BIOSERVICE
SCIENTIFIC LABORATORIES GmbH

In vitro Mammalian Cell Gene Mutation Assay

(Thymidine Kinase Locus/TK$^{+/-}$)

in Mouse Lymphoma L5178Y Cells

with

23081

Report

BSL BIOSERVICE Project No.: 010632

Sponsor:

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FÜR ARBEITSSCHUTZ,
ARBEITSMEDIZIN UND SICHERHEITSTECHNIK**

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LfAS

GLP - B E S C H E I N I G U N G

Bescheinigung

Hiermit wird bestätigt, daß die Prüfeinrichtung(en)

BSL Bioservice Scientific Laboratories GmbH
in 82152 Planegg (Ort, Anschrift)
Behringstraße 6
der Firma BSL Bioservice Scientific Laboratories GmbH (Firma)
am 29./30. November 1999 (Datum)

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

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

Certificate

It is hereby certified that the test facility(ies)

BSL Bioservice Scientific Laboratories GmbH
in 82152 Planegg (location, address)
Behringstraße 6
of Firma BSL Bioservice Scientific Laboratories GmbH (company name)
on 29./30. November 1999 (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.

Die Prüfungen von Stoffen und Zubereitungen betreffen folgende OECD-Prüfkategorie

Prüfkategorie 2: Prüfungen auf toxikologische Eigenschaften

Prüfkategorie 3: Prüfungen auf mutagene Eigenschaften (In vitro, in vivo)

Prüfkategorie 9: Sonstige Prüfungen; a) Mikrobiologische Sicherheitsprüfungen
b) Wirksamkeitsprüfungen an Zellkulturen

München, 04.08.2000
I.V.

Ritter
Leitender Gewerbedirektor

L.S.

Contents

Preface

General

Sponsor:	
Monitor:	Dr. M. Bracher
Testing Facility:	BSL BIOSERVICE GmbH Behringstrasse 6 D-82152 Planegg/Munich Germany
BSL BIOSERVICE Project No.:	010632
Test Item:	23081
Title:	*In vitro* Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/$TK^{+/-}$) in Mouse Lymphoma L5178Y Cells with 23081

Project Staff

Study Director:	Dipl.-Biol. Uwe Hamann
Deputy Director of the Testing Facility:	Dr. Angela Lutterbach
Quality Assurance Unit:	Dr. Margarete Hoechst Dipl.-Biol. Maike Führböter

Schedule

Arrival of the Test Item:	April 09, 2001
Date of Draft Project Protocol:	April 23, 2001
Date of Project Protocol:	May 17, 2001
Start of Experiments:	June 19, 2001
End of Experiments:	August 24, 2001
Date of Draft Report:	November 07, 2001
Date of Report	April 08, 2002

Project Staff Signatures

Study Director: Dipl.-Biol. Uwe Hamann

Date: 08.04.2002

Deputy Director of the Testing Facility: Dr. Angela Lutterbach

Date: 08.04.2002

Quality Assurance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated August 01, 1994 (BGBL. I, 1994, S. 1703).

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.

This study was assessed in compliance with the Project Protocol, the Study Plan and the Standard Operation Procedures of BSL BIOSERVICE. The study and/or the testing facility was periodically inspected by the Quality Assurance Unit and the dates and phases of the inspections are included in the Report. These inspections and audits were carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. The Final Report of the study will be audited. A Quality Assurance Statement, signed by the Quality Assurance, is included in the Report.

Guidelines

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

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 476, "In vitro Mammalian Cell Gene Mutation Tests" adopted 21st July, 1997

EEC Directive 2000/32, L 136, Annex 4E, B 17, dated June 08, 2000.

Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to the GLP-Regulations:

A copy of the Final Report, the Project Protocol, the Study Plan and a documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the project.

A sample will be stored according to the period fixed by the GLP-Regulations. Samples that are unstable may be disposed of before that time. Remaining test material will be sent back to the sponsor.

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

Statement of Compliance

BSL BIOSERVICE	
Project-No.:	010632
Test Item:	23081
Study Director:	Dipl.-Biol. Uwe Hamann
Title:	*In vitro* Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/$TK^{+/-}$) in Mouse Lymphoma L5178Y Cells with 23081

This study performed in the testing facility BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated August 01, 1994 (BGBL. I, 1994, S. 1703).

"OECD Principles of Good Laboratory Practice", as revised in 1997, Paris 1998.

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

Study Director: Dipl.-Biol. Uwe Hamann

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Date: 09.04.2002

Quality Assurance

BSL BIOSERVICE
Scientific Laboratories GmbH
Behringstr. 6, D-82152 Planegg

Statement

BSL BIOSERVICE

Project-No.:	010632
Test Item:	23081
Study Director:	Dipl.-Biol. Uwe Hamann
Title:	*In vitro* Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/$TK^{+/-}$) in Mouse Lymphoma L5178Y Cells with 23081

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

Audit	Phases and Dates of QAU Inspections	Dates of Reports to the Study Director and Management
Project Protocol/ Study Plan	June 05, 2001	June 05, 2001
Experimental Phase (Facility Audit)	February 15, 2001	February 18, 2001
Draft Report	December 13, 2001	December 13, 2001
Report	April 09, 2002	April 09, 2002

This Report reflects the raw data.

Quality Assurance

Dr. Margarete Hoechst (or)
Dipl.-Biol. Maike Führböter

Maike Führböter

Date: 09.04.2002

Summary

The test item 23081 was assessed for a possible potential to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

The selection of the concentrations was based on data from the pre-experiment. With metabolic activation 500 µg/ml and without metabolic activation 100 µg/ml were selected as the highest concentration. The test item was investigated at the following concentrations

with metabolic activation:

1.0, 2.5, 5.0, 10, 25, 50, 100 and 500 µg/ml

without metabolic activation:

0.5, 1.0, 2.5, 5.0, 10, 25, 50 and 100 µg/ml

In the experiment with metabolic activation the relative total growth (RTG) was 20.61 % for the highest concentration (500 µg/ml) evaluated. Without metabolic activation the highest evaluated concentration was 100 µg/ml with a RTG of 23.09 %.

With and without metabolic activation the mutation frequencies found in the groups treated with the test item did show a biologically relevant increase of mutants/10^6 cells as compared to the controls. Additionally, colony sizing showed clastogenic effects induced by the test item under the experimental conditions.

Precipitation could be observed without metabolic activation at concentrations starting from 50 µg/ml.

EMS (0.7 mg/ml) and B[a]P (2.5 µg/ml) were used as positive controls and showed distinct and biologically relevant effects in mutation frequency as well as in colony sizing.

Conclusion

In the described mutagenicity test under the experimental conditions reported, the test item 23081 has to be classified as mutagenic in the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

Objective

Mammalian cell culture systems are used to detect mutations induced by chemical substances. This *in vitro* experiment will be carried out to assess the potential of the test item to induce gene mutations by means of a Thymidine Kinase assay using the mouse lymphoma cell line L5178Y. The Thymidine Kinase (TK) system detects base pair mutations, frameshift mutations, small deletions as well as large, non lethal deletions and rearrangements of the relevant chromosomes (1, 6, 7).

TK catalyses the conversion of TFT (Trifluorothymidine) to its cytostatic and cytotoxic trifluoro-thymidine-monophosphate derivative. Cells deficient in the heterozygous TK-locus due to the forward mutation $TK^{+/-} \rightarrow TK^{-/-}$ are resistant to the cytotoxic effects of pyrimidine analogues such as TFT. The defiance of the „salvage" enzyme TK means that these antimetabolites are not incorporated into cellular nucleotides and the nucleotides needed for cellular metabolism are obtained solely from de novo synthesis. However, in the presence of TK TFT is incorporated into the nucleotides, resulting in inhibition of cellular metabolism and cytotoxicity. Thus mutant cells are able to proliferate in the presence of TFT, whereas normal cells which contain TK, are not. Cells as suspension cultures are exposed to the test item for a defined period of time (4 h for a short time exposure and 20 h for a long time exposure). Cytotoxicity is determined by measuring the colony-forming ability and the growth rate of cultures. The treated cultures are maintained 48-72 hours to allow near optimal phenotypic expression of induced mutations.

Mutant frequency is determined by seeding defined numbers of cells in medium containing the selective agent (TFT) to detect mutant cells, and in medium without selective medium to determine the cloning efficiency. After a suitable incubation time all colonies are counted. The number of mutant colonies in selective medium is adjusted by the number of colonies in non-selective medium to derive the mutant frequency (4).

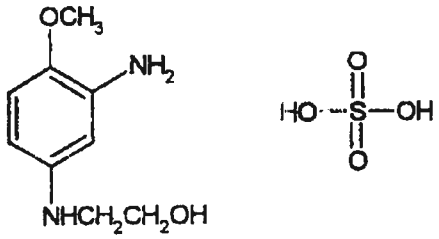
To establish a concentration response of the test item, at least eight concentrations are tested. These concentration levels should yield a concentration related toxic effect. The highest concentration should induce a reduced level of survival of approximately 10-20 % relative survival. The lowest concentration should be in the range of the negative control with respect to cell viability and proliferation. For soluble, non-toxic test compounds the recommended maximum test concentration will be 5 mg/ml, 5 µl/ml or 10 mM, whichever is the lowest. Solvent and negative controls will be tested in duplicate.

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

Materials and Methods

Characterisation of the Test Item

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

Code:	23081
Chemical name:	5-((2-Hydroxyethyl)amino)-2-methoxy-anilin-sulfat (1:1)
Batch No.:	101
Sample-No.:	R00056178
Aggregate State at RT:	solid, powder
Colour:	grey-blue
Structural Formula:	
Molecular Formula:	$C_9H_{14}N_2O_2.H_2O_4S$
Molecular Weight:	280.30
Certificate of Analysis:	12.07.2000
Stability:	warranted for 3 years
Storage:	room temperature
Safety precautions	Routine hygienic procedures were sufficient to assure personnel health and safety.

The test item was dissolved in cell culture medium (RPMI + 3 % HS) and diluted prior to treatment. The solvent was compatible with the survival of the cells and the S9 activity.

Controls

Controls

Positive and negative (solvent) controls were included in every experiment.

Positive Control Substances

Without metabolic activation

Name	EMS; Ethylmethanesulfonate
Supplier	SIGMA, D-82024 Taufkirchen
Catalogue No.	M 0880
Batch No.	99H0662
Dissolved in	Medium
Final concentration	0.7 mg/ml

With metabolic activation

Name	B[a]P, Benzo[a]pyrene
Supplier	SIGMA, D-82024 Taufkirchen
Catalogue No.	B 1760
Batch No.	059H3441
Dissolved in	DMSO, Dimethylsulfoxide; final concentration in nutrient medium 1%
Final concentration	2.5 µg/ml

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

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

Test System

Cells

Mouse Lymphoma L5178Y cells have been used successfully in *in vitro* experiments for many years. These cells are characterised by their high proliferation rate (10-12 h doubling time of the BSL BIOSERVICE stock cultures) and their cloning efficiency, usually more than 50 %. The cells maintain a near diploid karyotype (40 ± 2 chromosomes). They are constructed heterozygous at the Thymidine Kinase (TK) locus in order to detect mutation events at the TK-locus.

To prevent high backgrounds arising from spontaneous mutations, cells lacking TK can be killed by culturing in RPMI 1640 medium supplemented with:

9.0	µg/ml	Thymidine
15.0	µg/ml	Hypoxanthine
22.5	µg/ml	Glycine
0.1	µg/ml	Methotrexate

The cells are resuspended in medium containing thymidine, hypoxanthine and glycine but without methotrexate for 1-3 days.

Large stock cultures of the cleansed L5178Y cell line (supplied by ATCC, USA) are stored at <-130°C (vapour phase liquid nitrogen) in the cell bank of BSL BIOSERVICE which allows the repeated use of the same cell batch in experiments. Every cell batch is routinely checked for mycoplasma contamination.

Thawed stock cultures are maintained in plastic culture flasks (Greiner, D-72632 Frickenhausen) in RPMI 1640 complete medium and subcultured three times a week.

Mammalian Microsomal Fraction S9 Mix

One advantage of using *in vitro* cell cultures is the accurate control of the concentration and exposure time of cells to the test item under study. However, due to the limited capacity of cells growing *in vitro* for metabolic activation of potential mutagens an exogenous metabolic activation system is necessary (2). Many substances only develop mutagenic potential when they are metabolized by the mammalian organism. Metabolic activation of substances can be achieved by supplementing the cell cultures with liver microsome preparations (S9 mix).

The S9 liver microsomal fraction was prepared at BSL BIOSERVICE GmbH. Male Wistar rats were induced with Phenobarbital (80 mg/kg b.w.) and ß-Naphtoflavone (100 mg/kg b.w.).

The following quality control determinations were performed by the supplier:

a) Biological activity in the Salmonella assay
b) Sterility Test

A stock of the supernatant containing the microsomes was frozen in ampoules of 2 and 5 ml and stored at <-130°C (vapour phase liquid nitrogen).

The protein concentration in the S9 preparation (Lot: 310501) was 28.0 mg/ml. The S9 mix preparation was performed according to Ames et al. (2).

S9 Mix

An appropriate quantity of the S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/ml in the cultures. Cofactors were added to the S9 mix to reach the concentrations below:

8	mM	$MgCl_2$
33	mM	KCl
5	mM	Glucose-6-phosphate
4	mM	NADP

in 100 mM sodium-phosphate-buffer pH 7.4. During the experiment the S9 mix was stored on ice.

Pre-test for Toxicity

The toxicity of the test item was determined in a pre-experiment. Five concentrations [30, 100, 300, 1000 and 3000 µg/ml] were tested without metabolic activation. The experimental conditions in this pre-experiment were the same as described below for the main experiment.

Exposure Concentrations

The selection of the concentrations was based on data from the pre-experiment. With metabolic activation 500 µg/ml and without metabolic activation 100 µg/ml were selected as the highest concentration. The test item was investigated at the following concentrations

with metabolic activation:

1.0, 2.5, 5.0, 10, 25, 50, 100 and 500 µg/ml

without metabolic activation:

0.5, 1.0, 2.5, 5.0, 10, 25, 50 and 100 µg/ml

Precipitation could be observed without metabolic activation at concentrations starting from 50 µg/ml.

According to OECD Guidelines at least 8 concentrations of the test item were set up in the experiments with and without metabolic activation. The concentration range covered more than one logarithmic decade.

Experimental Performance

$1x10^6$ cells/ml (80 cm^2 flasks) suspended in 10 ml RPMI medium with 3 % horse serum were exposed to designated concentrations of the test item either in the presence or absence of metabolic activation in the mutation experiment. After 4 hours the test item was removed by centrifugation (200 x g, 10 min) and the cells were washed twice with PBS. Subsequently the cells were suspended in 30 ml complete culture medium and incubated for an expression and growth period of totally 72 hours at 37 °C in 5 % CO_2/95 % humidified air. The cell density was determined each day and adjusted to $3x10^5$ cells/ml, if necessary.

After the expression period the relative cloning efficiency (RCE; percentage cloning efficiency of the test group in relation to the solvent control) of the cells was determined by seeding a statistical number of 1.6 cells/well in two 96-well-plates (GREINER, D-72632 Frickenhausen). The cells were incubated for 7 days at 37 °C in a humidified atmosphere with 5% CO_2. Analysis of the results is based on the number of cultures with cell growth (positive cultures) and/or those without cell growth (negative cultures) compared to the total number of cultures seeded. Relative suspension and total growth (RSG and RTG; RTG= [RSG x RCE]/100) of the treated cell cultures were calculated according to the method of Clive and Spector (3). Additionally, cultures were seeded in selective medium. Cells from each experimental group were seeded in four 96-well-plates at a density of approximately 2000 cells/well in 200 µl selective medium (see below) with TFT (SERVA, D-69115 Heidelberg). The plates were scored after an incubation period of 11 to 14 days at 37 °C in 5 % CO_2/95 % humidified air. The mutation frequencies then were calculated from the data obtained from cultures used for the cloning efficiency (cultures with non selective medium) and those used for selection (cultures with selective medium) in the following manner:

$$\text{Mutation frequency} = \frac{\text{-ln [NC / TC (selective medium)]}}{\text{-ln [NC / TC (non selective medium)]}} \times 800$$

NC: number of negative cultures

TC: total number of cultures seeded

Complete Culture Medium

RPMI 1640 medium supplemented with 15 % horse serum (HS), 100 U/100 µg/ml Penicillin/Streptomycin and 220 µg/ml sodium pyruvate.

Treatment Medium

RPMI 1640 medium supplemented with 3 % horse serum (HS) without antibiotics and 220 µg/ml sodium pyruvate

Selective Medium

RPMI 1640 complete culture medium supplemented with TFT (3 µg/ml)

Phosphate buffered saline (PBS)

The PBS solution is composed as follows (per litre):

8000	mg	NaCl
200	mg	KCl
1150	mg	Na_2HPO_4
200	mg	KH_2PO_4

Data Recording

The data generated are recorded in the raw data. The results are presented in tables, including experimental groups with the test item, negative and positive controls. Individual colony counts for the treated and control groups are presented for both mutation induction and survival. Small (induced predominantly by chromosomal rearrangements) and large (induced predominantly by point mutations) colonies were determined.

Acceptability of the Assay

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

- At least three out of four 96-well-plates from the TFT resistance-testing portion of the experiment are scorable.
- The absolute cloning efficiency:
  ([number of positive cultures x 100] / total number of seeded cultures)
  of the negative and/or solvent controls is > 50 %.
- The spontaneous mutant frequency in the negative and/or solvent controls is in the range of BSL BIOSERVICE historical control data: about 20-150 mutants per 10^6 cells
- The positive controls (EMS and B[a]P) induce significant increases (at least 2-fold increase of mutant frequencies related to the comparable negative control values and higher than the historical range of negative controls) in the mutant frequencies.

Evaluation of Results

There are several criteria for determining a positive result:

- clear and dose-related increase in the mutant frequency,
- biologically relevant response (at least 2-fold increase of mutant frequencies related to the comparable negative control values and higher than the historical range of negative controls) for at least one of the dose groups.
- An increased occurrence of small colonies (slow growth colonies) indicated by a low large / small colonies ratio (< 4), is associated with clastogenic effects and / or chromosomal aberrations.

According to the OECD guidelines, the biological relevance is one criterion for the interpretation of results, a statistical evaluation of the results is not regarded as necessary.

A test item is considered to be negative if there is no biological relevant increase in the induction of mutant cells above concurrent control levels, at any dose level.

Deviations to Project Protocol

Concerning: Positive Control Substances

Before:

Without metabolic activation

Name	EMS; Ethylmethanesulfonate
Dissolved in	A. dest.
Final concentration	800 µg/ml

With metabolic activation

Name	BP, Benzo[a]pyrene
Final concentration	2.0 or 3.0 µg/ml

New:

Without metabolic activation

Name	EMS; Ethylmethanesulfonate
Dissolved in	Medium
Final concentration	0.7 mg/ml

With metabolic activation

Name	B[a]P, Benzo[a]pyrene
Final concentration	2.5 µg/ml

Reason for alteration

Updating the experimental conditions.

Concerning: Characterisation of the Test Item

Before:

The solvent/vehicle will be compatible with the survival of the bacteria and the S9 activity.

New:

The solvent was compatible with the survival of the cells and the S9 activity.

Reason for alteration:

Typing error

Concerning: Test System

Before:

Supplier of the cells D. Clive, Wellcome Research Laboratories, Research Triangle Park, NC.

New:

Supplier of the cells ATCC, USA.

Reason for alteration:

Change to build up a new cell stock.

Concerning: Acceptability of the Assay

Before:

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

- The spontaneous mutant frequency in the negative and/or solvent controls is in the range of BSL BIOSERVICE historical control data: 20-150 mutants per 10^6 cells
- The positive controls (EMS and B[a]P) induce significant increases (at least 2-fold; > historical range of the negative controls) in the mutant frequencies.

New:

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

- The spontaneous mutant frequency in the negative and/or solvent controls is in the range of BSL BIOSERVICE historical control data: about 20-150 mutants per 10^6 cells
- The positive controls (EMS and B[a]P) induce significant increases (at least 2-fold increase of mutant frequencies related to the comparable negative control values and higher than the historical range of negative controls) in the mutant frequencies.

Concerning: Evaluation of Results

Before:

There are several criteria for determining a positive result:

- biologically relevant response (at least 2-fold increase, mutant frequency > historical range of the negative controls) for at least one of the test points.

New:

There are several criteria for determining a positive result:

- biologically relevant response (at least 2-fold increase of mutant frequencies related to the comparable negative control values and higher than the historical range of negative controls) for at least one of the dose groups.
- An increased occurrence of small colonies (slow growth colonies) indicated by a low large / small colonies ratio (< 4), is associated with clastogenic effects and / or chromosomal aberrations.

Reason for alteration

More detailed description of the acceptability and evaluation criteria.

These deviations did not affect the quality and integrity of the study.

Results

Pre-Experiment, without metabolic activation

Table 1: Pre-test for toxicity, without metabolic activation

Test Group	Concen-tration [µg/ml]	Number of Cells Seeded	Number of Cells 4 h after Treatment	Number of Cells 24 h after Treatment	Number of Cells 48 h after Treatment	Relative Suspension Growth (RSG) [%]
S-	**0**	300000	334000	856000	1140000	100.00
1-	**30**	300000	274000	664000	662000	54.91
2-	**100**	300000	215000	317000	359000	18.12
3-	**300**	300000	90500	23300	23400	2.65
4-	**1000**	300000	85800	18600	15000	1.80
5-	**3000**	300000	238000	64300	50200	2.17

S = solvent control
RSG = [(value TSG / value TSG of corresponding controls) x 100]

Main Experiment, with metabolic activation

Table 2: Toxicity Data, with metabolic activation

Test Group	Concentration [µg/ml]	Number of Cells Seeded	Number of Cells 4 h after Treatment	Number of Cells 24 h after Treatment	Number of Cells 48 h after Treatment	Total Suspension Growth (TSG)[a]	Relative Suspension Growth (RSG)[b] [%]	Relative Cloning Efficiency (RCE)[c] [%]	Relative Total Growth (RTG)[d] [%]
S1+	0	300000	350000	1130000	1250000	13.45	100.00	100.00	100.00
S2+		300000	355000	1070000	1400000	14.07			
3+	1.0	300000	349000	1170000	1320000	14.75	107.21	96.25	103.19
4+	2.5	300000	353000	1050000	1390000	13.78	100.17	98.75	98.91
5+	5.0	300000	358000	1180000	1360000	14.94	108.60	96.25	104.53
6+	10	300000	367000	1150000	1320000	13.79	100.21	98.75	98.95
7+	25	300000	334000	1000000	1290000	12.87	93.57	106.25	99.42
8+	50	300000	332000	933000	1280000	11.99	87.15	91.25	79.52
9+	100	300000	276000	630000	1380000	10.50	76.31	90.00	68.68
10+	500	300000	239000	250000	753000	3.15	22.90	90.00	20.61
B[a]P	2.5	300000	342000	577000	1350000	7.59	55.18	90.00	49.66

S: solvent control
a: TSG = [(value 24 h x value 48 h) / (value 4 h x value 24 h*)]; * : for value 24 h > $3x10^5$ then value 24 h = $3x10^5$
b: RSG = [(value TSG / value TSG of corresponding controls) x 100]
c: RCE = [(mean value positive cultures / mean value positive cultures of corresponding controls) x 100]
d: RTG = (RSG x RCE)/100

Table 3: Mutagenicity Data, with metabolic activation

Test Group	Concentration [µg/ml]	Cloning Efficiency (CE) Plate 1[e]	Plate 2[e]	RCE (%)[f]	Mutagenicity Data Number of cultures / 96 wells Plate 1[e]	Plate 2[e]	Plate 3[e]	Plate 4[e]	Mean	Mutants/ 10^6 cells[g]	Mutation factor
S1+	0	81	82	100.00	27	30	27	25	27.25	141.31	
S2+		83	74		23	26	19	23	22.75	127.12	
3+	1.0	77	77	96.25	20	28	22	24	23.50	138.66	1.03
4+	2.5	79	79	98.75	25	30	25	30	27.50	155.97	1.16
5+	5.0	77	77	96.25	22	22	29	25	24.50	145.51	1.08
6+	10	78	80	98.75	21	29	17	29	24.00	132.94	0.99
7+	25	83	87	106.25	25	29	24	24	25.50	114.01	0.85
8+	50	73	73	91.25	25	22	23	31	25.25	170.88	1.27
9+	100	68	76	90.00	31	42	25	30	32.00	233.99	1.74
10+	500	73	71	90.00	31	41	45	43	40.00	311.04	2.32
B[a]P	2.5	71	73	90.00	50	47	52	52	50.25	427.71	3.19

S: solvent control
e: Number of cultures with cell growth.
f: RCE = [(mean value positive cultures / mean value positive cultures of corresponding controls) x 100]
g: Mutation Frequency = {-ln [negative cultures/total wells (selective medium)] / -ln [negative cultures/total wells (non selective medium)]} x800

Table 4: Colony Sizing, with metabolic activation

Test Group	Concentration [µg/ml]	Wells with at least 1 colony	Large colonies	Small colonies	Quotient Large/ Small
S1+	0	109	80	29	2.8
S2+		91	69	22	3.1
8+	50	101	68	33	2.1
9+	100	128	75	53	1.4
10+	500	160	92	68	1.4
B[a]P	2.5	201	124	77	1.6

S: solvent control

Main Experiment, without metabolic activation

Table 5: Toxicity Data, without metabolic activation

Test Group	Concentration [µg/ml]	Number of Cells Seeded	Number of Cells 4 h after Treatment	Number of Cells 24 h after Treatment	Number of Cells 48 h after Treatment	Total Suspension Growth (TSG)[a]	Relative Suspension Growth (RSG)[b] [%]	Relative Cloning Efficiency (RCE)[c] [%]	Relative Total Growth (RTG)[d] [%]
S1-	0	300000	308000	804000	1410000	12.27	100.00	100.00	100.00
S2-		300000	269000	720000	1160000	10.35			
3-	0.5	300000	306000	943000	1250000	12.84	113.54	112.76	128.03
4-	1.0	300000	301000	970000	1230000	13.21	116.83	94.36	110.24
5-	2.5	300000	300000	887000	1240000	12.22	108.06	107.42	116.08
6-	5.0	300000	295000	951000	1240000	13.32	117.82	100.30	118.17
7-	10	300000	287000	900000	1200000	12.54	110.92	96.14	106.64
8-	25	300000	276000	884000	1200000	12.81	113.29	84.87	96.14
9-	50 (P)*	300000	264000	771000	1040000	10.12	89.52	81.90	73.32
10-	100 (P)*	300000	255000	557000	728000	5.30	46.87	49.26	23.09
EMS	700	300000	249000	457000	810000	4.96	43.82	26.71	11.70

S: solvent control
a: TSG = [(value 24 h x value 48 h) / (value 4 h x value 24 h*)]; * : for value 24 h > $3x10^5$ then value 24 h = $3x10^5$
b: RSG = [(value TSG / value TSG of corresponding controls) x 100]
c: RCE = [(mean value positive cultures / mean value positive cultures of corresponding controls) x 100]
d: RTG = (RSG x RCE)/100
(P)*: visible precipitation of test item in cell pellet after centrifugation

Table 6: Mutagenicity Data, without metabolic activation

Test Group	Concentration [µg/ml]	Cloning Efficiency (CE) Plate 1[e]	Plate 2[e]	RCE (%)[f]	Mutagenicity Data Number of cultures / 96 wells Plate 1[e]	Plate 2[e]	Plate 3[e]	Plate 4[e]	Mean	Mutants/ 10^6 cells[g]	Mutation factor
S1-	0	72	79	100.00	19	24	27	18	22.00	134.87	
S2-		94	92		14	22	28	27	22.75	62.43	
3-	0.5	94	96	112.76	20	17	23	19	19.75	40.37	0.41
4-	1.0	77	82	94.36	25	14	15	14	17.00	88.54	0.90
5-	2.5	91	90	107.42	20	18	10	20	17.00	54.53	0.55
6-	5.0	88	81	100.30	19	18	20	19	19.00	83.15	0.84
7-	10	80	82	96.14	15	23	23	21	20.50	103.52	1.05
8-	25	67	76	84.87	18	12	21	15	16.50	110.47	1.12
9-	50 (P)*	70	68	81.90	9	12	15	15	12.75	89.87	0.91
10-	100 (P)*	39	44	49.26	18	14	10	11	13.25	209.87	2.13
EMS	700	24	21	26.71	71	72	67	66	69.00	3799.89	38.52

S: solvent control
e: Number of cultures with cell growth.
f: RCE = [(mean value positive cultures / mean value positive cultures of corresponding controls) x 100]
g: Mutation Frequency = {-ln [negative cultures / total wells (selective medium)] / -ln [negative cultures / total wells (non selective medium)]}x800
(P)*: visible precipitation of test item in cell pellet after centrifugation

Table 7: Colony Sizing, without metabolic activation

Test Group	Concentration [µg/ml]	Wells with at least 1 colony	Large colonies	Small colonies	Quotient Large/ Small
S1-	0	88	78	10	7.8
S2-		91	82	9	9.1
8-	25	66	51	15	3.4
9-	50 (P)*	51	41	10	4.1
10-	100 (P)*	53	43	10	4.3
EMS	700	276	212	64	3.3

S: solvent control
(P)*: visible precipitation of test item in cell pellet after centrifugation

Discussion

The test item 23081 was assessed for a possible potential to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

The selection of the concentrations was based on data from the pre-experiment.

With metabolic activation 500 µg/ml and without metabolic activation 100 µg/ml were selected as the highest concentration. The test item was investigated at the following concentrations

with metabolic activation:

1.0, 2.5, 5.0, 10, 25, 50, 100 and 500 µg/ml

without metabolic activation:

0.5, 1.0, 2.5, 5.0, 10, 25, 50 and 100 µg/ml

Toxicity:

With and without metabolic activation growth inhibition could be observed at the highest concentrations.

In the experiments with metabolic activation the relative total growth (RTG) was 20.61 % for the highest concentration (500 µg/ml) evaluated. Without metabolic activation the highest evaluated concentration was 100 µg/ml with a RTG of 23.09 %.

Precipitation could be observed without metabolic activation at concentrations starting from 50 µg/ml.

Mutagenicity:

With metabolic activation all mutant values of the negative control were found within the historical control data of the testing facility BSL BIOSERVICE (about 20 - 150 mutants per 10^6 cells, Table 8). Mutation frequencies with the negative controls were found to be 127.12 - 141.31 mutants/10^6 cells and 114.01 - 311.04 mutants/10^6 cells with the test item, respectively. The highest mutation factor (compared to the negative control values) of 2.32 was found at a concentration of 500 µg/ml with a RTG of 20.61 %. A dose dependent increase of mutants/10^6 cells could be observed at the higher concentrations (50 µg/ml – 500 µg/ml). Therefore, the groups treated with the test item did show a biologically relevant increase as compared to the controls.

Without metabolic activation all mutant values of the negative control were found within the historical control data of the testing facility BSL BIOSERVICE (about 20 - 150 mutants per 10^6 cells, Table 8). Mutation frequencies of the negative controls were found to be 62.43 - 134.87 mutants/10^6 cells and 40.37 - 209.87 mutants/10^6 cells with the test item, respectively. The highest mutation factor (compared to the negative control values) of 2.13 was found at a concentration of 100 µg/ml with a RTG of 23.09 %. The value of mutants/10^6 cells at this concentration is above the

range of the historical laboratory control data of BSL BIOSERVICE and is considered to be biologically relevant.

Relationship of large to small colonies:

Colony sizing was performed for the highest concentrations of the test item and for the negative and positive controls. An increased occurrence of small colonies (defined by slow growth and/or morphological alteration of the cell clone), indicated by a low large / small colonies ratio (< 4), is known to be associated with clastogenic effects and/or chromosomal aberrations.

With metabolic activation the quotient of large/small colonies of the negative controls were found to be 2.8 and 3.1. Compared to the historical data of BSL BIOSERVICE (ratio large/small colonies >4), the ratio of large/small colonies evaluated in the negative control groups were below the value of 4. The assay meets the a. m. acceptability criteria (page 17) and therefore this result is considered not to be biologically relevant. The quotient at the highest dose groups were found to be 2.1 (50 µg/ml), 1.4 (100 µg/ml) and 1.4 (500 µg/ml). The ratio of large/small colonies at the higher concentrations is also below the value of 4 and additionally below the values of the negative and even of the positive control. This result may indicate mutagenic or clastogenic effects of the test item at the higher concentrations.

Without metabolic activation the quotient of large/small colonies of the negative controls were found to be 7.8 and 9.1. The quotient of the highest dose groups were found to be 3.4 (25 µg/ml), 4.1 (50 µg/ml) and 4.3 (100 µg/ml). Therefore, at a concentration of 25 µg/ml the quotient of large/small colonies was below the value of 4. This may reflect mutagenic or clastogenic effects of the test item.

EMS (0.7 mg/ml) and B[a]P (2.5 µg/ml) were used as positive controls and showed distinct and biologically relevant effects in mutation frequency as well as in colony sizing.

Conclusion

In conclusion, in the described mutagenicity test under the experimental conditions reported, the test item 23081 has to be classified as mutagenic in the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

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Annex

Table 8: Historical Laboratory Control Data (in 2001)

	NC		PC	
	-S9	**+S9**	**-S9**	**+S9**
mean	79.79	98.83	2180.22	348.59
min.	30.86	43.08	833.25	173.23
max.	136.20	145.46	3799.89	630.81
SD	34.98	31.84	1048.16	123.90
RSD [%]	43.78	32.21	48.08	35.54
n =	24	26	12	13

NC: Negative Control
PC: Positive Control
S9: metabolic activation
mean: mean of mutants/10^6 cells
min.: minimum of mutants/10^6 cells
max.: maximum of mutants/10^6 cells
SD: Standard Deviation
RSD: relativ Standard Deviation
n: Number of control values