CCR · Cytotest Cell Research GmbH & Co. KG

CCR PROJECT 326417

SALMONELLA TYPHIMURIUM

REVERSE MUTATION ASSAY

WITH

[redacted]

Study Completion Date:

February 17, 1993

REPORT

CCR · In den Leppsteinswiesen 19 · D-6101 Roβdorf · F.R.G.
Telephone: 0 61 54 - 80 7-0 · Telefax 0 61 54 - 8 33 99

COPY OF GLP CERTIFICATE

HESSISCHES MINISTERIUM
FÜR UMWELT, ENERGIE
UND BUNDESANGELEGENHEITEN

GLP-Bescheinigung

Bescheinigung

Hiermit wird bestätigt, daß die Prüfungseinrichtung(en)
Cytotest Cell Research GmbH & Co KG
In den Leppsteinswiesen 19
in 6101 Roßdorf
(Ort, Anschrift)
der RCC Holding Verwaltung GmbH
(Firma)
am 03.08., 04.08., 05.08. und 06.08.92
(Datum)
von der für die Überwachung zuständigen Behörde 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.

Toxikologische Eigenschaften

Im Auftrag
Dr. Hecker
(Dr. Hecker)

Wiesbaden, den 28.09.1992

Certificate

It is hereby certified that the test facility(ies)
Cytotest Cell Research GmbH & Co KG
In den Leppsteinswiesen 19
in 6101 Roßdorf
(location, address)
of RCC Holding Verwaltung GmbH
(company name)
on 03.08., 04.08., 05.08. and 06.08.92
(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.

Toxicological properties

CONTENTS

PREFACE

GENERAL

Sponsor:	I S E G A Forschungs- u. Untersuchungs- Gesellschaft mbH Zeppelinstr. 3-5 D-8750 Aschaffenburg
Monitoring:	Heike Krämer
Testing Facility:	C C R CYTOTEST CELL RESEARCH GMBH & CO. KG D-6101 Roßdorf, F.R.G.
CCR Project No.:	326417
Test Article:	[redacted]
Title:	Salmonella typhimurium Reverse Mutation Assay with [redacted]

PROJECT STAFF

Management:	Dr. H.- E. Knoell
Study Director:	Dr. Albrecht Poth
Quality Assurance Unit:	Dr. Ch. Helmrich

SCHEDULE

Date of Protocol:	November 10, 1992
Date of 1st Amendment to Protocol:	January 18, 1993
Start of Pre-Experiment:	November 27, 1992
End of Pre-Experiment:	December 04, 1992
Start of Experiment I:	December 15, 1992
End of Experiment I:	December 18, 1992
Start of Experiment II:	January 05, 1993
End of Experiment II:	January 15, 1993
Date of Draft:	January 18, 1993
Date of Report:	February 17, 1993

PROJECT STAFF SIGNATURES

Study Director: Dr. Albrecht Poth

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Date: February 17, 1993

Management: Dr. H.- E. Knoell

.....

Date: February 17, 1993

QUALITY ASSURANCE

The study was performed in compliance with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated March 14, 1990 (BGBL. I S. 521).

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

GUIDELINES

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

First Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Salmonella typhimurium, Reverse Mutation Assay", adopted May 26, 1983 and

EEC Directive 79/831, Annex V, B 14.

ARCHIVING

C C R, D-6101 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 12 years:
Sample of 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: 326417

Test Article : [redacted]

Study Director: Dr. Albrecht Poth

Title : Salmonella typhimurium Reverse Mutation Assay with [redacted]

To the best of my knowledge and belief, this study performed in the testing facility of CCR was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated March 14, 1990

"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. Albrecht Poth

Albrecht Poth
.....

Date: February 18, 1993

QUALITY ASSURANCE UNIT

C C R, Cytotest Cell Research GmbH & Co. KG,
In den Leppsteinswiesen 19, D-6101 Roßdorf, F.R.G.

STATEMENT

Project Number: 326417

Test Article : [redacted]

Study Director: Dr. Albrecht Poth

Title : Salmonella typhimurium Reverse Mutation Assay with [redacted]

This report was audited by the Quality Assurance Unit and the study and/or testing facility were inspected on the following dates.

Dates of QAU Inspections/ Audits	Dates of Reports to the Study Director and to Management
November 11, 1992	November 11, 1992
December 15, 1992	December 15, 1992
January 21, 1993	January 21, 1993

Head of Quality Assurance Unit: Dr. Ch. Helmrich

.....Ch. Helmrich.....
Date: February 18, 1993

SUMMARY

This study was performed to investigate the potential of [redacted] to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test article was tested at the following concentrations:

33.3; 100.0; 333.3; 1000.0; 2500.0 and 5000.0 µg/plate

No relevant toxic effects occurred in the test groups with and without metabolic activation in experiment I and II in all strains used.

The plates incubated with the test article showed normal background growth up to 5000.0 µg/plate with and without S9 mix in all strains used.

No substantial increases in revertant colony numbers of any of the five tester strains were observed following treatment with [redacted] at any dose level, either in the presence or absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of significance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant 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 point mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, [redacted] is considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

OBJECTIVE

AIMS OF THE STUDY

The experiments were performed to assess the potential of the test article to induce gene mutations by means of two independent Salmonella typhimurium reverse mutation assays. Experiment I was performed as a plate incorporation assay. As a negative result was obtained in this experiment, experiment II was performed as a pre-incubation assay.

REASONS FOR THE STUDY

The most widely used assays for detecting gene mutations are those using bacteria. They are relatively simple and rapid to perform, and give reliable data on the ability of an agent to interact with DNA and produce mutations.

However, the bacteria most commonly used in these assays do not possess the enzyme systems which, in mammals, are known to convert promutagens into active DNA damaging metabolites. In order to overcome this major drawback an exogenous metabolic system is added in form of mammalian microsome enzyme activation mixture.

In spite of great differences between bacterial and eucaryotic cells with respect to structure and function there is an association between mutagenicity in bacteria and carcinogenicity in mammals described in literature (7,8).
Reverse mutation assays determine the frequency at which an agent abolishes or suppresses the effect of the forward mutation. The genetic target presented to an agent is therefore small, specific and selective. Several bacterial strains, or a single strain with multiple markers are necessary to overcome the effects of mutagen specificity. The reversions of bacteria from growth-dependence on a particular amino acid to growth in the absence of that amino acid (reversion from auxothrophy to prototrophy) is the most widely used marker.
The Salmonella typhimurium histidine (his) reversion system measures $his^- \longrightarrow his^+$ reversions. The S. typhimurium strains are constructed to differentiate between base pair (TA 1535, TA 100, TA 102) and frameshift (TA 1537, TA 98) mutations.

According to the direct plate incorporation and the pre-incubation method the bacteria were exposed to the test article with and without metabolic activation and plated on selective medium. After a suitable period of incubation, revertant colonies were counted.

To establish a dose response effect five dose levels with adequately spaced intervals were tested. The maximum dose level was 5000.0 µg/plate, unless limited by toxicity or solubility of the test article.

To validate the test, reference mutagens were tested in parallel to the test article.

MATERIALS AND METHODS

THE TEST ARTICLE

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

Name:	[redacted]
Batch No.:	[redacted]
Aggregate State at RT:	liquid
Colour:	[redacted]
Analysis:	[redacted]
Purity:	[redacted]
Stability:	Pure: [redacted]
	In solvent:
Storage:	4°C
Expiration Date:	not indicated by the sponsor

On the day of the experiment, the test article ZONYL RP 18 was dissolved in Ethanol. The solvent was chosen because of its solubility properties and its relative nontoxicity for the bacteria.

THE CONTROLS

The Negative Controls

Concurrent untreated and solvent controls were performed.

The Positive Control Substances

Without metabolic activation

Strains:	TA 1535, TA 100
Name:	sodium azide, NaN_3
Supplier:	SERVA, D-6900 Heidelberg, F.R.G.
Catalogue No.:	30175
Purity:	at least 99 %
Dissolved in:	aqua dest.
Concentration:	10 µg/plate

Strains:	TA 1537, TA 98
Name:	4-nitro-o-phenylene-diamine, 4-NOPD
Supplier:	SIGMA, D-8024 Deisenhofen, F.R.G.
Catalogue No.:	N 9504
Purity:	> 99.9 %
Dissolved in:	DMSO
Concentration:	50 µg/plate

Strain:	TA 102
Name:	methyl methane sulfonate, MMS
Supplier:	MERCK-SCHUCHARDT, D-8011 Hohenbrunn, F.R.G.
Catalogue No.:	820775
Purity:	> 99.0 %
Dissolved in:	aqua dest.
Concentration:	1.0, µl/plate

With metabolic activation

Strains:	TA 1535, TA 1537, TA 98, TA 100, TA 102
Name:	2-aminoanthracene, 2-AA
Supplier:	SIGMA, D-8024 Deisenhofen, F.R.G.
Catalogue No.:	A 1381
Purity:	97.5 %
Dissolved in:	DMSO
Concentration:	2.5 µg/plate

The stability of the positive control substances in solution was unknown but a mutagenic response in the expected range is sufficient evidence of biological stability.

THE TEST SYSTEM

Characterisation of the Salmonella typhimurium Strains

The strains are derived from S. typhimurium strain LT2 and due to a mutation in the histidine locus are histidine dependent. Additionally due to the "deep rough" (rfa-minus) mutation they possess a faulty lipopolysaccharide envelope which enables substances to penetrate the cell wall more easily. A further mutation causes a reduction in the activity of an excision repair system. The latter alteration includes mutational processes in the nitrate reductase and biotin genes produced in a UV-sensitive area of the gene named "uvrB-minus". In the strains TA 98, TA 100 and TA 102 the R-factor plasmid pKM 101 carries the ampicillin resistance marker. The strain TA 102 does not contain the uvrB$^-$-mutation. Additionally TA 102 contains the multicopy plasmid pAQ1, which carries the hisG428 mutation and a tetracyclin resistance gene. TA 102 contains the ochre mutation in hisG gene.

In summary, the mutations of the TA strains used in this study can be described as follows:

Salmonella typhimurium

TA 1537:	his C 3076; rfa$^-$; uvrB$^-$;	: frame shift mutations
TA 98:	his D 3052; rfa$^-$; uvrB$^-$;R-factor:	" "
TA 1535:	his G 46; rfa$^-$; uvrB$^-$;	:base-pair substitutions
TA 100:	his G 46; rfa$^-$; uvrB$^-$;R-factor:	" "
TA 102:	his G 428; rfa$^-$; uvrB$^+$; R-factor:	" "

Regular checking of the properties of the strains with regard to membrane permeability, ampicillin- and tetracyclin-resistance as well as normal spontaneous mutation rates is performed in the laboratory of C C R according to Ames et al. (1). In this way it was ensured that the experimental conditions set down by Ames were fulfilled.

The bacterial strains were obtained from Dr. Heinz Träger, Knoll AG, D-6700 Ludwigshafen, F.R.G.

Storage

The strain cultures were stored as stock cultures in ampoules with nutrient broth + 5 % DMSO in liquid nitrogen.

Precultures

From the thawed ampoules of the strains 0.5 ml bacterial suspension was transferred to 250 ml Erlenmeyer flasks containing 20 ml nutrient medium. This nutrient medium contains per litre:

8 g Merck Nutrient Broth
5 g NaCl

The bacterial culture was incubated in a shaking water bath for 10 hours at 37° C.

Selective Agar

2.0 % Vogel-Bonner-Glucose-Minimal-Agar was used as selective agar. Each petri dish was filled with 20 ml of this nutrient medium. Sterilisations were performed at 121° C in an autoclave.

Overlay Agar

The overlay agar contains per litre:

6.0 g Merck Agar Agar
6.0 g NaCl
10.5 mg L-histidine x HCl x H_2O
12.2 mg biotin

Sterilisations were performed at 121° C in an autoclave.

MAMMALIAN MICROSOMAL FRACTION S9 MIX

S9 (Preparation by C C R)

The S9 liver microsomal fraction was obtained from the liver of 8 - 12 weeks old male Wistar rats, strain WU (SAVO-Ivanovas, med. Versuchstierzuchten GmbH, D-7964 Kisslegg, F.R.G.; weight approx. 150 - 200 g) which received a single i.p. injection of 500 mg/kg b.w. Aroclor 1254 (Antechnika, D-7500 Karlsruhe, F.R.G.) in olive oil 5 days previously.

After cervical dislocation the livers of the animals were removed, washed in 150 mM KCl and homogenised. The homogenate, diluted 1+3 in KCl was centrifuged cold at 9,000 g for 10 minutes. A stock of the supernatant containing the microsomes was frozen in ampoules of 2, 3 or 5 ml and stored at -70° C. Small numbers of the ampoules are kept at -20° C for only several weeks before use. The standardisation of the protein content was made using the analysis kit of Bio-Rad Laboratories, D-8000 München: Bio-Rad protein assay, Catalogue 500 000 6 (6).

The protein concentration in the S9 preparation was 31.6 mg/ml (lot 060792).

S9 Mix

Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution. The amount of S9 supernatant was 15% v/v. The composition of the cofactor solution was concentrated to yield the following concentrations in the S9 mix:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
5 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.(2).

PRE-EXPERIMENT FOR TOXICITY

To evaluate the toxicity of the test article a prestudy was performed with strains TA 98 and TA 100. 8 concentrations were tested for toxicity and mutation induction with each 3 plates. The experimental conditions in this pre-experiment were the same as described below for the experiment I (plate incorporation test).

Toxicity of the test article may be evidenced by a reduction in the number of spontaneous revertants, a clearing of the bacterial background lawn, or by degree of survival of treated cultures.

DOSE SELECTION

According to the results of the pre-experiment the concentrations applied in the main experiments were chosen.

The maximum concentration was 5000.0 µg/plate. The concentration range included two logarithmic decades. In this study six adequately spaced concentrations were tested. Two independent experiments were performed.

As the results of the pre-experiment are in accordance with the criteria described above, these data are reported as a part of the main experiment I.

EXPERIMENTAL PERFORMANCE

For each strain and dose level, including the controls, a minimum of three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

100 µl	Test solution at each dose level, solvent control, negative control, or reference mutagen solution (positive control),
500 µl	S9 mix (for test with metabolic activation) or S9 mix substitution-buffer (for test without metabolic activation),
100 µl	Bacteria suspension (cf. test system, pre-culture of the strains),
2000 µl	Overlay agar

In the pre-incubation assay 100 µl test solution, 500 µl S9 mix / S9 mix substitution buffer and 100 µl bacteria suspension were mixed in a test tube and incubated at 37°C for 60 minutes. After pre-incubation 2.0 ml overlay agar was added to each tube. The mixture was poured on minimal agar plates.

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

DATA RECORDING

The colonies were counted using the AUTOCOUNT (Artek Systems Corporation, BIOSYS GMbH, D-6367 Karben; F.R.G.). The counter was connected to an IBM AT compatible PC with printer which printed out the individual values and the means from the plates for each concentration together with standard deviations and enhancement factors as compared to the spontaneous reversion rates (see tables of results). If precipitation of the test article precluded automatic counting the revertant colonies were counted by hand.

EVALUATION OF RESULTS

The generally accepted conditions for the evaluation of the results are:

- corresponding background growth on both negative control and test plates
- normal range of spontaneous reversion rates.

Range of spontaneous reversion frequencies (5,9)*				
1535	1537	98	100	102 (+)
3 - 37	4 - 31	15 - 60	75 - 200	120 - 300

* These values refer to the negative control without metabolic activation

(+) The range of strain TA 102 was determined from our historical control datas

Due to international guidelines a statistical evaluation of the results is recommended. However, no evaluated statistical procedure can be recommended for analysis of data from the bacterial assays at this time (5).

A test article is considered as positive if either a dose related and reproducible increase in the number of revertants or a significant and reproducible increase for at least one test concentration is induced.

A test article producing neither a dose related and reproducible increase in the number of revertants nor a significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

A significant response is described as follows:

A test article is considered as mutagenic if in strain TA 100 and TA 102 the number of reversions is at least twice as high and in strains TA 1535, TA 1537, and TA 98 it is at least three times higher as compared to the spontaneous reversion rate (4).

Also, a dose-dependent and reproducible increase in the number of revertants is regarded as an indication of possibly existing mutagenic potential of the test article regardless whether the highest dose induced the above described enhancement factors or not.

BIOMETRY

No appropriate statistical method is available (5).

RESULTS

PRE-EXPERIMENT FOR TOXICITY

To evaluate the toxicity of the test article a pre-study was performed with strains TA 98 and TA 100.

The results are given in the following table:

test groups	concentration per plate µg	revertants per plate TA 98 -	TA 98 +	TA 100 -	TA 100 +*
Negative control	-	37	42	76	104
Solvent control	-	30	40	82	106
4-NOPD	50.0	2542	/	/	/
Sodium azide	10.0	/	/	925	/
2-aminoanthracene	2.5	/	575	/	1119
	3.3	22	33	73	108
	10.0	34	23	72	98
	33.3	25	35	76	84
	100.0	34	43	72	85
	333.3	33	39	64	88
	1000.0	25	24	50	84
	2500.0	28	32	50	76
	5000.0	30	25	57	64

* - = without S9 mix; + = with S9 mix
/ = not performed

The plates with the test article showed normal background growth up to 5000.0 µg/plate in strain TA 98 and TA 100, respectively.

According to the dose selection criteria, the test article was tested at the following concentrations:

33.3; 100.0; 333.3; 1000.0; 2500.0 and 5000.0 µg/plate

TABLES OF RESULTS

EXPERIMENT I

PLATE INCORPORATION TEST

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

Test strain : TA 1535

Dose µg/plate	Plate 1	2	3	Revertants/plate mean	s.d.	factor+
without S9 mix						
Negative control	14	15	13	14	1.0	
Solvent control	20	15	15	17	2.9	1.0
33.3	19	22	23	21	2.1	1.3
100.0	18	16	14	16	2.0	1.0
333.3	11	13	17	14	3.1	0.8
1000.0	17	14	13	15	2.1	0.9
2500.0	17	7	15	13	5.3	0.8
5000.0	12	15	19	15	3.5	0.9
Positive control Sodium azide (10µg/plate)	1142	1178	1135	1152	23.1	69.1
with S9 mix						
Negative control	14	15	19	16	2.6	
Solvent control	21	22	27	23	3.2	1.0
33.3	26	21	21	23	2.9	1.0
100.0	27	26	22	25	2.6	1.1
333.3	22	26	20	23	3.1	1.0
1000.0	19	21	19	20	1.2	0.8
2500.0	21	30	28	26	4.7	1.1
5000.0	24	21	21	22	1.7	0.9
Positive control 2-Aminoanthracene (2.5µg/plate)	135	153	190	159	28.0	6.8

+ enhancement factor = $\frac{\Sigma \text{ revertants/concentr. test article}}{\Sigma \text{ revertants/solvent control}}$

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

Test strain : TA 1537

Dose µg/plate	Plate 1	2	3	Revertants/plate mean	s.d.	factor+
without S9 mix						
Negative control	8	10	5	8	2.5	
Solvent control	4	8	7	6	2.1	1.0
33.3	7	8	7	7	0.6	1.2
100.0	6	8	11	8	2.5	1.3
333.3	4	6	6	5	1.2	0.8
1000.0	5	4	6	5	1.0	0.8
2500.0	8	7	5	7	1.5	1.1
5000.0	9	4	5	6	2.6	0.9
Positive control 4-Nitro-o-phenylene-diamine (50µg/plate)	307	311	350	323	23.8	50.9
with S9 mix						
Negative control	12	15	11	13	2.1	
Solvent control	14	11	12	12	1.5	1.0
33.3	13	19	8	13	5.5	1.1
100.0	10	13	13	12	1.7	1.0
333.3	8	12	12	11	2.3	0.9
1000.0	15	11	11	12	2.3	1.0
2500.0	6	14	6	9	4.6	0.7
5000.0	8	11	10	10	1.5	0.8
Positive control 2-Aminoanthracene (2.5µg/plate)	115	110	93	106	11.5	8.6

+ enhancement factor = $\frac{\Sigma \text{ revertants/concentr. test article}}{\Sigma \text{ revertants/solvent control}}$

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

Test strain : TA 98

Dose µg/plate	Plate 1	2	3	Revertants/plate mean	s.d.	factor+
without S9 mix						
Negative control	42	35	35	37	4.0	
Solvent control	27	33	31	30	3.1	1.0
33.3	28	24	24	25	2.3	0.8
100.0	31	35	36	34	2.6	1.1
333.3	24	37	37	33	7.5	1.1
1000.0	32	26	17	25	7.5	0.8
2500.0	19	26	39	28	10.1	0.9
5000.0	28	28	33	30	2.9	1.0
Positive control 4-nitro-o-phenylene-diamine (50 µl/plate)	2464	2501	2660	2542	104.1	83.8
with S9 mix						
Negative control	38	44	43	42	3.2	
Solvent control	47	34	40	40	6.5	1.0
33.3	29	30	45	35	9.0	0.9
100.0	43	40	45	43	2.5	1.1
333.3	42	41	33	39	4.9	1.0
1000.0	25	26	21	24	2.6	0.6
2500.0	30	34	31	32	2.1	0.8
5000.0	29	23	24	25	3.2	0.6
Positive control 2-Aminoanthracene (2.5µg/plate)	520	454	751	575	156.0	14.3

+ enhancement factor = $\frac{\Sigma \text{ revertants/concentr. test article}}{\Sigma \text{ revertants/solvent control}}$

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

Test strain : TA 100

Dose µg/plate	Plate 1	2	3	Revertants/plate mean	s.d.	factor+
without S9 mix						
Negative control	78	82	68	76	7.2	
Solvent control	80	76	89	82	6.7	1.0
33.3	63	86	80	76	11.9	0.9
100.0	63	79	75	72	8.3	0.9
333.3	55	66	70	64	7.8	0.8
1000.0	50	50	51	50	0.6	0.6
2500.0	38	53	58	50	10.4	0.6
5000.0	45	63	64	57	10.7	0.7
Positive control Sodium azide (10µg/plate)	946	942	886	925	33.5	11.3
with S9 mix						
Negative control	103	111	99	104	6.1	
Solvent control	89	114	116	106	15.0	1.0
33.3	90	82	81	84	4.9	0.8
100.0	94	89	73	85	11.0	0.8
333.3	96	71	98	88	15.0	0.8
1000.0	93	102	58	84	23.2	0.8
2500.0	77	80	71	76	4.6	0.7
5000.0	56	67	68	64	6.7	0.6
Positive control 2-Aminoanthracene (2.5µg/plate)	1356	1083	919	1119	220.8	10.5

$$+ \text{ enhancement factor} = \frac{\Sigma \text{ revertants/concentr. test article}}{\Sigma \text{ revertants/solvent control}}$$

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

Test strain : TA 102

Dose µg/plate	Plate 1	2	3	Revertants/plate mean	s.d.	factor+
without S9 mix						
Negative control	200	207	208	205	4.4	
Solvent control	206	225	211	214	9.8	1.0
33.3	181	178	154	171	14.8	0.8
100.0	179	244	199	207	33.3	1.0
333.3	109	179	188	159	43.2	0.7
1000.0	171	151	190	171	19.5	0.8
2500.0	237	228	212	226	12.7	1.1
5000.0	199	201	209	203	5.3	0.9
Positive control methyl methan sulfonat (1.0µl/plate)	827	1107	1010	981	142.2	4.6
with S9 mix						
Negative control	280	296	243	273	27.2	
Solvent control	253	318	357	309	52.5	1.0
33.3	252	312	343	302	46.3	1.0
100.0	318	262	324	301	34.2	1.0
333.3	364	291	292	316	41.9	1.0
1000.0	204	289	251	248	42.6	0.8
2500.0	284	272	289	282	8.7	0.9
5000.0	214	284	295	264	43.9	0.9
Positive control 2-Aminoanthracene (2.5µg/plate)	752	861	864	826	63.8	2.7

+ enhancement factor = $\frac{\Sigma \text{ revertants/concentr. test article}}{\Sigma \text{ revertants/solvent control}}$

TABLES OF RESULTS

EXPERIMENT II

PRE-INCUBATION TEST

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

Test strain : TA 1535

Dose µg/plate	Plate 1	2	3	Revertants/plate mean	s.d.	factor+
without S9 mix						
Negative control	19	20	22	20	1.5	
Solvent control	21	18	21	20	1.7	1.0
33.3	23	18	17	19	3.2	1.0
100.0	15	13	24	17	5.9	0.9
333.3	17	14	23	18	4.6	0.9
1000.0	10	12	12	11	1.2	0.6
2500.0	13	9	15	12	3.1	0.6
5000.0	16	16	17	16	0.6	0.8
Positive control Sodium azide (10µg/plate)	1044	1096	1091	1077	28.7	53.9
with S9 mix						
Negative control	24	17	13	18	5.6	
Solvent control	31	24	27	27	3.5	1.0
33.3	24	26	18	23	4.2	0.8
100.0	21	24	25	23	2.1	0.9
333.3	31	26	31	29	2.9	1.1
1000.0	28	28	29	28	0.6	1.0
2500.0	28	27	30	28	1.5	1.0
5000.0	22	20	17	20	2.5	0.7
Positive control 2-Aminoanthracene (2.5µg/plate)	163	187	167	172	12.9	6.3

$$+ \text{ enhancement factor} = \frac{\Sigma \text{ revertants/concentr. test article}}{\Sigma \text{ revertants/solvent control}}$$

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

Test strain : TA 1537

Dose µg/plate	Plate 1	2	3	Revertants/plate mean	s.d.	factor+
without S9 mix						
Negative control	4	4	7	5	1.7	
Solvent control	8	7	9	8	1.0	1.0
33.3	9	6	11	9	2.5	1.1
100.0	8	9	12	10	2.1	1.2
333.3	6	11	9	9	2.5	1.1
1000.0	11	9	6	9	2.5	1.1
2500.0	13	7	9	10	3.1	1.2
5000.0	11	8	8	9	1.7	1.1
Positive control 4-Nitro-o-phenylene-diamine (50µg/plate)	212	245	176	211	34.5	26.4
with S9 mix						
Negative control	7	13	10	10	3.0	
Solvent control	13	14	13	13	0.6	1.0
33.3	12	15	13	13	1.5	1.0
100.0	14	13	9	12	2.6	0.9
333.3	11	18	13	14	3.6	1.1
1000.0	12	12	7	10	2.9	0.8
2500.0	10	8	10	9	1.2	0.7
5000.0	11	11	9	10	1.2	0.8
Positive control 2-Aminoanthracene (2.5µg/plate)	113	94	91	99	11.9	7.5

+ enhancement factor = $\frac{\Sigma \text{ revertants/concentr. test article}}{\Sigma \text{ revertants/solvent control}}$

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

Test strain : TA 98

Dose µg/plate	Plate 1	2	3	Revertants/plate mean	s.d.	factor+
without S9 mix						
Negative control	22	30	25	26	4.0	
Solvent control	23	27	26	25	2.1	1.0
33.3	18	29	31	26	7.0	1.0
100.0	19	23	25	22	3.1	0.9
333.3	19	18	20	19	1.0	0.8
1000.0	20	25	20	22	2.9	0.9
2500.0	17	19	19	18	1.2	0.7
5000.0	17	13	26	19	6.7	0.7
Positive control 4-nitro-o-phenylene-diamine (50 µg/plate)	1856	1874	1940	1890	44.2	74.6
with S9 mix						
Negative control	42	39	33	38	4.6	
Solvent control	39	45	43	42	3.1	1.0
33.3	43	37	45	42	4.2	1.0
100.0	39	44	42	42	2.5	1.0
333.3	33	46	45	41	7.2	1.0
1000.0	38	41	33	37	4.0	0.9
2500.0	37	41	33	37	4.0	0.9
5000.0	45	33	44	41	6.7	1.0
Positive control 2-Aminoanthracene (2.5µg/plate)	448	352	335	378	60.9	8.9

$$+ \text{ enhancement factor} = \frac{\Sigma \text{ revertants/concentr. test article}}{\Sigma \text{ revertants/solvent control}}$$

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

Test strain : TA 100

Dose µg/plate	Plate 1	2	3	Revertants/plate mean	s.d.	factor+
without S9 mix						
Negative control	82	95	81	86	7.8	
Solvent control	79	93	86	86	7.0	1.0
33.3	89	99	79	89	10.0	1.0
100.0	79	86	91	85	6.0	1.0
333.3	72	87	76	78	7.8	0.9
1000.0	82	86	78	82	4.0	1.0
2500.0	70	89	78	79	9.5	0.9
5000.0	98	86	78	87	10.1	1.0
Positive control Sodium azide (10µg/plate)	1133	1214	1196	1181	42.5	13.7
with S9 mix						
Negative control	106	91	90	96	9.0	
Solvent control	109	97	89	98	10.1	1.0
33.3	84	92	80	85	6.1	0.9
100.0	103	98	97	99	3.2	1.0
333.3	87	87	105	93	10.4	0.9
1000.0	78	75	74	76	2.1	0.8
2500.0	92	88	84	88	4.0	0.9
5000.0	78	84	78	80	3.5	0.8
Positive control 2-Aminoanthracene (2.5µg/plate)	1190	845	1089	1041	177.4	10.6

$$+ \text{ enhancement factor} = \frac{\Sigma \text{ revertants/concentr. test article}}{\Sigma \text{ revertants/solvent control}}$$

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

Test strain : TA 102

Dose µg/plate	Plate 1	2	3	Revertants/plate mean	s.d.	factor+
without S9 mix						
Negative control	151	171	179	167	14.4	
Solvent control	158	175	167	167	8.5	1.0
33.3	178	166	169	171	6.2	1.0
100.0	188	175	176	180	7.2	1.1
333.3	198	177	187	187	10.5	1.1
1000.0	188	179	189	185	5.5	1.1
2500.0	190	156	178	175	17.2	1.0
5000.0	159	167	189	172	15.5	1.0
Positive control methyl methan sulfonat (1.0 µl/plate)	998	1034	897	976	71.0	5.9
with S9 mix						
Negative control	260	219	270	250	27.0	
Solvent control	197	218	245	220	24.1	1.0
33.3	233	210	233	225	13.3	1.0
100.0	217	219	245	227	15.6	1.0
333.3	194	201	206	200	6.0	0.9
1000.0	224	199	213	212	12.5	1.0
2500.0	240	216	230	229	12.1	1.0
5000.0	237	256	174	222	42.9	1.0
Positive control 2-Aminoanthracene (2.5µg/plate)	811	924	702	812	111.0	3.7

$$+ \text{ enhancement factor} = \frac{\Sigma \text{ revertants/concentr. test article}}{\Sigma \text{ revertants/solvent control}}$$

SUMMARY OF RESULTS

Test article: [redacted]

S9 mix from : rat liver (Batch R 060792)

without S9 mix

Revertants/plate
mean from three plates

Dose µg/plate	TA 1535 I	TA 1535 II	TA 1537 I	TA 1537 II	TA 98 I	TA 98 II	TA 100 I	TA 100 II	TA 102 I	TA 102 II
Neg. contr.	14	20	8	5	37	26	76	86	205	167
Solv. contr.	17	20	6	8	30	25	82	86	214	167
33.3	21	19	7	9	25	26	76	89	171	171
100.0	16	17	8	10	34	22	72	85	207	180
333.3	14	18	5	9	33	19	64	78	159	187
1000.0	15	11	5	9	25	22	50	82	171	185
2500.0	13	12	7	10	28	18	50	79	226	175
5000.0	15	16	6	9	30	19	57	87	203	172
Positive controls										
Sodium azide (10µg/plate)	1152	1077					925	1181		
4-Nitro-o-phenylene-diamine (50µg/plate)			323	211	2542	1890				
methyl methan sulfonat (1.0µL/plate)									981	976

with S9 mix

Revertants/plate
mean from three plates

Dose µg/plate	TA 1535 I	TA 1535 II	TA 1537 I	TA 1537 II	TA 98 I	TA 98 II	TA 100 I	TA 100 II	TA 102 I	TA 102 II
Neg. contr.	16	18	13	10	42	38	104	96	273	250
Solv. contr.	23	27	12	13	40	42	106	98	309	220
33.3	23	23	13	13	35	42	84	85	302	225
100.0	25	23	12	12	43	42	85	99	301	227
333.3	23	29	11	14	39	41	88	93	316	200
1000.0	20	28	12	10	24	37	84	76	248	212
2500.0	26	28	9	9	32	37	76	88	282	229
5000.0	22	20	10	10	25	41	64	80	264	222
Positive control										
2-Amino-anthracene (2.5µg/plate)	159	172	106	99	575	378	1119	1041	826	812

CONCLUSIONS

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

The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test article was tested at the following concentrations:

33.3; 100.0; 333.3; 1000.0; 2500.0 and 5000.0 µg/plate

No relevant toxic effects occurred in the test groups with and without metabolic activation in experiment I and II in all strains used.

The plates incubated with the test article showed normal background growth up to 5000.0 µg/plate with and without S9 mix in all strains used.

No substantial increases in revertant colony numbers of any of the five tester strains were observed following treatment with [redacted] at any dose level, either in the presence or absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of significance.

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

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test article did not induce point mutations by base pair changes or frameshifts in the genome of the strains used.

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