citoxlab
Safety and Health Research

FINAL REPORT

C6OLF:
In Vitro Mammalian Cell Gene Mutation Test
(HPRT Assay)

Study code: 17/019-015C

Study Director:

25 January 2018

STATEMENT OF THE STUDY DIRECTOR

This study has been performed in accordance with the study plan, the OECD Guidelines for Testing of Chemicals No. 476 (2016), Commission Regulation (EC) No. 440/2008 of 30 May 2008, B.17., and the Principles of Good Laboratory Practice as specified by national Hungarian GLP Regulations of 42/2014. (VIII. 19.) EMMI decree of the Ministry of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM (98)17.

I, the undersigned, declare that this report constitutes a true record of the actions undertaken and the results obtained in the course of this study. By virtue of my dated signature I accept the responsibility for the validity of the data and the conclusion drawn from them.

No chemical analysis of the dose formulation was performed as part of this study. Traceability (equipment used, quantities of test item weighed) of dosing form preparations was checked and revealed no abnormalities of consequences. Furthermore, for this study, the formulations were prepared just before the treatment. Consequently, the absence of dose formulation analysis data is considered not to prejudice the overall GLP status of the study and the scientific reliability of the study conclusions.

Signature: _ Date: 25 january 2018

Study Director

STATEMENT OF THE MANAGEMENT

According to the conditions of the research and development assignment between Daikin industries ltd. (as Sponsors) and Citoxlab Hungary Ltd. (as Test Facility), the study titled "C6OLF: *In vitro* Mammalian Cell Gene Mutation Test (HPRT Assay)" has been performed according to the GLP requirements.

Signature: _ Date: 25 January 2018

Director of Science and Regulatory Affairs

QUALITY ASSURANCE STATEMENT

Study Code: 17/019-015C

Study Title: C6OLF: *In vitro* Mammalian Cell Gene Mutation Test (HPRT Assay)

Test Item: C6OLF

In order to enable the results of this study to meet legal requirements including regulatory submission and/or safety assessment, this study has been inspected and this report audited by the Quality Assurance Unit in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established the methods described and the results incorporated in this report accurately reflect the raw data produced during this study.

All inspections, data reviews and the report audit were reported in written form to the study director and to management. The dates of such inspections and of the report audit are given below:

Date of Inspection	Phase (s) Inspected/Audited	Date of report to	
		Management	Study Director
31 August 2017	Study Plan	31 August 2017	31 August 2017
19 October 2017	Amendment 1 to the Study Plan	19 October 2017	19 October 2017
24 October 2017	Formulation	24 October 2017	24 October 2017
10 January 2018	Draft Report	10 January 2018	10 January 2018
25 January 2018	Final Report	25 January 2018	25 January 2018

Signature: __ Date: 25 January 2018

On behalf of QA

GENERAL INFORMATION

STUDY TITLE:	C6OLF: *In vitro* Mammalian Cell Gene Mutation Test (HPRT Assay)
TEST ITEM:	C6OLF
SPONSOR:	Daikin industries ltd. Address: 1-1, Nishi-Hitotsuya, Settsu, Osaka 566-8585 Japan
TEST FACILITY:	Citoxlab Hungary Ltd. Address: H-8200 Veszprém, Szabadságpuszta, Hungary Phone: +36 88 545 300 Fax: +36 88 545 301
TEST FACILITY MANAGEMENT:	., Managing Director ., Scientific Director ., Director of Science and Regulatory Affairs

STUDY DIRECTOR: , M.Sc.
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QUALITY ASSURANCE: , M.Sc., Senior Director of QA
, B.Sc., Senior QA Inspector

RESPONSIBLE PERSONS*: , M.Sc., Assistant Scientist
, Technical team leader
, Ph.D., Head of Pharmacy
, statistical analysis

*Other trained, competent personnel for general work were also involved in the study as documented in the raw data.

BASIS OF STUDY: OECD Guidelines for Testing of Chemicals, No.476 (2016)
Commission Regulation (EC) No. 440/2008, B.17. (2008)

STUDY SCHEDULE**:

STUDY PLAN:	01 September 2017
AMENDMENT 1 TO THE STUDY PLAN:	20 October 2017
PRELIMINARY EXPERIMENT:	05-11 September 2017
START OF EXPERIMENT:	24 October 2017
END OF EXPERIMENT:	01 December 2017
DRAFT REPORT:	10 January 2018
FINAL REPORT:	25 January 2018

**Detailed study schedule is shown in Appendix 1.

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1. SUMMARY

An *in vitro* mammalian cell assay [1-2] was performed in CHO K1 Chinese hamster ovary cells at the *hprt* locus to evaluate the potential of C6OLF to cause gene mutation. Treatments were carried out for 5 hours with and without metabolic activation (±S9-mix) and for 24 hours without metabolic activation (-S9-mix).

Acetone was used as the vehicle (solvent) of the test item in this study. Treatment concentrations for the mutation assays of the main tests were selected based on the results of a preliminary toxicity test as follows:

Assay 1

5-hour treatment in the presence of S9-mix:

2000, 1000, 500, 250, 125, 62.5 and 31.25 µg/mL

5-hour treatment in the absence of S9-mix:

2000, 1000, 500, 250, 125, 62.5 and 31.25 µg/mL

Assay 2

5-hour treatment in the presence of S9-mix:
2000, 1000, 500, 250, 125, 62.5 and 31.25 µg/mL

24-hour treatment in the absence of S9-mix:
2000, 1000, 500, 250, 125, 62.5 and 31.25 µg/mL

In the main assays, a measurement of the survival (colony-forming ability at the end of the treatment period) and viability (colony-forming ability at the end of the 7 day expression period following the treatment) and mutagenicity (colony forming ability at the end of the 7 day expression period following the treatment, in the presence of 6-thioguanine as a selective agent) was determined.

In Assay 1 and 2, insolubility (precipitate / minimal amount of precipitate) was detected at higher concentrations in the final treatment medium at the end of the treatment in the experiments with and without metabolic activation. Discoloured medium caused by the presence of the test item was observed in Assay 1 and 2 with and without metabolic activation at several concentrations. There were no large changes in pH and osmolality after treatment in any cases.

In Assay 1 and 2, in the presence of S9-mix (5-hour treatment), no marked cytotoxicity of the test item was observed. An evaluation was made using data of all seven concentrations. No statistically significant increases in the mutation frequency were observed at any examined concentrations when compared to the negative (vehicle) control data and there was no dose response to the treatment (a trend analysis showed no effect of treatment).

In Assay 1, in the absence of S9-mix (5-hour treatment), no marked cytotoxicity of the test item was observed. An evaluation was made using data of all seven concentrations. No statistically significant increases in the mutation frequency were observed at any examined concentrations when compared to the negative (vehicle) control data and there was no dose response to the treatment (a trend analysis showed no effect of treatment)

In Assay 2, in the absence of S9-mix (24-hour treatment), no marked cytotoxicity of the test item was observed, thus an evaluation was made using data of all seven concentrations. No statistically significant increases in the mutation frequency were observed at any examined concentrations when compared to the negative (vehicle) control data and there was no dose response to the treatment (a trend analysis showed no effect of treatment). This experiment confirmed the negative results seen in Assay 1 without metabolic activation.

The spontaneous mutation frequency of the negative (vehicle) control was in accordance with the historical control range in all assays. The positive controls gave the anticipated increases in mutation frequency over the controls and were in good harmony with the historical data in all assays. Seven evaluated concentrations were presented in all assays. The cloning efficiencies for the negative controls at the beginning and end of the expression period were within the target range. The evaluated concentration ranges were considered to be adequate (concentrations were tested up to the cytotoxic range in each test). The overall study was considered to be valid.

In conclusion, no mutagenic effect of C6OLF was observed either in the presence or absence of a metabolic activation system under the conditions of this HPRT assay.

2. INTRODUCTION

Many mammalian cell gene mutation systems are available which may give a measure of the intrinsic response of the mammalian genome or its maintenance processes to mutagens.

The system used in this study has been extensively validated [3-6]. The method is based on the detection of mutations (either induced or spontaneously generated) in the hypoxanthine-guanine phosphoribosyl transferase (hprt) enzyme locus located on the X chromosome.

HPRT is a cellular enzyme that allows cells to salvage hypoxanthine and guanine from surrounding medium for use in DNA synthesis. If a toxic base analogue 6-thioguanine (6-TG) is present in the medium, then the analogue will be phosphorylated via the HPRT pathway and incorporated into the nucleic acid. Thus, the cells die unless the enzyme is rendered inactive, by mutation.

3. OBJECTIVE OF STUDY

To evaluate the potential of the C6OLF test item to induce forward mutation at the hypoxanthine-guanine phosphoribosyl transferase (hprt) enzyme locus in CHO K1 Chinese hamster ovary cells in the absence and presence of a rat liver metabolising system.

4. GUIDELINES

This study followed the procedures indicated by the following internationally accepted guidelines [1, 2] and recommendations:

- OECD Guidelines for Testing of Chemicals No. 476, "*In Vitro* Mammalian Cell Gene Mutation Tests using the *Hprt* and *xprt* genes" (adopted 29 July 2016)
- Commission Regulation (EC) No 440/2008 of 30 May 2008, B.17. "*In vitro* Mammalian Cell Gene Mutation Test", (Official Journal L 142, 31/05/2008)

5. MATERIALS AND METHODS

5.1. TEST ITEM

5.1.1. Name and Data of Test Item

Name:	C6OLF
Other name:	C6Olefin
Batch/Lot number:	C2160215
CAS Number:	25291-17-2
Chemical Name:	3,3,4,4,5,5,6,6,7,7,8,8,8-Tridecafluorooct-1-ene
Appearance:	Colourless liquid
Purity:	99.95%
Expiry date:	31 December 2020
Storage conditions:	Controlled room temperature (15-25°C, below 70 RH%), protected from humidity
Safety precautions:	Routine safety precautions (lab coat, gloves, safety glasses and face mask) for unknown materials were applied to assure personnel health and safety.

No correction for purity of the test item was applied as agreed by the Sponsor.

5.1.2. Identification, Receipt

The test item of a suitable chemical purity was provided by the Sponsor. All precautions required in the handling and disposal of the test item were outlined by the Sponsor. These documents are part of the raw data. Identification of the test item was performed based on the information provided by the Sponsor in the Pharmacy of Citoxlab Hungary Ltd. Certificate of Analysis of the test item is shown in Appendix 2.

5.1.3. Archive Sampling

An adequate sample of the test item was collected and retained under the same storage conditions as applied in the study in the Archives of Citoxlab Hungary Ltd.

5.1.4. Formulation

Based on the results of a short solubility test, Distilled water and Dimethyl sulfoxide (DMSO) were not suitable solvents for the test, however the test item was soluble at 400 mg/mL concentrations in Acetone which was suitable for the test. The formulation of 400 mg/mL concentration using this vehicle (solvent) was suitable for the test and this vehicle (solvent) was also compatible with the survival of the mammalian cells and the metabolic activation system. Therefore, it was selected for vehicle (solvent) of the study.

The test item was formulated in the selected vehicle (solvent) to provide a suitably concentrated stock solution as follows. The necessary amount of test item was weighed into a calibrated volumetric flask. Approximately 80% of the required volume of vehicle (solvent) was added and the formulation was stirred until homogeneity was reached, then the volume was adjusted to the required final level. From the stock solution, several dilutions were prepared using the selected vehicle (solvent) to prepare dosing solutions for lower doses. The stock solution and the vehicle (solvent) were filtered sterile using a 0.22 µm syringe filter (Supplier: Millipore, Lot No.: R6NA19212, Expiry date: October 2019 was used in this study) before the preparation of the dosing formulations in each case. The stock solutions as well as all dilutions (dosing solutions) were prepared freshly at the beginning of the experiments in the testing laboratory in a sterile hood. The formats were protected from light during formulation.

Analytical determination of the test item concentration, stability and homogeneity was not performed because of the character and short period of the study.

5.2. NEGATIVE AND POSITIVE CONTROLS

Negative (vehicle) and positive controls were included in the experiments. In addition, untreated control sample was also used to demonstrate that the selected vehicle (solvent) had no mutagenic effects. Routine safety precautions for controls (lab coat, gloves, safety glasses and face mask) were applied to assure personnel health and safety.

5.2.1. Negative (Vehicle) Control

Based on the available data, Acetone as vehicle was suitable for the test. The negative control cultures were treated with the vehicle (solvent) alone in the same way as the test item treated cultures.

Data of the chemical used for vehicle (solvent) control of the study are shown below:

Name:	Acetone
Supplier:	VWR
Lot No.:	15J060514 / 17B284019
Appearance:	Clear colourless liquid
Expiry date:	31 October 2020 / 28 February 2022
Storage conditions:	Room temperature

*Note: Lot No. 15J060514 was used in the preliminary experiment and in Assay 1; Lot No. 17B284019 was used in Assay 2.

Vehicle of the Positive controls

Dimethyl sulfoxide (DMSO) was used as vehicle of the positive control materials in the study. Data of this chemical are shown below:

Name:	Dimethyl sulfoxide
Abbreviation:	DMSO
Supplier:	VWR
Lot No.:	16F304002
Appearance:	Clear colourless liquid
Expiry date:	31 May 2021
Storage conditions:	Room temperature, protected from humidity

5.2.2. Positive Controls

Ethyl methanesulfonate, a widely used positive control in the absence of metabolic activation, was dissolved in DMSO and used at a final concentration of 0.4 µL/mL.

Name:	Ethyl methanesulfonate
Abbreviation:	EMS
CAS No.:	62-50-0
Supplier:	Sigma-Aldrich Co.
Lot No.:	BCBS6100V
Expiry date:	31 August 2019
Storage conditions:	Room temperature, under N_2

7,12-Dimethylbenz[a]anthracene, a mutagen that requires metabolic transformation by microsomal enzymes, was dissolved in DMSO and used as a positive control substance for the experiments with metabolic activation at a final concentration of 15 µg/mL.

Name:	7,12-Dimethylbenz[a]anthracene
Abbreviation:	DMBA
CAS No.:	57-97-6
Supplier:	Sigma-Aldrich Co.
Lot No.:	SLBS1630V
Expiry date:	30 September 2019
Storage condition:	Room temperature

Positive control solutions were freshly prepared at the beginning of the experiments in the testing laboratory in a sterile hood and were filtered sterile using a 0.22 µm syringe filter before use (Supplier: Millipore, Lot No.: R7CA72998, Expiry date: March 2020).

5.3. INDICATOR CELLS

CHO K1:	Sub-line (K1) of Chinese hamster ovary cell line CHO
ATCC No.:	CCL-61
Lot No.:	58244452
Date of Arrival:	10 August 2010
Date of Reconstitution:	03 January 2011
Supplier:	American Type Culture Collection (Manassas,Virginia, United States)

The CHO cell line was originally derived from the ovary of a female Chinese hamster (Puck and Kao, 1967). The CHO K1 is a sub-line of CHO cell line. The CHO K1 cell line was purchased from American Type Culture Collection (ATCC). Prior to use in this test, the culture was cleansed of pre-existing mutant cells by culturing in HAT medium on 22 April 2016. Cells were stored as frozen stocks in a liquid nitrogen tank. Checking of mycoplasma infection was carried out for each batch of frozen stock; the cell line was tested negative.

For each experiment, one or more vials were thawed rapidly, the cells were diluted in F12-10 medium (“culture medium”, the content of the medium is listed in Section 5.4.) and incubated at 37ºC (± 0.5 C) in a humidified atmosphere (5± 0.3% CO_2 in air). When cells were growing well, subcultures were established in an appropriate number of flasks. Trypsin-EDTA (0.25% Trypsin, 1 mM EDTA) solution was used for cell detachment to subculture.

5.4. GROWTH MEDIA AND CONDITIONS

Four types of Ham's F12 medium were prepared as follows:

	Final concentration in:			
	F12-1	F12-5	F12-10	F12-SEL**
Foetal bovine serum (FBS, heat inactivated)	1 % v/v	5 % v/v	10 % v/v	10 % v/v
L-Glutamine	0.01 mL/mL	0.01 mL/mL	0.01 mL/mL	0.01 mL/mL
Antibiotic-Antimycotic solution *	0.01 mL/mL	0.01 mL/mL	0.01 mL/mL	0.01 mL/mL

*: Standard content of the antibiotic-antimycotic solution is 10000 NE/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin-B.

**: Hypoxanthine-free Ham's F-12 medium was used for preparation of the selection culture medium

Notes: In some cases, L-glutamine was already included in the ready to use media. In those cases no additional L-glutamine was added according to the manufacturer's instruction.

5.5. EXTERNAL METABOLIC ACTIVATION SYSTEM

The post-mitochondrial fraction (S9) [7, 8] was prepared from rat liver by the Microbiological Laboratory of Citoxlab Hungary Ltd. The documentation of the preparation of this post-mitochondrial fraction is stored in the reagent notebook in the Microbiological Laboratory which is archived annually.

Induction of Liver Enzymes:

Male Wistar rats (389-479 g, animals were 14-17 weeks old at initiation of E12590, 321-412 g, animals were 9 weeks old at the initiation of E12713) were treated with phenobarbital (PB) and β-naphthoflavone (BNF) at 80 mg/kg/day by oral gavage for three consecutive days. Rats were given drinking water and food *ad libitum* until 12 hours before euthanasia when food was removed. Euthanasia was performed by ascending concentration of CO_2 and death was confirmed by cutting through major thoracic blood vessels. Initiation dates of the induction of liver enzymes used for preparation S9 used in this study were 16 January 2017 (E12590) and 11 September 2017 (E12713).

Preparation of Rat Liver Homogenate S9 Fraction:

On Day 4, the rats were euthanized and the livers were removed aseptically using sterile surgical tools. After excision, livers were weighed and washed several times in 0.15 M KCl. The washed livers were transferred to a beaker containing 3 mL of 0.15 M KCl per g of wet liver, and homogenized. Homogenates were centrifuged for 10 minutes at 9000 g, and then the supernatant was decanted and retained. The freshly prepared S9 fraction was aliquoted into 1-5 mL portions, frozen quickly and stored at -80 ± 10ºC. The dates of preparation of S9 fraction used in this study were 19 January 2017 (Citoxlab code: E12590) and 14 September 2017 (Citoxlab code: E12713).

*Note: Batch of E12590 was used in the preliminary experiment, batch of E12713 was used in the main tests.

The sterility of the preparation was confirmed. The protein concentration was determined by colorimetric test by chemical analyzer at 540 nm in the Clinical Chemistry Laboratory of Citoxlab Hungary Ltd. The protein concentration of the S9 fraction used was determined to be 29.6 g/L (E12590) and 28.9 g/L (E12713).

The biological activity in the *Salmonella* assay of each batch of S9 was characterized beside the 2-Aminoanthracene with another mutagen, Benzo(a)pyrene, that requires metabolic activation by microsomal enzymes. The batches of S9 used in this study functioned appropriately during the activity checking.

5.6. PREPARATION OF THE S9-MIX

Treatments were carried out both in the absence and presence of S9 mix. The S9-mix was prepared as follows:

	Concentration of the stock solution	Concentration in the mix
HEPES*	20 mM	0.2 mL/mL
KCl	330 mM	0.1 mL/mL
$MgCl_2$	50 mM	0.1 mL/mL
NADP**	40 mM	0.1 mL/mL
D-Glucose-6-phosphate (Monosodium salt)	50 mM	0.1 mL/mL
F12-10	-	0.1 mL/mL
S9 fraction	-	0.3 mL/mL

*HEPES = N-2-Hydroxyethylpiperazine-N-2-Ethane Sulphonic Acid
**NADP= β-Nicotinamide-adenine dinucleotide-phosphate

Prior to addition to the culture medium the S9-mix was kept in an ice bath.

For all cultures treated in the presence of S9-mix, a 1 mL aliquot of the mix was added to 9 mL of cell culture medium to give a total of 10 mL (the same ratio was applied in those cases when higher treatment volume was used). The final concentration of the liver homogenate in the test system was 3%.

5.7. TEST PROCEDURE

5.7.1. Principles of dose selection (Preliminary Toxicity Test)

Treatment concentrations for the mutation assays were selected based on the result of a short preliminary toxicity test. For the treatments in the preliminary toxicity test, a formulation of 400 mg/mL was prepared using the selected vehicle (solvent) and lower test concentrations were prepared by serial dilutions. The highest test concentration in the preliminary test was 2000 µg/mL (the maximal recommended concentration*).

*Note: For a relatively non-cytotoxic compound the maximum concentration is 2 µL/mL, 2 mg/mL or 10 mM, whichever is the lowest. When a test item is not of defined composition (e.g. substance of unknown or variable composition, complex reaction products or biological materials), the top concentration may need to be higher (e.g. 5 mg/mL) in the absence of sufficient toxicity, to increase the concentration of each of the components. For relatively insoluble substances, the highest dose is a concentration above the limit of solubility in the final culture medium at the end of the treatment. Where cytotoxicity occurs, the highest concentration selected is expected to result in approximately 10-20% relative survival (%RS). The lower test concentrations are normally separated by factors of two. The higher test concentrations may be spaced more closely in order to increase the chance of producing the required level of toxicity.

In the preliminary experiment, a 5-hour treatment in the presence and absence of S9-mix and a 24-hour treatment in the absence of S9-mix were performed with a range of test concentrations to determine toxicity immediately after the treatments.

Treatment of cell cultures was performed as described below for the main mutation assays. However, single cultures were tested and positive controls were not included. Following treatments (as cytotoxicity was observed on Day 1), cell number in the samples was adjusted to $2x10^5$ cells/mL after counting and cells (10 mL cell suspension) were transferred to dishes for growth some additional days. After the incubation period, cell concentrations were determined using a haemocytometer on Day 3 and 6 in the preliminary experiment.

Precipitation of the test item in the final culture medium was visually examined at the beginning and end of the treatments. The pH and osmolality of the treatment medium at the end of the treatment was also determined. Results of the preliminary toxicity test are given in Appendix 3.

5.7.2. Mutation Assays

In Assay 1, 5-hour treatment was performed with and without metabolic activation (in the presence and absence of S9-mix). In Assay 2, 5-hour treatment was performed with metabolic activation (in the presence of S9-mix) and 24-hour treatment without metabolic activation (in the absence of S9-mix). The following treatments were used in Assays 1 and Assay 2:

	Treatment period (hours)	**GROUPS**	**CONCENTRATIONS**
Assay 1	**5**	Untreated control	+S9 mix 100 µL/mL
		Negative (vehicle) control (0.5% (v/v) Acetone)	5 µL/mL +S9 mix 100 µL/mL
		Negative control for DMBA (DMSO)	10 µL/mL +S9 mix 100 µL/mL
		C6OLF	2000 µg/mL + S9 mix 100 µL/mL
		C6OLF	1000 µg/mL + S9 mix 100 µL/mL
		C6OLF	500 µg/mL + S9 mix 100 µL/mL
		C6OLF	250 µg/mL + S9 mix 100 µL/mL
		C6OLF	125 µg/mL + S9 mix 100 µL/mL
		C6OLF	62.5 µg/mL + S9 mix 100 µL/mL
		C6OLF	31.25 µg/mL + S9 mix 100 µL/mL
		7,12-Dimethylbenz[a]anthracene (DMBA)	15 µg/mL + S9 mix 100 µL/mL
	5	Untreated control	-

	Negative (vehicle) control (0.5% (v/v) Acetone)	5 µL/mL
	Negative control for EMS (DMSO)	10 µL/mL
	C6OLF	2000 µg/mL
	C6OLF	1000 µg/mL
	C6OLF	500 µg/mL
	C6OLF	250 µg/mL
	C6OLF	125 µg/mL
	C6OLF	62.5 µg/mL
	C6OLF	31.25 µg/mL
	Ethyl methanesulfonate (EMS)	0.4 µL/mL

Note: Seven concentrations were plated for mutagenicity testing in both cases.

	Treatment period (hours)	**GROUPS**	**CONCENTRATIONS**
Assay 2	**5**	Untreated control	+S9 mix 100 µL/mL
		Negative (vehicle) control (0.5% (v/v) Acetone)	5 µL/mL +S9 mix 100 µL/mL
		Negative control for DMBA (DMSO)	10 µL/mL +S9 mix 100 µL/mL
		C6OLF	2000 µg/mL + S9 mix 100 µL/mL
		C6OLF	1000 µg/mL + S9 mix 100 µL/mL
		C6OLF	500 µg/mL + S9 mix 100 µL/mL
		C6OLF	250 µg/mL + S9 mix 100 µL/mL
		C6OLF	125 µg/mL + S9 mix 100 µL/mL
		C6OLF	62.5 µg/mL + S9 mix 100 µL/mL
		C6OLF	31.25 µg/mL + S9 mix 100 µL/mL
		7,12-Dimethylbenz[a]anthracene (DMBA)	15 µg/mL + S9 mix 100 µL/mL
	24	Untreated control	-
		Negative (vehicle) control (0.5% (v/v) Acetone)	5 µL/mL
		Negative control for EMS (DMSO)	10 µL/mL
		C6OLF	2000 µg/mL
		C6OLF	1000 µg/mL
		C6OLF	500 µg/mL
		C6OLF	250 µg/mL
		C6OLF	125 µg/mL
		C6OLF	62.5 µg/mL
		C6OLF	31.25 µg/mL
		Ethyl methanesulfonate (EMS)	0.4 µL/mL

Note: Seven concentrations were plated for mutagenicity testing in both cases.

Treatment of the cells

For the 5-hour treatments, at least $2x10^6$ cells were placed in each of a series of sterile dishes (diameter approx. 100 mm) and incubated for about approximately 24 hours before treatment at 37°C (± 0.5°C) in a humidified atmosphere (5±0.3% CO_2 in air). On the treatment day, plating medium was removed and appropriate amount of fresh medium was added to the cells. Treatment medium for the 5-hour treatment contained 1% (v/v) serum (F12-1, for treatment without metabolic activation) or 5% (v/v) serum (F12-5, for treatment with metabolic activation). A suitable volume (50 µL) of vehicle (solvent) and test item solution or 100 µL positive control solution and their vehicles was added to the 10 mL final volume (higher volume using the same ratio was applied in those cases when higher than 10 mL final volume was used). In case of experiment with metabolic activation, 1.0 mL of S9-mix was added to the cultures.

After the 5-hour incubation period at 37°C (± 0.5°C) in a humidified atmosphere (5±0.3% CO_2 in air), the cultures were washed thoroughly with F12-10 medium (culture medium). Then, dishes were covered with appropriate amount of fresh F12-10 medium (10-60 mL) and incubated for 19 hours at 37°C (± 0.5°C) in a humidified atmosphere (5±0.3% CO_2 in air).

After the 19-hour incubation period, cells were washed twice with phosphate buffered saline (PBS), detached with trypsin-EDTA solution and counted using a haemocytometer. In samples where sufficient cells survived, cell number was adjusted to $2x10^5$ cells/mL. Cells (10 mL cell suspension) were transferred to dishes for growth through the expression period or diluted to be plated for survival.

For the 24-hour treatment, at least $2x10^6$ cells were placed in each of a series of sterile dishes (diameter approx. 100 mm) and incubated for approximately 24 hours before treatment at 37°C (± 0.5°C) in a humidified atmosphere (5±0.3% CO_2 in air). On the treatment day, plating medium was removed and appropriate amount of fresh medium was added to the cells. Treatment medium for the 24-hour treatment contained 5% serum (F12-5). A suitable volume (50 µL) of vehicle (solvent) and test item solution or 100 µL positive control solution and their vehicles was added to the 10 mL final volume (the same ratio was applied in those cases when higher than 10 mL final volume was used). After the 24-hour incubation period at 37°C (± 0.5°C) in a humidified atmosphere (5±0.3% CO_2 in air), cells were washed twice with phosphate buffered saline (PBS), detached with trypsin-EDTA solution and counted using a haemocytometer. In samples where sufficient cells survived, cell number was adjusted to $2x10^5$ cells/mL. Cells (10 mL cell suspension) were transferred to dishes for growth through the expression period or diluted to be plated for survival.

Duplicate cultures were used for each treatment. Solubility of the test item in the cultures was visually examined at the beginning and end of the treatments. Measurement of pH and osmolality was also performed after the treatment.

Plating for survival

Following adjustment of the cultures to $2x10^5$ cells/mL, samples from these cultures were diluted to 40 cells/mL using F12-10 medium as follows (typical ratio is shown, other dilutions using the same ratio were also acceptable):

	Initial cell conc	Dilution		Intermediate cell conc	Dilution		Final cell conc
	A	mL A	mL F12-10	(B)	mL B	mL F12-10	(C)
Survival	2×10^5 cells/mL*	0.1	9.9	2×10^3 cells/mL	0.4	19.6	40 cells/mL

* Where fewer than 2×10^5 cells/mL survive, an alternative dilution scheme was adopted to give 2×10^3 cells/mL

Five mL suspension (200 cells/dish) per each culture were plated into 3 parallel dishes (diameter was approx. 60 mm). The dishes were incubated at 37°C (± 0.5°C) in a humidified atmosphere (5±0.3% CO_2 in air) for 5 days for colony growing.

Expression period

Cultures were maintained in dishes for 7 days, during which time the HPRT-mutation was expressed. During this expression period, the cultures were sub-cultured and maintained at $2x10^5$ cells/dish twice (on Days 3, 6 and 8), to maintain logarithmic growth. At the end of the expression period the cell monolayers were trypsinised, cell density was determined by haemocytometer and cells were plated for viability and for selection of the mutant phenotype.

Plating for viability

At the end of the expression period (Day 8), cell number in the samples was adjusted to $4x10^5$ cells/mL, then further diluted to 40 cells/mL using F12-10 medium.

	Initial cell conc	Dilution		Intermediate cell conc	Dilution		Final cell conc
	A	mL A	mL F12-10	(B)	mL B	mL F12-10	(C)
Viability	4 x 10^5 cells/mL*	0.1	9.9	4 x 10^3 cells/mL	0.2	19.8	40 cells/mL

Five mL of cell suspension (200 cells/dish) per each culture were plated in F12-10 medium in 3 parallel dishes (diameter was approx. 60 mm) for a viability test. The dishes were incubated at 37°C (± 0.5°C) in a humidified atmosphere (5±0.3% CO_2 in air) for 5 days for colony growing.

Plating for selection of the mutant phenotype (6-TG resistance)

At the end of the expression period (Day 8), cell number in the samples was adjusted to $4x10^5$ cells/mL. 1 mL of the adjusted cell suspension and 4 mL of F12-SEL medium were added into Petri dishes (diameter approx. 100 mm, 5 parallels per sample) for each sample. An additional 5 mL of F12-SEL medium containing 20 µg/mL 6-thioguanine (abbreviation: 6-TG) was added to the dishes (final volume: 10 mL, final 6-TG concentration: 10 µg/mL) to determine mutation frequency. Dishes were incubated at 37°C (± 0.5°C) in a humidified atmosphere (5±0.3% CO_2 in air) for 7 days for colony growing.

Fixation and staining of colonies

After the growing or selection period, the culture medium was removed and colonies were fixed for 5 minutes with methanol. After fixation, colonies were stained using 10% Giemsa solution (diluted with distilled water) for 30 minutes, dried and manually counted.

5.8. ANALYSIS OF THE RESULTS

Relative survivals* were assessed by comparing the cloning efficiency of the treated groups to the negative (vehicle/solvent) control.

*Note: Other parameters measuring cytotoxicity like relative survival after treatment (comparing the cell number determined after treatment to the vehicle (solvent) control) or relative total growth (comparing the growth rate during the entire expression period to the vehicle (solvent) control) were also calculated.

The mutant frequency was calculated by dividing the total number of mutant colonies by the number of cells selected ($2x10^6$ cells: 5 plates at $4x10^5$ cells/plate), corrected for the cloning efficiency of cells prior to mutant selection (viability), and were expressed as 6-TG resistant mutants per 10^6 clonable cells.

The mutation frequencies were statistically analyzed. Statistical evaluation of data was performed with the SPSS PC+4.0 statistical program package (SPSS Hungary Ltd., Budapest, Hungary). The heterogeneity of variance between groups was checked by Bartlett`s test. Where no significant heterogeneity was detected, a one-way analysis of variance (ANOVA) was carried out. If the obtained result was significant, Duncan's Multiple Range test was used to assess the significance of inter-group differences. Where significant heterogeneity was found, the normal distribution of data was examined by Kolmogorow-Smirnow test. In the case of not normal distribution, the non-parametric method of Kruskal-Wallis One-Way analysis of variance was applied. If a positive result was detected, the inter-group comparisons were performed using Mann-Whitney U-test. Data also were checked for a trend in mutation frequency with treatment dose using Microsoft Excel 2010 software (R-squared values were calculated for the log concentration versus the mutation frequency).

In the statistical analysis, negative trends were not considered significant.

5.8.1. Acceptance criteria

The assay was considered valid if all the following criteria are met:

1. The mutant frequency in the negative (vehicle/solvent) control cultures was in accordance with the historical control data.
2. The positive control chemicals induced a clear increase in mutant frequency.
3. The cloning efficiency of the negative controls was in the range of 60-140% on Day 1 and 70-130% on Day 8.
4. At least four test item concentrations in duplicate cultures were presented.

5.8.2. Evaluation criteria

The test item was considered to be mutagenic in this assay if the following criteria are met:

1. The assay is valid.
2. The mutant frequency at one or more doses is significantly greater than that of the relevant negative (vehicle) control ($p<0.05$).
3. Increase of the mutant frequency is reproducible.
4. There is a dose-response relationship.

Results which only partially met the criteria were dealt with on a case-by-case basis (historical control data of untreated control samples was taken into consideration if necessary). Similarly, positive responses seen only at high levels of cytotoxicity required careful interpretation when assessing their biological significance. In cases with survival lower than 10%, extreme caution is taken in the interpretation.

According to the relevant OECD guideline, the biological relevance of the results was considered first, statistical significance was not the only determination factor for a positive response.

5.9. ARCHIVES

As demanded by the principles of Hungarian GLP [9] and Test Facility SOPs, all the study documents and materials (such as, but not limited to, all raw data, the original Study Plan and its amendment, the original Final Report and any amendments, one retained sample of the test item and correspondence) will be archived for a period of 15 years in the Archives of Citoxlab Hungary Ltd. (8200 Veszprém-Szabadságpuszta, Hungary).

Samples that are unstable may be disposed of before the time required by Hungarian GLP [9], but no raw data or material relating to the study will be discarded without the Sponsor's prior written consent.

After the retention time at the Test Facility has elapsed, all the archived materials listed above will be offered to the Sponsor or retained for a further period if agreed by a contract. Otherwise the materials will be discarded (with the exception of the original Study Plan and its amendment and the original Final Report and any amendments, which will be kept in the Archive of the Test Facility or transferred to external archiving.)

5.10. CHEMICALS USED IN THE EXPERIMENTS

The chemicals used in the independently performed experiments are summarized in the following table:

Materials	Lot No.	Supplier / Manufacturer	Expiry Date
Antibiotic-antimycotic solution	037M4876V	Sigma-Aldrich Co.	April 2019
Distilled water	805 0417	Hungaro-Gal	05 October 2017
Distilled water	808 0617	Hungaro-Gal	06 December 2017
Distilled water	811 0717	Hungaro-Gal	26 January 2018
Distilled water	815 0917	Hungaro-Gal	25 March 2018
Distilled water	71033Y25-2	B. Braun Pharmaceuticals SA	February 2020
EX-CELL(TM) CD CHO Medium*	SLBR4268V	Sigma-Aldrich Co.	November 2017
Fetal Bovine Serum**	17C438	SAFC	30 April 2022
Fetal Bovine Serum**	16C462	Sigma-Aldrich Co.	30 April 2021
Giemsa solution***	HX60731467	VWR	September 2019
D-Glucose-6-phosphate Na	SLBP2553V	Sigma-Aldrich Co.	October 2018
L-Glutamine	RNBF9654	Sigma-Aldrich Co.	March 2019
L-Glutamine	RNBG0877	Sigma-Aldrich Co.	June 2019
HEPES	SLBM2036V	Sigma-Aldrich Co.	December 2018
Magnesium chloride	SLBH7393V	Sigma-Aldrich Co.	October 2017
Magnesium chloride	SLBT5102	Sigma-Aldrich Co.	March 2021
Methanol	16L194011	VWR	December 2021
NADP (sodium salt)	SLBN0872V	Sigma-Aldrich Co.	November 2018
Nutrient Mixture F-12 HAM****	RNBF8873	Sigma-Aldrich Co.	January 2018
Nutrient Mixture F-12 HAM****	RNBG0928	Sigma-Aldrich Co.	May 2018
Phosphate buffered saline (PBS), 10X	SLBH9017	Sigma-Aldrich Co.	September 2017
Phosphate buffered saline (PBS), 10X	SLBS5708	Sigma-Aldrich Co.	January 2018
Potassium chloride	K45349336	MERCK	28 February 2019
6-Thioguanine	SLBQ5479V	Sigma-Aldrich Co.	30 September 2018
Trypsin-EDTA solution, 0.25%	SLBR8842V	Sigma-Aldrich Co.	April 2019
Trypsin-EDTA solution, 0.25%	SLBS8529	Sigma-Aldrich Co.	July 2019

*: serum-free, without L-glutamine, without hypoxanthine, without thymidine; with sodium bicarbonate, chemically defined
**: heat-inactivated, USA origin, sterile-filtered, cell culture tested
***: Giemsa's azur eosin methylene blue solution
****: with sodium bicarbonate, with L-glutamine, liquid, sterile-filtered, cell culture tested
HEPES: N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid
NADP: β-Nicotinamide adenine dinucleotide phosphate
6-Thioguanine: 2-amino-6-mercaptopurine
EDTA: Ethylenediaminetetraacetic acid

5.11. DEVIATIONS TO THE STUDY PLAN

Due to scientific reason, higher cell number (4 x 10^5 cells/dish) was used for plating of mutation plates than indicated in the Study Plan (2 x 10^5 cells/dish) in order to examine at least 2 million cells for each replicate as requested by the relevant OECD guideline. This suspension was used as starting point for the dilution of viability plates as well. This fact ensured that the study meet the criteria of the OECD guideline.

6. RESULTS AND DISCUSSION

6.1. PRELIMINARY EXPERIMENT

Treatment concentrations for the mutation assay were selected based on the results of a short preliminary experiment. 5-hour treatment in the presence and absence of S9-mix and 24-hour treatment in the absence of S9-mix was performed with a range of test item concentrations to determine toxicity immediately after the treatments. The highest test concentration in the preliminary test was 2000 µg/mL (the recommended maximum concentration). Tabulated results of the preliminary experiment are given in Appendix 3.

Insolubility were detected in the preliminary experiment. The concentrations selected for the main experiments were based on results of the performed Preliminary Toxicity Test according to the OECD guideline instructions (up to the maximum recommended concentration). Lower test concentrations were separated by factor of two. Seven concentrations were selected for the main experiments.

6.2. MUTATION ASSAYS

In the mutation assays, cells were exposed to the test item for 5 hours with and without metabolic activation system (±S9-mix) or for 24 hours without metabolic activation system (-S9-mix) then the cells were plated for determination of survival and in parallel sub-cultured without test item for 7 days to allow the expression of the genetic changes (if any occurred). At the end of the expression period, cells were allowed to grow and form colonies in culture dishes with and without selective agent (6-TG) for determination of mutations and viability.

Assay 1

In Assay 1, a 5-hour treatment with metabolic activation (in the presence of S9-mix) and a 5-hour treatment without metabolic activation (in the absence of S9-mix) were performed.

For the 5-hour treatment in the presence and absence of S9-mix, the following concentrations were examined: 2000, 1000, 500, 250, 125, 62.5 and 31.25 µg/mL.

In Assay 1, insolubility (precipitate / minimal amount of precipitate) was detected in 1000 µg/mL concentrations in the final treatment medium at the end of the treatment in the experiments with and without metabolic activation. The precipitation did not interfere with the reading of the results. Discoloured medium was observed with and without metabolic activation at several concentrations. There were no large changes in pH and osmolality after treatment in any cases.

In the presence of S9-mix (5-hour treatment), no marked cytotoxicity of the test item was observed (the highest concentration of 2000 µg/mL showed a relative survival of 76% after treatment and on the survival plates). An evaluation was made using data of all seven concentrations. No statistically significant increases in the mutation frequency were observed at any examined concentrations when compared to the negative (vehicle) control data (see Table 5) and there was no dose response to the treatment (a trend analysis showed no effect of treatment).

In the absence of S9-mix (5-hour treatment), no marked cytotoxicity of the test item was observed (the highest concentration of 2000 µg/mL showed a relative survival of 92% on the survival plates). An evaluation was made using data of all seven concentrations. No statistically significant increases in the mutation frequency were observed at any examined concentrations when compared to the negative (vehicle) control data (see Table 5) and there was no dose response to the treatment (a trend analysis showed no effect of treatment)

Data of Assay 1 are presented for survival (Table 1 of Appendix 4 and Appendix 7), viability (Table 3 of Appendix 5 and Appendix 9) and mutagenicity (Table 5 of Appendix 6 and Appendix 11). Observations after treatment are summarized in Appendix 13.

Assay 2

In Assay 2, 5-hour treatment with metabolic activation (in the presence of S9-mix) and 24-hour treatment without metabolic activation (in the absence of S9-mix) were performed.

For the 5-hour treatment in the presence of S9-mix, the following concentrations were examined: 2000, 1000, 500, 250, 125, 62.5 and 31.25 µg/mL.

For 24-hour treatment in the absence of S9-mix, the following concentrations were examined: 2000, 1000, 500, 250, 125, 62.5 and 31.25 µg/mL.

In Assay 2, insolubility (precipitate / minimal amount of precipitate) was detected in the 2000-500 µg/mL concentration range in the final treatment medium at the end of the treatment in the experiments with and without metabolic activation. Discoloured medium caused by the presence of the test item with and without metabolic activation at several concentrations. There were no large changes in pH and osmolality after treatment in any cases.

In the presence of S9-mix (5-hour treatment), similarly to the first test, no marked cytotoxicity of the test item was observed (the highest concentration of 2000 µg/mL showed a relative survival of 88% after treatment and on the survival plates), thus an evaluation was made using data of all seven concentrations. No statistically significant increases in the mutation frequency were observed at any examined concentrations when compared to the negative (vehicle) control data (see Table 6) and there was no dose response to the treatment (a trend analysis showed no effect of treatment). This experiment confirmed the negative results seen in Assay 1 with metabolic activation.

In the absence of S9-mix (24-hour treatment), no marked cytotoxicity of the test item was observed (the highest concentration of 2000 µg/mL showed a relative survival of 99% after treatment and on the survival plates), thus an evaluation was made using data of all seven concentrations. No statistically significant increases in the mutation frequency were observed at any examined concentrations when compared to the negative (vehicle) control data (see Table 6) and there was no dose response to the treatment (a trend analysis showed no effect of treatment). This experiment confirmed the negative results seen in Assay 1 without metabolic activation.

Data of Assay 2 are presented for survival (Table 2 of Appendix 4 and Appendix 8), viability (Table 4 of Appendix 5 and Appendix 10) and mutagenicity (Table 6 of Appendix 6 and Appendix 12). Observations after treatment are summarized in Appendix 13.

The other sporadic, statistically non-significant differences were examined for consistency; none of them were repeatable when comparing Assay 1 and Assay 2. Furthermore, all the observed mutation frequency values were within the general historical control range*. Together with the lack of correlation with dose level, this confirms that there were no biologically significant differences between treated samples and negative (vehicle) controls.

*Note: The spontaneous mutant frequency is generally between 5 and 20 $x10^{-6}$ based on the guideline.

6.3. VALIDITY OF THE MUTATION ASSAYS

The spontaneous mutation frequency of the negative (vehicle) control was in accordance with the general historical control range in all assays (Tables 5 and 6 of Appendix 6), and the observed values were in the expected range (5-20 x 10^{-6}) as shown in the OECD No. 476 guideline.

The positive controls (DMBA in the presence of metabolic activation and EMS in the absence of metabolic activation) gave the anticipated increases in mutation frequency over the controls (Tables 5 and 6 of Appendix 6) and were in good harmony with the historical data in all assays (for historical control data see Appendix 15).

The cloning efficiencies for the negative (vehicle) controls on Days 1 and 8 were within the target range of 60-140% and 70-130% in all assays (Tables 1-4).

The tested concentration range in the study was considered to be adequate as the highest evaluated concentration was the recommended maximum concentration were considered to be fulfilled.

Seven test item concentrations were evaluated in duplicate in each experiment.

The overall study was considered valid.

7. CONCLUSION

The HPRT Assay with C6OLF performed on CHO K1 Chinese hamster ovarian cells was considered to be valid and reflect the real potential of the test item to cause mutations in the cultured mammalian cells used in this study.

Treatment with the test item did not result in a statistically and biologically significant dose-dependent increase in mutation frequencies either in the presence or absence of a rat metabolic activation system (S9) in this study.

In conclusion, no mutagenic effect of C6OLF was observed either in the presence or absence of metabolic activation system under the conditions of this HPRT assay.

8. DISTRIBUTION OF THE FINAL REPORT

Sponsor: 1x PDF file

Archive: 1x original, bound

9. REFERENCES

1. OECD Guidelines for the Testing of Chemicals, Section 4, No. 476, "*In Vitro* Mammalian Cell Gene Mutation Tests using the *Hprt* and *xprt* genes" (adopted 29 July 2016)
2. Commission Regulation (EC) No 440/2008 of 30 May 2008, B.17. "*In vitro* Mammalian Cell Gene Mutation Test" (Official Journal L 142, 31/05/2008)
3. Hsie, A.W., Castiano, D.A., Couch, D.B., Krahn, D.F., Oneill, J.P.: The use of the Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. *Mutation Research* (1981) **86**, 193-214
4. Brusick, D.: Genetic Toxicology, Principles and Methods of Toxicology (1989), 424-426
5. Kao, F.T. and Puck, T.T.: Genetics of somatic mammalian cells. IV. Properties of somatic Chinese hamster cell mutants with respect to the requirement for proline. *Genetics* (1967) **55**, 513-524
6. Cole, J., Fox, M., Garner, C., McGregor, D.B., Thacker, J.: Gene mutation assays in cultured mammalian cells. In Basic Mutagenicity Tests UKEMS Recommended Procedures (Ed Kirkland D J) Cambridge University Press, (1990) 87-114
7. Ames, B. N., Joyce Mccann and Edith Yamasaki: Methods for Detecting Carcinogens and Mutagens with the *Salmonella* / Mammalian-Microsome Mutagenicity Test. *Mutation Research* (1975) **31**, 347-364
8. Maron, D. M. and Ames, B. N.: Revised Method for the *Salmonella* Mutagenicity Test. *Mutation Research* (1983) **113**: 173-215
9. Hungarian Good Laboratory Practice Regulations: 42/2014. (VIII. 19.) EMMI decree of the Ministry of Human Capacities which corresponds to the OECD GLP, 1997

A P P E N D I C E S

APPENDIX 1

Study Schedule

PRELIMINARY TOXICITY TEST

Plating of cells	:	04 September 2017
Treatment of the cells (Day 0)	:	05 September 2017
End of counting after treatment	:	11 September 2017

MUTATION ASSAYS

Assay 1

Plating of cells	:	23 October 2017
Treatment of the cells (Day 0)	:	24 October 2017
Start of expression period	:	25 October 2017
Staining of survival plates	:	30 October 2017
End of expression period	:	01 November 2017
Staining of viability plates	:	06 November 2017
Staining of mutagenicity plates	:	08 November 2017
End of colony counting	:	11 November 2017

Assay 2

Plating of cells	:	13 November 2017
Treatment of the cells (Day 0)	:	14 November 2017
Start of expression period	:	15 November 2017
Staining of survival plates	:	21 November 2017
End of expression period	:	22 November 2017
Staining of viability plates	:	27 November 2017
Staining of mutagenicity plates	:	29 November 2017
End of colony counting	:	01 December 2017

APPENDIX 2

Copy of the Certificate of Analysis

DAIKIN

CERTIFICATE OF ANALYSIS

DATE : 22nd Dec 2016
SAMPLE NAME : C6OLF
CHEMICAL NAME : 3,3,4,4,5,5,6,6,7,7,8,8,8-Tridecafluorooct-1-ene
Lot No. : C2163215

PURITY : 99.95 %
IMPURITIES : unknow

EXPIRY DATE : 31 Dec 2020

ANALYTICAL METHOD Gas chromatography

DAIKIN INDUSTRIES.LTD.

APPENDIX 3

Preliminary Toxicity Test Result

5-hour treatment in the presence of metabolic activation

Test item concentration	Cell number, cells/mL (Relative Survival*, %)			Observations at the beginning / after treatment
	after treatment (Day 1)	Day 3	Day 6	
Untreated control	8.70E+06 (123)	8.40E+06 (97)	8.20E+06 (99)	normal / normal (pH: 7.0; osm: 295 mmol/kg)
Negative (vehicle) control	7.05E+06 (100)	8.70E+06 (100)	8.30E+06 (100)	normal / normal (pH: 7.0; osm: 292 mmol/kg)
2000 µg/mL	8.15E+06 (116)	8.90E+06 (102)	8.80E+06 (106)	discoloured medium# / discoloured medium# (pH: 7.0; osm: 291 mmol/kg)
1000 µg/mL	7.95E+06 (113)	7.80E+06 (90)	7.80E+06 (94)	discoloured medium#, precipitate / discoloured medium#, precipitate# (pH: 7.0; osm: 292 mmol/kg)
500 µg/mL	7.05E+06 (100)	7.70E+06 (89)	7.80E+06 (94)	discoloured medium#, precipitate# / discoloured medium# (pH: 7.0; osm: 292 mmol/kg)
250 µg/mL	6.90E+06 (98)	7.90E+06 (91)	8.70E+06 (105)	discoloured medium# / discoloured medium# (pH: 7.0; osm: 290 mmol/kg)
125 µg/mL	7.25E+06 (103)	8.80E+06 (101)	7.60E+06 (92)	discoloured medium# / discoloured medium# (pH: 7.0; osm: 294 mmol/kg)
62.5 µg/mL	6.85E+06 (97)	8.30E+06 (95)	7.90E+06 (95)	normal/ discoloured medium# (pH: 7.0; osm: 292 mmol/kg)
31.25 µg/mL	7.00E+06 (99)	8.70E+06 (100)	7.30E+06 (88)	normal / discoloured medium# (pH: 7.0; osm: 291 mmol/kg)
15.625 µg/mL	6.95E+06 (99)	8.00E+06 (92)	8.00E+06 (96)	normal / discoloured medium# (pH: 7.0; osm: 292 mmol/kg)
7.813 µg/mL	7.20E+06 (102)	7.90E+06 (91)	7.70E+06 (93)	normal / discoloured medium# (pH: 7.0; osm: 292 mmol/kg)
3.906 µg/mL	7.25E+06 (103)	8.20E+06 (94)	8.60E+06 (90)	normal / discoloured medium# (pH: 7.0; osm: 298 mmol/kg)

*: compared to the negative (vehicle) control (0.5%(v/v) Acetone) #: minimal amount
osm: osmolality

APPENDIX 3 (continued)

Preliminary Toxicity Test Result

5-hour treatment in the absence of metabolic activation

Test item concentration	Cell number, cells/mL (Relative Survival*, %)			Observations at the beginning / after treatment
	after treatment (Day 1)	Day 3	Day 6	
Untreated control	9.90E+06 (111)	8.30E+06 (99)	8.50E+06 (109)	normal / normal (pH: 7.0; osm: 295 mmol/kg)
Negative (vehicle) control	8.95E+06 (100)	8.40E+06 (100)	7.80E+06 (100)	normal / normal (pH: 7.0; osm: 293 mmol/kg)
2000 µg/mL	1.07E+07 (120)	9.40E+06 (112)	6.30E+06 (81)	discoloured medium# / discoloured medium# (pH: 7.0; osm: 294 mmol/kg)
1000 µg/mL	1.02E+07 (113)	7.60E+06 (90)	7.90E+06 (101)	discoloured medium#, precipitate / discoloured medium#, precipitate# (pH: 7.0; osm: 294 mmol/kg)
500 µg/mL	9.80E+06 (109)	9.80E+06 (117)	7.00E+06 (90)	discoloured medium#, precipitate# / discoloured medium#, precipitate# (pH: 7.0; osm: 293 mmol/kg)
250 µg/mL	9.35E+06 (104)	1.00E+07 (119)	7.10E+06 (91)	discoloured medium# / discoloured medium# (pH: 7.0; osm: 295 mmol/kg)
125 µg/mL	8.70E+06 (97)	7.90E+06 (94)	7.20E+06 (92)	normal / discoloured medium# (pH: 7.0; osm: 294 mmol/kg)
62.5 µg/mL	8.80E+06 (98)	1.00E+07 (119)	6.80E+06 (87)	normal/ discoloured medium# (pH: 7.0; osm: 297 mmol/kg)
31.25 µg/mL	9.90E+06 (111)	8.60E+06 (102)	7.60E+06 (97)	normal / discoloured medium# (pH: 7.0; osm: 294 mmol/kg)
15.625 µg/mL	9.70E+06 (108)	9.20E+06 (110)	7.90E+06 (101)	normal / normal (pH: 7.0; osm: 297 mmol/kg)
7.813 µg/mL	1.02E+07 114)	7.90E+06 (94)	7.10E+06 (91)	normal / normal (pH: 7.0; osm: 295 mmol/kg)
3.906 µg/mL	9.25E+06 (103)	9.60E+06 (114)	6.20E+06 (79)	normal / normal (pH: 7.0; osm: 298 mmol/kg)

*: compared to the negative (vehicle) control (0.5% (v/v) Acetone) #: minimal amount
osm: osmolality

APPENDIX 3 (continued)

Preliminary Toxicity Test Result

24-hour treatment in the absence of metabolic activation

Test item concentration	Cell number, cells/mL (Relative Survival*, %)			Observations at the beginning / after treatment
	after treatment (Day 1)	Day 3	Day 6	
Untreated control	1.01E+07 (113)	8.30E+06 (93)	8.20E+06 (106)	normal / normal (pH: 7.0; osm: 293 mmol/kg)
Negative (vehicle) control	8.90E+06 (100)	8.90E+06 (100)	7.70E+06 (100)	normal / normal (pH: 7.0; osm: 296 mmol/kg)
2000 µg/mL	9.15E+06 (103)	8.50E+06 (96)	8.60E+06 (112)	discoloured medium# / discoloured medium# (pH: 7.0; osm: 296 mmol/kg)
1000 µg/mL	1.01E+07 (113)	8.60E+06 (97)	7.40E+06 (96)	discoloured medium#, precipitate / discoloured medium#, precipitate# (pH: 7.0; osm: 299 mmol/kg)
500 µg/mL	9.35E+06 (105)	8.90E+06 (100)	7.70E+06 (100)	discoloured medium#, precipitate# / discoloured medium#, precipitate# (pH: 7.0; osm: 296 mmol/kg)
250 µg/mL	8.45E+06 (95)	9.90E+06 (111)	8.50E+06 (110)	discoloured medium# / discoloured medium#, precipitate# (pH: 7.0; osm: 297 mmol/kg)
125 µg/mL	1.01E+07 (113)	9.30E+06 (104)	7.10E+06 (92)	normal / discoloured medium# (pH: 7.0; osm: 297 mmol/kg)
62.5 µg/mL	9.65E+06 (108)	9.00E+06 (101)	7.60E+06 (99)	normal/ discoloured medium# (pH: 7.0; osm: 294 mmol/kg)
31.25 µg/mL	9.65E+06 (108)	8.60E+06 (97)	7.60E+06 (99)	normal / discoloured medium# (pH: 7.0; osm: 294 mmol/kg)
15.625 µg/mL	8.10E+06 (91)	7.60E+06 (85)	7.30E+06 (95)	normal / discoloured medium# (pH: 7.0; osm: 297 mmol/kg)
7.813 µg/mL	9.35E+06 (105)	8.20E+06 (92)	7.00E+06 (91)	normal / normal (pH: 7.0; osm: 298 mmol/kg)
3.906 µg/mL	9.00E+06 (101)	9.20E+06 (103)	7.40E+06 (96)	normal / normal (pH: 7.0; osm: 300 mmol/kg)

*: compared to the negative (vehicle) control (0.5% (v/v) Acetone) #: minimal amount
osm: osmolality

APPENDIX 4

Summary of Survival Results

Table 1: Summarized Survival Results of Assay 1

S9 mix	Treatment period (hours)	Study phase	Test item or control concentration	Total number of colonies	Cloning Efficiency (CE)	Relative Survival (%) on plates
+	5	A1	2000 µg/mL	809	0.674	76
			1000 µg/mL	840	0.700	79
			500 µg/mL	828	0.690	78
			250 µg/mL	871	0.726	82
			125 µg/mL	892	0.743	84
			62.5 µg/mL	864	0.720	81
			31.25 µg/mL	884	0.737	83
			Negative control	1061	0.884	100
			Negative control for DMBA (DMSO)	929	0.774	88
			Untreated control	1087	0.906	102
			Positive control (DMBA)	41	0.034	4
-	5	A1	2000 µg/mL	1008	0.840	92
			1000 µg/mL	1081	0.901	99
			500 µg/mL	1001	0.834	91
			250 µg/mL	1019	0.849	93
			125 µg/mL	1064	0.887	97
			62.5 µg/mL	1100	0.917	100
			31.25 µg/mL	1040	0.867	95
			Negative control	1096	0.913	100
			Negative control for EMS (DMSO)	1112	0.927	101
			Untreated control	1052	0.877	96
			Positive control (EMS)	821	0.684	75

A1 = Assay 1
+ = in the presence of S9-mix
- = in the absence of S9-mix
Negative (vehicle) control = 0.5%(v/v) Acetone
DMSO = Dimethyl sulfoxide
DMBA = 7,12-Dimethylbenz[a]anthracene, 15 µg/mL
EMS = Ethyl methanesulfonate, 0.4 µL/mL

APPENDIX 4 (continued)

Summary of Survival Results

Table 2: Summarized Survival Results of Assay 2

S9 mix	Treatment period (hours)	Study phase	Test item or control concentration	Total number of colonies	Cloning Efficiency (CE)	Relative Survival (%) on plates
+	5	A2	2000 µg/mL	939	0.783	88
			1000 µg/mL	957	0.798	90
			500 µg/mL	980	0.817	92
			250 µg/mL	1046	0.872	98
			125 µg/mL	1001	0.834	94
			62.5 µg/mL	998	0.832	94
			31.25 µg/mL	1022	0.852	96
			Negative control	1067	0.889	100
			Negative control for DMBA (DMSO)	1058	0.882	99
			Untreated control	1045	0.871	98
			Positive control (DMBA)	31	0.026	3
-	24	A2	2000 µg/mL	1060	0.883	99
			1000 µg/mL	1106	0.992	103
			500 µg/mL	1112	0.927	103
			250 µg/mL	1018	0.848	95
			125 µg/mL	1033	0.861	96
			62.5 µg/mL	1080	0.900	100
			31.25 µg/mL	1099	0.916	102
			Negative control	1076	0.897	100
			Negative control for EMS (DMSO)	1074	0.895	100
			Untreated control	1041	0.868	97
			Positive control (EMS)	517	0.431	48

A2 = Assay 2
+ = in the presence of S9-mix
- = in the absence of S9-mix
Negative (vehicle) control = 0.5%(v/v) Acetone
DMSO = Dimethyl sulfoxide
DMBA = 7,12-Dimethylbenz[a]anthracene, 15 µg/mL
EMS = Ethyl methanesulfonate, 0.4 µL/mL

APPENDIX 5

Summary of Viability Results

Table 3: Summarized Viability Results of Assay 1

S9 mix	Treatment period (hours)	Study phase	Test item or control concentration	Total number of colonies	Cloning Efficiency (CE)
+	5	A1	2000 µg/mL	1174	0.978
			1000 µg/mL	1091	0.909
			500 µg/mL	1062	0.885
			250 µg/mL	1138	0.948
			125 µg/mL	1089	0.908
			62.5 µg/mL	1125	0.938
			31.25 µg/mL	1096	0.913
			Negative control	1236	1.030
			Negative control for DMBA (DMSO)	1152	0.960
			Untreated control	1218	1.015
			Positive control (DMBA)	1117	0.931
-	5	A1	2000 µg/mL	1282	1.068
			1000 µg/mL	1107	0.923
			500 µg/mL	1151	0.959
			250 µg/mL	1169	0.974
			125 µg/mL	1140	0.950
			62.5 µg/mL	1233	1.028
			31.25 µg/mL	1380	1.150
			Negative control	1253	1.044
			Negative control for EMS (DMSO)	1191	0.993
			Untreated control	1159	0.966
			Positive control (EMS)	1029	0.858

A1 = Assay 1
+ = in the presence of S9-mix
- = in the absence of S9-mix
DMBA = 7,12-Dimethylbenz[a]anthracene, 15 µg/mL
EMS = Ethyl methanesulfonate, 0.4 µL/mL
Negative (vehicle) control = 0.5%(v/v) Acetone
DMSO = Dimethyl sulfoxide

APPENDIX 5 (continued)

Summary of Viability Results

Table 4: Summarized Viability Results of Assay 2

S9 mix	Treatment period (hours)	Study phase	Test item or control concentration	Total number of colonies	Cloning Efficiency (CE)
+	5	A2	2000 µg/mL	962	0.802
			1000 µg/mL	1062	0.885
			500 µg/mL	1158	0.965
			250 µg/mL	1023	0.853
			125 µg/mL	961	0.801
			62.5 µg/mL	1085	0.904
			31.25 µg/mL	1096	0.913
			Negative control	1079	0.899
			Negative control for DMBA (DMSO)	1008	0.840
			Untreated control	1106	0.922
			Positive control (DMBA)	1159	0.966
-	24	A2	2000 µg/mL	1040	0.867
			1000 µg/mL	1206	1.005
			500 µg/mL	1009	0.841
			250 µg/mL	1044	0.870
			125 µg/mL	1228	1.023
			62.5 µg/mL	1028	0.857
			31.25 µg/mL	1037	0.864
			Negative control	1218	1.015
			Negative control for EMS (DMSO)	1044	0.870
			Untreated control	1095	0.913
			Positive control (EMS)	630	0.525

A2 = Assay 2
+ = in the presence of S9-mix
- = in the absence of S9-mix
DMBA = 7,12-Dimethylbenz[a]anthracene, 15 µg/mL
EMS = Ethyl methanesulfonate, 0.4 µL/mL
Negative (vehicle) control = 0.5%(v/v) Acetone
DMSO = Dimethyl sulfoxide

APPENDIX 6

Summary of Mutagenicity Results

Table 5: Summarized Mutagenicity Results of Assay 1

S9 mix	Treatment period (hours)	Study phase	Test item or control concentration	Total number of colonies	Mutant frequency
+	5	A1	2000 µg/mL	37	9.7
			1000 µg/mL	26	6.9
			500 µg/mL	18	5.1
			250 µg/mL	19	5.0
			125 µg/mL	15	4.1
			62.5 µg/mL	20	5.3
			31.25 µg/mL	16	4.4
			Negative control	19	4.6
			Negative control for DMBA (DMSO)	21	5.6
			Untreated control	20	5.0
			Positive control (DMBA)	1332	358.4**
-	5	A1	2000 µg/mL	19	4.4
			1000 µg/mL	22	6.2
			500 µg/mL	25	6.6
			250 µg/mL	17	4.4
			125 µg/mL	17	4.5
			62.5 µg/mL	13	3.2
			31.25 µg/mL	26	5.5
			Negative control	19	4.6
			Negative control for EMS (DMSO)	22	5.6
			Untreated control	22	5.7
			Positive control (EMS)	1041	303.7**

** = Statistically significant increase (at $p < 0.01$) compared to the relevant vehicle control

A1 = Assay 1
+ = in the presence of S9-mix
- = in the absence of S9-mix
DMBA = 7,12-Dimethylbenz[a]anthracene, 15 µg/mL
EMS = Ethyl methanesulfonate, 0.4 µL/mL
Negative (vehicle) control = 0.5%(v/v) Acetone
DMSO = Dimethyl sulfoxide

Mutant frequencies refer to 10^6 clonable cells.

APPENDIX 6 (continued)

Summary of Mutagenicity Results

Table 6: Summarized Mutagenicity Results of Assay 2

S9 mix	Treatment period (hours)	Study phase	Test item or control concentration	Total number of colonies	Mutant frequency
+	5	A2	2000 µg/mL	14	4.5
			1000 µg/mL	15	4.3
			500 µg/mL	16	4.1
			250 µg/mL	14	4.0
			125 µg/mL	17	5.3
			62.5 µg/mL	20	5.5
			31.25 µg/mL	21	5.7
			Negative control	30	8.3
			Negative control for DMBA (DMSO)	17	5.1
			Untreated control	17	4.6
			Positive control (DMBA)	1378	362.0**
-	24	A2	2000 µg/mL	16	4.6
			1000 µg/mL	19	4.7
			500 µg/mL	31	9.4
			250 µg/mL	28	8.6
			125 µg/mL	18	4.6
			62.5 µg/mL	16	4.7
			31.25 µg/mL	16	4.7
			Negative control	29	7.2
			Negative control for EMS (DMSO)	19	5.4
			Untreated control	18	4.9
			Positive control (EMS)	1659	796.4**

** = Statistically significant increase (at $p < 0.01$) compared to the relevant vehicle control

A2 = Assay 2
+ = in the presence of S9-mix
- = in the absence of S9-mix
DMBA = 7,12-Dimethylbenz[a]anthracene, 15 µg/mL
EMS = Ethyl methanesulfonate, 0.4 µL/mL
Negative (vehicle) control = 0.5%(v/v) Acetone
DMSO = Dimethyl sulfoxide

Mutant frequencies refer to 10^6 clonable cells.

APPENDIX 7

Individual Survival Data of Assay 1

Concentration	Assay 1					
	5-hour treatment, S9+					
	Number of colonies					
	A			B		
Untreated control	173	187	165	193	201	168
Negative (vehicle) control (0.5%(v/v) Acetone)	172	174	178	191	168	178
Negative control for DMBA (DMSO)	164	161	160	150	154	140
2000 µg/mL	147	149	114	131	131	137
1000 µg/mL	147	132	128	147	128	158
500 µg/mL	139	148	140	135	128	138
250 µg/mL	143	149	153	142	145	139
125 µg/mL	156	168	146	149	134	139
62.5 µg/mL	145	150	154	139	147	129
31.25 µg/mL	141	159	156	139	147	142
Positive control (15 µg/mL DMBA)	7	6	5	5	10	8

S9+: in the presence of S9-mix
DMSO: Dimethyl sulfoxide
DMBA: 7,12-Dimethylbenz[a]anthracene

Note: A and B were duplicate cultures.

APPENDIX 7 (continued)

Individual Survival Data of Assay 1

Concentration	Assay 1					
	5-hour treatment, S9-					
	Number of colonies					
	A			B		
Untreated control	162	172	159	178	203	178
Negative (vehicle) control (0.5%(v/v) Acetone)	169	192	182	201	172	180
Negative control for EMS (DMSO)	168	190	173	202	187	192
2000 µg/mL	156	160	157	160	177	198
1000 µg/mL	181	196	187	189	167	161
500 µg/mL	151	160	149	166	200	175
250 µg/mL	182	158	179	172	158	170
125 µg/mL	180	189	167	165	199	164
62.5 µg/mL	160	188	173	191	205	183
31.25 µg/mL	163	166	171	176	168	196
Positive control (0.4 µL/mL EMS)	132	131	129	129	143	157

S9-: in the absence of S9-mix
DMSO: Dimethyl sulfoxide
EMS: Ethyl methanesulfonate

Note: A and B were duplicate cultures.

APPENDIX 8

Individual Survival Data of Assay 2

Concentration	Assay 2					
	5-hour treatment, S9+					
	Number of colonies					
	A			B		
Untreated control	184	179	170	165	175	172
Negative (vehicle) control (0.5%(v/v) Acetone)	182	188	174	187	166	170
Negative control for DMBA (DMSO)	183	168	166	181	172	188
2000 µg/mL	166	151	157	160	147	158
1000 µg/mL	158	148	177	170	150	154
500 µg/mL	153	169	164	176	162	156
250 µg/mL	161	180	175	173	175	182
125 µg/mL	153	167	174	163	169	175
62.5 µg/mL	156	164	163	171	179	165
31.25 µg/mL	155	156	171	171	189	180
Positive control (15 µg/mL DMBA)	10	5	4	3	4	5

S9+: in the presence of S9-mix
DMSO: Dimethyl sulfoxide
DMBA: 7,12-Dimethylbenz[a]anthracene

Note: A and B were duplicate cultures.

APPENDIX 8 (continued)

Individual Survival Data of Assay 2

Concentration	Assay 2					
	24-hour treatment, S9-					
	Number of colonies					
	A			B		
Untreated control	170	190	172	165	173	171
Negative (vehicle) control (0.5%(v/v) Acetone)	193	179	173	179	172	180
Negative control for EMS (DMSO)	175	187	173	178	182	179
2000 µg/mL	170	176	185	171	182	176
1000 µg/mL	197	184	187	184	185	169
500 µg/mL	203	201	190	182	168	168
250 µg/mL	168	159	169	181	167	174
125 µg/mL	171	169	175	166	180	172
62.5 µg/mL	180	190	172	175	169	194
31.25 µg/mL	173	207	171	185	173	190
Positive control (0.4 µL/mL EMS)	97	88	87	92	71	82

S9-: in the absence of S9-mix
DMSO: Dimethyl sulfoxide
EMS: Ethyl methanesulfonate

Note: A and B were duplicate cultures.

APPENDIX 9

Individual Viability Data of Assay 1

Concentration	Assay 1					
	5-hour treatment, S9+					
	Number of colonies					
	A			B		
Untreated control	219	233	207	171	191	197
Negative (vehicle) control (0.5%(v/v) Acetone)	229	216	202	191	188	210
Negative control for DMBA (DMSO)	212	203	204	188	173	172
2000 µg/mL	189	210	215	172	187	201
1000 µg/mL	161	164	180	201	177	208
500 µg/mL	174	187	162	176	172	191
250 µg/mL	201	185	207	174	189	182
125 µg/mL	172	194	176	169	187	191
62.5 µg/mL	179	187	178	200	189	192
31.25 µg/mL	197	192	192	158	183	174
Positive control (15 µg/mL DMBA)	152	178	179	190	207	211

S9+: in the presence of S9-mix
DMSO: Dimethyl sulfoxide
DMBA: 7,12-Dimethylbenz[a]anthracene

Note: A and B were duplicate cultures.

APPENDIX 9 (continued)

Individual Viability Data of Assay 1

Concentration	Assay 1					
	5-hour treatment, S9-					
	Number of colonies					
	A			B		
Untreated control	213	193	181	194	194	184
Negative (vehicle) control (0.5%(v/v) Acetone)	222	211	215	201	196	208
Negative control for EMS (DMSO)	172	222	211	185	192	209
2000 µg/mL	230	226	210	198	197	221
1000 µg/mL	173	170	167	187	206	204
500 µg/mL	188	187	152	200	201	223
250 µg/mL	203	177	173	204	212	200
125 µg/mL	179	206	170	186	194	205
62.5 µg/mL	204	204	197	201	222	205
31.25 µg/mL	227	237	265	219	228	204
Positive control (0.4 µL/mL EMS)	159	179	187	161	172	171

S9-: in the absence of S9-mix
DMSO: Dimethyl sulfoxide
EMS: Ethyl methanesulfonate

Note: A and B were duplicate cultures.

APPENDIX 10

Individual Viability Data of Assay 2

Concentration	Assay 2					
	5-hour treatment, S9+					
	Number of colonies					
	A			B		
Untreated control	175	163	175	216	178	199
Negative (vehicle) control (0.5%(v/v) Acetone)	188	185	183	177	172	174
Negative control for DMBA (DMSO)	148	198	163	165	162	172
2000 µg/mL	162	174	187	128	154	157
1000 µg/mL	185	186	189	179	152	171
500 µg/mL	175	179	192	196	200	216
250 µg/mL	146	139	155	181	212	190
125 µg/mL	151	146	143	167	173	181
62.5 µg/mL	182	167	183	177	204	172
31.25 µg/mL	173	200	178	190	194	161
Positive control (15 µg/mL DMBA)	205	213	215	158	179	189

S9+: in the presence of S9-mix
DMSO: Dimethyl sulfoxide
DMBA: 7,12-Dimethylbenz[a]anthracene

Note: A and B were duplicate cultures.

APPENDIX 10 (continued)

Individual Viability Data of Assay 2

Concentration	Assay 2					
	24-hour treatment, S9-					
	Number of colonies					
	A			B		
Untreated control	182	186	191	190	162	184
Negative (vehicle) control (0.5%(v/v) Acetone	199	179	208	201	215	216
Negative control for EMS (DMSO)	188	177	197	150	162	170
2000 µg/mL	190	165	188	171	175	151
1000 µg/mL	196	202	198	205	207	198
500 µg/mL	176	183	160	152	153	185
250 µg/mL	158	148	149	197	185	207
125 µg/mL	177	191	190	225	224	221
62.5 µg/mL	152	182	161	179	177	177
31.25 µg/mL	144	138	156	200	208	191
Positive control (0.4 µL/mL EMS)	91	98	106	119	111	105

S9-: in the absence of S9-mix
DMSO: Dimethyl sulfoxide
EMS: Ethyl methanesulfonate

Note: A and B were duplicate cultures.

APPENDIX 11

Individual Mutagenicity Data of Assay 1

Concentration	Assay 1									
	5-hour treatment, S9+									
	Number of colonies									
	A					B				
Untreated control	2	2	2	1	3	2	3	1	1	3
Negative (vehicle) control (0.5%(v/v) Acetone)	2	2	2	1	2	1	3	1	2	3
Negative control for DMBA (DMSO)	2	2	2	2	1	3	4	1	1	3
2000 µg/mL	1	1	1	4	4	5	8	5	4	4
1000 µg/mL	1	2	2	1	1	3	4	4	4	4
500 µg/mL	2	0	1	2	2	2	2	3	2	2
250 µg/mL	2	2	3	2	2	2	2	2	1	1
125 µg/mL	2	1	1	3	2	2	2	0	1	1
62.5 µg/mL	1	2	1	2	1	4	2	3	3	1
31.25 µg/mL	3	0	2	2	0	2	1	3	1	2
Positive control (15 µg/mL DMBA)	111	130	132	127	121	161	153	149	123	125

S9+: in the presence of S9-mix
DMSO: Dimethyl sulfoxide
DMBA: 7,12-Dimethylbenz[a]anthracene

Note: A and B were duplicate cultures.

APPENDIX 11 (continued)

Individual Mutagenicity Data of Assay 1

Concentration	Assay 1									
	5-hour treatment, S9-									
	Number of colonies									
	A					B				
Untreated control	4	4	3	1	1	2	2	1	2	2
Negative (vehicle) control (0.5%(v/v) Acetone)	1	2	2	2	2	3	1	2	2	2
Negative control for EMS (DMSO)	2	2	2	2	1	2	2	2	3	4
2000 µg/mL	1	3	2	3	4	1	0	2	1	2
1000 µg/mL	3	3	2	4	4	1	1	2	1	1
500 µg/mL	4	2	3	2	2	2	3	0	2	5
250 µg/mL	2	1	3	1	3	1	2	1	2	1
125 µg/mL	1	1	1	0	4	2	2	2	1	3
62.5 µg/mL	2	3	0	1	2	0	0	2	1	2
31.25 µg/mL	6	2	2	4	6	1	1	2	1	1
Positive control (0.4 µL/mL EMS)	92	112	105	100	107	99	106	104	106	110

S9-: in the absence of S9-mix
DMSO: Dimethyl sulfoxide
EMS: Ethyl methanesulfonate

Note: A and B were duplicate cultures.

APPENDIX 12

Individual Mutagenicity Data of Assay 2

Concentration	Assay 2									
	5-hour treatment, S9+									
	Number of colonies									
	A					B				
Untreated control	2	2	1	1	2	2	1	3	1	2
Negative (vehicle) control (0.5%(v/v) Acetone)	1	6	1	3	4	1	7	3	3	1
Negative control for DMBA (DMSO)	1	2	2	2	1	1	2	2	2	2
2000 µg/mL	1	1	1	2	1	2	0	3	1	2
1000 µg/mL	1	1	3	0	1	0	4	2	1	2
500 µg/mL	3	0	1	1	2	1	2	2	3	1
250 µg/mL	2	1	0	1	1	2	3	1	0	3
125 µg/mL	1	2	2	1	2	2	2	2	2	1
62.5 µg/mL	3	0	2	1	2	4	2	1	2	3
31.25 µg/mL	3	1	1	2	4	1	2	2	3	2
Positive control (15 µg/mL DMBA)	123	130	139	145	105	150	144	144	142	156

S9+: in the presence of S9-mix
DMSO: Dimethyl sulfoxide
DMBA: 7,12-Dimethylbenz[a]anthracene

Note: A and B were duplicate cultures.

APPENDIX 12 (continued)

Individual Mutagenicity Data of Assay 2

Concentration	Assay 2									
	24-hour treatment, S9-									
	Number of colonies									
	A					B				
Untreated control	1	2	2	2	2	2	2	1	2	2
Negative (vehicle) control (0.5%(v/v) Acetone)	5	2	4	5	1	3	2	1	2	4
Negative control for EMS (DMSO)	2	3	1	3	2	2	2	1	2	1
2000 µg/mL	3	1	2	1	2	0	1	2	2	2
1000 µg/mL	1	0	3	6	1	1	2	4	1	0
500 µg/mL	2	2	1	1	2	6	6	4	4	3
250 µg/mL	5	4	1	5	5	1	3	1	2	1
125 µg/mL	2	1	3	3	4	1	1	0	2	1
62.5 µg/mL	2	1	1	5	1	2	1	0	1	2
31.25 µg/mL	2	2	0	1	2	3	3	1	1	1
Positive control (0.4 µL/mL EMS)	150	172	187	181	192	138	157	152	172	158

S9-: in the absence of S9-mix
DMSO: Dimethyl sulfoxide
EMS: Ethyl methanesulfonate

Note: A and B were duplicate cultures.

APPENDIX 13

Observations in the Main Tests

Assay 1: 5-hour treatment in the presence of metabolic activation

Test item (or vehicle) concentration	pH after treatment (Cultures A / B)		Osmolality after treatment (Cultures A / B)		Observations at the beginning / after treatment
Untreated control	7.0	7.0	303	301	normal / normal
Negative (vehicle) control (0.5%(v/v) Acetone)	7.0	7.0	299	300	normal / normal
Negative control for DMBA (DMSO)	7.0	7.0	447	445	normal / normal
2000 µg/mL	7.0	7.0	295	298	discoloured medium[#] / discoloured medium[#]
1000 µg/mL	7.0	7.0	300	301	discoloured medium[#], precipitate / discoloured medium[#], precipitate[#]
500 µg/mL	7.0	7.0	300	303	discoloured medium[#], precipitate[#] / discoloured medium[#]
250 µg/mL	7.0	7.0	293	295	discoloured medium[#] / discoloured medium[#]
125 µg/mL	7.0	7.0	302	300	discoloured medium[#] / discoloured medium[#]
62.5 µg/mL	7.0	7.0	298	301	discoloured medium[#] / discoloured medium[#]
31.25 µg/mL	7.0	7.0	303	299	discoloured medium[#] / discoloured medium[#]
Positive control (15 µg/mL DMBA)	7.0	7.0	434	436	normal / normal

#: minimal amount
DMSO: Dimethyl sulfoxide
DMBA: 7,12-Dimethylbenz[a]anthracene

APPENDIX 13 (continued)

Observations in the Main Tests

Assay 1: 5-hour treatment in the absence of metabolic activation

Test item (or vehicle) concentration	pH after treatment (Cultures A / B)		Osmolality after treatment (Cultures A / B)		Observations at the beginning / after treatment
Untreated control	7.0	7.0	305	303	normal / normal
Negative (vehicle) control (0.5%(v/v) Acetone)	7.0	7.0	301	300	normal / normal
Negative control for EMS (DMSO)	7.0	7.0	457	455	normal / normal
2000 µg/mL	7.0	7.0	298	297	discoloured medium[#]/ discoloured medium[#]
1000 µg/mL	7.0	7.0	305	302	discoloured medium[#], precipitate/ discoloured medium[#], precipitate[#]
500 µg/mL	7.0	7.0	308	306	discoloured medium[#], precipitate[#]/ discoloured medium[#]
250 µg/mL	7.0	7.0	302	303	discoloured medium[#] / discoloured medium[#]
125 µg/mL	7.0	7.0	303	303	discoloured medium[#] / discoloured medium[#]
62.5 µg/mL	7.0	7.0	302	301	normal / discoloured medium[#]
31.25 µg/mL	7.0	7.0	307	305	normal / discoloured medium[#]
Positive control (0.4 µl/mL EMS)	7.0	7.0	436	432	normal / normal

#: minimal amount
DMSO: Dimethyl sulfoxide
EMS: Ethyl methanesulfonate

APPENDIX 13 (continued)

Observations in the Main Tests

Assay 2: 5-hour treatment in the presence of metabolic activation

Test item (or vehicle) concentration	pH after treatment (Cultures A / B)		Osmolality after treatment (Cultures A / B)		Observations at the beginning / after treatment
Untreated control	7.0	7.0	297	302	normal / normal
Negative (vehicle) control (0.5%(v/v) Acetone)	7.0	7.0	306	302	normal / normal
Negative control for DMBA (DMSO)	7.0	7.0	452	450	normal / normal
2000 µg/mL	7.0	7.0	318	312	discoloured medium#, precipitate / discoloured medium#, precipitate
1000 µg/mL	7.0	7.0	306	306	discoloured medium#, precipitate / discoloured medium#, precipitate#
500 µg/mL	7.0	7.0	315	313	discoloured medium#, precipitate#/ discoloured medium#, precipitate#
250 µg/mL	7.0	7.0	307	315	discoloured medium#, precipitate#/ discoloured medium#
125 µg/mL	7.0	7.0	304	303	discoloured medium# / discoloured medium#
62.5 µg/mL	7.0	7.0	309	306	discoloured medium# / discoloured medium#
31.25 µg/mL	7.0	7.0	316	316	discoloured medium# / discoloured medium#
Positive control (15 µg/mL DMBA)	7.0	7.0	439	442	normal / normal

#: minimal amount
DMSO: Dimethyl sulfoxide
DMBA: 7,12-Dimethylbenz[a]anthracene

APPENDIX 13 (continued)

Observations in the Main Tests

Assay 2: 24-hour treatment in the absence of metabolic activation

Test item (or vehicle) concentration	pH after treatment (Cultures A / B)		Osmolality after treatment (Cultures A / B)		Observations at the beginning / after treatment
Untreated control	7.0	7.0	311	312	normal / normal
Negative (vehicle) control (0.5%(v/v) Acetone	7.0	7.0	307	305	normal / normal
Negative control for EMS (DMSO)	7.0	7.0	455	469	normal / normal
2000 µg/mL	7.0	7.0	301	307	discoloured medium#, precipitate / discoloured medium#, precipitate#
1000 µg/mL	7.0	7.0	303	301	discoloured medium#, precipitate / discoloured medium#, precipitate#
500 µg/mL	7.0	7.0	308	324	discoloured medium#, precipitate# / discoloured medium#, precipitate#
250 µg/mL	7.0	7.0	303	314	discoloured medium# / discoloured medium#
125 µg/mL	7.0	7.0	314	308	normal / discoloured medium#
62.5 µg/mL	7.0	7.0	300	306	normal / normal
31.25 µg/mL	7.0	7.0	304	315	normal / normal
Positive control (0.4 µl/mL EMS)	7.0	7.0	431	429	normal / normal

#: minimal amount
DMSO: Dimethyl sulfoxide
EMS: Ethyl methanesulfonate

APPENDIX 14

Cell Counting Results of the Main Tests

Assay 1: 5-hour treatment in the presence of metabolic activation

Test item (or vehicle) concentration	Cell number after treatment (Day 1) (Cultures A / B)		Cell number on Day 3 (Cultures A / B)		Cell number on Day 6 (Cultures A / B)		Cell number on Day 8 (Cultures A / B)	
Untreated control	5.45E+06	5.45E+06	1.30E+07	1.00E+07	8.20E+06	6.90E+06	4.86E+06	5.70E+06
Negative (vehicle) control (0.5%(v/v) Acetone)	4.70E+06	4.25E+06	8.60E+06	9.20E+06	7.40E+06	6.70E+06	4.89E+06	5.55E+06
Negative control for DMBA (DMSO)	5.00E+06	5.10E+06	9.10E+06	7.80E+06	6.80E+06	6.40E+06	5.01E+06	6.42E+06
2000 µg/mL	4.65E+06	4.65E+06	8.80E+06	7.50E+06	7.30E+06	6.50E+06	5.49E+06	5.94E+06
1000 µg/mL	4.70E+06	4.20E+06	7.30E+06	7.00E+06	6.50E+06	6.60E+06	6.66E+06	5.85E+06
500 µg/mL	4.25E+06	4.75E+06	7.80E+06	7.30E+06	6.60E+06	6.90E+06	5.85E+06	5.82E+06
250 µg/mL	4.80E+06	4.90E+06	8.90E+06	7.50E+06	6.70E+06	6.50E+06	5.19E+06	6.09E+06
125 µg/mL	4.05E+06	5.05E+06	8.70E+06	7.30E+06	6.80E+06	6.30E+06	5.70E+06	6.78E+06
62.5 µg/mL	4.35E+06	4.60E+06	7.80E+06	7.50E+06	7.20E+06	6.20E+06	5.64E+06	6.09E+06
31.25 µg/mL	4.40E+06	4.90E+06	8.90E+06	7.10E+06	5.60E+06	6.40E+06	6.24E+06	6.45E+06
Positive control (15 µg/mL DMBA)	1.45E+07	1.68E+07	6.75E+05	5.50E+05	3.70E+06	3.85E+06	7.32E+06	9.36E+06

DMSO: Dimethyl sulfoxide
DMBA: 7,12-Dimethylbenz[a]anthracene

APPENDIX 14 (continued)

Cell Counting Results of the Main Tests

Assay 1: 5-hour treatment in the absence of metabolic activation

Test item (or vehicle) concentration	Cell number after treatment (Day 1) (Cultures A / B)		Cell number on Day 3 (Cultures A / B)		Cell number on Day 6 (Cultures A / B)		Cell number on Day 8 (Cultures A / B)	
Untreated control	1.04E+07	9.80E+06	8.10E+06	8.10E+06	7.70E+06	7.30E+06	6.27E+06	6.60E+06
Negative (vehicle) control (0.5%(v/v) Acetone)	9.00E+06	9.05E+06	7.10E+06	7.80E+06	6.40E+06	6.60E+06	5.88E+06	6.39E+06
Negative control for EMS (DMSO)	9.60E+06	8.90E+06	6.70E+06	8.10E+06	7.90E+06	6.50E+06	8.49E+06	7.26E+06
2000 µg/mL	1.08E+07	8.70E+06	7.40E+06	9.40E+06	7.50E+06	6.50E+06	6.09E+06	6.90E+06
1000 µg/mL	8.50E+06	8.85E+06	8.90E+06	8.00E+06	7.10E+06	7.40E+06	7.56E+06	7.05E+06
500 µg/mL	1.12E+07	8.50E+06	8.10E+06	8.80E+06	7.40E+06	7.20E+06	7.35E+06	6.36E+06
250 µg/mL	9.25E+06	9.60E+06	8.10E+06	7.70E+06	7.00E+06	6.70E+06	7.53E+06	6.96E+06
125 µg/mL	9.15E+06	8.40E+06	8.10E+06	7.40E+06	6.80E+06	8.10E+06	7.32E+06	6.27E+06
62.5 µg/mL	1.00E+07	9.40E+06	7.30E+06	7.40E+06	7.00E+06	6.70E+06	5.82E+06	6.57E+06
31.25 µg/mL	1.01E+07	9.65E+06	8.60E+06	8.30E+06	7.10E+06	6.60E+06	5.64E+06	7.41E+06
Positive control (0.4 µl/mL EMS)	6.97E+07	6.42E+07	6.90E+06	6.10E+06	5.00E+06	5.90E+06	6.12E+06	5.46E+06

DMSO: Dimethyl sulfoxide
EMS: Ethyl methanesulfonate

APPENDIX 14 (continued)

Cell Counting Results of the Main Tests

Assay 2: 5-hour treatment in the presence of metabolic activation

Test item (or vehicle) concentration	Cell number after treatment (Day 1) (Cultures A / B)		Cell number on Day 3 (Cultures A / B)		Cell number on Day 6 (Cultures A / B)		Cell number on Day 8 (Cultures A / B)	
Untreated control	5.05E+06	5.05E+06	6.50E+06	6.40E+06	6.80E+06	8.00E+06	6.69E+06	5.88E+06
Negative (vehicle) control (0.5%(v/v) Acetone)	4.55E+06	5.15E+06	6.10E+06	7.00E+06	7.90E+06	7.40E+06	5.94E+06	6.06E+06
Negative control for DMBA (DMSO)	4.55E+06	4.25E+06	7.30E+06	7.20E+06	8.40E+06	5.60E+06	6.90E+06	6.96E+06
2000 µg/mL	4.25E+06	4.30E+06	6.50E+06	7.10E+06	7.50E+06	7.30E+06	6.36E+06	7.11E+06
1000 µg/mL	4.05E+06	4.05E+06	6.10E+06	7.00E+06	7.10E+06	6.80E+06	6.60E+06	6.48E+06
500 µg/mL	4.60E+06	4.60E+06	6.90E+06	6.10E+06	7.30E+06	7.00E+06	6.57E+06	5.55E+06
250 µg/mL	4.05E+06	4.00E+06	6.20E+06	6.70E+06	6.70E+06	6.10E+06	7.77E+06	6.12E+06
125 µg/mL	4.10E+06	4.15E+06	6.40E+06	7.20E+06	7.20E+06	7.40E+06	7.80E+06	6.51E+06
62.5 µg/mL	4.30E+06	4.25E+06	7.70E+06	6.50E+06	7.00E+06	6.30E+06	6.51E+06	7.20E+06
31.25 µg/mL	4.55E+06	4.70E+06	6.80E+06	7.40E+06	7.30E+06	6.10E+06	6.03E+06	6.60E+06
Positive control (15 µg/mL DMBA)	1.87E+07	2.21E+07	9.75E+05	5.25E+05	1.30E+06	1.00E+06	4.98E+06	5.55E+06

DMSO: Dimethyl sulfoxide
DMBA: 7,12-Dimethylbenz[a]anthracene

APPENDIX 14 (continued)

Cell Counting Results of the Main Tests

Assay 2: 24-hour treatment in the absence of metabolic activation

Test item (or vehicle) concentration	Cell number after treatment (Day 1) (Cultures A / B)		Cell number on Day 3 (Cultures A / B)		Cell number on Day 6 (Cultures A / B)		Cell number on Day 8 (Cultures A / B)	
Untreated control	7.55E+06	8.55E+06	7.40E+06	7.40E+06	9.10E+06	6.80E+06	5.82E+06	6.57E+06
Negative (vehicle) control (0.5%(v/v) Acetone)	9.20E+06	7.75E+06	8.10E+06	9.90E+06	6.50E+06	6.30E+06	6.06E+06	5.70E+06
Negative control for EMS (DMSO)	7.30E+06	7.75E+06	6.20E+06	8.30E+06	7.70E+06	7.20E+06	5.94E+06	6.39E+06
2000 µg/mL	7.55E+06	8.80E+06	7.80E+06	7.20E+06	6.30E+06	7.80E+06	6.48E+06	6.69E+06
1000 µg/mL	7.85E+06	9.25E+06	7.00E+06	8.20E+06	6.90E+06	7.10E+06	5.88E+06	6.21E+06
500 µg/mL	7.65E+06	9.15E+06	6.80E+06	8.40E+06	6.30E+06	7.20E+06	6.75E+06	6.12E+06
250 µg/mL	9.30E+06	8.50E+06	6.30E+06	7.80E+06	7.60E+06	6.10E+06	6.48E+06	6.18E+06
125 µg/mL	8.65E+06	1.00E+07	7.60E+06	6.90E+06	7.60E+06	7.30E+06	6.12E+06	5.79E+06
62.5 µg/mL	7.95E+06	8.20E+06	7.20E+06	9.00E+06	8.00E+06	6.90E+06	6.03E+06	5.88E+06
31.25 µg/mL	7.55E+06	8.05E+06	8.60E+06	6.70E+06	6.90E+06	7.80E+06	6.90E+06	6.57E+06
Positive control (0.4 µl/mL EMS)	5.52E+07	5.56E+07	5.20E+06	4.20E+06	4.10E+06	4.20E+06	2.94E+06	3.09E+06

DMSO: Dimethyl sulfoxide
EMS: Ethyl methanesulfonate

APPENDIX 15

Historical Control Data

(updated on 17 October 2017 using data of GLP studies)

	Mutation frequency (Number of 6-TG resistant mutants per 10^6 clonable cells)		
	Untreated control		
	5-hour, S9+	5-hour, S9-	24-hour, S9-
mean	18.3	20.7	19.0
standard deviation	15.1	16.4	17.2
minimum	5.1	5.5	3.3
maximum	64.1	55.5	58.0
n	27	13	14
	DMSO control		
	5-hour, S9+	5-hour, S9-	24-hour, S9-
mean	21.8	18.9	18.4
standard deviation	15.9	11.6	14.4
minimum	5.4	6.5	6.8
maximum	57.3	47.4	48.5
n	29	13	14
	Distilled water / Water based vehicle control		
mean	11.5	9.1	15.5
standard deviation	3.8	3.4	5.6
minimum	6.1	5.2	9.2
maximum	15.8	11.6	20.1
n	6	3	3
	Positive controls		
	DMBA	EMS	EMS
	5-hour, S9+	5-hour, S9-	24-hour, S9-
mean	905.2	445.6	1176.6
standard deviation	562.7	118.6	610.9
minimum	141.2	239.6	363.1
maximum	2119.4	636.6	2449.8
n	27	13	14

DMSO = Dimethyl sulfoxide
DMBA = 7,12-Dimethylbenz[a]anthracene
EMS = Ethyl methanesulfonate
S9+ = in the presence of S9-mix
S9- = in the absence of S9-mix

APPENDIX 16

Copy of the GLP Certificate

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Ref. no: OGYI/19440-7/2015
Admin.: Szatmári Andrea
Date: 22 September, 2015

GOOD LABORATORY PRACTICE (GLP)
CERTIFICATE

It is hereby certified that the test facility

CiToxLAB Hungary Ltd.

H-8200 Veszprém, Szabadságpuszta

is able to carry out

physico-chemical testing, toxicity studies, in vitro studies and mutagenicity studies, environmental toxicity studies on aquatic or terrestrial organisms, studies on behaviour in water, soil and air; bio-accumulation, reproduction toxicology, inhalation toxicology, analytical chemistry and contract archiving

in compliance with the Principles of GLP (Good Laboratory Practice) and also complies with the corresponding OECD/European Community requirements.

Date of the inspection: **02-04. June 2015.**

Dr. József Reiter
Deputy Director-General

Note: Translation of the Stamp on the official document ("Országos Gyógyszerészeti és Élelmezés-egészségügyi Intézet"): "National Institute of Pharmacy and Nutrition"