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Attn: 8(e) Coordinator
Office of Pollution Prevention and Toxics
US Environmental Protection Agency
1201 Constitution Ave., NW
Washington, DC 20460

8809000007775

**8(e) Submission: [epoxide substituted bicyclic olefin]**

RPW

The chemical identity of the substance is:
CAS Registry Number: 3188-75-8
Oxirane,2-[(bicyclo[2.2.1]hept-5-en-2-ylmethoxy)methyl]-

Dear 8(e) Coordinator:

This letter is to inform you of the results of (1) an *in vitro* Ames Test (Reverse Mutagenicity Test) using *Samonella Typhimrium* and *Escherichia Coli* and (2) an *in vitro* L5178Y +/-Mouse Lymphoma Assay (OECD476) Assay on [epoxide substituted bicyclic olefin]
We conducted the Mouse Lymphoma Assay after the positive Ames Test . The Ames Test was previously not reportable under 8(e) (in the absence of a second *in vitro* mutagenicity test) but it is reportable at this time.

The Ames Reverse Mutagenicity Test showed the substance to be mutagenic under the conditions of the test. In the Mouse Lymphoma Assay, a weak mutagenic response was observed in the absence of metabolic activation; however, a clear positive response was observed in the presence of metabolic activation, confirming the result of the Ames Test. We will continue mutagenicity testing by performing an *in vivo* Chromosome Aberration study.

When EPA approved the PMN for this chemical, they sent us a letter asking us to use conservative PPE (including organic cartridge respirator when exposure is possible) because EPA noted other epoxide compounds have shown to have serious health concerns. We have followed the PPE and respirator conditions and informed our employees and any companies working with this material of these conditions. We continue tests on this chemical as part of a REACH registration and will inform EPA of any other 8(e) reportable results.

The report and a sanitized letter and report are attached herein. If I can be of any assistance, please contact me at 440-922-1517.

Yours very truly,

**Company Sanitized**

8911000059

SafePharm Laboratories

[ ]

# REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

**SPL PROJECT NUMBER: 1518/0056**

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## QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress. In addition, inspection of general facilities not specifically related to this study are done monthly or annually in accordance with QA Standard Procedure.

This report has been audited by Safepharm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

| | | |
|---|---|---|
| | 14 May 2008 | Standard Test Method Compliance Audit |
| | 21 August 2008 | Test Material Preparation |
| | 19 August 2008 | Test System Preparation |
| | 22 August 2008 | Exposure |
| | 29 August 2008 | Assessment of Response |
| § | 14 October 2008 | Draft Report Audit |
| § | Date of QA Signature | Final Report Audit |

§ Evaluation specific to this study

G. Wren ..... DATE: 07 NOV 2008

For Safepharm Quality Assurance Unit*

***Authorised QA Signatures:**

| | |
|---|---|
| Deputy Head of Department: | JM Crowther MIScT MRQA |
| Senior Audit Staff: | G Wren ONC MRQA |

## GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

This report fully and accurately reflects the procedures used and data generated.

..... DATE: 06 NOV 2008

P W Thompson HNC
Study Director

This report may be presented in final form as a digital (pdf) document. Such documents produced by Safepharm are prepared by scanning the paper original, and are considered of equivalent integrity and authenticity to versions produced by optical photocopy. However, in all cases the hand-signed paper original, held in secure archives, is the definitive document.

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# REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

## SUMMARY

***Introduction.*** The method conforms to the guidelines for bacterial mutagenicity testing published by the major Japanese Regulatory Authorities including METI, MHLW and MAFF. It also meets the requirements of the OECD Guidelines for Testing of Chemicals No. 471 "Bacterial Reverse Mutation Test", Method B13/14 of Commission Directive 2000/32/EC and the USA, EPA (TSCA) OPPTS harmonised guidelines.

***Methods.*** *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and *Escherichia coli* strain WP2*uvrA*$^-$ were treated with the test material using the Ames plate incorporation method at five dose levels, in triplicate, both with and without the addition of a rat liver homogenate metabolising system (10% liver S9 in standard co-factors). The dose range was determined in a preliminary toxicity assay and was 50 to 5000 µg/plate in the first experiment. Large, dose-related and statistically significant increases in the frequency of TA100, TA1535 and WP2*uvrA*$^-$ revertant colonies were observed at the majority of test material dose levels in Experiment 1. The OECD 471 test guideline permits non-repetition of the experiment when a clear positive response is obtained in the first experiment, therefore, with the sponsor's approval, testing was suspended at the end of Experiment 1.

***Results.*** The vehicle (tetrahydrofuran) control plates gave counts of revertant colonies within the normal range. All of the positive control chemicals used in the test induced marked increases in the frequency of revertant colonies, both with or without metabolic activation. Thus, the sensitivity of the assay and the efficacy of the S9-mix were validated.

The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level and was, therefore, tested up to the maximum recommended dose level of 5000 µg/plate. No test material precipitate was observed on the plates at any of the doses tested in either the presence or absence of S9-mix.

Large, dose-related and statistically significant increases in the frequency of TA100, TA1535 and WP2*uvrA*$^-$ revertant colonies were observed at the majority of test material dose levels, initially from 50 and 150 µg/plate, in the absence and presence of S9, respectively. The increases in colony frequency at the upper dose levels of the test material were very large and in excess of the in-house historical control range of each strain (particularly TA100 and TA1535). A reproducible response was evident for bacterial strains TA100 and WP2uvrA$^-$ when taking the results of the

Preliminary Toxicity Test into consideration. Therefore, the test material was considered to be mutagenic in bacterial strains TA100, TA1535 and WP2*uvrA*$^-$. No increases were noted for the Frameshift detecting strains (TA98 and TA1537).

***Conclusion.*** The test material was considered to be mutagenic under the conditions of this test.

# REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

## 1. INTRODUCTION

This study was designed to assess the mutagenic potential of the test material using a bacterial/ microsome test system. The study was based on the *in vitro* technique described by Ames and his co-workers (1, 2, 3) and Garner et al (4) in which mutagenic activity is assessed by exposing histidine auxotrophs of *Salmonella typhimurium* and tryptophan auxotrophs of *Escherichia coli* to various concentrations of the test material. The method conforms to the guidelines for bacterial mutagenicity testing published by the major Japanese Regulatory Authorities including METI, MHLW and MAFF. It also meets the requirements of the OECD Guidelines for Testing of Chemicals No. 471 "Bacterial Reverse Mutation Test", Method B13/14 of Commission Directive 2000/32/EC and the USA, EPA (TSCA) OPPTS harmonised guidelines. A copy of the Certificate of Compliance with GLP, issued by the UK Department of Health, is included.

These mutant strains of *Salmonella* are incapable of synthesising histidine and are, therefore, dependent on an external source of this particular amino acid for normal growth. When exposed to a mutagenic agent these bacteria may undergo a reverse mutation to histidine independent forms which are detected by their ability to grow on a histidine deficient medium. Using various strains of this organism, revertants produced after exposure to a chemical mutagen may arise as a result of base-pair substitution in the genetic material (miscoding) or frame-shift mutation in which genetic material is either added or deleted. In order to make the bacteria more sensitive to mutation by chemical and physical agents, several additional traits have been introduced. These include a deletion through the excision repair gene (*uvrB*$^-$ *Salmonella* strains) which renders the organism incapable of DNA excision repair and deep rough mutation (*rfa*) which increases the permeability of the cell wall. A mutant strain of *Escherichia coli* (WP2*uvrA*$^-$), which requires tryptophan and which can be reverse mutated by base substitution to tryptophan independence was used to complement the *Salmonella* strains. This strain also has a deletion in an excision repair gene (*uvrA*$^-$). Since many compounds do not exert a mutagenic effect until they have been metabolised by enzyme systems not available in the bacterial cell, the test material and the bacteria are also incubated in the presence of a liver microsomal preparation (S9-mix) prepared from rats pre-treated with a mixture known to induce an elevated level of these enzymes.

The experimental phase of this study was performed between 15 August 2008 and 27 August 2008.

## 2. TEST MATERIAL

| | | |
|---|---|---|
| Sponsor's identification | : | [ ] |
| Description | : | Clear colourless liquid |
| Batch number | : | 08C10938 |
| Date received | : | 04 August 2008 |
| Storage conditions | : | Approximately 4°C in the dark under nitrogen |

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

## 3. METHODS

### 3.1 Tester Strains

*Salmonella typhimurium* TA1535, TA1537, TA98 and TA100

*Escherichia coli* WP2*uvrA*$^-$

The *Salmonella* strains were obtained from the University of California at Berkeley on culture discs on 4 August 1995 whilst *Escherichia coli* strain WP2*uvrA*$^-$ was obtained from the British Industrial Biological Research Association on 17 August 1987. All of the strains were stored at -196°C in a Statebourne liquid nitrogen freezer, model SXR 34. Prior to the master strains being used, characterisation checks were carried out to confirm the amino-acid requirement, presence of *rfa*, R factors, *uvrB* or *uvrA* mutation and the spontaneous reversion rate.

In this assay, overnight sub-cultures of the appropriate coded stock cultures were prepared in nutrient broth (Oxoid Limited; lot number 629250 02/13) and incubated at 37°C for approximately 10 hours. Each culture was monitored spectrophotometrically for turbidity with titres determined by viable count analysis on nutrient agar plates.

### 3.2 Preparation of Test and Control Materials

The test material was fully soluble in tetrahydrofuran (Sponsor's preferred vehicle) at 200 mg/ml in solubility checks performed in-house. Tetrahydrofuran was therefore selected as the vehicle.

The test material was accurately weighed and approximate half-log dilutions prepared in tetrahydrofuran by mixing on a vortex mixer on the day of each experiment. Tetrahydrofuran is

toxic to the bacterial cells at and above 50 µl, therefore all of the formulations were prepared at concentrations four times greater than required on Vogel-Bonner agar plates. To compensate, each formulation was dosed using 25 µl aliquots. Tetrahydrofuran is an acceptable vehicle for use in this test system (6). Analysis for concentration, homogeneity and stability of the test material formulations is not a requirement of the test guidelines and was, therefore, not determined. Prior to use, the solvent was dried using molecular sieves (sodium alumino-silicate) ie 2 mm pellets with a nominal pore diameter of $4 \times 10^{-4}$ microns.

Vehicle and positive controls were used in parallel with the test material. A solvent treatment group was used as the vehicle control and the positive control materials used in the series of plates without S9-mix were as follows:

| | |
|---|---|
| N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG): | 3 µg/plate for TA100, 5 µg/plate for TA1535 and 2 µg/plate for WP2*uvrA*$^-$ |
| 9-Aminoacridine (9AA): | 80 µg/plate for TA1537 |
| 4-Nitroquinoline-1-oxide (4NQO): | 0.2 µg/plate for TA98 |

In addition, 2-Aminoanthracene (2AA) and Benzo(a)pyrene (BP), which are non-mutagenic in the absence of metabolising enzymes, were used in the series of plates with S9-mix at the following concentrations:

2AA at 1 µg/plate for TA100
2AA at 2 µg/plate for TA1535 and TA1537
BP at 5 µg/plate for TA98
2AA at 10 µg/plate for WP2*uvrA*$^-$

### 3.3 Microsomal Enzyme Fraction

S9 was prepared in-house on 18 May 2008 from the livers of male Sprague-Dawley rats weighing ~ 250g. These had each orally received three consecutive daily doses of phenobarbitone/β-naphthoflavone (80/100 mg per kg per day) prior to S9 preparation on Day 4. Before use, each batch of S9 was assayed for its ability to metabolise appropriate indirect mutagens used in the Ames Test. The S9 was stored at -196°C.

### 3.4 S9-Mix and Agar

The S9-mix was prepared immediately before use using sterilised co-factors and maintained on ice for the duration of the test.

| | |
|---|---|
| S9 | 5.0 ml |
| 1.65 M KCl/0.4 M $MgCl_2$ | 1.0 ml |
| 0.1 M Glucose-6-phosphate | 2.5 ml |
| 0.1 M NADP | 2.0 ml |
| 0.2 M Sodium phosphate buffer (pH 7.4) | 25.0 ml |
| Sterile distilled water | 14.5 ml |

A 0.5 ml aliquot of S9-mix and 2 ml of molten, trace histidine or tryptophan supplemented, top agar were overlaid onto a sterile Vogel-Bonner Minimal agar plate in order to assess the sterility of the S9-mix. This procedure was repeated, in triplicate, on the day of each experiment.

Top agar was prepared using 0.6% Difco Bacto agar (lot number 7284259 08/12) and 0.5% sodium chloride with 5 ml of 1.0 mM histidine and 1.0 mM biotin or 1.0 mM tryptophan solution added to each 100 ml of top agar. Vogel-Bonner Minimal agar plates were purchased from ILS Limited (lot number 1045711-02 04/13).

### 3.5 Test Procedure

#### 3.5.1 Preliminary Toxicity Test

In order to select appropriate dose levels for use in the main test, a preliminary test was carried out to determine the toxicity of the test material. The concentrations tested were 0, 0.15, 0.5, 1.5, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate. The test was performed by mixing 0.1 ml of bacterial culture (TA100 or WP2*uvrA*$^-$), 2 ml of molten, trace histidine or tryptophan supplemented, top agar, 0.025 ml of test material formulation and 0.5 ml of S9-mix or phosphate buffer and overlaying onto sterile plates of Vogel-Bonner Minimal agar (30 ml/plate). Ten concentrations of the test material formulation and a vehicle control (tetrahydrofuran) were tested. In addition, 0.025 ml of the maximum concentration of the test material and 2 ml of molten, trace histidine or tryptophan supplemented, top agar were overlaid onto a sterile Nutrient agar plate in order to assess the sterility of the test material. After approximately 48 hours incubation at 37°C the plates were assessed for numbers of revertant colonies using a Domino colony counter and examined for effects on the growth of the bacterial background lawn.

### 3.5.2 Mutation Test - Experiment 1

Five concentrations of the test material (50, 150, 500, 1500 and 5000 µg/plate) were assayed in triplicate against each tester strain, using the direct plate incorporation method.

Measured aliquots (0.1 ml) of one of the bacterial cultures were dispensed into sets of test tubes followed by 2.0 ml of molten, trace histidine or tryptophan supplemented, top agar, 0.025 ml of vehicle or test material formulation or 0.1 ml of positive control and either 0.5 ml of S9-mix or phosphate buffer. The contents of each test tube were mixed and equally distributed onto the surface of Vogel-Bonner Minimal agar plates (one tube per plate). This procedure was repeated, in triplicate, for each bacterial strain and for each concentration of test material both with and without S9-mix.

All of the plates were incubated at 37°C for approximately 48 hours and the frequency of revertant colonies assessed using a Domino colony counter.

### 3.5.3 Mutation Test - Experiment 2

Large, dose-related and statistically significant increases in the frequency of revertant colonies were observed at the majority of test material dose levels in both the Preliminary Toxicity Test and Experiment 1. The OECD 471 test guideline permits non-repetition of the experiment when a clear positive response is obtained in the first experiment, therefore, with the sponsor's approval, testing was suspended at the end of Experiment 1.

## 3.6 Acceptance Criteria

The reverse mutation assay may be considered valid if the following criteria are met:

All tester strain cultures exhibit a characteristic number of spontaneous revertants per plate in the vehicle and untreated controls. Acceptable ranges are presented in the standard test method section 3 with historical control ranges for 2006 and 2007 in Appendix 1.

The appropriate characteristics for each tester strain have been confirmed, eg *rfa* cell-wall mutation and pKM101 plasmid R-factor etc.

All tester strain cultures should be in the approximate range of 1 to 9.9 x $10^9$ bacteria per ml.

Each mean positive control value should be at least twice the respective vehicle control value for each strain, thus demonstrating both the intrinsic sensitivity of the tester strains to mutagenic exposure and the integrity of the S9-mix. The historical control ranges for 2006 and 2007 are presented in Appendix 1.

There should be a minimum of four non-toxic test material dose levels.

There should be no evidence of excessive contamination.

### 3.7 Evaluation Criteria

There are several criteria for determining a positive result, such as a dose-related increase in revertant frequency over the dose range tested and/or a reproducible increase at one or more concentrations in at least one bacterial strain with or without metabolic activation. Biological relevance of the results will be considered first, statistical methods, as recommended by the UKEMS (5) can also be used as an aid to evaluation, however, statistical significance will not be the only determining factor for a positive response.

A test material will be considered non-mutagenic (negative) in the test system if the above criteria are not met.

Although most experiments will give clear positive or negative results, in some instances the data generated will prohibit a definitive judgement about the test material activity. Results of this type will be reported as equivocal.

## 4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

# 5. RESULTS

## 5.1 Preliminary Toxicity Test

The test material was non-toxic to the strains of bacteria used (TA100 and WP2*uvrA*⁻). The test material formulation and S9-mix used in this experiment were both shown to be sterile.

The numbers of revertant colonies for the toxicity assay were:

| With (+) or without (-) S9-mix | Strain | Dose (µg/plate) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 0.15 | 0.5 | 1.5 | 5 | 15 | 50 | 150 | 500 | 1500 | 5000 |
| - | TA100 | 94 | 63 | 83 | 82 | 92 | 74 | 102 | 199 | 385 | 762 | 993 |
| + | TA100 | 76 | 74 | 79 | 88 | 79 | 63 | 90 | 127 | 141 | 626 | 1218 |
| - | WP2*uvrA*⁻ | 21 | 27 | 26 | 30 | 35 | 26 | 30 | 61 | 94 | 170 | 163 |
| + | WP2*uvrA*⁻ | 26 | 33 | 36 | 21 | 31 | 39 | 28 | 48 | 30 | 151 | 160 |

## 5.2 Mutation Test

Prior to use, the master strains were checked for characteristics, viability and spontaneous reversion rate (all were found to be satisfactory). These data are not given in the report. The amino acid supplemented top agar and the S9-mix used in both experiments was shown to be sterile.

Results for the negative controls (spontaneous mutation rates) are presented in Table 1 and were considered to be acceptable. These data are for concurrent untreated control plates performed on the same day as the Mutation Test.

The individual plate counts, the mean number of revertant colonies and the standard deviations, for the test material, positive and vehicle controls, both with and without metabolic activation, are presented in Table 2 and Table 3 for Experiment 1.

A history profile of vehicle and positive control values for 2006 and 2007 is presented in Appendix 1.

The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level and was, therefore, tested up to the maximum recommended dose level of 5000 µg/plate. No test material precipitate was observed on the plates at any of the doses tested in either the presence or absence of S9-mix.

Large, dose-related and statistically significant increases in the frequency of TA100, TA1535 and WP2*uvrA*⁻ revertant colonies were observed at the majority of test material dose levels, initially from 50 and 150 µg/plate, in the absence and presence of S9, respectively. The increases in colony frequency at the upper dose levels of the test material were very large and in excess of the in-house historical control range of each strain (particularly TA100 and TA1535). A reproducible response was evident for bacterial strains TA100 and WP2*uvrA*⁻ when taking the results of the Preliminary Toxicity Test into consideration. Therefore, the test material was considered to be mutagenic in bacterial strains TA100, TA1535 and WP2*uvrA*⁻. No increases were noted for the Frameshift detecting strains (TA98 and TA1537).

All of the positive control chemicals used in the test induced marked increases in the frequency of revertant colonies thus confirming the activity of the S9-mix and the sensitivity of the bacterial strains.

## 6. CONCLUSION

The test material was considered to be mutagenic under the conditions of this test.

## 7. REFERENCES

1. Ames B N, Durston W E, Yamasaki E and Lee F D (1973b) *Proc. Natl. Acad. Sci. (USA)*, **70**, 2281-2285.

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5. Kirkland D J (Ed) (1989) Statistical Evaluation of Mutagenicity Test Data. *UKEMS Sub-committee on Guidelines for Mutagenicity Testing*, **Report - Part III**, Cambridge University Press.

6. Maron D et al (1981) *Mutation Research*, **88**, 343-350.

REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

**Table 1 Spontaneous Mutation Rates (Concurrent Negative Controls)**

**EXPERIMENT 1**

| Number of revertants (mean number of colonies per plate) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Base-pair substitution type | | | | | | Frameshift type | | | |
| TA100 | | TA1535 | | WP2*uvrA*⁻ | | TA98 | | TA1537 | |
| 102 | | 16 | | 34 | | 14 | | 11 | |
| 91 | (100) | 20 | (17) | 35 | (31) | 21 | (19) | 13 | (13) |
| 108 | | 16 | | 25 | | 22 | | 14 | |

REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

## Table 2 Test Results: Experiment 1 – Without Metabolic Activation

| Test Period | | From: 24 August 2008 | | | | | | To: 27 August 2008 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| With or without S9-Mix | Test substance concentration (µg/plate) | Number of revertants (mean number of colonies per plate) | | | | | | | | | |
| | | Base-pair substitution type | | | | | | Frameshift type | | | |
| | | TA100 | | TA1535 | | WP2*uvrA*- | | TA98 | | TA1537 | |
| - | 0 | 95<br>117<br>92 | (101)<br>13.7# | 13<br>15<br>11 | (13)<br>2.0 | 37<br>29<br>42 | (36)<br>6.6 | 53<br>54<br>43 | (50)<br>6.1 | 15<br>14<br>14 | (14)<br>0.6 |
| - | 50 | 142<br>169<br>177 | (163)<br>18.3<br>* | 40<br>22<br>45 | (36)<br>12.1<br>$$$ | 36<br>42<br>47 | (42)<br>5.5 | 59<br>53<br>66 | (59)<br>6.5 | 12<br>19<br>18 | (16)<br>3.8 |
| - | 150 | 231<br>242<br>245 | (239)<br>7.4<br>$$$ | 47<br>52<br>48 | (49)<br>2.6<br>$$$ | 33<br>55<br>62 | (50)<br>15.1 | 62<br>64<br>49 | (58)<br>8.1 | 18<br>10<br>27 | (18)<br>8.5 |
| - | 500 | 510<br>499<br>494 | (501)<br>8.2<br>$$$ | 99<br>104<br>121 | (108)<br>11.5<br>$$$ | 67<br>69<br>58 | (65)<br>5.9<br>$$$ | 65<br>47<br>86 | (66)<br>19.5 | 33<br>15<br>25 | (24)<br>9.0 |
| - | 1500 | 829<br>857<br>861 | (849)<br>17.4<br>$$$ | 229<br>212<br>249 | (230)<br>18.5<br>$$$ | 99<br>91<br>84 | (91)<br>7.5<br>$$$ | 52<br>48<br>57 | (52)<br>4.5 | 26<br>13<br>16 | (18)<br>6.8 |
| - | 5000 | 1383<br>1612<br>1297 | (1431)<br>162.8<br>$$$ | 342<br>364<br>385 | (364)<br>21.5<br>$$$ | 154<br>131<br>159 | (148)<br>14.9<br>$$$ | 48<br>33<br>53 | (45)<br>10.4 | 22<br>18<br>7 | (16)<br>7.8 |
| Positive controls | Name | ENNG | | ENNG | | ENNG | | 4NQO | | 9AA | |
| | Concentration (µg/plate) | 3 | | 5 | | 2 | | 0.2 | | 80 | |
| S9-Mix<br>- | No. colonies per plate | 272<br>294<br>359 | (308)<br>45.2 | 260<br>269<br>201 | (243)<br>36.9 | 798<br>859<br>822 | (826)<br>30.7 | 158<br>169<br>167 | (165)<br>5.9 | 929<br>985<br>967 | (960)<br>28.6 |

ENNG N-ethyl-N'-nitro-N-nitrosoguanidine
4NQO 4-Nitroquinoline-1-oxide
9AA 9-Aminoacridine
* $p \leq 0.05$
$$$ $p \leq 0.005$
# Standard deviation

## REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

### Table 3 Test Results: Experiment 1 – With Metabolic Activation

| Test Period | | From: 24 August 2008 | | | | | | To: 27 August 2008 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| With or without S9-Mix | Test substance concentration (µg/plate) | Number of revertants (mean number of colonies per plate) | | | | | | | | | | | |
| | | Base-pair substitution type | | | | | | Frameshift type | | | | | |
| | | TA100 | | TA1535 | | WP2*uvrA*- | | TA98 | | TA1537 | | | |
| + | 0 | 99<br>122<br>107 | (109)<br>11.7# | 13<br>13<br>14 | (13)<br>0.6 | 46<br>37<br>44 | (42)<br>4.7 | 33<br>33<br>48 | (38)<br>8.7 | 16<br>14<br>14 | (15)<br>1.2 | | |
| + | 50 | 114<br>125<br>147 | (129)<br>16.8 | 18<br>13<br>25 | (19)<br>6.0 | 48<br>55<br>58 | (54)<br>5.1 | 40<br>48<br>26 | (38)<br>11.1 | 11<br>15<br>10 | (12)<br>2.6 | | |
| + | 150 | 140<br>134<br>150 | (141)<br>8.1 | 29<br>32<br>27 | (29)<br>2.5<br>$$$ | 46<br>41<br>56 | (48)<br>7.6 | 29<br>33<br>29 | (30)<br>2.3 | 13<br>12<br>13 | (13)<br>0.6 | | |
| + | 500 | 227<br>191<br>222 | (213)<br>19.5<br>$$$ | 54<br>66<br>76 | (65)<br>11.0<br>$$$ | 65<br>49<br>62 | (59)<br>8.5 | 33<br>45<br>33 | (37)<br>6.9 | 15<br>18<br>15 | (16)<br>1.7 | | |
| + | 1500 | 346<br>345<br>338 | (343)<br>4.4<br>$$$ | 120<br>125<br>139 | (128)<br>9.8<br>$$$ | 64<br>73<br>64 | (67)<br>5.2<br>* | 32<br>33<br>46 | (37)<br>7.8 | 15<br>10<br>14 | (13)<br>2.6 | | |
| + | 5000 | 1794<br>2013<br>1665 | (1824)<br>175.9<br>$$$ | 309<br>328<br>338 | (325)<br>14.7<br>$$$ | 166<br>173<br>241 | (193)<br>41.4<br>$$$ | 34<br>54<br>40 | (43)<br>10.3 | 15<br>10<br>12 | (12)<br>2.5 | | |
| Positive controls | Name | 2AA | | 2AA | | 2AA | | BP | | 2AA | | | |
| | Concentration (µg/plate) | 1 | | 2 | | 10 | | 5 | | 2 | | | |
| S9-Mix<br>+ | No. colonies per plate | 1375<br>1391<br>1195 | (1320)<br>108.8 | 291<br>298<br>324 | (304)<br>17.4 | 573<br>515<br>467 | (518)<br>53.1 | 420<br>335<br>308 | (354)<br>58.4 | 245<br>247<br>236 | (243)<br>5.9 | | |

2AA 2-Aminoanthracene
BP Benzo(a)pyrene
* $p \leq 0.05$
$$$ $p \leq 0.005$
# Standard deviation

REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

## Appendix 1 History Profile of Vehicle and Positive Control Values

### COMBINED VEHICLE AND UNTREATED CONTROL VALUES 2006

| Strain | TA100 | | TA1535 | | WP2uvrA⁻ | | TA102 | | TA98 | | TA1537 | | WP2uvrA⁻ pKM101 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S9-Mix | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
| Mean | 99 | 90 | 20 | 13 | 25 | 30 | 286 | 337 | 22 | 26 | 12 | 14 | 133 | 157 |
| SD | 15.6 | 13.7 | 5.6 | 3.2 | 4.5 | 5.4 | 34.7 | 31.2 | 5.7 | 5.7 | 3.2 | 4.6 | 31.3 | 28.4 |
| Min | 66 | 62 | 8 | 6 | 12 | 14 | 197 | 245 | 11 | 13 | 4 | 3 | 77 | 121 |
| Max | 160 | 147 | 39 | 31 | 48 | 50 | 381 | 393 | 77 | 48 | 28 | 27 | 234 | 216 |
| Values | 897 | 733 | 880 | 716 | 787 | 632 | 151 | 88 | 906 | 732 | 868 | 703 | 81 | 22 |

### POSITIVE CONTROL VALUES 2006

| Strain | TA100 | | TA1535 | | WP2uvrA⁻ | | TA102 | | TA98 | | TA1537 | | WP2uvrA⁻ pKM101 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S9-Mix | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
| Mean | 578 | 1585 | 352 | 245 | 855 | 541 | 1458 | 917 | 218 | 222 | 1023 | 498 | 1962 | 1850 |
| SD | 218.2 | 566.9 | 229.5 | 82.2 | 325.1 | 222.1 | 267.7 | 166.1 | 64.3 | 67.0 | 502.1 | 138.8 | 839.1 | 889.8 |
| Min | 228 | 363 | 111 | 108 | 276 | 199 | 919 | 621 | 101 | 102 | 175 | 186 | 553 | 110 |
| Max | 1955 | 2969 | 1448 | 621 | 1942 | 1991 | 2074 | 1430 | 480 | 417 | 2718 | 917 | 3438 | 3053 |
| Values | 162 | 162 | 164 | 164 | 159 | 158 | 64 | 64 | 164 | 164 | 164 | 164 | 19 | 20 |

### COMBINED VEHICLE AND UNTREATED CONTROL VALUES 2007

| Strain | TA100 | | TA1535 | | WP2uvrA⁻ | | TA102 | | TA98 | | TA1537 | | WP2uvrA⁻ pKM101 | | WP2pKM101 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S9-Mix | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
| Mean | 94 | 87 | 21 | 13 | 26 | 28 | 273 | 309 | 21 | 26 | 11 | 13 | 132 | 162 | 100 | 128 |
| SD | 16.0 | 14.8 | 5.2 | 3.6 | 5.8 | 6.4 | 40.4 | 43.2 | 5.9 | 6.0 | 3.8 | 4.1 | 48.5 | 47.8 | 21.8 | 21.4 |
| Min | 63 | 50 | 9 | 6 | 12 | 11 | 194 | 208 | 7 | 11 | 2 | 4 | 65 | 78 | 67 | 98 |
| Max | 156 | 152 | 38 | 28 | 46 | 49 | 390 | 395 | 99 | 57 | 28 | 27 | 278 | 263 | 136 | 165 |
| Values | 807 | 629 | 767 | 601 | 541 | 384 | 159 | 107 | 1282 | 609 | 1245 | 569 | 197 | 128 | 27 | 15 |

### POSITIVE CONTROL VALUES 2007

| Strain | TA100 | | TA1535 | | WP2uvrA⁻ | | TA102 | | TA98 | | TA1537 | | WP2uvrA⁻ pKM101 | | WP2pKM101 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S9-Mix | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
| Mean | 558 | 1231 | 343 | 231 | 652 | 505 | 1281 | 960 | 256 | 308 | 1697 | 339 | 960 | 1103 | 2134 | 997 |
| SD | 313.5 | 651.1 | 864.2 | 476.5 | 707.9 | 535.2 | 458.9 | 303.6 | 289.7 | 280.5 | 1193.6 | 254.4 | 1679.6 | 1010.6 | 1047.0 | 586.4 |
| Min | 258 | 320 | 79 | 73 | 138 | 139 | 567 | 401 | 78 | 106 | 117 | 107 | 332 | 355 | 1091 | 285 |
| Max | 1634 | 3777 | 2087 | 661 | 2023 | 1185 | 2422 | 1441 | 1483 | 2055 | 5618 | 677 | 2522 | 3023 | 3934 | 2024 |
| Values | 196 | 197 | 197 | 203 | 159 | 159 | 60 | 61 | 419 | 202 | 414 | 198 | 64 | 64 | 9 | 11 |

SD = Standard deviation
Min = Minimum value
Max = Maximum value

## Appendix 2 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

Department of Health

# THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

## GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

| TEST FACILITY | TEST TYPE |
|---|---|
| **SafePharm Laboratories Ltd.**<br>**Shardlow Business Park**<br>**Shardlow**<br>**Derbyshire**<br>**DE72 2GD** | **Analytical Chemistry**<br>**Environmental Fate**<br>**Environmental Toxicity**<br>**Mutagenicity**<br>**Phys/Chem testing**<br>**Toxicology** |

DATE OF INSPECTION

**21st August 2007**

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

15/10/07

**Dr. Andrew J. Gray**
**Head, UK GLP Monitoring Authority**

MHRA

harlan™

[ ]

# L5178Y TK +/- MOUSE LYMPHOMA ASSAY

## PROJECT NUMBER: 1518/0060

AUTHOR: [ ]

**STUDY SPONSOR:**

[ ]

**TEST FACILITY:**

Harlan Laboratories Ltd
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD
UK

Telephone: +44 (0) 1332 792896

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## QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress. In addition, inspection of general facilities not specifically related to this study are done monthly or annually in accordance with QA Standard Procedure.

This report has been audited by the Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, unless otherwise indicated, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

| | | |
|---|---|---|
| | 05 February 2008 | Standard Test Method Compliance Audit |
| | 11 November 2008 | Test Material Preparation |
| | 05 November 2008 | Test System Preparation |
| | 11 November 2008 | Exposure |
| | 06 November 2008 | Assessment of Response |
| | 07 November 2008 | Plating |
| § | 05 December 2008 | Draft Report Audit |
| § | Date of QA Signature | Final Report Audit |

§ Evaluation specific to this study

..... G. Wren ..... DATE: ..... 09 DEC 2008 .....

For the Quality Assurance Unit*

***Authorised QA Signatures:**
Deputy Head of Department: JM Crowther MIScT MRQA
Senior Audit Staff: G Wren ONC MRQA

## GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

This report fully and accurately reflects the procedures used and data generated.

L. Flanders ..... DATE: 08 DEC 2008

L Flanders
Study Director

This report may be presented in final form as a digital (pdf) document. Such documents produced are prepared by scanning the paper original, and are considered of equivalent integrity and authenticity to versions produced by optical photocopy. However, in all cases the hand-signed paper original, held in secure archives, is the definitive document.

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[ ]

# L5178Y TK +/- MOUSE LYMPHOMA ASSAY

## SUMMARY

***Introduction.*** The study was conducted according to a method that was designed to assess the potential mutagenicity of the test material on the thymidine kinase, TK +/-, locus of the L5178Y mouse lymphoma cell line.

***Methods.*** L5178Y TK +/- 3.7.2c mouse lymphoma cells (heterozygous at the thymidine kinase locus) were treated with the test material at eight dose levels, in duplicate, together with vehicle (solvent) and positive controls. Four-hour exposures were used both with and without metabolic activation. An unequivocal mutagenic response was obtained in Experiment 1 and, therefore, a repeat experiment was not performed. This is in accordance with the current OECD 476 test guideline.

The dose range of test material for Experiment 1 was selected following the results of a preliminary toxicity test and was 12.5 to 150 µg/ml in the absence of metabolic activation and 2.5 to 60 µg/ml in the presence of metabolic activation.

***Results.*** The maximum dose level used was limited by test material induced toxicity. No precipitate of test material was observed at any of the dose levels. The vehicle (solvent) controls had acceptable mutant frequency values that were within the normal range for the L5178Y cell line at the TK +/- locus. The positive control materials induced marked increases in the mutant frequency indicating the satisfactory performance of the test and of the activity of the metabolising system.

The test material induced marked statistically significant dose-related (linear-trend) increases in the mutant frequency in both the absence and presence of metabolic activation. The increases in mutant frequency in the presence of metabolic activation were predominantly due to small colony formation, indicating clastogenic activity resulting in structural chromosome damage.

***Conclusion.*** The test material was considered to be mutagenic to L5178Y cells under the conditions of the test.

[ ]

# L5178Y TK +/- MOUSE LYMPHOMA ASSAY

## 1. INTRODUCTION

This study was conducted according to a method that was designed to assess the potential mutagenicity of the test material on the thymidine kinase, TK +/-, locus of the L5178Y mouse lymphoma cell line.

The use of cultured mammalian cells for mutation studies may give a measure of the intrinsic response of the mammalian genome and its maintenance process to mutagens. Such techniques have been used for many years with widely different cell types and loci. The thymidine kinase heterozygote system, TK +/- to TK -/-, was described by Clive *et al.*, (1972) and is based upon the L5178Y mouse lymphoma cell line established by Fischer (1958). This system has been extensively validated (Clive *et al.*, 1979; Amacher *et al*, 1980; Jotz and Mitchell, 1981).

The protocol used meets the requirements of the OECD (476) Council Regulation (EC) No. 440/2008 of 30 May 2008 and the United Kingdom Environmental Mutagen Society. The technique used was a fluctuation assay using microtitre plates and trifluorothymidine as the selective agent and is based on that described by Cole and Arlett (1984). Two distinct types of mutant colonies can be recognised, i.e. large and small. Large colonies grow at a normal rate and represent events within the gene (base-pair substitutions or deletions) whilst small colonies represent large genetic changes involving chromosome 11b (indicative of clastogenic activity).

On the 9th November 2008 Safepharm Laboratories Ltd. changed its name to Harlan Laboratories Ltd. Therefore, this study has been reported and archived under the name Harlan Laboratories Ltd., and not Safepharm Laboratories Ltd. as stated in the protocol. This amendment was considered not to affect the purpose or integrity of the study as the facilities and the management structure remain the same.

The experimental phases of the study were performed between 29 September 2008 and 10 November 2008.

## 2. TEST MATERIAL

| | | |
|---|---|---|
| Sponsor's identification | : | [ ] |
| Description | : | Clear colourless liquid |
| Batch number | : | 08C10938 |
| Date received | : | 04 August 2008 |
| Storage conditions | : | Approximately 4°C in the dark under nitrogen |

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

## 3. METHODS

### 3.1 Cell Line

The L5178Y TK+/- 3.7.2c mouse lymphoma cell line was obtained from Dr J Cole of the MRC Cell Mutation Unit at the University of Sussex, Brighton, UK. The cells were originally obtained from Dr D Clive of Burroughs Wellcome (USA) in October 1978 and were frozen in liquid nitrogen at that time.

### 3.2 Cell Culture

The stocks of cells are stored in liquid nitrogen at -196°C. Cells were routinely cultured in RPMI 1640 medium with Glutamax-1 and HEPES buffer (20 mM) supplemented with Penicillin (100 units/ml), Streptomycin (100 µg/ml), Sodium pyruvate (1 mM), Amphotericin B (2.5 µg/ml) and 10% donor horse serum (giving R10 media) at 37°C with 5% $CO_2$ in air. The cells have a generation time of approximately 12 hours and were subcultured accordingly. RPMI 1640 with 20% donor horse serum (R20) and without serum (R0) are used during the course of the study. Master cultures of stock cells were checked and found to be mycoplasma free and were cultured using methods designed to reduce the risk of possible contamination.

### 3.3 Cell Cleansing

The TK +/- heterozygote cells grown in suspension spontaneously mutate at a low but significant rate. Before the stocks of cells were frozen they were cleansed of homozygous (TK -/-) mutants by culturing in THMG medium for 24 hours. This medium contained Thymidine (9 µg/ml), Hypoxanthine (15 µg/ml), Methotrexate (0.3 µg/ml) and

Glycine (22.5 µg/ml). For the following 24 hours the cells were cultured in THG medium (i.e. THMG without Methotrexate) before being returned to R10 medium.

### 3.4 Preparation of Test and Control Materials

The test material was accurately weighed and dissolved in Dimethyl sulfoxide (DMSO) before the appropriate dilutions were made. The molecular weight of the test material was 180.24, therefore the maximum dose level investigated in the Preliminary Toxicity Test was 1800 µg/ml which was approximately equivalent to 10 mM. There was no marked change in pH when the test material was dosed into media and the osmolality did not increase by more than 50 mOsm. Analysis for concentration, homogeneity and stability of the test material preparations were not a requirement of the test method and were therefore not performed.

Vehicle and positive controls were used in parallel with the test material. Solvent (DMSO) treatment groups were used as the vehicle controls. Ethylmethanesulphonate (EMS) Sigma batch 116K0765 at 400 µg/ml was used as the positive control in the absence of metabolic activation. Cyclophosphamide (CP) Acros batch A0164185 at 2 µg/ml was used as the positive control in the presence of metabolic activation.

### 3.5 Microsomal Enzyme Fractions

PB/βNF S9 was prepared in-house at Safepharm Laboratories on 18 May 2008 and 31 August 2008 from the livers of male Sprague-Dawley and Wistar Han™ rats respectively, weighing ~250g. These had each received, orally, three consecutive daily doses of phenobarbital/β-naphthoflavone (80/100 mg per kg per day) prior to S9 preparation on the fourth day. The S9 was stored at -196°C in a liquid nitrogen freezer.

20% S9-mix was prepared by mixing S9, NADP (5 mM), G6P (5 mM), KCl (33 mM) and $MgCl_2$ (8 mM) in R0.

The final concentration of S9 was 2% throughout the study.

### 3.6 Preliminary Toxicity Test

A preliminary toxicity test was performed on cell cultures at 5 x $10^5$ cells/ml, using a 4-hour exposure time both with and without metabolic activation (S9), and at 1.5 x $10^5$

cells/ml using a 24-hour exposure without S9. The dose range used in the preliminary toxicity test was 7.03 to 1800 µg/ml for all three of the exposure groups. Following the exposure period the cells were washed twice with R10, resuspended in R20 medium, counted using a coulter counter and then serially diluted to $2 \times 10^5$ cells/ml.

The cultures were incubated and sub-cultured after 24 hours by counting and diluting to $2 \times 10^5$ cells/ml. After a further 24 hours the cultures were counted and then discarded. The cell counts were then used to calculate Suspension Growth values (SG). The SG values were then adjusted to account for immediate post treatment toxicity, and a comparison of each treatment SG value to the concurrent vehicle control performed to give a % Relative Suspension Growth Value (%RSG).

Results from the preliminary toxicity test were used to set the test material dose levels for the mutagenicity experiments. Maximum dose levels were selected using the following criteria:

i) Maximum recommended dose level, 5000 µg/ml or 10 mM.

ii) The presence of excessive precipitate where no test material-induced toxicity was observed.

iii) Test material-induced toxicity, where the maximum dose level used should produce 10 to 20% survival (the maximum level of toxicity required). This optimum upper level of toxicity was confirmed by an IWGT meeting in New Orleans, USA (Moore et al 2002).

### 3.7 Mutagenicity Test

Several days before starting the experiment, an exponentially growing stock culture of cells was set up so as to provide an excess of cells on the morning of the experiment. The cells were counted and processed to give $1 \times 10^6$ cells/ml in 10 ml aliquots in R10 medium in sterile plastic universals. The treatments were performed in duplicate (A + B), both with and without metabolic activation (S9-mix) at eight dose levels of the test material (12.5 to 150 µg/ml in the absence of metabolic activation, and 2.5 to 60 µg/ml in the presence of metabolic activation), vehicle and positive controls. To each universal was added 2 ml of S9-mix if required, 0.2 ml of the treatment dilutions, (0.2 ml for the positive control) and sufficient R0 medium to bring the total volume to 20 ml.

The treatment vessels were incubated at 37°C for 4 hours with continuous shaking using an orbital shaker within an incubated hood.

At the end of the treatment period the cells were washed twice using R10 medium then resuspended in R20 medium at a cell density of 2 x $10^5$ cells/ml. The cultures were incubated and subcultured every 24 hours for the expression period of two days, by counting and dilution to 2 x $10^5$ cells/ml.

On Day 2 of the experiment, the cells were counted, diluted to $10^4$ cells/ml and plated for mutant frequency (2000 cells/well) in selective medium containing 4 µg/ml 5-trifluorothymidine (TFT) in 96-well microtitre plates. Cells were also diluted to 10 cells/ml and plated (2 cells/well) for viability (%V) in non-selective medium.

The daily cell counts were used to obtain a Relative Suspension Growth (%RSG) value that gives an indication of post treatment toxicity during the expression period as a comparison to the vehicle control, and when combined with the Viability (%V) data a Relative Total Growth (RTG) value.

### 3.8 Plate Scoring

Microtitre plates were scored using a magnifying mirror box after ten to fourteen days incubation. The number of positive wells (wells with colonies) was recorded together with the total number of scorable wells (normally 96 per plate). The numbers of small and large colonies seen in the TFT mutation plates were also recorded. Colonies are scored manually by eye using qualitative judgement. Large colonies are defined as those that cover approximately ¼ to ¾ of the surface of the well and are generally no more than one or two cells thick. As a rule of thumb, all colonies less than 25% of the average area of the large colonies are scored as small colonies. Small colonies are normally observed to be more than two cells thick. To assist the scoring of the TFT mutant colonies 0.025 ml of MTT solution (2.5 mg/ml in PBS) was added to each well of the mutation plates. The plates were incubated for approximately two hours. MTT is a vital stain that is taken up by viable cells and metabolised to give a brown/black colour, thus aiding the visualisation of the mutant colonies, particularly the small colonies.

### 3.9 Calculation of % Relative Suspension Growth (%RSG)

The cell counts obtained immediately post treatment and over the 2-day expression period were used to calculate the % Relative Suspension Growth.

Suspension Growth (SG) = (24-hour cell count/2) x (48-hour cell count/2)

Day 0 Factor = dose 0-hour cell count/vehicle control 0-hour cell count

%RSG = [(dose SG x dose Day 0 Factor)/vehicle control SG] x 100

### 3.10 Calculation of Plating Efficiency (PE)

Since the distribution of colony-forming units over the wells is described by the Poisson distribution, the plating efficiency (PE), from which is derived the viability (%V), was calculated using the zero term of the Poisson distribution [P(0)] method.

$$P(0) = \frac{\text{number of negative wells}}{\text{total wells plated}}$$

$$PE\% = \frac{-\ln P(0) \times 100}{\text{number of cells/well}}$$

### 3.11 Calculation of Relative Total Growth (RTG)

For each culture, the relative cloning efficiency, RCE, was calculated:

$$RCE = \frac{PE}{\text{Mean Solvent Control PE}} \times 100\%$$

Finally, for each culture RTG is calculated:

RTG = (RCE x RSG)/100%

### 3.12 Calculation of Mutation Frequency (MF)

MF per survivor = [(-ln P(0) selective medium)/cells per well in selective medium)]/surviving fraction in non-selective medium.

The experimental data was analysed using a dedicated computer program which follows the statistical guidelines recommended by the UKEMS.

### 3.13 Interpretation of Results

The normal range for mutant frequency per survivor is 50-200 x $10^{-6}$ for the TK+/- locus in L5178Y cells at this laboratory. Vehicle controls results should ideally be within this range, although minor errors in cell counting and dilution or exposure to the metabolic activation system may cause this to be slightly elevated. Experiments where the vehicle control values are markedly greater than 250 x $10^{-6}$ mutant frequency per survivor are not normally acceptable and will be repeated.

Positive control chemicals should induce at least three to five fold increases in mutant frequency greater than the corresponding vehicle control.

During the course of the study dose selection for the mutagenicity experiments will be made using data from the preliminary toxicity test in an attempt to obtain the desired levels of toxicity. This optimum toxicity is approximately 20% survival (80% toxicity), but no less than 10% survival (90% toxicity). Both %RSG and RTG values are used either individually or combined to designate the level of toxicity achieved by the test material for any individual dose level. Dose levels that have survival values less than 10% are excluded from any statistical analysis, as any response they give would be considered to have no biological or toxicological relevance.

For a test material to demonstrate a mutagenic response it must produce a statistically significant increase in the induced mutant frequency (IMF) over the concurrent vehicle mutant frequency value. Following discussions at an International Workshop on Genotoxicity Test Procedures in Plymouth, UK, 2002 (Moore et al 2003) it was felt that the IMF must exceed some value based on the global background MF for each method (agar or microwell). This Global Evaluation Factor (GEF) value was set following a further meeting of the International Workshop in Aberdeen, Scotland, 2003 (Moore et al 2006) at 126 x $10^{-6}$ for the microwell method. Therefore any test material dose level that has a mutation frequency value that is greater than the corresponding vehicle control by the GEF of 126 x $10^{-6}$ will be considered positive. However, if a test material produces a modest increase in mutant frequency, which only marginally exceeds the GEF value and is not reproducible or part of a dose-related response, then it may be considered to have no toxicological significance. Conversely, when a test material induces modest reproducible increases in the mutation frequencies that do not exceed the GEF value

then scientific judgement will be applied. If the reproducible responses are significantly dose-related and include increases in the absolute numbers of mutant colonies then they may be considered to be toxicologically significant.

Small significant increases designated by the UKEMS statistical package will be reviewed using the above criteria, and may be disregarded at the Study Director's discretion.

## 4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Harlan Laboratories Ltd, Shardlow, UK archives for five years, after which instructions will be sought as to further retention or disposal.

# 5. RESULTS

## 5.1 Preliminary Toxicity Test

The dose range of the test material used in the preliminary toxicity test was 7.03 to 1800 µg/ml. The results for the Relative Suspension Growth (%RSG) were as follows:

| Dose (µg/ml) | % RSG (-S9) 4-Hour Exposure | % RSG (+S9) 4-Hour Exposure | % RSG (-S9) 24-Hour Exposure |
|---|---|---|---|
| 0 | 100 | 100 | 100 |
| 7.03 | 110 | 92 | 113 |
| 14.06 | 105 | 92 | 110 |
| 28.13 | 105 | 80 | 67 |
| 56.25 | 78 | 35 | 26 |
| 112.5 | 50 | 8 | 1 |
| 225 | 1 | 3 | 0 |
| 450 | 1 | 0 | 0 |
| 900 | 2 | 1 | 0 |
| 1800 | 0 | 0 | 0 |

In all three of the exposure groups there was a marked reduction in the Relative Suspension Growth (%RSG) of cells treated with the test material when compared to the concurrent vehicle controls. A greasy / oily precipitate of the test material was observed at 1800 µg/ml at the end of the exposure period. In the subsequent Mutagenicity Test the maximum dose level was limited by toxicity.

## 5.2 Mutagenicity Test

A summary of the results from the test is presented in Table 1.

The results of the microtitre plate counts and their analysis are presented in Tables 2 to 7.

There was evidence of toxicity following exposure to the test material in both the absence and presence of metabolic activation, as indicated by the %RSG and RTG values. There was also evidence of a marked reduction in (%V) viabilities in the presence of metabolic activation, therefore indicating that residual toxicity had occurred. Optimum levels of toxicity were achieved in both the absence and presence of metabolic

activation. The excessive toxicity observed at 150 µg/ml in the absence of metabolic activation resulted in this dose level not being plated for viability or TFT resistance. The toxicity observed at 60 µg/ml in the presence of metabolic activation exceeded the upper acceptable limit of 90%; therefore, this dose level was excluded from the statistical analysis. Acceptable levels of toxicity were seen with both positive control substances (Table 3 and Table 6).

Neither of the vehicle control mutant frequency values were outside the range of 50 to 200 x $10^{-6}$ viable cells that is acceptable for L5178Y cells at Harlan Laboratories Ltd, Shardlow, UK. Both of the positive controls produced marked increases in the mutant frequency per viable cell indicating that the test system was operating satisfactorily and that the metabolic activation system was functional (Tables 3 and 6).

The test material induced marked statistically significant dose related (linear-trend) increases in the mutant frequency x $10^{-6}$ per viable cell in both the absence and presence of metabolic activation (Tables 3 and 6). In the absence of metabolic activation, statistically significant increases in mutant frequency were observed at the upper two surviving dose levels, dose levels at or approaching the toxic limit of the test material. However, the increases in mutant frequency approached the GEF at 110 µg/ml and greatly exceeded it at 130 µg/ml, where there was also evidence of an increase in absolute numbers of mutant colonies (Table 3). A much greater response was observed in the presence of S9. Increases in mutant frequency that greatly exceeded the GEF were observed at dose levels with much lower levels of toxicity than those observed in the absence of metabolic activation (Table 6). There were also increases in the absolute numbers of mutant colonies at these dose levels. No precipitate of test material was observed at any of the dose levels.

The numbers of small and large colonies and their analysis are presented in Tables 4 and 7. Whilst the increase in mutant frequency was only partly due to small colony formation in the absence of metabolic activation, a marked shift towards small colony formation was observed in the presence of metabolic activation, therefore indicating a clastogenic response.

It was considered not necessary to perform the scheduled second experiment as an unequivocal positive mutagenic response was obtained in the first mutagenicity test. This is in accordance with the current OECD 476 Test Guideline and, therefore, did not affect the integrity of the study.

## 6. CONCLUSION

The test material induced toxicologically significant increases in the mutant frequency at the TK +/- locus in L5178Y cells and is therefore considered to be mutagenic under the conditions of the test.

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L5178Y TK +/- MOUSE LYMPHOMA ASSAY

**Key to Tables 1 to 7**

| | | |
|---|---|---|
| $ | = | Cell counts ($x10^5$ cells/ml). Set up on previous day to $2 \times 10^5$ cells/ml unless otherwise stated in parenthesis. |
| %RSG | = | Relative Suspension Growth |
| RTG | = | Relative Total Growth |
| %V | = | Viability Day 2 |
| § or # | = | Positive wells per tray, 96 wells plated unless otherwise stated in parenthesis |
| A,B | = | Replicate cultures |
| CP | = | Cyclophosphamide |
| EMS | = | Ethylmethanesulphonate |
| MF§ | = | 5-TFT resistant mutants/$10^6$ viable cells 2 days after treatment |
| NP | = | Not plated due to toxicity or surplus to requirements |
| Ø | = | Not plated for viability or TFT resistance |
| Nv | = | Number of wells scored, viability plates |
| Yv | = | Number of wells without colonies, viability plates |
| Ym | = | Number of wells without colonies, mutation plates |
| Nm | = | Number of wells scored, mutation plates |
| X | = | Treatment excluded from test statistics due to toxicity |
| * | = | $p<0.05$ |
| *** | = | $p<0.001$ |

[ ] L5178Y TK +/- MOUSE LYMPHOMA ASSAY

**Table 1** **Summary of Results**

**Experiment 1**

| Treatment (µg/ml) | | 4-Hours-S-9 | | | | Treatment (µg/ml) | | 4-Hours+S-9 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | %RSG | RTG | MF§ | | | | %RSG | RTG | MF§ | |
| 0 | | 100 | 1.00 | 139.38 | | 0 | | 100 | 1.00 | 185.72 | |
| 12.5 | Ø | 97 | | | | 2.5 | Ø | 96 | | | |
| 25 | | 97 | 1.04 | 190.73 | | 5 | Ø | 91 | | | |
| 50 | | 79 | 0.69 | 193.81 | | 10 | | 89 | 0.91 | 201.54 | |
| 70 | | 68 | 0.78 | 165.09 | | 20 | | 92 | 0.98 | 271.47 | |
| 90 | | 53 | 0.51 | 217.03 | | 30 | | 74 | 0.55 | 521.06 | * |
| 110 | | 43 | 0.28 | 253.32 | * | 40 | | 62 | 0.43 | 790.10 | * |
| 130 | | 15 | 0.15 | 404.81 | * | 50 | | 47 | 0.18 | 1449.70 | * |
| 150 | Ø | 5 | | | | 60 | X | 32 | 0.07 | 2395.41 | |
| Linear trend | | | | | | Linear trend | | | | | |
| EMS 400 | | 81 | 0.73 | 651.73 | | CP 2 | | 67 | 0.42 | 774.68 | |

L5178Y TK +/- MOUSE LYMPHOMA ASSAY

**Table 2 Cell and 96-Well Plate Counts: Experiment 1 (-S9) 4-Hour Exposure**

| Treatment (µg/ml) | | Cell counts $ | | | Viability § after day 2 2 cells/well | | | | Resistant mutants § after day 2 2000 cells/well | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0h | 24h | 48h | | | | | | | | |
| 0 | A | 8.33 | 5.42 | 7.19 | 68 | 68 | 69 | 65 | 19 | 14 | 19 | 17 |
| | B | 9.28 | 5.69 | 5.76 | 70 | 75 | 72 | 78 | 14 | 15 | 16 | 16 |
| 12.5 | A | 8.88 | 5.59 | 6.53 | NP | NP | | | NP | NP | | |
| | B | 9.10 | 5.42 | 5.89 | NP | NP | | | NP | NP | | |
| 25 | A | 8.67 | 5.84 | 6.63 | 64 | 68 | | | 14 | 19 | | |
| | B | 8.60 | 4.72 | 6.87 | 82 | 77 | | | 30 | 28 | | |
| 50 | A | 8.49 | 4.34 | 7.27 | 64 | 56 | | | 17 | 16 | | |
| | B | 7.61 | 4.85 | 6.27 | 73 | 72 | | | 21 | 24 | | |
| 70 | A | 7.98 | 4.06 | 6.65 | 78 | 76 | | | 17 | 18 | | |
| | B | 7.41 | 4.93 | 5.72 | 73 | 76 | | | 27 | 25 | | |
| 90 | A | 8.08 | 3.76 | 6.18 | 62 | 70 | | | 18 | 19 | | |
| | B | 7.19 | 4.03 | 5.13 | 71 | 74 | | | 31 | 25 | | |
| 110 | A | 6.93 | 2.78 | 7.55(2.78) | 69 | 68 | | | 22 | 27 | | |
| | B | 6.79 | 2.57 | 7.15(2.57) | 64 | 63 | | | 27 | 22 | | |
| 130 | A | 7.32 | 1.89 | 3.88(1.89) | 67 | 61 | | | 26 | 33 | | |
| | B | 7.65 | 1.63 | 3.52(1.63) | 67 | 65 | | | 48 | 34 | | |
| 150 | A | 6.79 | 1.23 | 1.84(1.23) | NP | NP | | | NP | NP | | |
| | B | 7.14 | 1.07 | 1.99(1.07) | NP | NP | | | NP | NP | | |
| Positive control EMS (µg/ml) | | | | | | | | | | | | |
| 400 | A | 8.33 | 4.68 | 6.59 | 62 | 67 | | | 45 | 53 | | |
| | B | 8.88 | 5.41 | 5.18 | 66 | 73 | | | 54 | 56 | | |

L5178Y TK +/- MOUSE LYMPHOMA ASSAY

## Table 3 Statistical Analysis: Experiment 1 (-S9) 4-Hour Exposure

| Treatment (µg/ml) | | %RSG | %V | RTG | MF§ |
|---|---|---|---|---|---|
| 0 | | 100 | 66.53 | 1.00 | 139.38 |
| 12.5 | Ø | 97 | | | |
| 25 | | 97 | 70.90 | 1.04 | 190.73 |
| 50 | | 79 | 58.58 | 0.69 | 193.81 |
| 70 | | 68 | 77.81 | 0.78 | 165.09 |
| 90 | | 53 | 63.89 | 0.51 | 217.03 |
| 110 | | 43 | 58.16 | 0.28 | 253.32 |
| 130 | | 15 | 56.52 | 0.15 | 404.81 |
| 150 | Ø | 5 | | | |
| Positive control EMS | | | | | |
| Treatment (µg/ml) | | %RSG | %V | RTG | MF§ |
| 400 | | 81 | 59.85 | 0.73 | 651.73 |

**Test for linear trend**

| | |
|---|---|
| Slope | 1.075E-006 |
| Variance | 6.938E-014 |
| $b^2/S_b$ | 16.644*** |

[ ] L5178Y TK +/- MOUSE LYMPHOMA ASSAY

**Table 4** **Large and Small Colonies Analysis: Experiment 1 (-S9) 4-Hour Exposure**

| Treatment (µg/ml) | | Viability # after day 2 | | | | Small colonies # after day 2 | | | | Large colonies # after day 2 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | A | 68 | 68 | 69 | 65 | 8 | 6 | 5 | 6 | 11 | 8 | 14 | 11 |
| | B | 70 | 75 | 72 | 78 | 4 | 7 | 9 | 6 | 10 | 8 | 7 | 10 |
| 25 | A | 64 | 68 | | | 4 | 11 | | | 10 | 8 | | |
| | B | 82 | 77 | | | 8 | 11 | | | 22 | 17 | | |
| 50 | A | 64 | 56 | | | 4 | 7 | | | 13 | 9 | | |
| | B | 73 | 72 | | | 9 | 15 | | | 12 | 9 | | |
| 70 | A | 78 | 76 | | | 6 | 8 | | | 11 | 10 | | |
| | B | 73 | 76 | | | 4 | 11 | | | 23 | 14 | | |
| 90 | A | 62 | 70 | | | 6 | 7 | | | 12 | 12 | | |
| | B | 71 | 74 | | | 18 | 11 | | | 13 | 14 | | |
| 110 | A | 69 | 68 | | | 11 | 13 | | | 11 | 14 | | |
| | B | 64 | 63 | | | 13 | 10 | | | 14 | 12 | | |
| 130 | A | 67 | 61 | | | 12 | 7 | | | 14 | 26 | | |
| | B | 67 | 65 | | | 26 | 16 | | | 22 | 18 | | |
| 400 EMS | A | 62 | 67 | | | 10 | 10 | | | 35 | 43 | | |
| | B | 66 | 73 | | | 20 | 20 | | | 34 | 36 | | |

**Mutation frequencies**

| Treatment (µg/ml) | Viable | | Small colonies | | | Large colonies | | | Proportion small colony mutants |
|---|---|---|---|---|---|---|---|---|---|
| | | | Mutants | | | Mutants | | | |
| | Yv | Nv | Ym | Nm | MF§ | Ym | Nm | MF§ | |
| 0 | 203 | 768 | 717 | 768 | 51.6 | 689 | 768 | 81.6 | 0.39 |
| 25 | 93 | 384 | 350 | 384 | 65.4 | 327 | 384 | 113.3 | 0.37 |
| 50 | 119 | 384 | 349 | 384 | 81.6 | 341 | 384 | 101.4 | 0.45 |
| 70 | 81 | 384 | 355 | 384 | 50.5 | 326 | 384 | 105.2 | 0.33 |
| 90 | 107 | 384 | 342 | 384 | 90.6 | 333 | 384 | 111.5 | 0.45 |
| 110 | 120 | 384 | 337 | 384 | 112.2 | 333 | 384 | 122.5 | 0.48 |
| 130 | 124 | 384 | 323 | 384 | 153.0 | 304 | 384 | 206.7 | 0.43 |
| 400 EMS | 116 | 384 | 324 | 384 | 141.9 | 236 | 384 | 406.7 | 0.29 |

L5178Y TK +/- MOUSE LYMPHOMA ASSAY

**Table 5** **Cell and 96-Well Plate Counts: Experiment 1 (+S9) 4-Hour Exposure**

| Treatment (µg/ml) | | Cell counts $ | | | Viability § after day 2 2 cells/well | | | | Resistant mutants § after day 2 2000 cells/well | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0h | 24h | 48h | | | | | | | | |
| 0 | A | 9.15 | 6.11 | 7.76 | 79 | 68 | 69 | 67 | 22 | 16 | 22 | 24 |
| | B | 8.98 | 6.04 | 6.93 | 75 | 70 | 75 | 81 | 20 | 26 | 23 | 26 |
| 2.5 | A | 9.69 | 5.55 | 8.05 | NP | NP | | | NP | NP | | |
| | B | 8.63 | 6.03 | 6.64 | NP | NP | | | NP | NP | | |
| 5 | A | 8.45 | 5.99 | 7.09 | NP | NP | | | NP | NP | | |
| | B | 8.20 | 6.52 | 7.06 | NP | NP | | | NP | NP | | |
| 10 | A | 8.49 | 5.78 | 7.38 | 77 | 79 | | | 26 | 30 | | |
| | B | 8.78 | 6.27 | 6.50 | 72 | 67 | | | 20 | 22 | | |
| 20 | A | 8.74 | 5.45 | 7.51 | 84 | 69 | | | 32 | 29 | | |
| | B | 8.78 | 5.81 | 7.59 | 75 | 71 | | | 27 | 41 | | |
| 30 | A | 8.48 | 5.10 | 6.87 | 56 | 65 | | | 32 | 47 | | |
| | B | 8.59 | 4.84 | 7.31 | 68 | 62 | | | 39 | 45 | | |
| 40 | A | 8.68 | 4.94 | 5.64 | 59 | 53 | | | 52 | 60 | | |
| | B | 8.90 | 4.00 | 7.09 | 65 | 66 | | | 48 | 50 | | |
| 50 | A | 8.49 | 4.41 | 5.29 | 45 | 32 | | | 60 | 59 | | |
| | B | 8.12 | 3.83 | 5.73 | 46 | 43 | | | 47 | 49 | | |
| X 60 | A | 8.79 | 3.38 | 4.29 | 27 | 24 | | | 56 | 57 | | |
| | B | 8.68 | 3.09 | 4.82 | 28 | 20 | | | 45 | 38 | | |
| Positive control CP (µg/ml) | | | | | | | | | | | | |
| 2 | A | 9.06 | 4.89 | 6.01 | 49 | 59 | | | 44 | 47 | | |
| | B | 8.44 | 4.75 | 6.75 | 60 | 58 | | | 51 | 49 | | |

L5178Y TK +/- MOUSE LYMPHOMA ASSAY

## Table 6 Statistical Analysis: Experiment 1 (+S9) 4-Hour Exposure

| Treatment (µg/ml) | | %RSG | %V | RTG | MF§ |
|---|---|---|---|---|---|
| 0 | | 100 | 71.44 | 1.00 | 185.72 |
| 2.5 | Ø | 96 | | | |
| 5 | Ø | 91 | | | |
| 10 | | 89 | 73.10 | 0.91 | 201.54 |
| 20 | | 92 | 75.40 | 0.98 | 271.47 |
| 30 | | 74 | 53.01 | 0.55 | 521.06 |
| 40 | | 62 | 50.09 | 0.43 | 790.10 |
| 50 | | 47 | 28.31 | 0.18 | 1449.70 |
| 60 | X | 32 | 14.91 | 0.07 | 2395.41 |
| Positive control CP | | | | | |
| Treatment (µg/ml) | | %RSG | %V | RTG | MF§ |
| 2 | | 67 | 44.40 | 0.42 | 774.68 |

**Test for linear trend**

| | |
|---|---|
| Slope | 1.096E-005 |
| Variance | 2.039E-012 |
| $b^2/S_b$ | 58.933*** |

[ ] L5178Y TK +/- MOUSE LYMPHOMA ASSAY

## Table 7 Large and Small Colonies Analysis: Experiment 1 (+S9) 4-Hour Exposure

| Treatment (µg/ml) | | Viability # after day 2 | | | | Small colonies # after day 2 | | | | Large colonies # after day 2 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | A | 79 | 68 | 69 | 67 | 10 | 9 | 11 | 12 | 12 | 7 | 11 | 12 |
| | B | 75 | 70 | 75 | 81 | 7 | 9 | 12 | 8 | 13 | 17 | 11 | 18 |
| 10 | A | 77 | 79 | | | 10 | 16 | | | 16 | 14 | | |
| | B | 72 | 67 | | | 12 | 6 | | | 8 | 16 | | |
| 20 | A | 84 | 69 | | | 19 | 18 | | | 13 | 11 | | |
| | B | 75 | 71 | | | 18 | 24 | | | 9 | 17 | | |
| 30 | A | 56 | 65 | | | 19 | 32 | | | 13 | 15 | | |
| | B | 68 | 62 | | | 24 | 28 | | | 15 | 17 | | |
| 40 | A | 59 | 53 | | | 39 | 43 | | | 13 | 17 | | |
| | B | 65 | 66 | | | 30 | 35 | | | 18 | 15 | | |
| 50 | A | 45 | 32 | | | 46 | 44 | | | 14 | 15 | | |
| | B | 46 | 43 | | | 30 | 34 | | | 17 | 15 | | |
| X 60 | A | 27 | 24 | | | 43 | 40 | | | 13 | 17 | | |
| | B | 28 | 20 | | | 38 | 31 | | | 7 | 7 | | |
| 2 CP | A | 49 | 59 | | | 27 | 32 | | | 17 | 15 | | |
| | B | 60 | 58 | | | 36 | 31 | | | 15 | 18 | | |

**Mutation frequencies**

| Treatment (µg/ml) | Viable | | Small colonies | | | Large colonies | | | Proportion small colony mutants |
|---|---|---|---|---|---|---|---|---|---|
| | | | Mutants | | | Mutants | | | |
| | Yv | Nv | Ym | Nm | MF§ | Ym | Nm | MF§ | |
| 0 | 184 | 768 | 690 | 768 | 75.0 | 667 | 768 | 98.7 | 0.44 |
| 10 | 89 | 384 | 340 | 384 | 83.2 | 330 | 384 | 103.7 | 0.45 |
| 20 | 85 | 384 | 305 | 384 | 152.7 | 334 | 384 | 92.5 | 0.61 |
| 30 | 133 | 384 | 281 | 384 | 294.5 | 324 | 384 | 160.2 | 0.63 |
| 40 | 141 | 384 | 237 | 384 | 481.7 | 321 | 384 | 178.9 | 0.70 |
| 50 | 218 | 384 | 230 | 384 | 905.4 | 323 | 384 | 305.6 | 0.72 |
| 60 | 285 | 384 | 232 | 384 | 1690.1 | 340 | 384 | 408.2 | 0.78 |
| 2 CP | 158 | 384 | 258 | 384 | 447.8 | 319 | 384 | 208.8 | 0.66 |

[ ] L5178Y TK +/- MOUSE LYMPHOMA ASSAY

## Appendix 1 Historical Vehicle and Positive Control Mutation Frequencies

**Experiments -S9**

| Vehicle control | Positive control |
|---|---|
| MF | MF |
| 103.26 | 750.93 |
| 148.69 | 1389.62 |
| 76.01 | 644.25 |
| 111.60 | 859.93 |
| 59.06 | 404.08 |
| 98.89 | 390.47 |
| 74.10 | 867.79 |
| 148.29 | 1479.67 |
| 140.59 | 1120.00 |
| 140.85 | 1120.17 |
| 114.32 | 985.94 |
| 77.86 | 918.45 |
| 88.16 | 805.87 |
| 90.63 | 662.16 |
| 110.33 | 801.69 |
| 116.66 | 1046.34 |
| 148.68 | 646.27 |
| 86.16 | 918.42 |
| 139.43 | 991.46 |
| 111.51 | 925.06 |

[ ] L5178Y TK +/- MOUSE LYMPHOMA ASSAY

## Appendix 1 (Continued) Historical Vehicle and Positive Control Mutation Frequencies

**Experiments +S9**

| Vehicle control | Positive control |
|---|---|
| MF | MF |
| 145.05 | 648.22 |
| 115.97 | 918.59 |
| 94.43 | 808.63 |
| 107.44 | 602.27 |
| 110.35 | 876.39 |
| 74.26 | 618.05 |
| 86.27 | 456.57 |
| 137.59 | 756.60 |
| 58.57 | 311.15 |
| 67.55 | 617.94 |
| 141.27 | 825.53 |
| 147.39 | 1062.58 |
| 117.16 | 545.28 |
| 80.93 | 825.04 |
| 121.67 | 633.01 |
| 107.42 | 633.92 |
| 195.82 | 992.73 |
| 102.03 | 599.73 |
| 107.13 | 1214.46 |
| 137.07 | 615.33 |

## Appendix 2 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

Department of Health

# THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

## GOOD LABORATORY PRACTICE

### STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

| TEST FACILITY | TEST TYPE |
|---|---|
| **SafePharm Laboratories Ltd.**<br>**Shardlow Business Park**<br>**Shardlow**<br>**Derbyshire**<br>**DE72 2GD** | **Analytical Chemistry**<br>**Environmental Fate**<br>**Environmental Toxicity**<br>**Mutagenicity**<br>**Phys/Chem testing**<br>**Toxicology** |

DATE OF INSPECTION

**21st August 2007**

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

15/10/07

**Dr. Andrew J. Gray**
**Head, UK GLP Monitoring Authority**

MHRA

On 9th November 2008, Safepharm Laboratories Ltd changed its name to Harlan Laboratories Ltd. Therefore, until a new GLP Certificate is issued from the UK GLP monitoring authority the certificate in this report still states the name as Safepharm Laboratories Ltd.