FINAL REPORT

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

Study code: 17/082-015C

Study Director:

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 were 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 was considered not to prejudice the overall GLP status of the study and the scientific reliability of the study conclusions.

Signature: _____ Date: 25 January 2018

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 "APFHx: *In vitro* Mammalian Cell Gene Mutation Test (HPRT Assay)" has been performed according to the GLP requirements.

Signature: _ Date: 23 January 2018

Director of Science and Regulatory Affairs

QUALITY ASSURANCE STATEMENT

Study Code: 17/082-015C

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

Test Item: APFHx

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
07 June 2017	Study Plan	07 June 2017	07 June 2017
27 September 2017	Amendment 1 to the Study Plan	27 September 2017	27 September 2017
09 November 2017	Formulation	09 November 2017	09 November 2017
23 January 2018	Draft Report	23 January 2018	23 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: APFHx: *In Vitro* Mammalian Cell Gene Mutation Test (HPRT Assay)

TEST ITEM: APFHx

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, Technical assistant
Head of Pharmacy
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*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:	08 June 2017
PRELIMINARY EXPERIMENT:	26 June - 02 July 2017
AMENDMENT 1 TO THE STUDY PLAN:	27 September 2017
START OF EXPERIMENT:	17 October 2017
END OF EXPERIMENT:	28 November 2017
DRAFT REPORT:	24 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 APFHx 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).

Distilled water 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

5-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, insolubility (minimal amount of precipitate) was detected at 2000 – 125 µg/mL concentration range in the final treatment medium at the end of the treatment in the experiments with metabolic activation. The precipitation did not interfere with the reading of the results. There were no large changes in pH and osmolality after treatment in any cases.

In Assay 2, insolubility (minimal amount of precipitate) was detected at 2000 and 1000 µg/mL concentrations in the final treatment medium at the end of the treatment in the experiments with metabolic activation. The precipitation did not interfere with the reading of the results. 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. Statistically significant increase (at $p<0.01$ level) was observed in this experiment at 2000 µg/mL concentration, although the observed value was within the general historical control range. Furthermore, the observed mutant frequencies (7.9×10^{-6}) was within the expected range of the negative control samples according to the relevant OECD guideline (expected range: $5\text{-}20 \times 10^{-6}$). No dose response to the treatment was observed (a trend analysis showed no effect of treatment). The negative result was confirmed by the Assay 1 without metabolic activation

The spontaneous mutation frequency of the negative (vehicle) control was in accordance with the general 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 APFHx 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 APFHx 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:	APFHx
Batch/Lot No.:	C150E57002
CAS number:	21615-47-4
Expiry date:	December 2020
Description:	White solid
Purity:	99.8%
Storage condition:	Room temperature, protected from humidity
Safety precautions:	Routine safety precautions (lab coat, gloves, safety glasses and face mask) for unknown materials will be applied to assure personnel health and safety.

Data are based on the Test Item Data Sheet (dated 17 February 2017), Material Safety Data Sheet (dated 21 February 2017) and additional information provided by the Sponsors.

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. Test Item Data Sheet 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 available information provided by the Sponsor and the performed trial formulation, the test item was soluble in Distilled water. The formulation of 200 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 (no correction for purity of the test item was applied). 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.: R6SA39062, Expiry date: December 2019 was used in the Preliminary experiment and Lot No.: R7CA72998, Expiry date: March 2020 was used in the main assays) 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.

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, Distilled water 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:	Distilled water
Supplier:	B. Braun Pharmaceuticals SA
Lot No.:	63352Y25-2 / 71033Y25-2
Appearance:	Clear colourless liquid
Expiry date:	31 July 2019 / 29 February 2020
Storage conditions:	Room temperature

*Note: Lot 63352Y25-2 was used in the preliminary experiment; lot 71033Y25-2 was used in Assays 1-2.

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
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 (as detailed in 5.1.4.).

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 37°C (± 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, 398-621 g, animals were 11-14 weeks old at initiation of E12591 and 321-412 g and 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), 16 January 2017 (E12591) 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), 19 January 2017 (Citoxlab code: E12591) and 14 September 2017 (Citoxlab code: E12713)*.

*Note: Batch of E12590 was used in the preliminary experiment, batch E12591 was used in the assay 1 and batch E12713 was used in the assay 2.

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), 29.4 g/L (E12591) 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 200 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 1, 3 and 6.

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 (Distilled water)	10 µL/mL +S9 mix 100 µL/mL
		Negative control for DMBA (DMSO)	10 µL/mL + S9 mix 100 µL/mL
		APFHx	2000 µg/mL + S9 mix 100 µL/mL
		APFHx	1000 µg/mL + S9 mix 100 µL/mL
		APFHx	500 µg/mL + S9 mix 100 µL/mL
		APFHx	250 µg/mL + S9 mix 100 µL/mL
		APFHx	125 µg/mL + S9 mix 100 µL/mL
		APFHx	62.5 µg/mL + S9 mix 100 µL/mL
		APFHx	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 (Distilled water)	10 µL/mL
		Negative control for EMS (DMSO)	10 µL/mL
		APFHx	2000 µg/mL
		APFHx	1000 µg/mL
		APFHx	500 µg/mL
		APFHx	250 µg/mL
		APFHx	125 µg/mL
		APFHx	62.5 µg/mL
		APFHx	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 (Distilled water)	10 µL/mL +S9 mix 100 µL/mL
		Negative control for DMBA (DMSO)	10 µL/mL + S9 mix 100 µL/mL
		APFHx	2000 µg/mL + S9 mix 100 µL/mL
		APFHx	1000 µg/mL + S9 mix 100 µL/mL
		APFHx	500 µg/mL + S9 mix 100 µL/mL
		APFHx	250 µg/mL + S9 mix 100 µL/mL
		APFHx	125 µg/mL + S9 mix 100 µL/mL
		APFHx	62.5 µg/mL + S9 mix 100 µL/mL
		APFHx	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 (Distilled water)	10 µL/mL
		Negative control for EMS (DMSO)	10 µL/mL
		APFHx	2000 µg/mL
		APFHx	1000 µg/mL
		APFHx	500 µg/mL
		APFHx	250 µg/mL
		APFHx	125 µg/mL
		APFHx	62.5 µg/mL
		APFHx	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 (100 µL) of vehicle (solvent), test item solution or positive control solution 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 (100 µL) of vehicle (solvent), test item solution or positive control solution 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 x 10^5 cells/mL	0.1	9.9	2 x 10^3 cells/mL	0.4	19.6	40 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, or on Days 4, 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^n	Dilution		Intermediate cell conc^n	Dilution		Final cell conc^n
	A	mL A	mL F12-10	(B)	mL B	mL F12-10	(C)
Viability	4×10^5 cells/mL	0.1	9.9	4×10^3 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*.

*Note: The counting was assisted with inverted microscope in the Assay 2, half area of the dishes was counted and this was multiplied by 2, therefore even numbers were obtained. However, no marked cytotoxicity of the test item was observed therefore this fact had no impact on the study on the results or integrity of the study.

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
Antibiotic-antimycotic solution	096M4760V	Sigma-Aldrich Co.	October 2018
Distilled water	805 0417	HungaroPharma	05 October 2017
Distilled water	803 0117	HungaroPharma	30 July 2017
Distilled water	808 0617	HungaroPharma	06 December 2017
Distilled water	811 0717	HungaroPharma	26 January 2018
Distilled water	815 0917	HungaroPharma	25 March 2018
Distilled water	71033Y25-2	B. Braun	29 February 2020
EX-CELL(TM) CD CHO Medium*	SLBR4268V	Sigma-Aldrich Co.	November 2017
Fetal Bovine Serum**	17C438	Sigma-Aldrich Co.	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	RNBG0877	Sigma-Aldrich Co.	June 2019
L-Glutamine	RNBF9654	Sigma-Aldrich Co.	March 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****	RNBF7902	Sigma-Aldrich Co.	December 2017
Nutrient Mixture F-12 HAM****	RNBG0928	Sigma-Aldrich Co.	May 2018
Nutrient Mixture F-12 HAM****	RNBF8873	Sigma-Aldrich Co.	January 2018
Phosphate buffered saline (PBS), 10X	SLBS5708	Sigma-Aldrich Co.	January 2018
Phosphate buffered saline (PBS), 10X	SLBH9017	Sigma-Aldrich Co.	September 2017
Potassium chloride	K45349336	MERCK	28 February 2019
6-Thioguanine	SLBQ5479V	Sigma-Aldrich Co.	30 September 2018
Trypsin-EDTA solution, 0.25%	SLBR8841V	Sigma-Aldrich Co.	February 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 (Lot: SLBR4268V)
**: 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 was 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 of S9-mix, the following concentrations were examined: 2000, 1000, 500, 250, 125, 62.5 and 31.25 µg/mL.

For the 5-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 1, insolubility (minimal amount of precipitate) was detected at 2000 – 125 µg/mL concentration range in the final treatment medium at the end of the treatment in the experiments with metabolic activation. The precipitation did not interfere with the reading of the results. 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 103% 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 of Appendix 6) 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 88% 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 of Appendix 6) 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 (minimal amount of precipitate) was detected at 2000 and 1000 µg/mL concentrations in the final treatment medium at the end of the treatment in the experiments with metabolic activation. The precipitation did not interfere with the reading of the results. 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% 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 of Appendix 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 84% on the survival plates), thus an evaluation was made using data of all seven concentrations. Statistically significant increase (at $p<0.01$ level) was observed in this experiment at 2000 µg/mL concentration (see Table 6 of Appendix 6), although the observed value was within the general historical control range. Furthermore, the observed mutant frequencies (7.9×10^{-6}) was within the expected range of the negative control samples according to the relevant OECD guideline (expected range: $5\text{-}20 \times 10^{-6}$). No dose response to the treatment was observed (a trend analysis showed no effect of treatment). The negative result was confirmed by the 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 $x10^{-6}$ according to 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 ($5\text{-}20 \times 10^{-6}$) 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 APFHx 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 APFHx 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

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A P P E N D I C E S

APPENDIX 1

Study Schedule

PRELIMINARY TOXICITY TEST

Plating of cells	:	25 June 2017
Treatment of the cells (Day 0)	:	26 June 2017
End of counting after treatment	:	02 July 2017

MUTATION ASSAYS

Assay 1

Plating of cells	:	16 October 2017
Treatment of the cells (Day 0)	:	17 October 2017
Start of expression period	:	18 October 2017
Staining of survival plates	:	23 October 2017
End of expression period	:	25 October 2017
Staining of viability plates	:	30 October 2017
Staining of mutagenicity plates	:	01 November 2017
End of colony counting	:	04 November 2017

Assay 2

Plating of cells	:	08 November 2017
Treatment of the cells (Day 0)	:	09 November 2017
Start of expression period	:	10 November 2017
Staining of survival plates	:	15 November 2017
End of expression period	:	17 November 2017
Staining of viability plates	:	22 November 2017
Staining of mutagenicity plates	:	24 November 2017
End of colony counting	:	28 November 2017

APPENDIX 2

Copy of the Test Item Data Sheet

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TEST ITEM DATA SHEET
CiToxLAB Hungary Ltd.

This questionnaire allows us to safely store, handle and properly administer your test substance. All data will be treated as strictly confidential. Please also send a Certificate of Analysis (CoA) and Safety Data Sheet (SDS) if available.

1. SPONSOR (as defined by GLP)

Company *	Daikin industries ltd.	**Contact Person ***	
Address *	1-1, Nishi-Hitotsuya, Settsu, Osaka 566-8585, japan	**Phone**	81-6-6349-5336
		Fax	
		E-mail *	

2. TEST ITEM INFORMATION

Name * (As it should be used in report)	APFHx			
Substance Classification*	☐ Pharmaceutical	☒ Industrial Chemical	☐ Agrochemical	☐ Other
Chemical Name (IUPAC, CAS if applicable)	ammonium undecafluorohexanoate			
Other Names, Synonyms (if applicable)	C-1500N			
Batch (Lot) Number *	C150E57002			☐ See CoA / Container
Description * (Colour, Appearance at 20 °C)	solid			
Expiry (Retest) date * (day-month-year)	December 2020	☐ See CoA / Container	**If not indicated, 1 year from date of receipt is applied.**	
CAS number (if available)	21615-47-4	**Molecular Weight** (if applicable)	331.08	☐ Salt form
Molecular Formula (if applicable)	C6H4F11NO2	**Quantity *** (Sent by Sponsor)	20g	☐ See label
Purity / Concentration * (Otherwise treated as 100%)	99.8%			☐ See CoA / Container
Storage conditions * Test item will be stored at **Room temperature (15-25°C, ≤ 70 RH%)** unless specified otherwise (Storeroom is well ventilated and protected from direct sunlight.)	☐ Refrigerated (2-8°C)	☐ Frozen (≤ -15°C)	☐ Ultra-low (≤ -70°C)	
	☐ Protected from humidity (tight closed container)		☒ Protected from humidity (beside silica)	
	☐ Protected from light	☐ Under inert gas (e.g. N_2)	☐ See CoA / Container	
	☐ Other:			
Hazards information Please provide SDS or indicate known or suspected hazards, if no SDS is available	☐ Unknown	☐ Flammable	☐ Oxidising	☐ Corrosive
	☒ Irritant (eye)	☐ Oral toxicity	☐ Inhalation toxicity	☐ Teratogenic
	☐ Mutagenic	☐ Carcinogenic	☐ Hazardous to the aquatic environment	
	☐ Other:			
Safety precautions (if no SDS is available)				

* = *mandatory fields*

APPENDIX 2 (continued)

Copy of the Test Item Data Sheet

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3. PHYSICAL-CHEMICAL PARAMETERS AND SOLUBILITY INFORMATION					
pH (at g/L at °C)		Density (g/cm³ at °C)		☐ See CoA / SDS	
Other known parameters (e.g. melting point, boiling point, vapour pressure, viscosity, etc)					
Solution / suspension preparation The following may be used as vehicles, solvents or suspending agents. Please indicate suitability (mark with X)	☒ Water ☐ Saline ☐ 1% Aq. Methylcellulose ☒ Ethanol ☐ Methanol ☐ Propylene glycol ☐ Polyethylene glycol ☐ Methyl ethyl ketone ☐ Corn oil ☐ Olive oil ☐ Acetone ☐ Acetonitrile ☐ Dimethyl sulfoxide ☐ *N,N*-Dimethylformamide ☐ Other:				
Preferred vehicle(s) and known stability data (concentration, temperature, duration, pH value, light conditions, etc.)					
Analytical method for quantitative analysis	☐ Not available ☐ Available and attached ☐ Can be provided upon request				
4. FORMULATION, SAMPLE PREPARATION AND OTHER INSTRUCTIONS					
Test item can be heated	☒ Yes (up to 100°C) ☐ No	Grinding in a mortar or in a mill is acceptable	☒ Yes ☐ Not applicable		
Dose formulation or solution can be heated	☒ Yes (up to 100°C) ☐ No	Sonication can be used to aid dissolution	☒ Yes ☐ Not applicable		
Incompatible or may interfere with material(s)					
Other test item handling instructions					
5. TEST ITEM SHIPMENT (If no information is provided, test item can be shipped as a normal package)					
Shipment conditions (please mark with X)	☒ Normal package ☐ Icepacks ☐ Dry ice ☐ Other:				
Shipping restrictions (if no SDS is available)					
6. DISPOSAL (If no information provided, test item will be disposed after finalisation of all studies)					
Remaining Test item * (please mark with X)	☒ Dispose ☐ Return ☐ Specified at General remarks				

- Unless required otherwise, test item will be disposed in a safe and proper manner after finalization of all studies or 3 months after sending the (draft) reports.
- In accordance with GLP regulations CiToxLAB Hungary Ltd. reserves the right to retain a reference sample per each test item for archiving purpose.
- Returning test item will be at the expense of the Sponsor. If additional charges are applicable the Sponsor's agreement will be obtained prior to dispatch.

General remarks			
On behalf of Sponsor * (Signature)		Date * (day-month-year)	17-2-2017

* = *mandatory fields*

Delivery Address:
Pharmacy Department
CiToxLAB Hungary Ltd.
H-8200 Veszprem, Szabadsagpuszta,
HUNGARY
Tel: +36 88 545 300
Fax: +36 88 545 301
E-mail: test.item@hu.citoxlab.com

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	5.90E+06 (92)	8.40E+06 (114)	7.80E+06 (110)	normal / normal (pH: 7.0; osm: 301 mmol/kg)
Negative (vehicle) control	6.40E+06 (100)	7.40E+06 (100)	7.10E+06 (100)	normal / normal (pH: 7.0; osm: 295 mmol/kg)
2000 µg/mL	5.80E+06 (91)	7.60E+06 (103)	6.80E+06 (96)	precipitate# / discoloured medium#, precipitate# (pH: 7.0; osm: 304 mmol/kg)
1000 µg/mL	6.05E+06 (95)	8.50E+06 (115)	7.60E+06 (107)	precipitate# / discoloured medium#, precipitate# (pH: 7.0; osm: 298 mmol/kg)
500 µg/mL	5.90E+06 (92)	7.10E+06 (96)	8.00E+06 (113)	precipitate# / discoloured medium#, precipitate# (pH: 7.0; osm: 297 mmol/kg)
250 µg/mL	5.70E+06 (89)	7.50E+06 (101)	7.20E+06 (101)	precipitate# / discoloured medium#, precipitate# (pH: 7.0; osm: 294 mmol/kg)
125 µg/mL	6.10E+06 (95)	6.40E+06 (86)	7.40E+06 (104)	precipitate# / precipitate# (pH: 7.0; osm: 296 mmol/kg)
62.5 µg/mL	5.60E+06 (88)	7.20E+06 (97)	7.00E+06 (99)	precipitate# / precipitate# (pH: 7.0; osm: 293 mmol/kg)
31.25 µg/mL	6.15E+06 (96)	7.60E+06 (103)	6.60E+06 (93)	precipitate# / precipitate# (pH: 7.0; osm: 296 mmol/kg)
15.625 µg/mL	6.50E+06 (102)	8.40E+06 (114)	7.80E+06 (110)	precipitate# / precipitate# (pH: 7.0; osm: 296 mmol/kg)
7.813 µg/mL	5.55E+06 (87)	7.20E+06 (97)	6.60E+06 (93)	precipitate# / precipitate# (pH: 7.0; osm: 297 mmol/kg)
3.906 µg/mL	5.80E+06 (91)	6.90E+06 (93)	7.00E+06 (99)	precipitate# / precipitate# (pH: 7.0; osm: 297 mmol/kg)

*: compared to the negative (vehicle) control (Distilled water)

#: 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	7.55E+06 (103)	7.10E+06 (88)	7.80E+06 (116)	normal / normal (pH: 7.0; osm: 301 mmol/kg)
Negative (vehicle) control	7.30E+06 (100)	8.10E+06 (100)	6.70E+06 (100)	normal / normal (pH: 7.0; osm: 297 mmol/kg)
2000 µg/mL	5.95E+06 (82)	7.70E+06 (95)	6.30E+06 (94)	precipitate# / discoloured medium# (pH: 7.0; osm: 307 mmol/kg)
1000 µg/mL	6.65E+06 (91)	6.80E+06 (84)	6.20E+06 (93)	normal / discoloured medium# (pH: 7.0; osm: 299 mmol/kg)
500 µg/mL	6.90E+06 (95)	8.60E+06 (106)	7.50E+06 (112)	normal / discoloured medium# (pH: 7.0; osm: 297 mmol/kg)
250 µg/mL	8.45E+06 (116)	7.40E+06 (91)	5.90E+06 (88)	normal / discoloured medium# (pH: 7.0; osm: 296 mmol/kg)
125 µg/mL	8.05E+06 (110)	8.00E+06 (99)	6.90E+06 (103)	normal / discoloured medium # (pH: 7.0; osm: 297 mmol/kg)
62.5 µg/mL	7.50E+06 (103)	7.90E+06 (98)	6.40E+06 (96)	normal / normal (pH: 7.0; osm: 296 mmol/kg)
31.25 µg/mL	7.70E+06 (105)	7.40E+06 (91)	6.30E+06 (94)	normal / normal (pH: 7.0; osm: 295 mmol/kg)
15.625 µg/mL	7.80E+06 (107)	6.70E+06 (83)	7.00E+06 (104)	normal / normal (pH: 7.0; osm: 295 mmol/kg)
7.813 µg/mL	8.00E+06 (110)	7.40E+06 (91)	6.30E+06 (94)	normal / normal (pH: 7.0; osm: 295 mmol/kg)
3.906 µg/mL	8.20E+06 (112)	7.10E+06 (88)	7.50E+06 (112)	normal / normal (pH: 7.0; osm: 296 mmol/kg)

*: compared to the negative (vehicle) control (Distilled water)

#: 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	6.90E+06 (90)	7.50E+06 (97)	6.60E+06 (94)	normal / normal (pH: 7.0; osm: 301 mmol/kg)
Negative (vehicle) control	7.70E+06 (100)	7.70E+06 (100)	7.00E+06 (100)	normal / normal (pH: 7.0; osm: 297 mmol/kg)
2000 µg/mL	4.30E+06 (56)	6.50E+06 (84)	6.90E+06 (99)	precipitate# / discoloured medium# (pH: 7.0; osm: 309 mmol/kg)
1000 µg/mL	6.15E+06 (80)	6.30E+06 (82)	6.70E+06 (96)	normal / normal (pH: 7.0; osm: 302 mmol/kg)
500 µg/mL	6.15E+06 (80)	8.30E+06 (108)	7.10E+06 (101)	normal / normal (pH: 7.0; osm: 300 mmol/kg)
250 µg/mL	7.65E+06 (99)	8.30E+06 (108)	7.30E+06 (104)	normal / normal (pH: 7.0; osm: 299 mmol/kg)
125 µg/mL	8.45E+06 (110)	6.80E+06 (88)	6.90E+06 (99)	normal / normal (pH: 7.0; osm: 297 mmol/kg)
62.5 µg/mL	7.35E+06 (95)	7.80E+06 (101)	6.00E+06 (86)	normal / normal (pH: 7.0; osm: 297 mmol/kg)
31.25 µg/mL	7.15E+06 (93)	8.10E+06 (105)	7.50E+06 (107)	normal / normal (pH: 7.0; osm: 296 mmol/kg)
15.625 µg/mL	6.15E+06 (80)	7.50E+06 (97)	7.00E+06 (100)	normal / normal (pH: 7.0; osm: 298 mmol/kg)
7.813 µg/mL	6.95E+06 (90)	7.30E+06 (95)	6.50E+06 (93)	normal / normal (pH: 7.0; osm: 298 mmol/kg)
3.906 µg/mL	7.05E+06 (92)	6.70E+06 (87)	7.50E+06 (107)	normal / normal (pH: 7.0; osm: 299 mmol/kg)

*: compared to the negative (vehicle) control (Distilled water)

#: 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	1178	0.982	103
			1000 µg/mL	1006	0.838	88
			500 µg/mL	1082	0.902	95
			250 µg/mL	1100	0.917	96
			125 µg/mL	1159	0.966	101
			62.5 µg/mL	1297	1.081	113
			31.25 µg/mL	1255	1.046	110
			Negative control	1144	0.953	100
			Negative control for DMBA (DMSO)	1090	0.908	95
			Untreated control	1075	0.896	94
			Positive control (DMBA)	38	0.032	3
-	5	A1	2000 µg/mL	1113	0.928	88
			1000 µg/mL	1261	1.051	99
			500 µg/mL	1049	0.874	83
			250 µg/mL	1143	0.953	90
			125 µg/mL	1312	1.093	103
			62.5 µg/mL	1270	1.058	100
			31.25 µg/mL	1200	1.000	94
			Negative control	1271	1.059	100
			Negative control for EMS (DMSO)	1189	0.991	94
			Untreated control	1113	0.928	88
			Positive control (EMS)	893	0.744	70

A1 = Assay 1
+ = in the presence of S9-mix
- = in the absence of S9-mix
Negative (vehicle) control = Distilled water
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	916	0.763	88
			1000 µg/mL	1188	0.990	114
			500 µg/mL	984	0.820	94
			250 µg/mL	1180	0.983	113
			125 µg/mL	1076	0.897	103
			62.5 µg/mL	1048	0.873	100
			31.25 µg/mL	1296	1.080	124
			Negative control	1044	0.870	100
			Negative control for DMBA (DMSO)	1056	0.880	101
			Untreated control	1288	1.073	123
			Positive control (DMBA)	27	0.023	3
-	24	A2	2000 µg/mL	888	0.740	84
			1000 µg/mL	1132	0.943	107
			500 µg/mL	1232	0.027	117
			250 µg/mL	1084	0.903	103
			125 µg/mL	1180	0.983	112
			62.5 µg/mL	1032	0.860	98
			31.25 µg/mL	1268	1.057	120
			Negative control	1056	0.880	100
			Negative control for EMS (DMSO)	1004	0.837	95
			Untreated control	1168	0.973	111
			Positive control (EMS)	588	0.490	56

A2 = Assay 2
+ = in the presence of S9-mix
- = in the absence of S9-mix
Negative (vehicle) control = Distilled water
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	952	0.793
			1000 µg/mL	964	0.803
			500 µg/mL	1088	0.907
			250 µg/mL	1071	0.893
			125 µg/mL	1112	0.927
			62.5 µg/mL	1021	0.851
			31.25 µg/mL	1077	0.898
			Negative control	1158	0.965
			Negative control for DMBA (DMSO)	1046	0.872
			Untreated control	1053	0.878
			Positive control (DMBA)	1057	0.881
-	5	A1	2000 µg/mL	1029	0.858
			1000 µg/mL	1113	0.928
			500 µg/mL	948	0.790
			250 µg/mL	943	0.786
			125 µg/mL	953	0.794
			62.5 µg/mL	1003	0.836
			31.25 µg/mL	1098	0.915
			Negative control	1143	0.953
			Negative control for EMS (DMSO)	1160	0.967
			Untreated control	1039	0.866
			Positive control (EMS)	797	0.664

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

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	1193	0.994
			1000 µg/mL	1225	1.021
			500 µg/mL	1180	0.983
			250 µg/mL	1087	0.906
			125 µg/mL	1089	0.908
			62.5 µg/mL	1121	0.934
			31.25 µg/mL	1193	0.994
			Negative control	1237	1.031
			Negative control for DMBA (DMSO)	1170	0.975
			Untreated control	1289	1.074
			Positive control (DMBA)	1224	1.020
-	24	A2	2000 µg/mL	1142	0.952
			1000 µg/mL	1198	0.998
			500 µg/mL	1132	0.943
			250 µg/mL	1274	1.062
			125 µg/mL	1150	0.958
			62.5 µg/mL	1167	0.973
			31.25 µg/mL	1120	0.933
			Negative control	1157	0.964
			Negative control for EMS (DMSO)	1185	0.988
			Untreated control	1028	0.857
			Positive control (EMS)	586	0.488

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

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	25	8.2
			1000 µg/mL	21	6.5
			500 µg/mL	23	6.4
			250 µg/mL	14	3.9
			125 µg/mL	16	4.3
			62.5 µg/mL	20	5.9
			31.25 µg/mL	21	5.9
			Negative control	21	5.5
			Negative control for DMBA (DMSO)	19	5.5
			Untreated control	20	5.7
			Positive control (DMBA)	1022	293.3**
-	5	A1	2000 µg/mL	17	5.0
			1000 µg/mL	18	5.0
			500 µg/mL	16	5.1
			250 µg/mL	19	6.1
			125 µg/mL	17	5.3
			62.5 µg/mL	19	5.7
			31.25 µg/mL	13	3.7
			Negative control	23	6.0
			Negative control for EMS (DMSO)	20	5.2
			Untreated control	21	6.1
			Positive control (EMS)	740	279.4**

** = 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
Negative (vehicle) control = Distilled water
DMSO = Dimethyl sulfoxide
DMBA = 7,12-Dimethylbenz[a]anthracene, 15 µg/mL
EMS = Ethyl methanesulfonate, 0.4 µL/mL

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	21	5.3
			1000 µg/mL	23	5.6
			500 µg/mL	27	6.9
			250 µg/mL	19	5.2
			125 µg/mL	17	4.7
			62.5 µg/mL	21	5.7
			31.25 µg/mL	19	4.8
			Negative control	20	4.9
			Negative control for DMBA (DMSO)	23	5.9
			Untreated control	17	4.0
			Positive control (DMBA)	1445	353.6**
-	24	A2	2000 µg/mL	30	7.9**
			1000 µg/mL	22	5.6
			500 µg/mL	19	5.1
			250 µg/mL	21	5.0
			125 µg/mL	19	4.9
			62.5 µg/mL	16	4.1
			31.25 µg/mL	19	5.2
			Negative control	18	4.7
			Negative control for EMS (DMSO)	20	5.1
			Untreated control	18	5.3
			Positive control (EMS)	1399	722.6**

** = 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
Negative (vehicle) control = Distilled water
DMSO = Dimethyl sulfoxide
DMBA = 7,12-Dimethylbenz[a]anthracene, 15 µg/mL
EMS = Ethyl methanesulfonate, 0.4 µL/mL

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	187	198	167	163	179	181
Negative (vehicle) control (Distilled water)	184	193	180	211	198	178
Negative control for DMBA (DMSO)	187	171	182	193	171	186
2000 µg/mL	185	157	165	231	221	219
1000 µg/mL	156	160	165	160	184	181
500 µg/mL	191	214	200	160	160	157
250 µg/mL	191	172	169	201	194	173
125 µg/mL	177	176	195	202	217	192
62.5 µg/mL	208	185	207	241	239	217
31.25 µg/mL	223	208	209	209	207	199
Positive control (15 µg/mL DMBA)	8	5	6	4	8	7

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	191	190	173	194	178	187
Negative (vehicle) control (Distilled water)	204	218	213	220	199	217
Negative control for EMS (DMSO)	210	210	181	197	212	179
2000 µg/mL	181	189	172	187	181	203
1000 µg/mL	224	221	191	229	199	197
500 µg/mL	177	174	153	172	194	179
250 µg/mL	209	214	208	165	202	145
125 µg/mL	231	221	208	243	195	214
62.5 µg/mL	178	183	195	240	239	235
31.25 µg/mL	204	177	196	196	212	215
Positive control (0.4 µL/mL EMS)	132	143	159	131	167	161

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	228	196	200	224	232	208
Negative (vehicle) control (distilled water)	200	172	184	172	156	160
Negative control for DMBA (DMSO)	164	200	168	160	172	192
2000 µg/mL	152	144	152	144	160	164
1000 µg/mL	196	224	208	180	180	200
500 µg/mL	156	172	188	168	152	148
250 µg/mL	212	224	204	200	168	172
125 µg/mL	172	180	192	164	192	176
62.5 µg/mL	184	156	172	160	192	184
31.25 µg/mL	236	232	204	204	212	208
Positive control (15 µg/mL DMBA)	3	4	3	6	7	4

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	188	216	208	204	184	168
Negative (vehicle) control (Distilled water)	164	192	176	172	184	168
Negative control for EMS (DMSO)	156	164	168	184	160	172
2000 µg/mL	144	152	140	152	136	164
1000 µg/mL	168	192	196	212	188	176
500 µg/mL	200	216	192	204	224	196
250 µg/mL	184	176	172	168	196	188
125 µg/mL	212	208	200	192	196	172
62.5 µg/mL	160	184	168	156	180	184
31.25 µg/mL	212	236	204	196	208	212
Positive control (0.4 µL/mL EMS)	100	124	80	80	92	112

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	162	166	162	190	189	184
Negative (vehicle) control (Distilled water)	207	199	198	188	171	195
Negative control for DMBA (DMSO)	197	176	181	163	167	162
2000 µg/mL	189	178	164	135	146	140
1000 µg/mL	157	151	159	163	175	159
500 µg/mL	199	174	204	181	166	164
250 µg/mL	184	204	191	154	162	176
125 µg/mL	181	170	182	184	195	200
62.5 µg/mL	169	174	194	166	171	147
31.25 µg/mL	173	191	188	170	168	187
Positive control (15 µg/mL DMBA)	194	183	197	181	155	147

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	162	160	157	196	188	176
Negative (vehicle) control (DMSO)	194	194	178	187	187	203
Negative control for EMS (DMSO)	195	202	201	194	167	201
2000 µg/mL	185	182	178	158	155	171
1000 µg/mL	177	172	167	185	200	212
500 µg/mL	174	178	178	135	146	137
250 µg/mL	154	129	153	161	180	166
125 µg/mL	169	168	157	160	159	140
62.5 µg/mL	195	187	183	153	144	141
31.25 µg/mL	208	209	197	157	163	164
Positive control (0.4 µL/mL EMS)	128	151	109	129	152	128

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	198	211	192	232	234	222
Negative (vehicle) control (Distilled water)	205	204	215	201	219	193
Negative control for DMBA (DMSO)	174	194	191	208	188	215
2000 µg/mL	211	186	185	206	214	191
1000 µg/mL	206	203	196	192	217	211
500 µg/mL	201	214	198	176	202	189
250 µg/mL	152	181	171	178	211	194
125 µg/mL	163	182	172	177	214	181
62.5 µg/mL	177	184	179	201	199	181
31.25 µg/mL	188	173	194	200	207	231
Positive control (15 µg/mL DMBA)	195	200	189	214	223	203

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	170	162	163	185	183	165
Negative (vehicle) control (Distilled water)	197	197	190	188	201	184
Negative control for EMS (DMSO)	188	200	190	185	211	211
2000 µg/mL	197	183	171	177	201	213
1000 µg/mL	172	174	188	234	206	224
500 µg/mL	162	167	171	196	209	227
250 µg/mL	205	223	221	214	209	202
125 µg/mL	200	206	203	183	182	176
62.5 µg/mL	200	187	181	209	188	202
31.25 µg/mL	193	169	174	192	199	193
Positive control (0.4 µL/mL EMS)	90	87	101	95	117	96

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	3	1	2	1	1	2	3	3	2
Negative (vehicle) control (Distilled water)	2	2	2	1	3	2	1	4	2	2
Negative control for DMBA (DMSO)	1	3	2	2	1	1	4	2	2	1
2000 µg/mL	2	2	1	1	4	1	2	3	5	4
1000 µg/mL	2	2	1	1	2	2	2	4	3	2
500 µg/mL	1	1	3	2	3	2	3	2	4	2
250 µg/mL	2	2	3	0	1	1	1	1	2	1
125 µg/mL	2	1	2	2	1	2	0	2	2	2
62.5 µg/mL	2	2	1	1	3	1	5	2	2	1
31.25 µg/mL	2	1	2	1	2	2	1	3	3	4
Positive control (15 µg/mL DMBA)	101	104	92	106	86	130	90	106	117	90

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	1	2	2	3	2	4	2	2	1	2
Negative (vehicle) control (Distilled water)	3	2	3	3	2	3	2	2	1	2
Negative control for EMS (DMSO)	2	1	2	3	3	1	2	2	3	1
2000 µg/mL	1	1	2	2	2	1	4	2	1	1
1000 µg/mL	2	2	3	2	2	2	2	2	0	1
500 µg/mL	2	1	2	0	3	1	2	1	1	3
250 µg/mL	1	3	2	2	2	2	1	2	2	2
125 µg/mL	3	1	2	2	2	2	1	2	1	1
62.5 µg/mL	1	1	4	2	2	2	1	4	0	2
31.25 µg/mL	1	1	1	2	0	1	2	1	2	2
Positive control (0.4 µL/mL EMS)	70	90	85	69	88	50	69	77	80	62

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	2	2	1	1	2	1	2	2
Negative (vehicle) control (Distilled water)	2	2	1	2	2	3	1	2	3	2
Negative control for DMBA (DMSO)	2	2	3	2	1	4	1	2	2	4
2000 µg/mL	3	1	2	2	2	4	1	1	2	3
1000 µg/mL	2	1	5	3	2	1	2	2	2	3
500 µg/mL	3	2	2	3	2	5	1	3	3	3
250 µg/mL	2	1	1	1	3	3	3	2	2	1
125 µg/mL	1	2	2	2	2	2	1	0	4	1
62.5 µg/mL	3	2	3	0	4	3	2	1	2	1
31.25 µg/mL	4	2	2	1	1	1	2	2	3	1
Positive control (15 µg/mL DMBA)	132	143	136	126	128	151	146	164	149	170

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	3	2	1	3	1	2	2	2	1	1
Negative (vehicle) control (Distilled water)	2	2	1	2	2	1	2	2	2	2
Negative control for EMS (DMSO)	2	2	2	2	3	2	2	2	2	1
2000 µg/mL	3	2	4	2	2	5	4	3	2	3
1000 µg/mL	2	2	1	3	4	5	1	1	1	2
500 µg/mL	1	2	1	3	2	2	2	2	2	2
250 µg/mL	1	2	2	2	2	3	2	4	2	1
125 µg/mL	2	3	3	1	2	2	2	1	1	2
62.5 µg/mL	1	2	1	0	1	4	2	2	1	2
31.25 µg/mL	4	2	3	1	2	1	4	0	1	1
Positive control (0.4 µL/mL EMS)	160	168	159	163	135	125	132	129	110	118

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	7.2	7.0	305	297	normal / normal
Negative (vehicle) control (Distilled water)	7.2	7.0	307	291	normal / normal
Negative control for DMBA (DMSO)	7.0	7.0	458	442	normal / normal
2000 µg/mL	7.0	7.0	309	301	precipitate[#] / discoloured medium[#], precipitate[#]
1000 µg/mL	7.0	7.0	297	307	precipitate[#] / discoloured medium[#], precipitate[#]
500 µg/mL	7.0	7.0	294	305	precipitate[#] / discoloured medium[#], precipitate[#]
250 µg/mL	7.0	7.0	293	298	precipitate[#] / discoloured medium[#], precipitate[#]
125 µg/mL	7.0	7.0	307	295	precipitate[#] / precipitate[#]
62.5 µg/mL	7.0	7.0	301	295	normal / normal
31.25 µg/mL	7.2	7.0	296	323	normal / normal
Positive control (15 µg/mL DMBA)	7.0	7.0	435	435	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	7.2	7.0	300	301	normal / normal
Negative (vehicle) control (Distilled water)	7.0	7.0	297	298	normal / normal
Negative control for EMS (DMSO)	7.2	7.0	449	458	normal / normal
2000 µg/mL	7.0	7.0	310	308	precipitate# / discoloured medium#
1000 µg/mL	7.0	7.2	315	322	normal / discoloured medium#
500 µg/mL	7.0	7.0	297	296	normal / discoloured medium#
250 µg/mL	7.0	7.0	297	300	normal / discoloured medium#
125 µg/mL	7.0	7.2	295	298	normal / discoloured medium#
62.5 µg/mL	7.0	7.0	297	304	normal / normal
31.25 µg/mL	7.0	7.0	303	305	normal / normal
Positive control (0.4 µl/mL EMS)	7.2	7.0	433	433	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	296	290	normal / normal
Negative (vehicle) control (Distilled water)	7.0	7.0	295	290	normal / normal
Negative control for DMBA (DMSO)	7.0	7.0	448	449	normal / normal
2000 µg/mL	7.0	7.0	321	307	discoloured medium#, precipitate# / discoloured medium#, precipitate#
1000 µg/mL	7.0	7.0	305	303	precipitate# / discoloured medium#, precipitate#
500 µg/mL	7.0	7.0	297	295	normal / discoloured medium#
250 µg/mL	7.0	7.0	289	294	normal / discoloured medium#
125 µg/mL	7.0	7.0	294	296	normal / discoloured medium#
62.5 µg/mL	7.0	7.0	298	292	normal / normal
31.25 µg/mL	7.0	7.0	297	302	normal / normal
Positive control (15 µg/mL DMBA)	7.0	7.0	439	437	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	297	normal / normal
Negative (vehicle) control (Distilled water)	7.0	7.0	303	309	normal / normal
Negative control for EMS (DMSO)	7.0	7.0	458	455	normal / normal
2000 µg/mL	7.0	7.0	312	316	discoloured medium#, precipitate# / discoloured medium#
1000 µg/mL	7.0	7.0	311	309	normal / normal
500 µg/mL	7.0	7.0	307	299	normal / normal
250 µg/mL	7.0	7.0	304	299	normal / normal
125 µg/mL	7.0	7.0	302	298	normal / normal
62.5 µg/mL	7.0	7.0	303	304	normal / normal
31.25 µg/mL	7.0	7.0	305	303	normal / normal
Positive control (0.4 µl/mL EMS)	7.0	7.0	437	442	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	8.05E+06	7.45E+06	7.90E+06	9.30E+06	6.50E+06	6.00E+06	7.05E+06	6.84E+06
Negative (vehicle) control (Distilled water)	6.85E+06	6.25E+06	7.70E+06	6.90E+06	6.70E+06	7.60E+06	6.87E+06	6.18E+06
Negative control for DMBA (DMSO)	6.35E+06	6.70E+06	9.60E+06	8.20E+06	6.30E+06	7.60E+06	6.60E+06	6.18E+06
2000 µg/mL	5.25E+06	4.15E+06	8.70E+06	8.20E+06	6.90E+06	7.40E+06	6.96E+06	7.68E+06
1000 µg/mL	7.20E+06	6.55E+06	8.30E+06	6.80E+06	6.60E+06	6.70E+06	8.28E+06	7.08E+06
500 µg/mL	5.90E+06	7.20E+06	7.80E+06	6.10E+06	6.40E+06	9.90E+06	6.36E+06	6.51E+06
250 µg/mL	6.80E+06	6.25E+06	9.30E+06	6.30E+06	6.60E+06	8.40E+06	5.37E+06	7.02E+06
125 µg/mL	7.15E+06	6.15E+06	1.02E+07	7.50E+06	6.50E+06	9.30E+06	6.66E+06	6.09E+06
62.5 µg/mL	5.95E+06	5.50E+06	9.50E+06	6.60E+06	6.20E+06	7.90E+06	6.66E+06	6.09E+06
31.25 µg/mL	6.25E+06	6.35E+06	7.80E+06	8.90E+06	6.60E+06	7.40E+06	6.24E+06	6.21E+06
Positive control (15 µg/mL DMBA)	1.69E+07	2.37E+07	6.50E+05	6.75E+05	1.50E+06	1.78E+06	7.08E+06	8.88E+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 4 (Cultures A / B)		Cell number on Day 6 (Cultures A / B)		Cell number on Day 8 (Cultures A / B)	
Untreated control	1.02E+07	1.10E+07	7.70E+06	7.10E+06	8.50E+06	7.90E+06	7.17E+06	6.33E+06
Negative (vehicle) control (Distilled water)	9.05E+06	8.45E+06	7.30E+06	8.70E+06	7.10E+06	6.90E+06	6.33E+06	6.24E+06
Negative control for EMS (DMSO)	9.40E+06	9.45E+06	7.80E+06	7.20E+06	6.80E+06	7.80E+06	6.27E+06	5.97E+06
2000 µg/mL	8.15E+06	8.10E+06	7.10E+06	8.90E+06	8.50E+06	7.20E+06	6.75E+06	6.36E+06
1000 µg/mL	8.70E+06	8.50E+06	6.60E+06	7.40E+06	7.00E+06	6.70E+06	6.45E+06	6.18E+06
500 µg/mL	1.06E+07	9.45E+06	8.00E+06	8.30E+06	7.70E+06	6.10E+06	5.55E+06	8.79E+06
250 µg/mL	9.05E+06	1.14E+07	6.90E+06	7.00E+06	6.20E+06	7.20E+06	8.64E+06	7.50E+06
125 µg/mL	8.20E+06	8.70E+06	7.20E+06	8.00E+06	6.50E+06	6.50E+06	7.89E+06	7.08E+06
62.5 µg/mL	9.00E+06	8.00E+06	8.80E+06	8.40E+06	6.90E+06	6.80E+06	5.58E+06	8.16E+06
31.25 µg/mL	9.80E+06	8.80E+06	6.90E+06	8.30E+06	5.90E+06	6.40E+06	6.57E+06	7.11E+06
Positive control (0.4 µl/mL EMS)	5.08E+07	6.39E+07	5.90E+06	8.00E+06	5.60E+06	4.80E+06	5.55E+06	5.76E+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 4 (Cultures A / B)		Cell number on Day 6 (Cultures A / B)		Cell number on Day 8 (Cultures A / B)	
Untreated control	8.40E+06	9.30E+06	8.90E+06	8.20E+06	6.80E+06	8.00E+06	6.57E+06	5.52E+06
Negative (vehicle) control (Distilled water)	8.70E+06	8.70E+06	7.90E+06	8.80E+06	7.50E+06	6.60E+06	5.97E+06	6.03E+06
Negative control for DMBA (DMSO)	7.75E+06	7.35E+06	9.40E+06	8.10E+06	7.20E+06	7.70E+06	6.51E+06	5.52E+06
2000 µg/mL	5.60E+06	5.55E+06	8.10E+06	8.40E+06	7.10E+06	7.00E+06	6.51E+06	6.03E+06
1000 µg/mL	6.55E+06	7.10E+06	8.20E+06	8.20E+06	6.60E+06	8.20E+06	5.79E+06	5.46E+06
500 µg/mL	7.05E+06	7.25E+06	7.80E+06	7.90E+06	5.90E+06	7.30E+06	6.09E+06	6.33E+06
250 µg/mL	7.95E+06	8.20E+06	8.50E+06	8.20E+06	7.20E+06	8.30E+06	5.94E+06	6.09E+06
125 µg/mL	8.20E+06	8.00E+06	8.60E+06	8.60E+06	7.60E+06	7.70E+06	6.69E+06	5.88E+06
62.5 µg/mL	8.60E+06	7.85E+06	8.10E+06	9.10E+06	6.80E+06	6.70E+06	5.94E+06	6.18E+06
31.25 µg/mL	8.50E+06	9.35E+06	9.10E+06	8.10E+06	6.50E+06	7.70E+06	6.03E+06	5.07E+06
Positive control (15 µg/mL DMBA)	2.78E+07	2.63E+07	2.15E+06	3.35E+06	9.40E+06	9.30E+06	6.72E+06	5.64E+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 4 (Cultures A / B)		Cell number on Day 6 (Cultures A / B)		Cell number on Day 8 (Cultures A / B)	
Untreated control	1.13E+07	1.24E+07	9.90E+06	7.80E+06	6.80E+06	6.70E+06	8.10E+06	6.15E+06
Negative (vehicle) control (Distilled water)	1.35E+07	1.16E+07	8.30E+06	7.40E+06	7.60E+06	6.30E+06	6.36E+06	6.51E+06
Negative control for EMS (DMSO)	1.06E+07	1.21E+07	8.70E+06	8.30E+06	6.20E+06	6.40E+06	6.36E+06	5.79E+06
2000 µg/mL	6.70E+06	6.20E+06	8.00E+06	8.50E+06	6.90E+06	7.20E+06	7.23E+06	6.63E+06
1000 µg/mL	9.40E+06	1.02E+07	8.50E+06	8.00E+06	7.20E+06	7.50E+06	6.87E+06	5.82E+06
500 µg/mL	1.16E+07	1.17E+07	7.40E+06	9.30E+06	6.80E+06	7.20E+06	7.20E+06	6.27E+06
250 µg/mL	9.85E+06	1.06E+07	8.90E+06	7.70E+06	7.50E+06	7.40E+06	5.91E+06	5.82E+06
125 µg/mL	1.18E+07	1.16E+07	8.50E+06	7.20E+06	6.90E+06	6.20E+06	6.18E+06	7.44E+06
62.5 µg/mL	1.02E+07	1.09E+07	9.20E+06	8.60E+06	6.60E+06	5.60E+06	6.51E+06	6.48E+06
31.25 µg/mL	1.35E+07	1.06E+07	8.00E+06	8.00E+06	7.20E+06	6.30E+06	6.45E+06	6.15E+06
Positive control (0.4 µl/mL EMS)	6.88E+07	7.00E+07	7.60E+06	4.90E+06	4.20E+06	4.30E+06	3.24E+06	2.73E+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.:
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"