.181
test article	300.000	0.688	0.063	64.724

* corrected with the blanc

RESULTS AND DISCUSSION

The test articl[redacted] 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 main experiments, using identical procedures, both with and without liver microsomal activation.

No relevant toxic effects occurred up to the limit of solubility and above.

In both experiments the numbers of mutant colonies per 10^6 cells did not exceed the values of the corresponding controls substantially and remained well within the historical range. Furthermore, there was no indication of a concentration depend increase of mutant colonies.

In both experiments of this study (with and without S9 mix) the range of the negative controls was from 3.0 up to 17.9 mutant colonies per 10^6 cells; the range of the groups treated with the test article was from 5.7 up to 20.2 mutant colonies per 10^6 cells.

EMS (0.6 mg/ml) and DMBA (3.85 µg/ml) were used as positive controls and showed a distinct increase in induced mutant colonies.

In conclusion, it can be stated that in this mutagenicity assay and under the experimental conditions reported the test article did not induce gene mutations at the HPRT locus in V79 cells.

REFERENCES

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DISTRIBUTION OF THE REPORT

Sponsor	2x (original, copy)
Study Director	1x (copy)

Annex: Tables of Results Experiment 1

Table I: Toxicity data; experiment I

	conc.	S9	number of cells per flask*				CE%**	CE***	cells/ml	cell den-
	per ml	mix	seeded	found		mean	absolute	relative	at 1st sub-	sity; % of
			I/II	I	II				cultivation	control
column	1	2	3	4	5	6	7	8	9	10
Negative control (Untreated cells)			523	306	301	303.5	58.0			
Negative control	0.00 µg	-	523	236	274	255.0	48.8	100	2011333	100
Solvent control with Ethanol	0.00 µg	-	523	214	266	240.0	45.9	100	2098000	100
Positive control with EMS	0.6 mg	-	523	189	201	195.0	37.3	76.5	1779333	88.5
Test article	1.00 µg	-	523	253	245	249.0	47.6	103.8	2258667	107.7
Test article	10.00 µg	-	523	203	244	223.5	42.7	93.1	2312000	110.2
Test article	100.00 µg	-	523	234	214	224.0	42.8	93.3	1848667	88.1
Test article	200.00 µg	-	523	197	184	190.5	36.4	79.4	1880667	89.6
Test article	250.00 µg	-	523	193	183	188.0	35.9	78.3	1876667	89.5
Test article	300.00 µg	-	523	175	204	189.5	36.2	79.0	1875333	89.4
Negative control	0.00 µg	+	523	269	214	241.5	46.2	100.0	2190000	100.0
Solvent control with DMSO	0.00 µg	+	523	252	214	233.0	44.6	100.0	2272000	100.0
Solvent control with Ethanol	0.00 µg	+	523	224	255	239.5	45.8	100.0	2468000	100.0
Positive control with DMBA	3.85 µg	+	523	158	179	168.5	32.2	72.3	1542667	67.9
Test article	1.00 µg	+	523	200	195	197.5	37.8	82.5	2346667	95.1
Test article	10.00 µg	+	523	186	211	198.5	38.0	82.9	2476667	100.4
Test article	100.00 µg	+	523	207	226	216.5	41.4	90.4	2646000	107.2
Test article	200.00 µg	+	523	241	223	232.0	44.4	96.9	2497333	101.2
Test article	250.00 µg	+	523	237	222	229.5	43.9	95.8	2060667	83.5
Test article	300.00 µg	+	523	229	233	231.0	44.2	96.5	2444667	99.1

* only colonies with more than 50 cells 7 days after seeding were scored

** CE absolute (value column 6 / value column 3 x 100

*** CE relative (value column 6 / value column 6 of corresponding control x 100)

Table II: Mutagenicity data; experiment I (part 1: cell survival)

	conc.	S9	number of cells per flask*				factor**	cells	cells***
	per ml	mix	seeded	found		mean	calculated	seeded	survived
			I/II	I	II				
column	1	2	3	4	5	6	7	8	9
Negative control	0.00 µg	-	505	296	301	298.5	0.59	435000	257124
Solvent control with DMSO	0.00 µg	-	513	303	353	328.0	0.64	414000	264702
Positive control with EMS	0.6 mg	-	504	318	325	321.5	0.64	414000	264089
Test article	1.00 µg	-	504	347	365	356.0	0.71	450000	317857
Test article	10.00 µg	-	culture was not continued#						
Test article	100.00 µg	-	500	325	360	342.5	0.69	408000	279480
Test article	200.00 µg	-	511	398	406	402.0	0.79	402000	316250
Test article	250.00 µg	-	culture was not continued#						
Test article	300.00 µg	-	503	380	410	395.0	0.79	426000	334533
Negative control	0.00 µg	+	503	381	409	395.0	0.79	414000	325109
Solvent control with DMSO	0.00 µg	+	500	336	328	332.0	0.66	478500	317724
Solvent control with Ethanol	0.00 µg	+	509	362	372	367.0	0.72	396000	285525
Positive control with DMBA	3.85 µg	+	502	291	302	296.5	0.59	432000	255155
Test article	1.00 µg	+	507	345	342	343.5	0.68	463500	314028
Test article	10.00 µg	+	culture was not continued#						
Test article	100.00 µg	+	511	366	349	357.5	0.70	420000	293836
Test article	200.00 µg	+	502	375	362	368.5	0.73	486000	356755
Test article	250.00 µg	+	culture was not continued#						
Test article	300.00 µg	+	507	358	317	337.5	0.67	522000	347485

* only colonies with more than 50 cells 7 days after seeding were scored

** factor calculated (value column 6 / value column 3)

*** cells survived after plating in TG containing medium (value column 8 / value column 7)

\# concerning concentration range and toxicity four concentrations were selected to be evaluated at the end of the experiment

Table III: Mutagenicity data; experiment I (part 2: mutation rates)

	conc.	S9	number of mutant colonies per flask*					mean	standard	mutant**
	per ml	mix	found after plating in TG medium						deviation	colonies
			I	II	III	IV	V			per 10^6 cells
column	1	2	3	4	5	6	7	8	9	10
Negative control	0.00 µg	-	6	7	2	5	3	4.6	2.1	17.9
Solvent control with DMSO	0.00 µg	-	5	2	4	3	3	3.4	1.1	12.8
Positive control with EMS	0.6 mg	-	137	118	117	151	136	131.8	14.3	499.1
Test article	1.00 µg	-	5	7	8	4	4	5.6	1.8	17.6
Test article	10.00 µg	-	culture was not continued#							
Test article	100.00 µg	-	6	3	5	6	3	4.6	1.5	16.5
Test article	200.00 µg	-	3	5	6	11	3	5.6	3.3	17.7
Test article	250.00 µg	-	culture was not continued#							
Test article	300.00 µg	-	7	5	4	5	9	6.0	2.0	17.9
Negative control	0.00 µg	+	2	6	3	4	4	3.8	1.5	11.7
Solvent control with DMSO	0.00 µg	+	9	8	1	6	4	5.6	3.2	17.6
Solvent control with Ethanol	0.00 µg	+	4	4	5	2	5	4.0	1.2	14.0
Positive control with DMBA	3.85 µg	+	107	117	103	103	89	103.8	10.1	406.8
Test article	1.00 µg	+	5	4	3	3	5	4.0	1.0	12.7
Test article	10.00 µg	+	culture was not continued#							
Test article	100.00 µg	+	1	4	4	3	2	2.8	1.3	9.5
Test article	200.00 µg	+	7	10	6	6	7	7.2	1.6	20.2
Test article	250.00 µg	+	culture was not continued#							
Test article	300.00 µg	+	4	1	6	5	3	3.8	1.9	10.9

* only colonies with more than 50 cells 7 days after seeding were scored

** value column 8 (this page) x 10^6 / value column 9 of table II

\# concerning concentration range and toxicity four concentrations were selected to be evaluated at the end of the experiment

Tables of Results: Experiment II

Table I: Toxicity data; experiment II

	conc.	S9	number of cells per flask*				CE%**	CE***	cells/ml	cell den-
	per ml	mix	seeded	found		mean	absolute	relative	at 1st sub-	sity; % of
			I/II	I	II				cultivation	control
column	1	2	3	4	5	6	7	8	9	10
Negative control (Untreated cells)			552	377	402	389.5	70.6			
Negative control	0.00 µg	-	552	308	304	306.0	55.4	100	2329333	100
Solvent control with Ethanol	0.00 µg	-	552	334	261	297.5	53.9	100	2437333	100
Positive control with EMS	0.6 mg	-	552	263	229	246.0	44.6	80.4	2336667	100.3
Test article	1.00 µg	-	552	326	310	318.0	57.6	106.9	2626667	107.8
Test article	100.00 µg	-	552	283	251	267.0	48.4	89.7	2175333	89.3
Test article	200.00 µg	-	552	297	281	289.0	52.4	97.1	1870000	76.7
Test article	250.00 µg	-	552	256	334	295.0	53.4	99.2	2176000	89.3
Test article	300.00 µg	-	552	293	291	292.0	52.9	98.2	2710667	111.2
Negative control	0.00 µg	+	501	244	196	220.0	43.9	100.0	2995333	100.0
Solvent control with DMSO	0.00 µg	+	501	201	197	199.0	39.7	100.0	3155333	100.0
Solvent control with Ethanol	0.00 µg	+	501	207	213	210.0	41.9	100.0	3060000	100.0
Positive control with DMBA	3.85 µg	+	501	146	152	149.0	29.7	74.9	2293333	72.7
Test article	1.00 µg	+	501	194	208	201.0	40.1	95.7	2958000	96.7
Test article	100.00 µg	+	501	215	200	207.5	41.4	98.8	2173333	71.0
Test article	200.00 µg	+	501	217	222	219.5	43.8	104.5	1767333	57.8
Test article	250.00 µg	+	501	195	219	207.0	41.3	98.6	2904667	94.9
Test article	300.00 µg	+	501	214	226	220.0	43.9	104.8	3184667	104.1

* only colonies with more than 50 cells 7 days after seeding were scored
** CE absolute (value column 6 / value column 3 x 100
*** CE relative (value column 6 / value column 6 of corresponding control x 100)

Table II: Mutagenicity data; experiment II (part 1: cell survival)

	conc.	S9	number of cells per flask*				factor**	cells	cells***
	per ml	mix	seeded	found		mean	calculated	seeded	survived
			I/II	I	II				
column	1	2	3	4	5	6	7	8	9
Negative control	0.00 µg	-	501	347	341	344.0	0.69	390000	267784
Solvent control with DMSO	0.00 µg	-	519	400	423	411.5	0.79	414000	328249
Positive control with EMS	0.6 mg	-	512	314	362	338.0	0.66	456000	301031
Test article	1.00 µg	-	500	366	348	357.0	0.71	499000	356286
Test article	100.00 µg	-	512	355	370	362.5	0.71	390000	276123
Test article	200.00 µg	-	511	385	356	370.5	0.73	408000	295820
Test article	250.00 µg	-	culture was not continued#						
Test article	300.00 µg	-	510	394	399	396.5	0.78	390000	303206
Negative control	0.00 µg	+	501	419	389	404.0	0.81	414000	333844
Solvent control with DMSO	0.00 µg	+	502	370	355	362.5	0.72	385500	278374
Solvent control with Ethanol	0.00 µg	+	501	353	348	350.5	0.70	387000	270746
Positive control with DMBA	3.85 µg	+	508	338	306	322.0	0.63	390000	247205
Test article	1.00 µg	+	502	364	349	356.5	0.71	387000	274832
Test article	100.00 µg	+	502	381	385	383.0	0.76	414500	316242
Test article	200.00 µg	+	500	409	383	396.0	0.79	445000	352440
Test article	250.00 µg	+	culture was not continued#						
Test article	300.00 µg	+	504	357	377	367.0	0.73	433500	315664

* only colonies with more than 50 cells 7 days after seeding were scored
** factor calculated (value column 6 / value column 3)
*** cells survived after plating in TG containing medium (value column 8 / value column 7)
# concerning concentration range and toxicity four concentrations were selected to be evaluated at the end

Table III: Mutagenicity data; experiment II (part 2: mutation rates)

	conc.	S9	number of mutant colonies per flask*					mean	standard	mutant**
	per ml	mix	found after plating in TG medium						deviation	colonies
			I	II	III	IV	V			per 10^6 cells
column	1	2	3	4	5	6	7	8	9	10
Negative control	0.00 µg	-	2	2	1	4	4	2.6	1.3	9.7
Solvent control with DMSO	0.00 µg	-	5	8	1	5	4	4.6	2.5	14.0
Positive control with EMS	0.6 mg	-	122	123	136	140	142	132.6	9.5	440.5
Test article	1.00 µg	-	1	3	1	0	3	1.6	1.3	4.5
Test article	100.00 µg	-	1	2	1	4	1	1.8	1.3	6.5
Test article	200.00 µg	-	7	3	2	1	2	3.0	2.3	10.1
Test article	250.00 µg	-	culture was not continued#							
Test article	300.00 µg	-	3	4	3	2	4	3.2	0.8	10.6
Negative control	0.00 µg	+	0	1	1	1	2	1.0	0.7	3.0
Solvent control with DMSO	0.00 µg	+	1	1	2	1	1	1.2	0.4	4.3
Solvent control with Ethanol	0.00 µg	+	2	2	1	2	2	1.8	0.4	6.6
Positive control with DMBA	3.85 µg	+	119	106	116	130	133	120.8	10.9	488.7
Test article	1.00 µg	+	0	1	3	2	2	1.6	1.1	5.8
Test article	100.00 µg	+	5	6	6	4	2	4.6	1.7	14.5
Test article	200.00 µg	+	1	2	3	1	3	2.0	1.0	5.7
Test article	250.00 µg	+	culture was not continued#							
Test article	300.00 µg	+	5	4	2	5	6	4.4	1.5	13.9

* only colonies with more than 50 cells 7 days after seeding were scored

** value column 8 (this page) x 10^6 / value column 9 of table II

\# concerning concentration range and toxicity four concentrations were selected to be evaluated at the end

DEVIATIONS TO THE PROTOCOL

There were no deviations to the protocol.

BIOMETRY

Since the distribution of mutant cells does not follow known statistical models, an adequate statistical method is not available.

Die Übereinstimmung mit dem Original wird bestätigt.

end J-11932