



STUDY NUMBER 1305602

**CELL MUTATION ASSAY AT THE
THYMIDINE KINASE LOCUS (TK ^{+/-})
IN MOUSE LYMPHOMA L5178Y CELLS
WITH
cC6O4 AMMONIUM SALT**

REPORT

**STUDY COMPLETION DATE:
JULY 14, 2010**

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 address)

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

[Signature]

Referent, Wiesbaden, den 30. März 2009

(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)



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)

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3 PREFACE

3.1 General

Title: Cell Mutation Assay
at the Thymidine Kinase Locus (TK^{+/+})
in Mouse Lymphoma L5178Y Cells
with cC6O4 AMMONIUM SALT

Sponsor: SOLVAY SOLEXIS S.p.A.
Viale Lombardia 20
20021 Bollate (MI)
Italy

Study Monitor:

Test Facility: Harlan Cytotest Cell Research GmbH
(Harlan CCR)
In den Leppsteinswiesen 19
64380 Rossdorf
Germany

3.2 Responsibilities

Study Director:

Deputy Study Director:

Management:

Head of Quality Assurance Unit:

3.3 Schedule

Experimental Starting Date: January 05, 2010

Experimental Completion Date: February 22, 2010

3.4 Project Staff Signatures

Study Director

[REDACTED]

[REDACTED]

[REDACTED]

Management

[REDACTED]

[REDACTED]

[REDACTED]

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), dated July 25, 1994 ("BGBl. I 1994", pp. 1703), last revision dated June 27, 2002, and amended version dated July 02, 2008 ("BGBl.", p. 1146).

"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.

United States Environmental Protection Agency „Health Effects Test Guidelines, OPPTS 870.5300, *In Vitro* Mammalian Cell Gene Mutation Test“, EPA 712-C-98-221, August 1998.

3.7 Archiving

Harlan CCR will archive the following data for 15 years:

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

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

3.8 Deviations from the Study Plan

There were no deviations to the study plan.

4 STATEMENT OF COMPLIANCE

Study Number: 1305602
Test Item: cC6O4 AMMONIUM SALT
Study Director: [REDACTED]
Title: Cell Mutation Assay
at the Thymidine Kinase Locus (TK^{+/+})
in Mouse Lymphoma L5178Y Cells
with cC6O4 AMMONIUM SALT

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), dated July 25, 1994 ("BGBl. I 1994", pp. 1703), last revision dated June 27, 2002, and amended version dated July 02, 2008 ("BGBl.", p. 1146).

"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

[REDACTED]
[REDACTED]
[REDACTED]

5 QUALITY ASSURANCE UNIT STATEMENT

Study Number: 1305602
Test Item: cC6O4 AMMONIUM SALT
Study Director: [REDACTED]
Title: Cell Mutation Assay
at the Thymidine Kinase Locus (TK^{+/+})
in Mouse Lymphoma L5178Y Cells
with cC6O4 AMMONIUM SALT

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:	November 18, 2009	November 18, 2009
1 st Amendment to Study Plan:	November 25, 2009	November 25, 2009
2 nd Amendment to Study Plan:	December 07, 2009	December 07, 2009
3 rd Amendment to Study Plan:	July 09, 2010	July 09, 2010
<u>Process Inspection</u> Preparation for Application and Application: Preparation for Application:	January 27, 2010 February 17, 2010	January 27, 2010 February 17, 2010
Report:	March 31, 2010 July 14, 2010	March 31, 2010 July 14, 2010

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

Head of Quality Assurance Unit [REDACTED]

Date [REDACTED]

6 SUMMARY

The study was performed to investigate the potential of cC6O4 AMMONIUM SALT to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

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 h. The second experiment was performed with a treatment period of 24 hours in the absence of metabolic activation and 4 hours in the presence of metabolic activation.

The highest concentration (3640 µg/mL) applied in the pre-experiment was chosen with regard to the molecular weight of the test item corresponding to a molar concentration of about 10 mM. The concentration range of the main experiments was limited by cytotoxic effects and precipitation of the test item.

No substantial and reproducible dose dependent increase in mutant colony numbers was observed in both main experiments. No relevant shift of the ratio of small versus large colonies was observed up to the maximal concentration of the test item.

Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

The tested concentrations are described in table 2 (page 17). The evaluated experimental points and the results are summarised in table 1 (page 11).

Conclusion

In conclusion it can be stated that during the mutagenicity test described and under the experimental conditions reported the test item did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation.

Therefore, cC6O4 AMMONIUM SALT is considered to be non-mutagenic in this mouse lymphoma assay.

Table 1: Summary of results

			relative	mutant		relative	mutant	
	conc. µg	S9	total	colonies/		total	colonies/	
	per mL	mix	growth	10 ⁶ cells	threshold	growth	10 ⁶ cells	threshold
Column	1	2	3	4	5	6	7	8
Experiment I / 4 h treatment			culture I			culture II		
Solv. control with water		-	100.0	138	264	100.0	124	250
Pos. control with MMS	19.5	-	14.9	468	264	25.6	310	250
Test item	56.9	-	88.7	131	264	64.6	178	250
Test item	113.8	-	85.9	124	264	76.5	149	250
Test item	227.5	-	28.4	306	264	67.0	145	250
Test item	455.0	-	5.8	370	264	17.0	138	250
Test item	910.0	-	culture was not continued [#]			culture was not continued [#]		
Test item	1365.0	-	culture was not continued [#]			culture was not continued [#]		
Solv. control with water		+	100.0	182	308	100.0	186	312
Pos. control with CPA	3.0	+	58.6	327	308	42.3	295	312
Pos. control with CPA	4.5	+	46.6	339	308	14.2	628	312
Test item	56.9	+	100.2	130	308	65.1	196	312
Test item	113.8	+	122.5	152	308	65.8	212	312
Test item	227.5	+	92.8	170	308	80.7	197	312
Test item	455.0 (p)	+	91.5	141	308	34.6	394	312
Test item	910.0 (p)	+	50.8	158	308	10.2	764	312
Test item	1365.0 (p)	+	culture was not continued [#]			culture was not continued [#]		
Experiment II / 24 h treatment			culture I			culture II		
Solv. control with water		-	100.0	142	268	100.0	175	301
Pos. control with MMS	13.0	-	12.2	520	268	11.3	630	301
Test item	28.1	-	72.7	212	268	69.6	238	301
Test item	56.3	-	97.9	141	268	72.7	200	301
Test item	112.5	-	79.6	132	268	75.4	170	301
Test item	225.0	-	23.9	254	268	22.5	214	301
Test item	337.5	-	0.7	952	268	1.1	535	301
Test item	450.0	-	culture was not continued [#]			culture was not continued [#]		
Experiment II / 4 h treatment			culture I			culture II		
Solv. control with water		+	100.0	182	308	100.0	196	322
Pos. control with CPA	3.0	+	98.0	194	308	34.4	442	322
Pos. control with CPA	4.5	+	39.3	394	308	26.1	349	322
Test item	56.3	+	109.5	141	308	119.8	256	322
Test item	112.5	+	104.0	187	308	105.3	197	322
Test item	225.0	+	48.5	271	308	71.7	211	322
Test item	450.0 (p)	+	31.7	271	308	48.2	345	322
Test item	675.0 (p)	+	culture was not continued [#]			2.6	373	322
Test item	900.0 (p)	+	culture was not continued [#]			culture was not continued [#]		

Threshold = number of mutant colonies per 10⁶ cells of each solvent control plus 126

culture was not continued due to exceedingly severe cytotoxic effects

(p) precipitation visible to the unaided eye

The values printed in bold are judged as invalid, since the acceptance criteria (page 21) are not met (RTG < 10% in both parallel cultures).

7 OBJECTIVE

7.1 Aims of the Study

The assay was performed to assess the potential of the test item to induce mutations by means of two independent mouse lymphoma thymidine kinase locus experiments using the cell line L5178Y. In the first experiment the treatment duration was 4 hours with and without metabolic activation. Experiment II was performed with a treatment time of 24 hours in the absence and 4 hours in the presence of metabolic activation.

7.2 Relevance of the Test System

In vitro methods are valuable to accurately control the concentration and exposure time of cells to the test item 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 at the autosomal thymidine kinase (TK) locus of heterozygous L5178Y/TK^{+/+} cells to TK^{-/-} mutants. In addition, evidence has been obtained that small TK^{-/-} colonies may result from chromosomal damage to the TK locus and adjacent genes (1). Gene and chromosome mutations are considered as an initial step in the carcinogenic process (2).

The L5178Y/TK^{+/+} cells are exposed to the test item with and without exogenous metabolic activation. Following an expression time the descendants of the treated cell population are monitored for the loss of functional TK enzyme.

TK catalyses the conversion of TFT (Trifluorothymidine) to its cytostatic and cytotoxic trifluorothymidine-monophosphate derivative. Therefore, cells deficient in TK due to a forward mutation are resistant to TFT. These cells are able to proliferate in the presence of TFT 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 48 hours.

The mutant frequency is determined by cloning known numbers of cells in 96-well plates containing the selective agent to detect mutant cells, and in 96-well plates without selective agent to determine the surviving cells (cloning efficiency). After a suitable period of time the colonies are counted. Mutation rates are calculated from the number of mutant colonies corrected for cell survival (4, 5).

In order to establish a dose effect relationship at least four concentrations of the test item are tested. These concentrations should yield a dose related toxic effect. The highest concentration level should produce a low level (10 - 20 %) of cell culture growth.

Reference mutagens are tested in parallel to the test item in order to demonstrate the sensitivity of the test system.

8 MATERIALS AND METHODS

8.1 Test Item

Internal Test Item Number: S1069711

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

Identity: cC6O4 ammonium salt (dry salt)
Batch No.: 156/39
Molecular Weight: 357 g/mol
Purity: 97.5 %
Stability in solvent: Not indicated by the sponsor
Storage: In the refrigerator (+2 to +8 °C)
Expiration Date: December 31, 2020

On the day of the experiment (immediately before treatment), the test item was dissolved in deionised water. The final concentration of deionised water in the culture medium was 10 % v/v.

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

	Solvent control	cC6O4 ammonium salt 3640 µg/mL
Osmolarity mOsm	272	296
pH-value	7.63	7.62

8.2 Controls

8.2.1 Solvent Controls

Concurrent solvent controls (deionised water) were performed.

8.2.2 Positive Control Substances

8.2.2.1 Without metabolic activation

Name:	Methyl Methane Sulfonate (MMS)
Supplier:	Sigma, 82024 Taufkirchen, Germany
Charge:	76296KJ
Expiration Date:	May 2010
Purity:	> 99%
Dissolved in:	Nutrient medium
Final Concentration:	19.5 µg/mL = 0.18 mM (experiment I) 13.0 µg/mL = 0.12 mM (experiment II)

The solutions were prepared on the day of experiment.

8.2.2.2 With metabolic activation

Name:	Cyclophosphamide (CPA)
Supplier:	Aldrich Chemie, 89555 Steinheim, Germany
Charge:	097K1311
Expiration Date:	March 2011
Purity:	≥ 98 %
Dissolved in:	0.9 % saline
Final Concentration:	3.0 µg/mL = 10.7 µM 4.5 µg/mL = 16.1 µM

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

The stability of both positive control substances in solution is proven by the mutagenic response in the expected range.

8.3 Test System

8.3.1 Reasons for the Choice of the Cell Line L5178Y

The L5178Y cell line has successfully been used in *in vitro* experiments for many years. L5178Y cells are characterised by a high proliferation rate (doubling time 10 - 12 h in stock cultures) and cloning efficiencies of untreated cells of usually more than 50 % both necessary for the appropriate performance of the study. The cells have a stable karyotype with a near diploid (40 ± 2) chromosome number (2).

A low metabolic activity of cells under *in vitro* conditions is a disadvantage of assays with cell cultures as many chemicals develop a mutagenic potential only after metabolic activation by the mammalian organism. However, metabolic activation of chemicals can at least partially be achieved by supplementing the cell cultures with liver microsome preparations (S9 mix).

8.4 Cell Cultures

Prior to mutagenicity testing the amount of spontaneous mutants is reduced by growing the cells for one day in RPMI 1640-HAT medium supplemented with:

hypoxanthine	1.0×10^{-4} M
aminopterin	2.0×10^{-7} M
thymidine	1.6×10^{-5} M

The incubation of the cells in HAT-medium is followed by a recovery period of 2 days in RPMI 1640 medium containing:

hypoxanthine	1.0×10^{-4} M
thymidine	1.6×10^{-5} M

After this incubation the L5178Y cells are returned to normal RPMI 1640 medium (complete culture medium).

Large stocks of the cleansed L5178Y cell line are stored in liquid nitrogen in the cell bank of Harlan CCR allowing the repeated use of the same cell culture batch in many experiments. Before freezing, each batch was screened for mycoplasma contamination and checked for karyotype stability. Consequently, the parameters of the experiments remain similar because of the reproducible characteristics of the cells.

Thawed stock cultures are propagated in plastic flasks (Greiner, 72632 Frickenhausen, Germany) in RPMI 1640 complete culture medium (see page 18). The cells are subcultured two times prior to treatment. The cell cultures are incubated at $37 \pm 1.5^\circ\text{C}$ in a humidified atmosphere with 4.5 % carbon dioxide and 95.5 % ambient air.

8.5 Mammalian Microsomal Fraction S9 Mix

8.5.1 S9 (Preparation by Harlan CCR)

Phenobarbital/ β -naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 is prepared from 8 - 12 weeks old male Wistar HsdCpb:WU rats (Harlan Laboratories GmbH, 33178 Borcheln, Germany), weight approx. 220 - 320 g induced by applications of 80 mg/kg b.w. phenobarbital i.p. (Desitin; 22335 Hamburg, Germany) and β -naphthoflavone p.o. (Aldrich, 89555 Steinheim, 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 150 mM KCl solution (1+3) followed by centrifugation at 9000 g. Aliquots of the supernatant were frozen and stored in ampoules at -80° C. Small numbers of the ampoules were kept at -20°C for up to one week.

The protein concentration of the S9 preparation was 31.7 mg/mL (Lot. No.: 021009) in the pre-experiment and 32.7 mg/mL (Lot. No.: 210809) in experiment I and 35.0 mg/mL (Lot. No.: 070110) in experiment II.

8.5.2 S9 Mix

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to give a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the 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. (3). The concentration in the final test medium was 5 % (v/v).

8.6 Pre-Test on Toxicity

A pre-test was performed in order to determine the concentration range of the mutagenicity experiments. Both, pH value and osmolarity were determined at the maximal concentration of the test item and in the solvent control without metabolic activation.

1×10^7 cells (3×10^6 cells at the beginning of 24 h treatment) were exposed to each concentration of the test item for 4 and 24 hours without and 4 hours with metabolic activation. During the 4 h treatment period the serum concentration was reduced from 15 % to 3 %. Following treatment the cells were washed twice by centrifugation (425 g, 10 min) and resuspended in "saline G". Subsequently the cells were resuspended in 30 mL complete culture medium for a 2-day growth period. The cell density was determined immediately after treatment and at each day of the growth period and adjusted to 3×10^5 cells/mL, if necessary. The relative suspension growth (RSG) of the treated cell cultures was calculated at the end of the growth period according to the method of Clive and Spector (1).

8.7 Dose selection

According to the results of the pre-test at least four adequate concentrations were chosen for the mutation experiments.

The highest concentration should be 10 mM, but not higher than 5 mg/mL, unless limited by the solubility or toxicity of the test item.

RSG (Relative Suspension Growth) or RTG (Relative Total Growth) values (main experiment) below 50 % are considered toxic. In case of toxic effects, the highest test item concentration of the main experiment should reduce the RSG or RTG value to approximately 10 - 20 %.

The pre-experiment was performed in the presence (4 h treatment) and absence (4 h and 24 h treatment) of metabolic activation. Test item concentrations between 28.4 µg/mL and 3640 µg/mL were used. The highest concentration in the pre-experiment was chosen with regard to the purity (97.5 %) and the molecular weight (357 g/mol) of the test item. Strong toxic effects were observed at 1820 µg/mL and above in the absence of metabolic activation (4 h treatment). In the presence of metabolic activation (4 hours treatment) toxic effects occurred at 455 µg/mL and above. Following continuous treatment (24 hours) strong toxic effects were determined at 227.5 µg/mL and above.

The test medium was checked for precipitation at the end of each treatment period (4 or 24 hours) before the test item was removed. After 4 hours treatment precipitation of the test item was observed at 910 µg/mL and above in the presence and absence of metabolic activation. Following continuous treatment no precipitation was noted.

There was no relevant shift of the pH value or the osmolarity of the medium even at the maximum concentration of the pre-experiment.

The dose range of the main experiments was limited by the occurrence of toxicity and precipitation. The individual concentrations of the first experiment with and without metabolic activation and the second experiment without metabolic activation were generally spaced by a factor of 2.0. A narrower spacing was used in the upper concentration range to cover the limit of solubility or cytotoxic effects more closely.

To overcome problems with possible deviations in toxicity both main experiments were started with more than four concentrations.

Table 2: Doses applied in the Mouse Lymphoma Assay with cC6O4 AMMONIUM SALT

	concentrations in µg/mL					
	Experiment I					
without S9 mix*	56.9	113.8	227.5	455	910	1365
with S9 mix*	56.9	113.8	227.5	455 ^P	910 ^P	1365 ^P
	Experiment II					
without S9 mix**	28.1	56.3	112.5	225.0	337.5	450
with S9 mix*	56.3	112.5	225	450 ^P	675 ^P	900 ^P

* 4 h treatment; ** 24 h treatment

Following the expression phase of 48 hours the cultures at the concentrations of 910 and 1365 µg/mL (printed in bold letters) in experiment I without metabolic activation and at

1365 µg/mL with metabolic activation were not analysed due to exceedingly severe toxic effects. In the second experiment the cultures at 450 µg/mL without metabolic activation and 675 µg/mL (culture I) and 900 µg/mL with metabolic activation were not continued for the same reason.

8.8 Experimental Performance

In the mutation experiment 1×10^7 (3×10^6 during 24 h exposure) cells/flask (80 cm² flasks) suspended in 10 ml RPMI medium with 3 % horse serum (15 % horse serum during 24 h exposure) were exposed to various concentrations of the test item either in the presence or absence of metabolic activation. After 4 h (24 h in the second experiment) the test item was removed by centrifugation (425 x g, 10 min) and the cells were washed twice with "saline G". Subsequently the cells were resuspended in 30 ml complete culture medium and incubated for an expression and growth period of 48 h.

The cell density was determined each day and adjusted to 3×10^5 cells/ml, if necessary. The relative suspension growth (RSG) of the treated cell cultures was calculated by the day 1 fold-increase in cell number multiplied by the day 2 fold-increase in cell number according to the method of Clive and Spector (1).

After the expression period the cultures were selected. Cells from each experimental group were seeded into 2 microtiter plates so that each well contained approximately 4×10^3 cells in selective medium (see below) with TFT (Serva, 69042 Heidelberg, Germany). The viability (cloning efficiency) was determined by seeding about 2 cells per well into microtiter plates (same medium without TFT). The plates were incubated at $37^\circ \pm 1.5^\circ \text{C}$ in 4.5 % CO₂/95.5 % water saturated air for 10 - 15 days. Then the plates were evaluated.

8.8.1 Complete Culture Medium

RPMI 1640 medium (GIBCO, invitrogen) supplemented with 15 % horse serum (HS, GIBCO, invitrogen) (3 % HS during 4 hour treatment), 1% of 100 U/100 µg/mL Penicillin/Streptomycin, 220 µg/mL Sodium-Pyruvate, and 0.5 – 0.75 % Amphotericin used as antifungal (6).

8.8.2 Selective Medium

RPMI 1640 (complete culture medium) by addition of 5 µg/mL TFT.

8.8.3 Saline G Solution

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

NaCl	8000 mg
KCl	400 mg
Glucose	1100 mg
Na ₂ HPO ₄ ·7H ₂ O	290 mg
KH ₂ PO ₄	150 mg
pH: 7.2	

8.9 Size Distribution of the Colonies

Colonies were counted manually. In accordance with their size the colonies were classified into two groups. The colony size distribution was determined in the controls and at all concentrations of the test item. Criteria to determine colony size were the absolute size of the colony (more than 1/3 of a well for large colonies) and the optical density of the colonies (the optical density of the small colonies is generally higher than the optical density of the large ones).

8.10 Data Recording

The data generated were recorded in the raw data. The results were presented in tabular form, including experimental groups with the test item, negative, solvent, and positive controls. The survival rate and viability were determined based on the Poisson distribution method. The zero term of the Poisson distribution, $[P(0)]$ method, was used (7, 8). The mutation frequency was derived from the cloning efficiency under selective conditions compared to the corresponding viability under non-selective conditions.

8.11 Calculation and Processing of the Data

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

Pre-test	
total suspension growth (4 h treatment):	(cell number at 24 h / cell number at 4 h) × (cell number at 48 h / if cell number at 24 h > 300000 then 300000, if cell number at 24 h < 300000 then cell number at 24 h)
total suspension growth (24 h treatment):	(cell number at 24 h / cell number of seeded cells per mL (100000)) × (cell number at 48 h / if cell number at 24 h > 300000 then 300000, if cell number at 24 h < 300000 then cell number at 24 h)
relative suspension growth:	total suspension growth × 100 / total suspension growth of corresponding control
Main test	
total suspension growth (4 h treatment):	(cell number at 24 h / cell number at 4 h) × (cell number at 48 h / if cell number at 24 h > 300000 then 300000, if cell number at 24 h < 300000 then cell number at 24 h)
total suspension growth (24 h treatment):	(cell number at 24 h / cell number of seeded cells per mL (100000)) × (cell number at 48 h / if cell number at 24 h > 300000 then 300000, if cell number at 24 h < 300000 then cell number at 24 h) × (cell number at 72 h / if cell number at 48 h > 300000 then 300000, if cell number at 48 h < 300000 then cell number at 48 h)
relative suspension growth:	total suspension growth × 100 / total suspension growth of corresponding control
relative total growth:	relative suspension growth × relative cloning efficiency / 100
cloning efficiency (viability):	$-\ln(\text{mean number of empty wells per plate} / 96) / \text{cells seeded per well}$
relative cloning efficiency:	cloning efficiency × 100 / cloning efficiency of corresponding control
cells survived:	cloning efficiency × cell number seeded in TFT medium
mutant colonies / 10 ⁶ cells:	(small mutant colonies (column 14) + large mutant colonies (column 15))
small mutant colonies / 10 ⁶ cells:	$(-\ln(\text{mean number of wells not containing small colonies col. 10} / 96) / \text{cells survived Table "Mutagenicity data/part I", col. 11}) \times 10^6$
large mutant colonies / 10 ⁶ cells:	$(-\ln(\text{mean number of wells not containing large colonies col. 9} / 96) / \text{cells survived Table "Mutagenicity data/part I", col. 11}) \times 10^6$
threshold:	number of mutant colonies per 10 ⁶ cells of each solvent control plus 126
Cloning efficiency (viability):	cloning efficiency determined after the expression period to measure viability of the cells without selective agent

8.12 Acceptability of the Assay

A mutation assay is considered acceptable if it meets the following criteria (the current recommendations of the IWGT (ref. 9, 10, 11, 12, 13) are considered):

1. All plates, from either the cloning efficiency or the TFT resistance-testing portion of the experiment are analysable.
2. The absolute cloning efficiency at the time of mutant selection (CE) of the solvent controls is 65 – 120 %.
3. The total suspension growth of the solvent control calculated by the day 1 fold-increase in cell number multiplied by the day 2 fold-increase in cell number is 8 – 32. Following 24 h treatment the total suspension growth is 32 – 180 (ref. 13).
4. The range of the solvent control mutant frequency is in the range of 50 – 170 x 10⁻⁶ cells.
5. The positive controls (MMS and CPA) should yield an absolute increase in total MF, that is an increase above spontaneous background MF (an induced MF [IMF]) of at least 300 x 10⁻⁶ cells. At least 40 % of the induced mutation frequency (IMF) should be reflected in the small colony MF. Alternatively, the positive controls should induce at least 150 small colonies.
6. The upper limit of cytotoxicity observed in the positive control culture should be the same as for the experimental cultures (i.e. the relative total growth – RTG- should be greater than 10 % of the concurrent selective control group).
7. The highest concentration of the test item should be 10 mM or 5000 µg/mL, unless limited by toxicity or solubility of the test item. If toxicity occurred, the highest concentration should lower the relative total growth to approximately 10 to 20 % of survival. If precipitation is noted, the highest analysed concentration should be the lowest concentration where precipitation is observed by the naked eye.

8.13 Evaluation of Results

A test item is classified as mutagenic if the induced mutation frequency reproducibly exceeds a threshold of 126 colonies per 10⁶ cells above the corresponding solvent control.

A relevant increase of the mutation frequency should be dose-dependent.

A mutagenic response is considered to be reproducible if it occurs in both parallel cultures.

However, in the evaluation of the test results the historical variability of the mutation rates in the solvent controls of this study are taken into consideration.

Results of test groups are generally rejected if the relative total growth is less than 10 % of the vehicle control unless the exception criteria specified by the IWGT recommendations are fulfilled.

Whenever a test item is considered mutagenic according to the above mentioned criteria, the ratio of small versus large colonies is used to differentiate point mutations from clastogenic effects. If the increase of the mutation frequency is accompanied by a reproducible and dose dependent shift in the ratio of small versus large colonies clastogenic effects are indicated.

8.14 Statistical Analysis

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using SYSTAT®11 (SYSTAT Software, Inc., 501, Canal Boulevard, Suite C, Richmond, CA 94804, USA) statistics software. The number of mutant colonies obtained for the groups treated with the test item was compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05. However, both, biological relevance and statistical significance were considered together.

experimental group	p-value
experiment I, culture I without S9 mix	0.024
experiment I, culture II without S9 mix	0.788
experiment I, culture I with S9 mix	0.867
experiment I, culture II with S9 mix	0.001
experiment II, culture I without S9 mix	0.284
experiment II, culture II without S9 mix	0.861
experiment II, culture I with S9 mix	0.081
experiment II, culture II with S9 mix	0.103

9 RESULTS AND DISCUSSION

The study was performed to investigate the potential of cC6O4 AMMONIUM SALT to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

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 h. The second experiment was performed in the absence of metabolic activation with a treatment period of 24 hours and in the presence of metabolic activation with a treatment period of 4 hours.

The main experiments were evaluated at the following concentrations:

Experiment I:

without S9 mix:	56.9; 113.8; 227.5; and 455 µg/mL
with S9 mix:	56.9; 113.8; 227.5; 455; and 910 µg/mL

Experiment II:

without S9 mix:	28.1; 56.3; 112.5; 225; and 337.5 µg/mL
with S9 mix:	56.3; 112.5; 225; 450; and 675 µg/mL

Precipitation of the test item visible to the naked eye was noted at 455 µg/mL and above in the first experiment with metabolic activation and at 450 µg/mL and above in the second experiment with metabolic activation.

Relevant toxic effects indicated by a relative total growth of less than 50 % of relative total growth were observed in experiment I starting at 227.5 µg/mL in the presence and absence of metabolic activation. In the second experiment toxic effects as described above occurred at 225 µg/mL and above with and without metabolic activation. The data generated in the second experiment without metabolic activation at 337.5 µg/mL were not judged as acceptable since the RTG dropped far below the 10% limit in both parallel cultures. The same was true for the data generated in the second experiment with metabolic activation at 675 µg/mL. The RTG of the second culture was only 2.6, the first culture was not analysable at all due to exceedingly severe cytotoxic effects.

No substantial and reproducible dose dependent increase of the mutation frequency was observed in both experiments up to the maximum concentration with and without metabolic activation. The mutation frequency exceeded the threshold of 126 above each solvent control in the first culture of the first experiment without metabolic activation at 227.5 and 455.0 µg/mL. In the second culture of the first experiment with metabolic activation the threshold was exceeded at precipitating concentrations of 450 and 910 µg/mL. However, this increase was not reproduced in the parallel culture under identical conditions and consequently judged as artefact. In the second experiment the threshold of 126 above each solvent control count was again exceeded in one of the parallel cultures at a precipitating concentration of 450 µg/mL with metabolic activation. Again, no comparable increase was noted in the parallel culture under identical conditions so this isolated increase was judged as precipitation artefact. Precipitation is a problem in the mouse lymphoma assay since L5178Y cells grow in suspension. The test item is removed by centrifugation at the end of treatment. At precipitating concentrations the test item can not completely be removed at the intended end of treatment since the precipitate sediments together with the cells upon centrifugation. Arbitrary amounts of the test item are carried over to the next steps of the assay leading to irreproducible artefacts.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using SYSTAT® statistics software. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was detected in the first culture of the first experiment without metabolic activation and in the second culture of the first experiment with metabolic activation. These apparent trends were considered irrelevant since the trend was not reproduced in the second culture without metabolic activation under identical experimental conditions. In the second culture with metabolic activation the trend was based on precipitation artefacts as discussed above.

In this study the range of the solvent controls was from 124 up to 196 mutant colonies per 10^6 cells; the range of the groups treated with the test item was from 124 up to 764 mutant colonies per 10^6 cells. The solvent controls exceeded the recommended upper limit of 170 per 10^6 cells somewhat in the presence of metabolic activation and in one of both cultures following long term treatment for 24 hours. However, the data are judged as acceptable since the IWGT recommendations published in 2003 (11) stated an upper limit of 200 colonies per 10^6 cells and all solvent controls remained within the historical control range. MMS (19.5 µg/mL in experiment I and 13.0 µg/mL in experiment II) and CPA (3.0 and 4.5 µg/mL) were used as positive controls and showed a distinct increase in induced total mutant colonies at acceptable levels of toxicity with at least one of the concentrations of the controls.

Conclusion

In conclusion it can be stated that under the experimental conditions reported the test item did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation.

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12 ANNEX I: TABLES OF RESULTS

12.1 Pre-Experiment

Table 3: Toxicity data

	conc. µg per mL	S9 mix	treatment period h	number of cells per mL found 4 h after treatment	number of cells per mL found 24 h after treatment	number of cells per mL found 48 h after treatment	total suspension growth TSG	relative suspension growth RSG	precipitation
Column	1	2	3	4	5	6	7	8	9
Solvent control with water		-	4	332400	478000	220800	1.1	100.0	
Test item	28.4	-	4	290200	724000	1503200	12.5	1181.1	
Test item	56.9	-	4	303400	1169000	1507800	19.4	1829.7	
Test item	113.8	-	4	246000	589000	1703800	13.6	1284.8	
Test item	227.5	-	4	157200	369200	1594200	12.5	1179.2	
Test item	455.0	-	4	37000	43000	250200	6.8	638.9	
Test item	910.0	-	4	55800	40200	39400	0.7	66.7	precipitation
Test item	1820.0	-	4	13200	13000	0	0.0	0.0	precipitation
Test item	3640.0	-	4	3200	6400	0	0.0	0.0	precipitation
Solvent control with water		+	4	380400	787400	1621600	11.2	100.0	
Test item	28.4	+	4	324400	636800	1556400	10.2	91.0	
Test item	56.9	+	4	342000	861600	1267600	10.6	95.1	
Test item	113.8	+	4	405400	824600	1475400	10.0	89.4	
Test item	227.5	+	4	255400	565400	1553000	11.5	102.4	
Test item	455.0	+	4	137600	149600	745200	5.4	48.4	
Test item	910.0	+	4	31000	27200	148000	4.8	42.7	precipitation
Test item	1820.0	+	4	15200	16000	0	0.0	0.0	precipitation
Test item	3640.0	+	4	3400	6400	0	0.0	0.0	precipitation
Solvent control with water		-	24		354200	1845000	21.8	100.0	
Test item	28.4	-	24		357000	1608600	19.1	87.9	
Test item	56.9	-	24		355400	1236400	14.6	67.2	
Test item	113.8	-	24		257000	1311800	13.1	60.2	
Test item	227.5	-	24		125000	683600	6.8	31.4	
Test item	455.0	-	24		23000	70800	0.7	3.3	
Test item	910.0	-	24		55000	66800	0.7	3.1	
Test item	1820.0	-	24		30600	0	0.0	0.0	
Test item	3640.0	-	24		74000	0	0.0	0.0	

12.2 Main Experiment

12.2.1 Experiment I, Culture I

Table 4: Toxicity data

			number of cells			total suspension growth	relative suspension growth	relative total growth
			per mL	found 4 h	found 24 h	found 48 h		
	conc. µg	S9						
	per mL	mix			after treatment			
Column	1	2	3	4	5	6	7	8
Solvent control with water	-	-	415400	2014000	1279400	20.7	100.0	100.0
Pos. control with MMS	19.5	-	424000	1481400	921600	10.7	51.9	14.9
Test item	56.9	-	435400	1645800	1133800	14.3	69.1	88.7
Test item	113.8	-	414600	1730200	1020600	14.2	68.7	85.9
Test item	227.5	-	382600	1348400	1146400	13.5	65.1	28.4
Test item	455.0	-	152600	123800	324600	2.1	10.3	5.8
Test item	910.0	-	144000	67400	culture was not continued [#]			
Test item	1365.0	-	154600	70200	culture was not continued [#]			
Solvent control with water	+	+	433000	1500200	1519400	17.5	100.0	100.0
Pos. control with CPA	3.0	+	270000	713800	1483200	13.1	74.5	58.6
Pos. control with CPA	4.5	+	309600	771200	1551800	12.9	73.4	46.6
Test item	56.9	+	404400	1153000	1290200	12.3	69.9	100.2
Test item	113.8	+	421200	1317800	1406800	14.7	83.6	122.5
Test item	227.5	+	397200	1050600	1453800	12.8	73.0	92.8
Test item	455.0	+	199600	382400	1400200	8.9	51.0	91.5
Test item	910.0	+	22800	59200	150800	6.6	37.7	50.8
Test item	1365.0	+	21800	20400	culture was not continued [#]			

culture was not continued due to strong toxic effects

Table 5: Mutagenicity data (part 1); experiment I; culture I

	conc. µg per mL	S9 mix	cells seeded	number of empty wells		viability		cloning efficiency	relative cloning efficiency	cells seeded in TFT-medium	cells survived	total number of colonies plate 1	total number of colonies plate 2
Column	1	2	3	4	5	6	7	8	9	10	11	12	13
Solvent control with water		-	2.0	14	11	12.5	2.12	1.02	100.0	4452	4538	49	49
Pos. control with MMS	19.5	-	2.0	55	52	53.5	2.12	0.29	28.7	4920	1438	47	49
Test item	56.9	-	2.0	5	9	7.0	2.83	1.31	128.4	4364	5713	59	51
Test item	113.8	-	2.0	12	3	7.5	6.36	1.27	125.1	4520	5762	50	55
Test item	227.5	-	2.0	38	41	39.5	2.12	0.44	43.6	3916	1739	41	43
Test item	455.0	-	2.0	30	31	30.5	0.71	0.57	56.2	3880	2224	62	54
Test item	910.0	-	2.0	culture was not continued [#]									
Test item	1365.0	-	2.0	culture was not continued [#]									
Solvent control with water		+	2.0	22	19	20.5	2.12	0.77	100.0	4192	3236	47	43
Pos. control with CPA	3.0	+	2.0	29	28	28.5	0.71	0.61	78.7	4280	2599	60	56
Pos. control with CPA	4.5	+	2.0	36	36	36.0	0.00	0.49	63.5	4140	2030	47	51
Test item	56.9	+	2.0	11	10	10.5	0.71	1.11	143.3	4588	5077	52	47
Test item	113.8	+	2.0	11	9	10.0	1.41	1.13	146.5	4116	4655	58	49
Test item	227.5	+	2.0	13	14	13.5	0.71	0.98	127.1	4320	4237	54	51
Test item	455.0	+	2.0	7	5	6.0	1.41	1.39	179.6	4680	6488	65	61
Test item	910.0	+	2.0	13	11	12.0	1.41	1.04	134.7	4116	4279	48	52
Test item	1365.0	+	2.0	culture was not continued [#]									

culture was not continued due to strong toxic effects

Table 6: Mutagenicity data (part 2); experiment I; culture I

			number of empty wells found after plating in TFT medium*																			
			calculated for plate 1				calculated for plate 2				mean	mean	mean	of all		mutant	small	large				
	conc. µg per mL	S9 mix	large colonies	small colonies	sum.	large colonies	small colonies	sum.	large colonies	small colonies	mean	mean	empty wells	standard deviation	colonies per 10 ⁵ cells	colonies/ mutant 10 ⁶ cells	colonies/ mutant 10 ⁶ cells					
Column	1	2	3	4	5	6	7	8	9	10			11	12	13	14	15					
Solvent control with water		-	81	62	47	89	54	47	85	58		47.0	0.00		138	111	27					
Pos. control with MMS	19.5	-	95	50	49	93	50	47	94	50		48.0	1.41		468	454	15					
Test item	56.9	-	88	45	37	86	55	45	87	50		41.0	5.66		131	114	17					
Test item	113.8	-	88	54	46	89	48	41	89	51		43.5	3.54		124	110	14					
Test item	227.5	-	92	59	55	87	62	53	90	61		54.0	1.41		306	266	40					
Test item	455.0	-	86	44	34	90	48	42	88	46		38.0	5.66		370	331	39					
Test item	910.0	-	culture was not continued [#]																			
Test item	1365.0	-	culture was not continued [#]																			
Solvent control with water		+	89	56	49	92	57	53	91	57		51.0	2.83		182	164	18					
Pos. control with CPA	3.0	+	91	41	36	90	46	40	91	44		38.0	2.83		327	305	23					
Pos. control with CPA	4.5	+	95	50	49	92	49	45	94	50		47.0	2.83		339	326	13					
Test item	56.9	+	89	51	44	89	56	49	89	54		46.5	3.54		130	115	15					
Test item	113.8	+	85	49	38	85	58	47	85	54		42.5	6.36		152	126	26					
Test item	227.5	+	86	52	42	93	48	45	90	50		43.5	2.12		170	154	17					
Test item	455.0	+	84	43	31	88	43	35	86	43		33.0	2.83		141	124	17					
Test item	910.0	+	88	56	48	92	48	44	90	52		46.0	2.83		158	143	15					
Test item	1365.0	+	culture was not continued [#]																			

* 96 wells minus number of wells containing large or small colonies

culture was not continued due to strong toxic effects

12.2.2 Experiment I, Culture II

Table 7: Toxicity data

			number of cells					
			per mL			total	relative	relative
	conc. µg	S9	found 4 h	found 24 h	found 48 h	suspension	suspension	total
Column	per mL	mix	after treatment			growth	growth	growth
	1	2	3	4	5	6	7	8
Solvent control with water	-	-	432000	1510000	1633200	19.0	100.0	100.0
Pos. control with MMS	19.5	-	394000	945200	1280800	10.2	53.8	25.6
Test item	56.9	-	448000	1425800	1657200	17.6	92.4	64.6
Test item	113.8	-	423200	1641400	1314800	17.0	89.3	76.5
Test item	227.5	-	349000	1021800	1347800	13.2	69.1	67.0
Test item	455.0	-	162000	157200	466400	2.9	15.1	17.0
Test item	910.0	-	159600	91400	culture was not continued [#] culture was not continued [#]			
Test item	1365.0	-	150000	78000				
Solvent control with water		+	324000	1124800	1867400	21.6	100.0	100.0
Pos. control with CPA	3.0	+	318000	761400	1884000	15.0	69.6	42.3
Pos. control with CPA	4.5	+	346400	662800	1605800	10.2	47.4	14.2
Test item	56.9	+	447400	1198800	1927600	17.2	79.7	65.1
Test item	113.8	+	387400	1077600	1876800	17.4	80.5	65.8
Test item	227.5	+	361000	1188000	1945600	21.3	98.8	80.7
Test item	455.0	+	179000	349000	1836200	11.9	55.2	34.6
Test item	910.0	+	24600	54800	139800	5.7	26.3	10.2
Test item	1365.0	+	21400	28600	culture was not continued [#]			

culture was not continued due to strong toxic effects

Table 8: Mutagenicity data (part 1); experiment I; culture II

			viability						relative				
	conc. µg per mL	S9 mix	cells seeded	number of empty wells plate 1	plate 2	mean	standard deviation	cloning efficiency	cloning efficiency	seeded in TFT-medium	cells survived	total number of colonies plate 1	total number of colonies plate 2
Column	1	2	3	4	5	6	7	8	9	10	11	12	13
Solvent control with water		-	2.0	19	20	19.5	0.71	0.80	100.0	4056	3232	40	26
Pos. control with MMS	19.5	-	2.0	48	42	45.0	4.24	0.38	47.5	4616	1749	39	43
Test item	56.9	-	2.0	35	28	31.5	4.95	0.56	69.9	4440	2474	39	34
Test item	113.8	-	2.0	24	25	24.5	0.71	0.68	85.7	4344	2966	43	30
Test item	227.5	-	2.0	19	22	20.5	2.12	0.77	96.9	4312	3329	36	40
Test item	455.0	-	2.0	17	15	16.0	1.41	0.90	112.4	4340	3888	44	38
Test item	910.0	-	2.0	culture was not continued [#]									
Test item	1365.0	-	2.0	culture was not continued [#]									
Solvent control with water		+	2.0	8	11	9.5	2.12	1.16	100.0	4120	4765	60	61
Pos. control with CPA	3.0	+	2.0	20	27	23.5	4.95	0.70	60.8	4352	3062	58	63
Pos. control with CPA	4.5	+	2.0	51	45	48.0	4.24	0.35	30.0	4440	1539	64	58
Test item	56.9	+	2.0	13	16	14.5	2.12	0.95	81.7	4156	3928	54	59
Test item	113.8	+	2.0	11	18	14.5	4.95	0.95	81.7	4036	3814	54	64
Test item	227.5	+	2.0	18	11	14.5	4.95	0.95	81.7	4124	3898	59	54
Test item	455.0	+	2.0	24	21	22.5	2.12	0.73	62.7	3928	2849	67	71
Test item	910.0	+	2.0	39	39	39.0	0.00	0.45	38.9	2780	1252	65	58
Test item	1365.0	+	2.0	culture was not continued [#]									

culture was not continued due to strong toxic effects

Table 9: Mutagenicity data (part 2); experiment I; culture II

			number of empty wells found after plating in TFT medium*																		
			calculated for plate 1					calculated for plate 2					mean		mean	mean	of all		mutant	small	large
	conc. µg per mL	S9 mix	large colonies	small colonies	sum.	large colonies	small colonies	sum.	large colonies	small colonies	sum.	large colonies	small colonies	mean	empty wells	standard deviation	10 ⁶ cells	mutant colonies/ 10 ⁶ cells	mutant colonies/ 10 ⁶ cells	mutant colonies/ 10 ⁶ cells	
Column	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15						
Solvent control with water	-	-	91	61	56	93	73	70	92	67	63.0	9.90	124	111	1						
Pos. control with MMS	19.5	-	94	59	57	94	55	53	94	57	55.0	2.83	310	298	1						
Test item	56.9	-	90	63	57	87	71	62	89	67	59.5	3.54	178	145	3						
Test item	113.8	-	87	62	53	91	71	66	89	67	59.5	9.19	149	124	2						
Test item	227.5	-	90	66	60	95	57	56	93	62	58.0	2.83	145	134	1						
Test item	455.0	-	92	56	52	94	60	58	93	58	55.0	4.24	138	130	1						
Test item	910.0	-	culture was not continued [#]																		
Test item	1365.0	-	culture was not continued [#]																		
Solvent control with water	+	+	92	40	36	85	46	35	89	43	35.5	0.71	186	169	1						
Pos. control with CPA	3.0	+	86	48	38	94	35	33	90	42	35.5	3.54	295	274	2						
Pos. control with CPA	4.5	+	94	34	32	93	41	38	94	38	35.0	4.24	628	611	1						
Test item	56.9	+	85	53	42	86	47	37	86	50	39.5	3.54	196	166	2						
Test item	113.8	+	87	51	42	82	46	32	85	49	37.0	7.07	212	179	3						
Test item	227.5	+	82	51	37	89	49	42	86	50	39.5	3.54	197	167	3						
Test item	455.0	+	88	37	29	91	30	25	90	34	27.0	2.83	394	369	2						
Test item	910.0	+	91	36	31	93	41	38	92	39	34.5	4.95	764	730	3						
Test item	1365.0	+	culture was not continued [#]																		

* 96 wells minus number of wells containing large or small colonies

culture was not continued due to strong toxic effects

12.2.3 Graphical summary of experiment I / Culture I

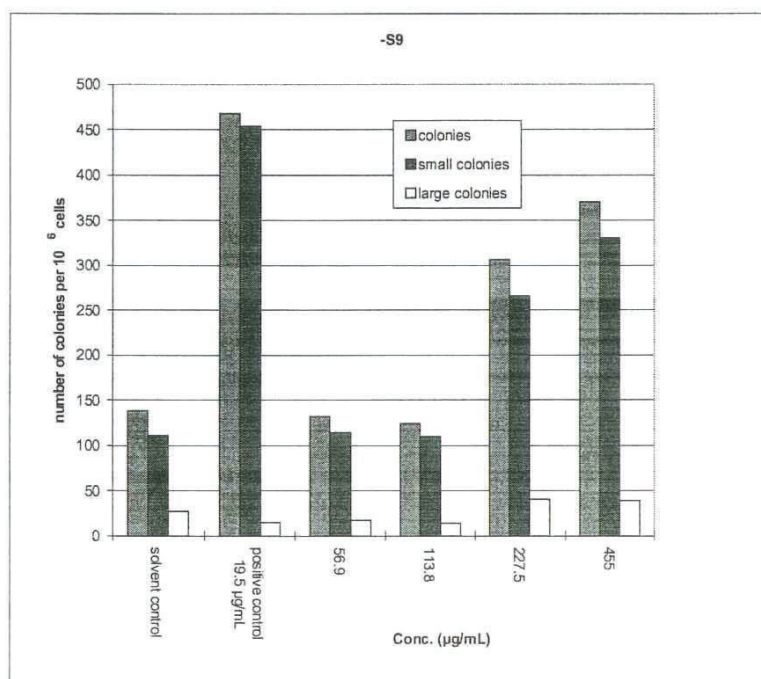


Fig. 1: Summary of experiment I, culture I without metabolic activation

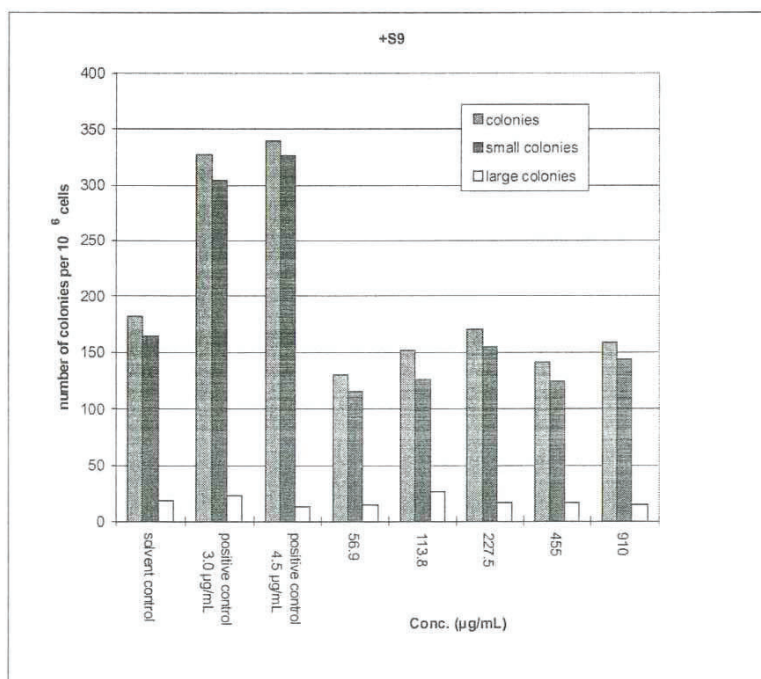


Fig. 2: Summary of experiment I, culture I with metabolic activation

12.2.4 Graphical summary of experiment I / Culture II

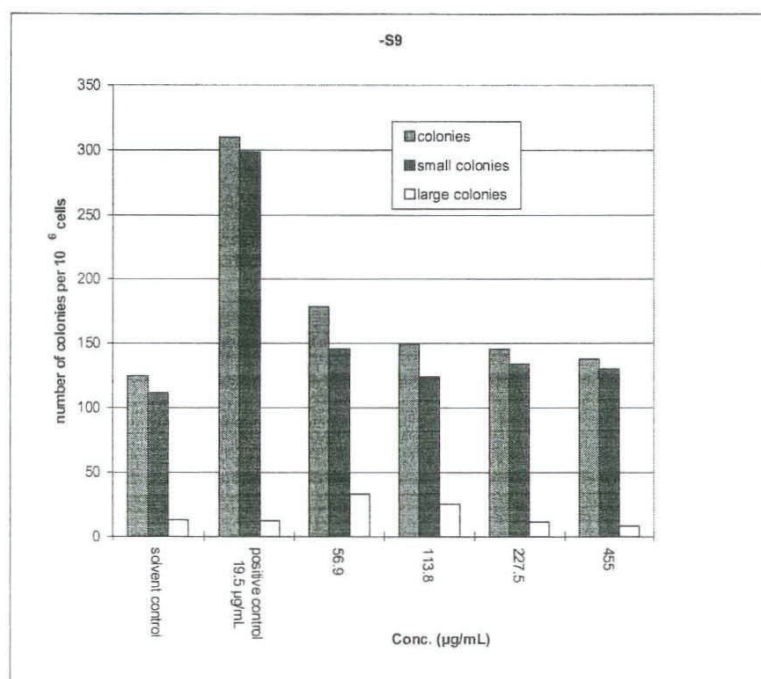


Fig. 3: Summary of experiment I, culture II without metabolic activation

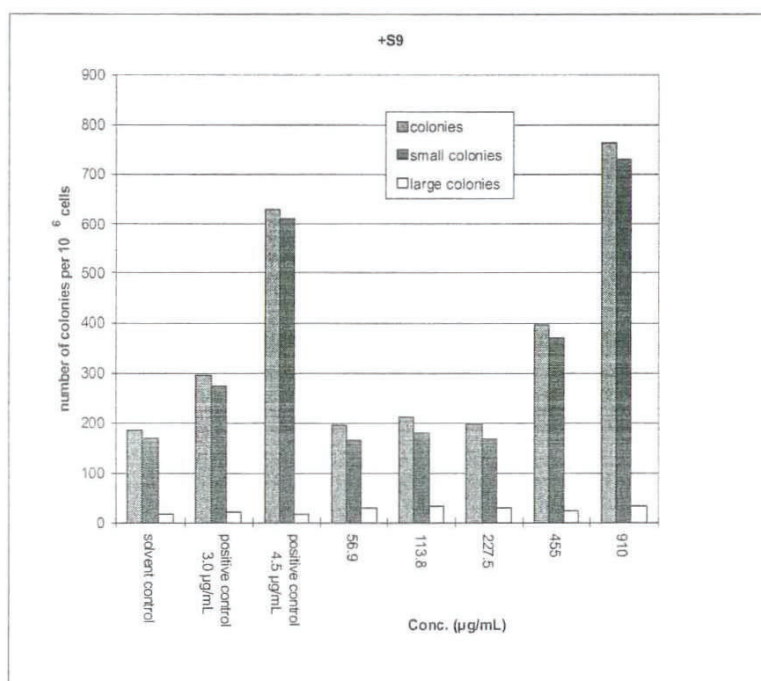


Fig. 4: Summary of experiment I, culture II with metabolic activation

12.2.5 Experiment II, Culture I

Table 10: Toxicity data

			number of cells			total suspension growth	relative suspension growth	relative total growth
			per mL	found 24 h	found 48 h	found 72 h		
	conc. µg per mL	S9 mix						
Column	1	2	7	8	9	10	11	12
Solvent control with water		-	211000	1786000	1212200	72.2	100.0	100.0
Pos. control with MMS	13.0	-	338400	1009200	981000	37.2	51.6	12.2
Test item	28.1	-	315400	1605600	1177800	66.3	91.8	72.7
Test item	56.3	-	316200	1884600	1145000	75.8	105.1	97.9
Test item	112.5	-	254800	1646400	1047000	57.5	79.6	79.6
Test item	225.0	-	151200	632400	1048000	22.1	30.6	23.9
Test item	337.5	-	78800	175200	374000	3.7	5.2	0.7
Test item	450.0	-	30200	49000		culture was not continued [#]		
			found 4 h	found 24 h	found 48 h			
			after treatment					
Solvent control with water		+	535400	861000	1411000	7.6	100.0	100.0
Pos. control with CPA	3.0	+	422000	600800	1536200	7.3	96.4	98.0
Pos. control with CPA	4.5	+	611400	778000	1392200	5.9	78.1	39.3
Test item	56.3	+	529600	848400	1304400	7.0	92.1	109.5
Test item	112.5	+	457400	759200	1468000	8.1	107.4	104.0
Test item	225.0	+	439800	522800	1403000	5.6	73.5	48.5
Test item	450.0	+	226200	122000	473400	2.1	27.7	31.7
Test item	675.0	+	62800	25600	34600	0.6	7.3	#
Test item	900.0	+	27000	28200		culture was not continued [#]		

culture was not continued due to strong toxic effects

Table 11: Mutagenicity data (part 1); experiment II; culture I

	conc. µg per mL	S9 mix	cells seeded	number of plate 1	empty wells plate 2	viability mean	standard deviation	cloning efficiency	relative cloning efficiency	cells seeded in TFT-medium	cells survived	total number of colonies plate 1	total number of colonies plate 2
Column	1	2	3	4	5	6	7	8	9	10	11	12	13
Solvent control with water		-	2.0	13	16	14.5	2.1	0.9	100.0	4616	4363	53	49
Pos. control with MMS	13.0	-	2.0	66	57	61.5	6.4	0.2	23.6	4180	931	41	40
Test item	28.1	-	2.0	24	19	21.5	3.5	0.7	79.2	3956	2960	46	57
Test item	56.3	-	2.0	13	20	16.5	4.9	0.9	93.2	4020	3540	32	51
Test item	112.5	-	2.0	16	13	14.5	2.1	0.9	100.0	4204	3973	38	50
Test item	225.0	-	2.0	21	23	22.0	1.4	0.7	77.9	4052	2985	62	59
Test item	337.5	-	2.0	75	74	74.5	0.7	0.1	13.4	3132	397	30	36
Test item	450.0	-	2.0	culture was not continued [#]									
Solvent control with water		+	2.0	17	21	19.0	2.8	0.8	100.0	4584	3713	54	52
Pos. control with CPA	3.0	+	2.0	17	20	18.5	2.1	0.8	101.6	4596	3784	45	63
Pos. control with CPA	4.5	+	2.0	40	45	42.5	3.5	0.4	50.3	4400	1793	51	54
Test item	56.3	+	2.0	14	14	14.0	0.0	1.0	118.9	4280	4120	41	50
Test item	112.5	+	2.0	18	22	20.0	2.8	0.8	96.8	4000	3137	44	48
Test item	225.0	+	2.0	31	35	33.0	2.8	0.5	65.9	4380	2339	40	55
Test item	450.0	+	2.0	12	18	15.0	4.2	0.9	114.6	3908	3627	65	60
Test item	675.0	+	2.0	culture was not continued [#]									
Test item	900.0	+	2.0	culture was not continued [#]									

culture was not continued due to strong toxic effects

Table 12: Mutagenicity data (part 2): experiment II; culture I

			number of empty wells found after plating in TFT medium*										mean		mutant	small	large
			calculated for plate 1			calculated for plate 2			mean	mean	of all	standard	colonies	colonies	colonies		
	conc. µg	S9	large	small	sum.	large	small	sum.	large	small	empty	deviation	per 10 ⁶ cells	per 10 ⁶ cells	per 10 ⁶ cells		
Column	per mL	mix	colonies	colonies	colonies	colonies	colonies	colonies	colonies	colonies	wells	deviation	10 ⁶ cells	10 ⁶ cells	10 ⁶ cells		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
Solvent control with water		-	68	71	43	71	72	47	70	72	45.0	2.83	142	68	7		
Pos. control with MMS		-	84	67	55	83	69	56	84	68	55.5	0.71	520	371	15		
Test item		-	71	75	50	64	71	39	68	73	44.5	7.78	212	93	11		
Test item		-	72	88	64	63	78	45	68	83	54.5	13.44	141	41	10		
Test item		-	75	79	58	63	79	46	69	79	52.0	8.49	132	49	8		
Test item		-	67	63	34	68	65	37	68	64	35.5	2.12	254	136	11		
Test item		-	80	82	66	75	81	60	78	82	63.0	4.24	952	412	53		
Test item		-	culture was not continued [#]														
Solvent control with water		+	78	60	42	85	55	44	82	58	43.0	1.41	182	138	4		
Pos. control with CPA		+	88	59	51	87	42	33	88	51	42.0	12.73	194	170	2		
Pos. control with CPA		+	88	53	45	87	51	42	88	52	43.5	2.12	394	342	5		
Test item		+	90	61	55	86	56	46	88	59	50.5	6.36	141	120	2		
Test item		+	88	60	52	86	58	48	87	59	50.0	2.83	187	155	3		
Test item		+	92	60	56	89	48	41	91	54	48.5	10.61	271	246	2		
Test item		+	94	33	31	90	42	36	92	38	33.5	3.54	271	259	1		
Test item		+	culture was not continued [#]														
Test item		+	culture was not continued [#]														
Test item		+	culture was not continued [#]														

* 96 wells minus number of wells containing large or small colonies
culture was not continued due to strong toxic effects

12.2.6 Experiment II, Culture II**Table 13: Toxicity data**

			number of cells			total suspension growth	relative suspension growth	relative total growth
			per mL	found 24 h	found 48 h	found 72 h		
	conc. µg per mL	S9 mix		found 24 h	found 48 h	found 72 h		
Column	1	2	7	8	9	10	11	12
Solvent control with water	-	-	320400	1840400	1580000	103.5	100.0	100.0
Pos. control with MMS	13.0	-	298800	1064400	1174600	41.7	40.3	11.3
Test item	28.1	-	344400	1371800	1686000	88.5	85.5	69.6
Test item	56.3	-	363600	1153600	1752000	81.7	78.9	72.7
Test item	112.5	-	329800	1470200	1425000	76.8	74.2	75.4
Test item	225.0	-	150800	580000	1327200	25.7	24.8	22.5
Test item	337.5	-	60600	154400	492800	4.9	4.8	1.1
Test item	450.0	-	30800	94400	culture was not continued [#]			
			found 4 h	found 24 h	found 48 h			
			after treatment					
Solvent control with water	+	+	552000	1089800	1725600	11.4	100.0	100.0
Pos. control with CPA	3.0	+	496600	718800	1826000	8.8	77.6	34.4
Pos. control with CPA	4.5	+	513600	602600	1982000	7.8	68.3	26.1
Test item	56.3	+	513600	1578800	1467400	15.0	132.4	119.8
Test item	112.5	+	514200	936200	1736200	10.5	92.8	105.3
Test item	225.0	+	459000	651200	1634000	7.7	68.0	71.7
Test item	450.0	+	188400	309200	1410200	7.7	67.9	48.2
Test item	675.0	+	80200	28000	64800	0.8	7.1	2.6
Test item	900.0	+	30600	21200	culture was not continued [#]			

[#] culture was not continued due to strong toxic effects

Table 14: Mutagenicity data (part 1): experiment II; culture II

			viability						relative	cells	cells	total	total	
	conc. µg per mL	S9 mix	cells seeded	number of plate 1	empty wells plate 2	standard mean	deviation	cloning efficiency	cloning efficiency	seeded in TFT-medium	survived	number of colonies plate 1	number o colonies plate 2	
Column	1	2	3	4	5	6	7	8	9	10	11	12	13	
Solvent control with water	-	-	2.0	18	17	17.5	0.7	0.9	100.0	4592	3908	56	53	
Pos. control with MMS	13.0	-	2.0	57	62	59.5	3.5	0.2	28.1	4232	1012	46	52	
Test item	28.1	-	2.0	22	26	24	2.8	0.7	81.4	4036	2798	54	52	
Test item	56.3	-	2.0	24	16	20	5.7	0.8	92.2	3988	3128	49	52	
Test item	112.5	-	2.0	14	20	17	4.2	0.9	101.7	4160	3601	50	52	
Test item	225.0	-	2.0	22	19	20.5	2.1	0.8	90.7	4180	3227	48	60	
Test item	337.5	-	2.0	63	67	65	2.8	0.2	22.9	3904	761	34	33	
Test item	450.0	-	2.0	culture was not continued [#]										
Solvent control with water		+	2.0	17	16	16.5	0.7	0.9	100.0	4824	4248	50	60	
Pos. control with CPA	3.0	+	2.0	42	46	44.0	2.8	0.4	44.3	4644	1812	56	53	
Pos. control with CPA	4.5	+	2.0	45	53	49.0	5.7	0.3	38.2	4660	1567	46	38	
Test item	56.3	+	2.0	18	21	19.5	2.1	0.8	90.5	4052	3229	52	62	
Test item	112.5	+	2.0	13	13	13.0	0.0	1.0	113.5	4176	4175	56	60	
Test item	225.0	+	2.0	13	17	15.0	2.8	0.9	105.4	4544	4218	64	55	
Test item	450.0	+	2.0	25	30	27.5	3.5	0.6	71.0	3940	2463	60	53	
Test item	675.0	+	2.0	52	48	50.0	2.8	0.3	37.0	2844	928	28	23	
Test item	900.0	+	2.0	culture was not continued [#]										

culture was not continued due to strong toxic effects

Table 15: Mutagenicity data (part 2): experiment II; culture II

			number of empty wells found after plating in T-F-T medium*										mean of all	standard deviation	mutant colonies per 10 ⁶ cells	small colonies per 10 ⁶ cells	large colonies per 10 ⁶ cells
			calculated for plate 1					calculated for plate 2					mean				
	conc. µg per mL	S9 mix	large colonies	small colonies	sum. colonies	large colonies	small colonies	sum. colonies	large colonies	small colonies	mean	mean	empty wells		10 ⁶ cells	10 ⁶ cells	10 ⁶ cells
Column	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
Solvent control with water	-	-	79	57	40	76	63	43	78	60	41.5	2.12	175	120	50		
Pos. control with MMS	13.0	-	88	58	50	86	54	44	87	56	47.0	4.24	630	532	97		
Test item	28.1	-	78	60	42	81	59	44	80	60	43.0	1.41	238	171	67		
Test item	56.3	-	72	71	47	66	72	42	69	72	44.5	3.54	200	94	100		
Test item	112.5	-	77	65	46	77	65	46	77	65	46.0	0.00	170	108	67		
Test item	225.0	-	81	63	48	81	51	36	81	57	42.0	8.49	214	162	55		
Test item	337.5	-	90	68	62	89	69	62	90	69	62.0	0.00	535	443	92		
Test item	450.0	-	culture was not continued [#]														
Solvent control with water	+		92	50	46	86	40	30	89	45	38.0	11.31	196	178	187		
Pos. control with CPA	+		94	42	40	94	46	44	94	44	42.0	2.83	442	431	121		
Pos. control with CPA	+		90	56	50	94	60	58	92	58	54.0	5.66	349	322	22		
Test item	56.3	+	93	47	44	88	42	34	91	45	39.0	7.07	256	238	181		
Test item	112.5	+	93	43	40	83	49	36	88	46	38.0	2.83	197	176	22		
Test item	225.0	+	84	44	32	90	43	37	87	44	34.5	3.54	211	188	33		
Test item	450.0	+	89	43	36	88	46	38	89	45	37.0	1.41	345	312	22		
Test item	675.0	+	94	70	68	95	68	67	95	69	67.5	0.71	373	356	17		
Test item	900.0	+	culture was not continued [#]														

* 96 wells minus number of wells containing large or small colonies

culture was not continued due to strong toxic effects

12.2.7 Graphical summary of experiment II, Culture I

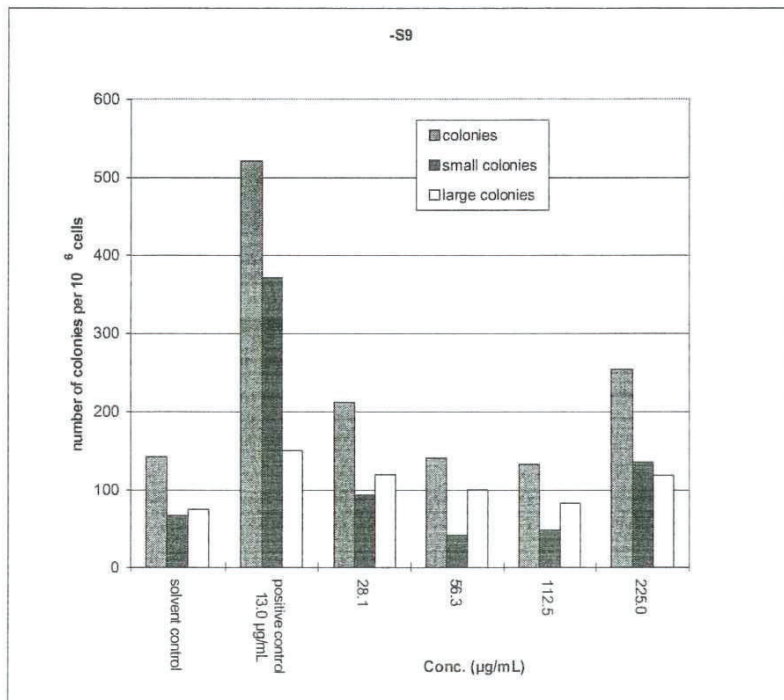


Fig. 5: Summary of experiment II, culture I without metabolic activation

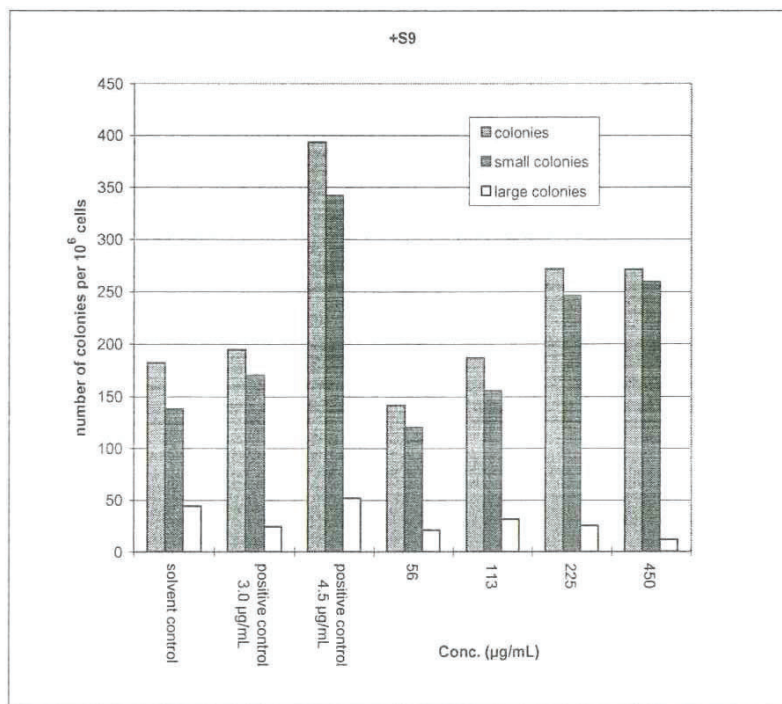


Fig. 6: Summary of experiment II, culture I with metabolic activation

Graphical summary of experiment II, Culture II

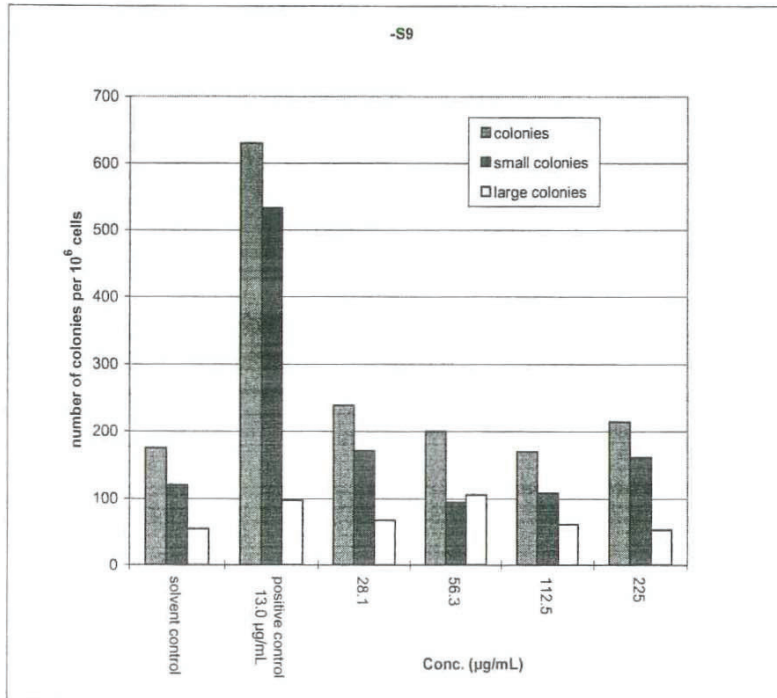


Fig. 7: Summary of experiment II, culture II without metabolic activation

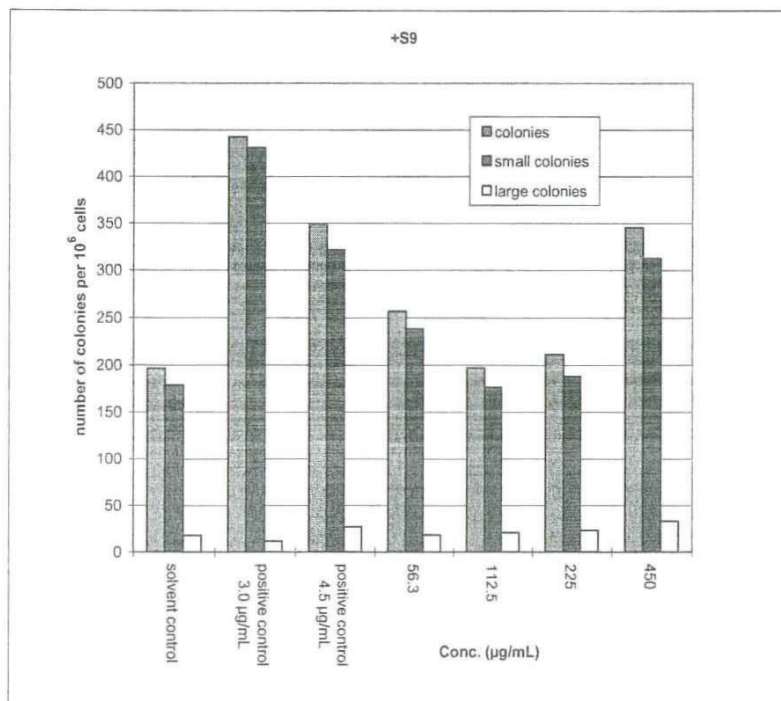


Fig. 8: Summary of experiment II, culture II with metabolic activation

13 ANNEX II: HISTORICAL DATA

These values represent the historical control data from 2006 to 2008

Number of mutant colonies per 10^6 cells		
4 h treatment / without metabolic activation		
	Solvent control (medium, DMSO, water, ethanol, acetone, THF)	Positive control (MMS)
range:	39 – 210	224 – 1582
Mean value:	105	395
Standard deviation:	37	143
Number of studies:	127	127
4 h treatment / with metabolic activation		
	Solvent control (medium, DMSO, water, ethanol, acetone, THF)	Positive control (CPA)
range:	40 – 209	222 – 6886
Mean value:	109	520
Standard deviation:	39	456
Number of studies:	123	123
24 h treatment / without metabolic activation		
	Solvent control (medium, DMSO, water, ethanol, acetone, THF)	Positive control (MMS)
range:	33 – 214	240 - 2062
Mean value:	102	586
Standard deviation:	39	259
Number of studies:	106	106