

PE Biosystems

A DIVISION OF PERKIN-ELMER

850 Lincoln Centre Drive
Foster City CA 94404
(650) 570-6667

8EHQ - 0199-14333

RECEIVED
OPPT CBIC

99 JAN 11 AM 11:40

January 8, 1999

VIA FEDERAL EXPRESS

Document Processing Center 7407
Room G-99, East Tower
(Attn: Section 8(e) Coordinator)
U.S. Environmental Protection Agency
401 "M" Street, S.W.
Washington, DC 20460

MR 15353



8EHQ-98-14333

Re: Additional Section 8(e) Submittal for HOAT

Dear Section 8(e) Coordinator:

In accordance with the requirements of Section 8(e) of the Toxic Substances Control Act (TSCA), 15 U.S.C. § 2607(e), PE Biosystems, a division of the Perkin-Elmer Corporation, is submitting additional information to the U.S. Environmental Protection Agency (EPA) concerning the *in vitro* mutagenicity of a chemical substance (1-Hydroxy-7-azabenzotriazole, HOAT) that is manufactured, imported, and distributed by PE Biosystems and its subsidiaries. This chemical substance is intended for use only in research and development (R&D) applications; specifically, it is used as an activator in peptide and peptide nucleic acid (PNA) synthesis by scientists in academic, governmental and industrial R&D laboratories.

In my Section 8(e) submittal letter to you dated December 8, 1998, I discussed the receipt of preliminary results of a mouse lymphoma assay of the *in vitro* mutagenic potential of HOAT (CAS Number: 148893-10-1), and committed to forwarding the report to EPA when it was finalized. The Final Report was issued by the contract testing laboratory on December 18, 1998. Accordingly, I am submitting it within the 15 working days as required by TSCA Section 8(e).

Name and Address of Person and Company Reporting

This information is being submitted on behalf of PE Biosystems, by:

Debora Van der Sluis
Chemical Regulatory Compliance Manager
PE Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404
Telephone: (650) 638-5277
Facsimile: (650) 638-6786

99 JAN 22 AM 11:06
RECEIVED
OPPT NCIC



89990000080

CONTAINS NO CBI

Summary of Adverse Effects and Source of Information

HOAT

Attached is a report entitled "1-Hydroxy-7-azabenzotriazole (HOAT), Mouse Lymphoma Mutation Assay", prepared by Inveresk Research in Edinburgh, Scotland (the HOAT Mouse Lymphoma Study). This study was prepared for a PE Biosystems subsidiary (PE Applied Biosystems) in Warrington, England.

The HOAT Mouse Lymphoma Study evaluated the mutagenic activity of HOAT in the mouse lymphoma L5178Y cell line, scoring for forward mutations at the thymidine kinase locus: tk⁺tk⁺ to tk⁻tk⁻. Tests were conducted both in the absence and presence of S9. In both experiments in the absence of S9 mix, HOAT gave a "weak" mutagenic response at or near to the pre-determined maximum concentration of 5000 µg/ml, at which there was little or no toxicity.

Based on the results of the HOAT Mouse Lymphoma Study, PE Biosystems is currently revising the MSDS for HOAT to reflect the associated *in vitro* mutagenic effects. I will submit a copy of this revised MSDS to EPA promptly after revision.

* * * * *

Please contact me at 650-638-5277 if you have any questions about this submittal.

Sincerely,



Debora Van der Sluis

Enclosure

CONTAINS NO CBI

Inveresk Report No. 16862

1-HYDROXY-7-AZABENZOTRIAZOLE (HOAT)
MOUSE LYMPHOMA MUTATION ASSAY

Inveresk Project No. 762819



Inveresk Research

TRANENT EH33 2NE SCOTLAND
TELEPHONE: +44 (0) 1875 614545
FAX: +44 (0) 1875 614555

CONFIDENTIAL

Inveresk Report No. 16862

**1-HYDROXY-7-AZABENZOTRIAZOLE (HOAT)
MOUSE LYMPHOMA MUTATION ASSAY**

Inveresk Project No. 762819

Sponsor's Monitoring scientist:

B Tarbit

Author:

C G Riach

Sponsor:

**Perkin-Elmer Applied Biosystems
7 Kingsland Grange
Woolston
Warrington
Cheshire
WA1 4SR**

Performing Laboratory:

**Inveresk Research
Tranent, EH33 2NE
Scotland**

Total Number of Pages: 87

AUTHENTICATION

'I, the undersigned, hereby declare that this work was performed under my direction and in accordance with the OECD Principles of Good Laboratory Practice. The study was conducted according to the procedures herein described and this report represents a true and accurate record of the results obtained.'



C G Riach BSc HNC
Study Director

Date: *Dec 18. 98*

QUALITY ASSURANCE STATEMENT

The execution of this type of short-term study is not individually inspected. The processes involved are inspected at intervals according to a predetermined schedule.

Inveresk Project No. 762819Report No. 16862

Date of QA Inspection	Phase	Date of Reporting to Management
7 October 1998	Day 0 Cloning	9 October 1998

This report has been audited by Inveresk Quality Assurance Personnel according to the appropriate Standard Operating Procedure and is considered to describe the methods and procedures used in the study. The reported results accurately reflect the original data of the study.

Signed: Gillian Farrell Date: 18 DEC 1998
(Quality Assurance)

CONTENTS

	<u>Page</u>
TITLE PAGE	1
STUDY DIRECTOR'S AUTHENTICATION	2
QUALITY ASSURANCE STATEMENT	3
CONTENTS	4
PERSONNEL INVOLVED	6
SUMMARY	7
INTRODUCTION	9
EXPERIMENTAL PROCEDURE	12
EVALUATION OF RESULTS	22
RESULTS AND DISCUSSION	28
CONCLUSION	33
REFERENCES	34

CONTENTS (continued)

	<u>Page</u>
TABLES	36
1-3 Toxicity Tests with HOAT	36
4-7 Summary Data from Mutation Assays with HOAT	39
 APPENDICES	 46
1 Cell Cultures	46
2 Preparation and Characterisation of the Metabolic Activation System	48
3-5 Toxicity Tests: Suspension Growth	49
6-9 Mutation Assays: Day 0/1 Relative Survival	52
10-13 Mutation Assays: Suspension Growth	60
14-17 Mutation Assays: Day 2/3 Cloning Efficiency of Non-mutants	67
18-21 Mutation Assays: Day 2/3 Mutant Selection	74
22 Worked Examples of Calculations	82
23 Summary of Control Data from Recent Experiments	86
 FINAL PAGE OF REPORT	 87

PERSONNEL INVOLVED IN PROJECT No. 762819

Study Director: C G Riach BSc HNC

Project Leader: W C Zajac

Statistical Analysis A N Graham BSc

Quality Assurance: G Farrell BSc GIBiol

1-HYDROXY-7-AZABENZOTRIAZOLE (HOAT):
MOUSE LYMPHOMA MUTATION ASSAY

SUMMARY

1-Hydroxy-7-azabenzotriazole (HOAT) was assayed for mutagenic potential in the mouse lymphoma L5178Y cell line, clone 3.7.2.C, scoring for forward mutations at the thymidine kinase locus: tk⁺tk⁻ to tk⁻tk⁻. All tests were conducted both in the absence and in the presence of a post-mitochondrial supernatant fraction obtained from the livers of adult, male rats (S9). The study was designed to be consistent with the guidelines of the International Committee on Harmonisation (ICH), EU, OECD and United States, and the study complies with Japanese requirements.

In preliminary cytotoxicity tests HOAT was dissolved in dimethylsulphoxide. When conducted over a 4 h exposure period, HOAT was toxic only at the highest concentration of 4167 µg.ml⁻¹, at which the pH was very low, and was likely to have been the cause of the toxicity. When HOAT was exposed to the cell cultures for 24 h in the absence of S9 mix, toxicity was seen at 1500 µg.ml⁻¹, at which the pH was normal. In all subsequent experiments, HOAT was formulated in tissue culture medium, adjusted with sodium hydroxide to give a neutral pH.

Four independent mutation experiments were conducted: 2 in the absence and 2 in the presence of S9 mix. In the first experiment in the absence of S9 mix, exposure to the cells was for 4 h. Results were obtained where the final concentrations of HOAT in the treatment medium ranged between 625 and 5000 µg.ml⁻¹ (the latter concentration was the prearranged maximum). In the second experiment in the absence of S9 mix, exposure to the cells was for 24 h, and results were obtained where the final concentrations of HOAT in the treatment medium ranged between 1000 and 2500 µg.ml⁻¹.

In both experiments in the presence of S9 mix, exposure to the cells was for 4 h. Results were obtained where the final concentrations of HOAT in the treatment medium ranged between 625 and 5000 $\mu\text{g}\cdot\text{ml}^{-1}$.

Positive control cultures were included, and the resultant mutant colonies from these provided proof of adequate recovery of "small" type colonies. Duplicate cultures were carried through the experiments for each treatment point. Vehicle control cultures were also included and were tested in quadruplicate. The results were analysed for comparison of the log mutant fraction between the vehicle controls and each concentration of HOAT. The results were also tested for linear trend of mutant fraction with concentration of HOAT.

In both experiments in the absence of S9 mix, HOAT gave a weak mutagenic response. The log mutant fractions at the highest concentrations were significantly higher than the vehicle controls, and there was a significant linear trend with increasing concentration of HOAT. These responses occurred at, or near to, the prearranged maximum concentration of 5000 $\mu\text{g}\cdot\text{ml}^{-1}$, at which there was little or no toxicity.

A similar weak response was obtained from HOAT in the first experiment in the presence of S9 mix, while the second experiment gave no response. This variability may have been due to a function of the S9 mix, or may have been due to the weakness of the response combining with sub-optimal sensitivity in the second experiment.

The colony size distribution data suggest that HOAT induces both large type and small type colonies. This suggests that both mutational events and chromosomal aberrations are occurring.

It is concluded that HOAT is weakly mutagenic in mouse lymphoma L5178Y cells, in the absence of S9 mix. In the presence of S9 mix, the evidence is inconclusive: HOAT gave a weak mutagenic response in one assay, and was not mutagenic in another assay.

INTRODUCTION

The test substance was 1-Hydroxy-7-azabenzotriazole (HOAT) (Batch No. 216696)

KEY DATES

Study Initiation:	14 September 1998
Experimental Start Date:	15 September 1998
Experimental Completion Date:	28 October 1998
Study Completion Date:	See Authentication page for date of Study Director's signature

STUDY OBJECTIVE

The objective of the study was to determine the potential of the test substance to induce forward mutations at the tk⁺tk⁻ locus of mouse lymphoma L5178Y cells.

JUSTIFICATION OF THE TEST SYSTEM

Specific-locus mutation tests with mammalian cells *in vitro* can be used to demonstrate and quantify genetic damage in these cells. Such tests can be used, for example, to confirm results obtained with bacterial cell tests. A positive result with the mammalian cell test as well as a bacterial cell assay increases the need for careful evaluation of the toxic potential of the test chemical. A negative result, while not reversing the interpretation of a positive result with bacteria, does reduce the value of that result and reduces the need for toxicity evaluation of the chemical.

Since 1964, mutations have been knowingly induced in cultured mammalian cells (Fischer and Sartorelli (1964); Chu and Malling (1968); Kao and Puck (1968)). The thymidine kinase heterozygote system, where tk⁺tk⁻ is mutated to tk⁻tk⁻, was described by Clive *et al* (1972) and is based upon the L5178Y mouse lymphoma cell line established by Fischer (1958). A more detailed description of the system as a test for

mutagens was published later (Clive and Spector (1975)). Among the published validation studies of the L5178Y cell system are those of Clive *et al* (1979); Amacher *et al* (1980); Jotz and Mitchell (1981); McGregor *et al* (1987, 1988a, 1988c, 1991a, 1991b); Myhr *et al* (1990).

Two principal methods of performing the mouse lymphoma assay exist: the soft agar cloning method and the microwell method (Cole *et al* (1983)). The latter method was used in this study.

Although some guidelines eg, ICH, do not dictate an automatic repetition of experiments, it is considered appropriate to prove the reproducibility of all findings in this assay system. Consequently, 2 experiments were conducted in both the absence and the presence of an exogenous enzyme supplement (S9 mix).

A recent validation study commissioned by the ICH (to be published) has shown that the detection (by the L5178Y assay) of a significant number of substances positive in the chromosome aberration assay was enhanced by an extended exposure period in the absence of S9 mix. Such substances included nucleoside analogues, base analogues and aneuploidy inducers. It is also recognised that some chemicals (for example caffeine and diethylstilbestrol) require longer exposure periods (typically 24 hours) to give clear positive responses in the cell mutation assay (MCA EuroDirect Publication No. 174/95). The study was designed, therefore, to include a 24 h exposure period in the second experiment in the absence of S9 mix, should the results of the first experiment (using a standard 4 h exposure) be negative.

COLONY SIZING

It has been widely shown (Hozier *et al* (1981) and others) that genetic damage involving the TK locus results in 2 phenotypes. Some mutant cells divide at the normal rate to produce large colonies, while other cells divide at a distinctly slower rate, producing small colonies. A high proportion of large type colonies are associated with small chromosomal deletions or point mutations, while a large proportion of the small

type colonies are associated with large chromosomal deletions. Assessment of the relative numbers of both colony types can provide information to support results obtained in bacterial mutation and chromosome aberration tests.

REGULATORY COMPLIANCE

This study was designed to comply with ICH Guidelines, OECD Guideline No. 476, Directive 97/302/EEC Part B and United States 40 CFR § 798.5300.

LOCATION OF STUDY

The study was conducted in the Elphinstone Research Centre Laboratories of Inveresk Research, Tranent, EH33 2NE, Scotland.

ARCHIVES

All data generated and recorded during this study will be stored in the Scientific Archives of Inveresk Research for 5 years after issue of the final report. After 5 years, the Sponsor will be consulted regarding the disposal or continued storage of raw data.

EXPERIMENTAL PROCEDURE

All routine activities conducted during this study are detailed in Inveresk's Standard Operating Procedures.

All experimental procedures were conducted using aseptic technique and under amber light.

TEST MATERIAL

The test substance, 1-Hydroxy-7-azabenzotriazole (HOAT) (Batch No. 216696), was received from Perkin Elmer on 10 June 1998. The substance, a light brown powder, was stored in the dark at ca 4°C.

Test Material Formulation

All handling of the test substance and its solutions was conducted under amber light. The test substance formulations were prepared within 1 hour of dosing. Detailed records of preparation of the dosing solutions were maintained to allow checking of procedures. Chemical analysis of the test substance formulations was not carried out.

All concentrations quoted are in terms of the material supplied for testing.

CELLS

The cells used were from the tk*tk⁻-3.7.2.C mouse lymphoma L5178Y cell line obtained from Dr D Clive, Burroughs Wellcome Co., Research Triangle Park, NC27709, USA, in December 1982. The cells grow in suspension culture, have a generation time of about 11 h, have a stable, near-diploid chromosome number and have a high cloning efficiency in a soft agar cloning medium.

CULTURE MEDIUM

The basic culture medium (R_0P) was RPMI 1640 medium, supplemented with penicillin (100 units. ml^{-1}), streptomycin (100 $\mu g. ml^{-1}$), sodium bicarbonate (1.125 g. l^{-1}) and pluronic acid (0.05% w/v). For cell growth, heat-inactivated horse serum (10% v/v) was added to R_0P to give $R_{10}P$.

The medium used during treatment was R_0P supplemented with 5% v/v heat-inactivated horse serum (R_5P).

For colony formation, cloning medium was used, consisting of R_0P supplemented with heat-inactivated horse serum (20% v/v), sodium pyruvate (1.9 mM), and amphotericin B (fungizone) (2.5 $\mu g. ml^{-1}$).

For selection of tk⁺tk⁻ cells, cloning medium was supplemented with trifluorothymidine (TFT) at 3 $\mu g. ml^{-1}$.

CELL CULTURES

Cell stocks for use in the mutation assays were prepared in suitable volumes according to the procedures detailed in Appendix 1.

S9 MIX (McGregor *et al* (1988b))

Aroclor 1254-induced S9 enzymes (the supernatant of the post-mitochondrial 9000 g fraction) were prepared from the livers of adult, male Fischer rats, as described by Ames *et al* (1975).

S9 was stored, as 2 ml or 5 ml samples, in sterile plastic tubes immersed in liquid nitrogen (ca -196°C).

The enzymic activity of each batch of S9 was characterised by testing selected pre-mutagens in an Ames test with *S. typhimurium* TA 1538. The results of the characterisation and preparation details of the batch used in the mutation experiments are presented in Appendix 2. S9 batches used must also have shown, within each test, a satisfactory mutagenic response in cells treated with 3-methylcholanthrene.

To prepare S9 mix, R₀P was added to preweighed cofactors: nicotinamide adenine dinucleotide phosphate (NADP) disodium salt and glucose-6-phosphate (G-6-P) disodium salt, giving final concentrations in the 'S9 mix' of:

NADP Na ₂	4 mM (= 3.150 mg.ml ⁻¹)
G-6-P Na ₂	25 mM (= 7.605 mg.ml ⁻¹)

This solution was immediately filter-sterilised by passage through a 0.2 µm disposable filter assembly and mixed 9:1 (v/v) with the S9.

PRELIMINARY AND ACCESSORY TESTING

Solubility of Test Material

Initial tests with HOAT showed it to have good solubility in dimethylsulphoxide (DMSO), which was chosen as the solvent for the toxicity tests. When performing these tests, a concentration of 416.7 mg.ml⁻¹ was achieved. This gave, after 1 in 100 dilution in the cell culture medium, a final concentration of 4167 µg.ml⁻¹. When this concentration proved excessively acidic, an alternative formulation was devised.

HOAT was dissolved in the basic culture medium, R₀P, and brought to pH 7 with 5 M sodium hydroxide. The concentration of the resulting stock solution was 12.5 mg.ml⁻¹. This gave, after a 4 in 10 dilution in the cell cultures, the prearranged maximum concentration of 5000 µg.ml⁻¹. All the required dilutions were prepared using R₀P. This method of formulation was used throughout the mutation experiments.

Toxicity Test

Doses in mutation experiments should extend into the toxic range, the maximum usable limit allowing a relative survival of at least 10% of the concurrent vehicle control values. It was necessary, therefore, to perform initial toxicity tests in the absence and presence of S9 mix.

The concentrations of HOAT tested were as follows:

0.5, 1.5, 5, 15, 50, 150, 500, 1500 and 4167 $\mu\text{g}.\text{ml}^{-1}$

On the day of the test (Day 0), samples of cell culture (in 5 ml R_{10}P) were dispensed to sterile tubes containing 3.9 ml R_0P . Freshly prepared S9 mix or R_0P (1 ml) was added to each tube followed by the test solution in 0.1 ml DMSO. Vehicle control cultures received 0.1 ml DMSO. The final reaction mixture in all cultures contained 10 ml of cells, at a population density of ca 6.0×10^5 cells. ml^{-1} , in R_5P medium.

All tubes were incubated on a rotating drum at ca 37°C , 10 rpm for 4 h. After this, the cells were gently sedimented by centrifugation at ca 200 g for 5 min and resuspended in R_{10}P medium (20 ml). This step was repeated to give a cell density of ca 3×10^5 . ml^{-1} .

The Day 0 Relative Survival (RS) and the relative suspension growth (RSG) over 2 days following treatment were then assessed for each cell culture, as described below in the mutation tests.

The toxicity test was performed using the standard, 4 h exposure period in the absence and presence of S9 mix. An additional toxicity test was performed in the absence of S9 mix with 24 h exposure to the test substance, as a contingency against the later requirement for a full experiment using this extended exposure period.

On the day of the test (Day 0), samples of cell culture (in 10 ml R₁₀P) were dispensed to sterile tubes containing 7.8 ml R₀P. Freshly prepared R₀P (2 ml) was added to each tube followed by the test solution in 0.2 ml DMSO. Vehicle control cultures received 0.2 ml DMSO. The final reaction mixture in all cultures contained 20 ml of cells, at a population density of ca 3.0 x 10⁵ cells.ml⁻¹, in R₅P medium. (The larger volumes allow the same numbers of cells to be treated as in the experiments conducted at 4 h exposure, but at half the density. The lower density is required to allow cell growth during the exposure period.)

All tubes were incubated on a rotating drum at ca 37°C, 10 rpm for 24 h. After this (on Day 1), the cells were gently sedimented by centrifugation at 200 g for 5 min and resuspended in R₁₀P medium (20 ml). This step was repeated. Cell counts were made and the densities adjusted (where higher) to give ca 3 x 10⁵ cells.ml⁻¹. The Day 1 RS for each cell culture was then assessed, as described below in the mutation tests. In addition, the cell population densities were recorded over 2 further days, then the RSG values were determined.

Physical Characteristics of the Dosing Solutions

Observations of precipitation were made on dosing and before cultures were washed out after the treatment period. High concentrations of HOAT were monitored for changes in osmolality.

DOSE SELECTION

Two full mutagenicity assays were performed in the absence and presence of S9 mix. The intention was to carry 4 concentrations of test material in each through to completion of the experiment.

The concentrations of HOAT tested were as follows ($\mu\text{g}\cdot\text{ml}^{-1}$):

Assay 1 (in the absence of S9 mix¹): 156, 313, 625, 1250, 2500 and 5000

Assay 2 (in the presence of S9 mix¹): 156, 313, 625, 1250, 2500 and 5000

Assay 3 (in the absence of S9 mix²): 63, 125, 250, 500, 1000, 1500, 2000 and
2500

Assay 4 (in the presence of S9 mix¹): 625, 1250, 2500 and 5000

¹ Experiment using a 4 h exposure period

² Experiment using a 24 h exposure period

Each concentration range selection was based on all results obtained to date.

MUTATION TESTS

Vehicle Controls

All toxicity and mutation assays included vehicle control cultures. These cultures were subjected to the same experimental manipulations as the treated cultures. In this study, the vehicle control cultures were treated with DMSO in the toxicity tests and R₀P in the mutation tests.

Positive Control Materials

The positive controls used in the absence of S9 mix (4 h exposure period) were: 250 $\mu\text{g}\cdot\text{ml}^{-1}$ ethyl methanesulphonate (EMS), a large colony inducer; and 15 $\mu\text{g}\cdot\text{ml}^{-1}$ methyl methanesulphonate (MMS), which usually induces greater numbers of small colonies. When performing an experiment using the extended, 24 h exposure period, these concentrations were reduced to 150 $\mu\text{g}\cdot\text{ml}^{-1}$ EMS and 5 $\mu\text{g}\cdot\text{ml}^{-1}$ MMS. In the presence of S9 mix, 3-methylcholanthrene (3-MC), a large and small colony inducer, was used at a concentration of 2.5 $\mu\text{g}\cdot\text{ml}^{-1}$. EMS and 3-MC were dissolved in DMSO, while MMS was dissolved in water.

The positive control cultures were subjected to the same experimental manipulations as the test substance-treated cultures.

Treatment (4 h exposure period)

Tests were conducted both in the absence and in the presence of S9 mix. All treatments were performed on duplicate cell cultures, except the vehicle control, which was tested in quadruplicate.

On the day of the test (Day 0), samples of cell culture (in 5 ml R₁₀P) were dispensed to sterile tubes. Freshly prepared S9 mix or R₀P (1 ml) was added to each tube followed by the test solution in 4 ml R₀P. Vehicle control cultures received 4 ml R₀P, while positive control cultures received 0.1 ml of the appropriate solution plus 3.9 ml R₀P. The final reaction mixture in all cultures contained 10 ml of cells, at a population density of ca 6.0 x 10⁵ cells.ml⁻¹, in R₅P medium.

All tubes were incubated on a rotating drum at ca 37°C, 10 rpm for 4 h. After this, the cells were gently sedimented by centrifugation at ca 200 g for 5 min and resuspended in R₁₀P medium (20 ml). This step was repeated to give a cell density of ca 3 x 10⁵.ml⁻¹.

A small sample (ca 0.5 ml) was removed from each culture before the cells were returned to the rotating drum and allowed to express their genetic lesions at ca 37°C for 2 days. Cell numbers were adjusted, after counting, to ca 3 x 10⁵ cells.ml⁻¹ on Day 1.

The samples of cells taken at the end of the treatment period were used to estimate the survival of the treated cultures, relative to the vehicle controls. The cell densities were assessed, the cultures diluted into cloning medium as appropriate, and two 96-well dishes seeded with an estimated 1.6 cells per well (in 200 µl). The numbers of empty wells after 9 days, plus the post-treatment cell densities, were used to calculate the Day 0 RS for each cell culture.

Extended Treatment (24 h exposure)

If the results of the first experiment in the absence of S9 mix were negative, the second experiment in the absence of S9 mix was conducted using an extended, 24 h exposure period. This facilitated continuous exposure to the test substance through >1 cell cycle.

On the day of the test (Day 0), samples of cell culture (in 10 ml R₁₀P) were dispensed to sterile tubes. Freshly prepared R₀P (2 ml) was added to each tube followed by the test solution in 8 ml R₀P. Vehicle control cultures received 8 ml R₀P, while positive control cultures received 0.2 ml of the appropriate solution plus 7.8 ml R₀P. The final reaction mixture in all cultures contained 20 ml of cells, at a population density of ca 3.0 x 10⁵ cells.ml⁻¹, in R₅P medium.

All tubes were incubated on a rotating drum at ca 37°C, 10 rpm for 24 h. After this (on Day 1), the cells were gently sedimented by centrifugation at 200 g for 5 min and resuspended in R₁₀P medium (20 ml). This step was repeated. Cell counts were made and the densities adjusted (where higher) to give ca 3 x 10⁵ cells.ml⁻¹. A small sample (ca 0.5 ml) was removed from each culture before the cells were returned to the rotating drum and allowed to express their genetic lesions at ca 37°C for 2 days. Cell numbers were adjusted, after counting, to ca 3 x 10⁵ cells.ml⁻¹ on Day 2.

The samples of cells taken at the end of the treatment period were used to estimate the survival of the treated cultures relative to the vehicle controls. The cultures were diluted into cloning medium as appropriate, and two 96-well dishes seeded with an estimated 1.6 cells per well (in 200 µl). The numbers of empty wells after 9 days, plus the post-treatment cell densities, were used to calculate the Day 1 RS for each cell culture.

Expression of Genetic Damage

The cell counts over the 2/3 days of the experiments provided a measure of suspension growth. This in turn provided an alternative measure of the level of toxicity of the test substance. This was used when choosing concentrations to carry through to final assessment, as the results from the Day 0/1 cloning efficiency assay were not available at the time the decision was made.

On Day 2 (4 h exposure) or Day 3 (24 h exposure), cell counts were determined. All vehicle and positive control cultures, plus those from the four highest concentrations giving acceptable suspension growth (generally, *ca* 10% of vehicle control cultures) were selected for expression of genetic damage. This was determined by performing two parallel cloning assays: the cloning efficiency assay and the mutant selection assay.

For the cloning efficiency assay, each culture was diluted into cloning medium to give an estimated 8 cells.ml^{-1} . Two 96-well dishes were filled with $200 \mu\text{l}$ cell culture per well, so giving an estimated 1.6 cells per well.

For the mutant selection assay, TFT stock solution was added to cloning medium to give a final concentration of $3 \mu\text{g.ml}^{-1}$. Into this medium, the cell cultures were diluted to give an estimated $1 \times 10^4 \text{ cells.ml}^{-1}$. Two 96-well dishes were filled with $200 \mu\text{l}$ cell culture per well, so giving an estimated 2000 cells per well.

All dishes were incubated at *ca* 37°C in an atmosphere of 5% CO_2 :95% air (v/v) until the colonies were fully developed (9 days for cloning efficiency assay; 12 days for mutant selection assay).

Plate Reading

The plates were scored using a dissecting microscope fitted to a light box with dark field illumination. The number of empty wells in each plate in the cloning efficiency

assay was counted. When scoring the mutant selection assay, separate counts were made of the numbers of wells containing large type and small type colonies. Large colonies are similar to those found on the cloning efficiency plates. They are flat colonies, filling about half the floor of the well by the end of the incubation period. Small colonies tend to look dense in comparison, and cover a much smaller area of the well floor. Any wells containing both colony types were scored as a large type. (The total number of empty wells is required for the calculation of mutant fraction, so each well can only be scored once).

EVALUATION OF RESULTS

CALCULATIONS

Assessment of Cell Survival

The recommended endpoint for assessing survival in the microwell assay is Day 0 relative survival (RS). Day 0 RS expresses the post-treatment cloning efficiency corrected for post-treatment cell counts. (Where an experiment has been performed using the extended 24 h exposure period, survival was assessed from Day 1 RS.) The cloning efficiency (CE) was calculated from the zero term of the Poisson distribution using the formula:

$$CE = \frac{-\ln(P(0))}{\text{number of cells per well}}$$

$$\text{where } P(0) = \frac{\text{empty wells}}{\text{total wells}}$$

The cloning efficiency value was then used to calculate survival (S). The survival calculation takes into account any reduction in post-treatment cell count for each treated culture. This is known as the cell count factor (CCF).

$$CCF = \frac{\text{individual treated post-treatment cell count}}{\text{mean vehicle control post-treatment cell count}}$$

$$S = CE \times CCF$$

$$RS = \frac{\text{individual survival value}}{\text{mean vehicle control survival value}}$$

Assessment of Mutant Fractions

The number of empty wells from the Day 2 cloning efficiency assay (Day 3 for 24 h exposure) and the number of empty wells from the TFT-resistance assay were used to calculate the mutant fraction, as below.

The cloning efficiency (CE) was calculated as above. The mutant fraction per viable cell was calculated as below:

$$\text{Mutant fraction per viable cell} = \frac{\text{CE in medium containing TFT}}{\text{CE in non - selective medium}}$$

Each mutant fraction was expressed per 10^6 viable cells.

Colony Size Fractions

The ratio of small type mutant colonies to large type mutant colonies was expressed for each culture.

A set of actual calculations based on the current study is given in Appendix 22.

HISTORICAL CONTROL DATA

The historical ranges of vehicle and positive controls are continually updated and are used as guides in the acceptance of each experiment. The summarised historical data are presented in Appendix 23.

This laboratory has recently changed the growth medium used in the assay, from Fischer's medium and donor horse serum to RPMI medium and heat-inactivated horse serum. This change was made on the basis that the mutant fractions being obtained with MMS were lower than those proposed by Mitchell *et al* (1997). Use of the new growth medium has resulted in changes to both the spontaneous mutant fractions and

the mutant fractions induced by all the positive controls. It has been necessary, therefore, to start a new historical control database. The previous database is also included, for comparison.

ANALYSIS OF DATA

General

Any plate containing either 0% or 100% empty wells was rejected.

The mean cloning efficiency of all cultures should be less than 130%. (Cloning efficiencies >100% may arise through natural sampling error or due to inevitable time delays between performing the cell density assessment and cloning the cell samples.)

Results from vehicle control cultures were rejected if the mean cloning efficiency was less than 60% post-treatment, or less than 70% after the expression period.

Results from any treatment were inadequate if there were less than 2 acceptable cultures. Where results were obtained from a single culture, they may have been included as supporting evidence.

Criteria for a Valid Assay

A valid assay should have met the following criteria.

The highest concentration was limited by solubility or toxicity, or, in the absence of either of these, was the maximum practical concentration of test compound based on the recommendations in current guidelines, viz, 5000 µg.ml⁻¹.

The positive and negative control mutant fractions were within or close to the historical ranges for the accumulated laboratory database.

There was an absence of confounding technical problems eg, contamination, outliers, excessive toxicity, osmolality and pH changes.

Interpretation of Toxicity

Mutagenic responses that occur only at survival values below 10%, while being statistically significant, are recognised as having questionable biological significance (Clive *et al* (1983), Scott *et al* (1991)). Furthermore, results arising from an initial high cell kill are also prone to high divergence and thus tend to be statistically less robust. As genetic damage naturally reduces cell viability, it is normal, however, for mutagenic responses to occur only at reduced levels of survival. If a test substance is toxic, results should be obtained from concentrations resulting in survival values down to 20%, if a conclusion of nonmutagenic is to be reached with confidence. It is important, therefore, to obtain results in the survival range *ca* 20%.

Normally, only the viability data are presented where a culture gave a survival value below 10% and the test substance was non-mutagenic. If one of a pair of duplicate cultures gave a survival of >10%, while the other gave a survival of <10% (due to higher toxicity), and both gave evidence of mutagenic responses, the full data from both cultures may have been presented. For test substances giving a mutagenic response at concentrations resulting in >10% survival, results were included for concentrations resulting in <10% survival.

Interpretation of Mutagenic Activity

The results for each experiment were subjected to statistical analysis by the recommended UKEMS method (Robinson *et al*, 1989). The data analysis included the following:

- 1 Determination of the heterogeneity factor for each concentration

- 2 Comparison of the heterogeneity factor with the historical control. Any concentration levels with a heterogeneity factor statistically higher than the historical control was excluded from all statistical analysis
- 3 Determination of the heterogeneity factor for the experiment
- 4 Calculation of a new historical control heterogeneity factor
- 5 Calculation of the log mutant fraction
- 6 Comparison of the log mutant fraction between the control and each concentration of the test substance (at $P < 0.05$)
- 7 A test for linear trend of mutant fraction with concentration of test substance

An experiment was considered positive if one or more concentrations were statistically significant and there was a significant linear trend. Absolute increases in numbers of mutant colonies provide supporting evidence of a positive response. An experiment may also have been classed as positive in the absence of a linear trend if there was mitigating evidence. This may have been, for example, the presence of a similar level of toxicity at all concentrations assessed. In such a case, the confirmatory experiment would have been expected to assess concentrations covering different levels of toxicity, to establish a linear trend.

Similarly, if an experiment was positive only at one concentration, mitigating evidence would have been expected; eg, the presence of a steep toxicity curve, preventing results from being obtained at more than one toxic concentration. In such a case, the repeat experiment would have been expected to assess concentrations covering a narrow range, to produce results at more than one toxic concentration.

A test substance was positive if 2 positive experiments out of 2 were recorded within the same activation condition. Test substances that gave a negative response in the standard exposure in the absence of S9 mix, but gave a positive response in the extended exposure, were subject to a confirmatory experiment with the extended exposure.

COLONY SIZING

The ratio of small to large type mutant colonies was stated for all cultures. These ratios provide evidence of adequate recovery of small type colonies from control cultures, and may provide additional information regarding the type of genetic damage being induced by positive test substances.

RESULTS AND DISCUSSION

TOXICITY TESTS (TABLES 1-3)

Appendices 3-5 show the relative suspension growth values of the cell cultures obtained in the toxicity tests.

The preliminary toxicity tests showed that at 4 h exposure, HOAT was not toxic up to a concentration of 1500 $\mu\text{g}\cdot\text{ml}^{-1}$. At the highest concentration of 4167 $\mu\text{g}\cdot\text{ml}^{-1}$, HOAT was completely toxic, and precipitated. The pH at this concentration was, however, unacceptably low (4.55/4.75), the maximum allowable shift being 1. Furthermore, the pH was sufficiently low to account for most or all of the toxicity.

Following consultation with the Sponsor, a decision was made to neutralise the solution of HOAT before use. When the solvent was changed to R₀P, it was found possible to achieve a non-precipitating solution at pH 7 that reached the prearranged maximum concentration of 5000 $\mu\text{g}\cdot\text{ml}^{-1}$ (see Solubility of Test Material, p 14).

At 24 h exposure, HOAT reduced the Day 1 RS to 9% of the control value. The pH at this concentration was not significantly reduced, and so it is likely that the toxicity on this occasion was due to the test material.

The monitoring of osmolality showed that HOAT did not cause a significant change in osmotic pressure at the concentrations used.

MUTATION ASSAYS (TABLES 4 TO 7)

The original data obtained in the generation of the Day 0/1 Relative Survival values in the 4 mutation assays is transcribed in Appendices 6-9. Similarly, the Relative Suspension Growth values are presented in Appendices 10-13, the Day 2/3 Cloning Efficiency of the non-mutants is presented in Appendices 14-17, and the Day 2/3 Mutant Selection data is presented in Appendices 18-21.

Vehicle Control Groups

The solvent control values were within the normal ranges experienced in this laboratory and reported in the literature with the L5178Y cell line. All values were within $\pm 2 \times$ SD of the new historical mean figures.

Positive Control Groups

With one exception, the mutant fractions obtained with EMS, MMS and 3-MC were within $\pm 2 \times$ SD of the new historical mean values, and all positive control log mutant fractions were significantly higher than the vehicle controls at $P < 0.05$. The exception was MMS in the 24 h exposure experiment (Assay 3). The mean value of 1226.5 mutants per million was below the range (1500-1605). With only 4, closely similar values in the new database, the SD was low, at 44. The result obtained with MMS in this study was considered natural variation, and was judged acceptable. 3-MC (which is not mutagenic in the absence of S9 mix) proved the efficacy of the S9.

In Assays 1 (4 h exposure) and 3 (24 h exposure), EMS gave a characteristic, low ratio of small to large colonies, indicating a preponderance of large type colonies were present in the cultures. MMS, by giving higher ratios, indicated it had induced a preponderance of small type colonies (again, characteristic). 3-MC induced a preponderance of small colonies in Assay 2, and approximately even numbers of small and large colonies in Assay 4.

HOAT

Assays 1 (in the Absence of S9 Mix - Table 4) and 2 (in the Presence of S9 Mix - Table 5)

HOAT was assessed for mutagenic activity at concentrations of: 625, 1250, 2500, and 5000 $\mu\text{g.ml}^{-1}$ in the absence and presence of S9 mix. (The 2 lowest concentrations were surplus to requirement.)

Weak mutagenic responses were obtained in both the absence and presence of S9 mix. In the absence of S9 mix, the mean mutant fraction at the highest concentration of 5000 $\mu\text{g.ml}^{-1}$ was 3.7-fold higher than the vehicle control value. At this concentration, there was a statistically significant difference in the log mutant fraction compared with the vehicle control ($P < 0.05$). The test for linear trend was also significant ($P < 0.001$).

In the presence of S9 mix, the mean mutant fraction at the second highest concentration of 2500 $\mu\text{g.ml}^{-1}$ was 2.2-fold higher than the vehicle control value, while that at the highest concentration of 5000 $\mu\text{g.ml}^{-1}$ was 2.9-fold. At both these concentrations, there was a statistically significant difference in the log mutant fraction compared with the vehicle control ($P < 0.05$). The test for linear trend was also significant ($P < 0.001$).

There was no toxicity at the highest, prearranged maximum concentration of 5000 $\mu\text{g.ml}^{-1}$, in either experiment.

Both these experiments were classed positive. As the magnitude of the responses was weak, and as the concentrations being tested were at their maximum limit (without achieving toxicity), the decision was taken to invoke the 24 h exposure in the second experiment in the absence of S9 mix.

Assays 3 (in the Absence of S9 Mix - Table 6) and 4 (in the Presence of S9 Mix - Table 7)

HOAT was assessed for mutagenic activity at concentrations of: 1000, 1500, 2000 and 2500 $\mu\text{g}\cdot\text{ml}^{-1}$ in the absence of S9 mix (24 h exposure). (All lower concentrations were surplus to requirement).

In the presence of S9 mix, HOAT was assessed for mutagenic activity at concentrations of: 625, 1250, 2500 and 5000 $\mu\text{g}\cdot\text{ml}^{-1}$.

In the absence of S9 mix, HOAT was less toxic than predicted by the toxicity test, and the highest concentration of 2500 $\mu\text{g}\cdot\text{ml}^{-1}$ resulted in a mean Day 1 RS of 61%. There was, however, a weak mutagenic response. (As the intended toxicity had not been achieved, it could be expected that the response would be no larger than that obtained in the 4 h exposure assay.) At the 2 highest concentrations of 2000 and 2500 $\mu\text{g}\cdot\text{ml}^{-1}$, there was a statistically significant difference in the log mutant fraction compared with the vehicle control ($P < 0.05$). The test for linear trend was also significant ($P = 0.004$). The experiment was classed positive.

On this occasion, there was no mutagenic response in the presence of S9 mix. The reason for this is not clear, although with the test material giving only a weak response at very high concentrations, the lack of a positive result in one out of 4 experiments does not give cause for concern. One possibility may be that S9 mix varies in its ability to modify the genotoxicity of HOAT. Another possibility is that at the time the second pair of experiments was conducted, the assay system was functioning at less than maximum sensitivity. The weak response would still have been detected in the absence of S9 mix due to the long exposure, but might not have been detected in the presence of S9 mix.

The colony size distribution data suggest that HOAT induces both large type and small type colonies. Although variable overall, there was some evidence that

more small type colonies were induced in the positive cultures. This suggests that both mutational events and chromosomal aberrations are occurring, with a slight tendency towards more chromosomal aberrations.

CONCLUSION

It is concluded that HOAT is weakly mutagenic in mouse lymphoma L5178Y cells, in the absence of S9 mix.

In the presence of S9 mix, the evidence is inconclusive. HOAT gave a weak mutagenic response in one assay, and was not mutagenic in another assay.

REFERENCES

- Amacher, D E, S C Paillet, G N Turner, V A Ray and D S Salzburg (1980). *Mutation Res*, 72, 447-474.
- Ames, B N, McCann, J and Yamasaki, E (1975). *Mutation Res*, 31, 347-404.
- Chu, E H Y and H V Malling (1968). *Proc Nat Acad Sci*, 61, 1306.
- Clive, D, W G Flamm, M R Machesko and N J Bernheim (1972). *Mutation Res*, 16, 77-87.
- Clive, D and J F S Spector (1975). *Mutation Res*, 31, 17-29.
- Clive, D, K O Johnson, J F S Spector, A G Batson and M M M Brown (1979). *Mutation Res*, 59, 61-108.
- Clive, D, McCuen, R, Spector, J F S, Piper, C and Mavournin, K H (1983). *Mutation Res*, 115, 225-256.
- Cole, J, Arlett, C F, Green, M H L, Lowe, J and Muriel, W (1983). *Mutation Res.*, 111, 371-386.
- Fischer, G A (1958). *Ann N Y Acad Sci*, 76, 673-680.
- Fischer, G A and A C Sartorelli (1964). *Methods Med Res*, 10, 247-267.
- Hozier, J, J Sawyer, M Moore, B Howard and D Clive (1981). *Mutation Res*, 84, 169-181.
- Jotz, M M and A D Mitchell (1981). In, 'Evaluation of Short-Term Tests for Carcinogens', F J de Serres and J Ashby (Eds). Elsevier, Amsterdam, 580-593.

- Kao, F T, and Puck, T T (1968). Proc Nat Acad Sci USA, 60, 1275-1281.
- McGregor, D B, Martin, R, Cattanach, P, Edwards, I, McBride, D and Caspary, W J (1987). Environmental Mutagenesis, 9, 143-160.
- McGregor, D B, Brown, A, Cattanach, P, Edwards, I, McBride, D, and Caspary, W J (1988a). Environmental and Molecular Mutagenesis, 11, 91-118.
- McGregor, D B, Edwards, I, Riach, C G, Cattanach, P, Martin, R, Mitchell, A and Caspary, W J (1988b). Mutagenesis, Vol 3, No. 6, 485-490.
- McGregor, D B, Brown, A, Cattanach, P, Edwards, I, McBride, D, Riach, C and Caspary, W J (1988c). Environmental and Molecular Mutagenesis, 12, 85-154.
- McGregor, D B, Brown, A G, Howgate, S, McBride, D, Riach, C G and Caspary, W J (1991a). Environmental and Molecular Mutagenesis, 17, 196-219.
- McGregor, D B, A Brown, P Cattanach, I Edwards, D McBride, C Riach, W Shepherd and W J Caspary (1991b). Environmental and Molecular Mutagenesis, 17, 122-129.
- Mitchell, A D, Auletta, A E, Clive, D, Kirby P E, Moore, M M, Myhr, B C (1997). Mutation Res., 394, 177-303.
- Myhr, B, D McGregor, L Bowers, C Riach, A G Brown, I Edwards, D McBride, R Martin and W J Caspary (1990). Environmental and Molecular Mutagenesis, 16, 38-167.
- Robinson, W D, Green, M H L, Cole, J, Healy, M J R, Garner, R C and Gatehouse, D (1989). Statistical Evaluation of Mutagenicity Test Data, 102-140.
- Scott, D, Galloway, S M, Marshall, R R, Ishidate, M, Brusick, D, Ashby, J and Myhr, B C (1991). A report from ICPEMC Task Group 9. Mutation Res, 257, 147-205.

TABLE 1

Mouse Lymphoma Toxicity Test in the Absence of S9 Mix: 4 h Exposure

Chemical	Concentration ($\mu\text{g. ml}^{-1}$) (100 μl added)	pH/ Osmolality (mMol.kg^{-1})	Post- Treatment Cell Count	Day 1 Survival		Total Wells Scored	Cell Count Factor (CCF)	Cloning Efficiency	Survival	Day 0 Relative Survival %
				Plate 1 Empty Wells	Plate 2 Empty Wells					
DMSO		7.7/403	4.2	26	26	192	1.00	0.82	0.82	100.0
HOAT	0.5	-	4.3	37	29	192	1.02	0.67	0.68	83.7
	1.5	-	4.0	29	16	192	0.95	0.91	0.86	105.8
	5.0	-	3.2	12	20	192	0.76	1.12	0.85	104.5
	15.0	-	4.5	33	19	192	1.07	0.82	0.87	107.1
	50.0	-	4.2	23	32	192	1.00	0.78	0.78	95.7
	150.0	-	3.9	20	18	192	0.93	1.01	0.94	115.2
	500.0	7.54/407	4.1	23	25	192	0.98	0.87	0.85	103.6
1500.0	7.15/396	3.0	14	13	192	0.71	1.23	0.88	107.3	
p4167.0	4.55/387	3.0	96	96	192	0.71	0.00	0.00	0.0	

p= Precipitation

TABLE 2
Mouse Lymphoma Toxicity Test in the Presence of S9 Mix

Chemical	Concentration ($\mu\text{g.ml}^{-1}$) (100 μl added)	pH/ Osmolality (mMol.kg^{-1})	Post- Treatment Cell Count	Day 0 Survival		Total Wells Scored	Cell Count Factor (CCF)	Cloning Efficiency	Survival	Day 0 Relative Survival %
				Plate 1 Empty Wells	Plate 2 Empty Wells					
DMSO		7.69/498	3.7	22	24	192	1.00	0.89	0.89	100.0
HOAT	0.5	-	3.3	34	15	192	0.89	0.85	0.76	85.2
	1.5	-	2.8	15	14	192	0.76	1.18	0.89	100.1
	5.0	-	3.9	22	21	192	1.05	0.94	0.99	110.4
	15.0	-	3.4	19	17	192	0.92	1.05	0.96	107.7
	50.0	-	4.2	14	25	192	1.14	1.00	1.13	126.6
	150.0	-	3.8	14	12	192	1.03	1.25	1.28	143.7
	500.0	7.52/408	4.3	20	32	192	1.16	0.82	0.95	106.2
	1500.0	7.09/472	3.2	18	14	192	0.86	1.12	0.97	108.5
	p4167.0	4.75/404	2.7	96	96	192	0.73	0.00	0.00	0.0

p= Precipitation

TABLE 3

Mouse Lymphoma Toxicity Test in the Absence of S9 Mix: 24 h Exposure

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	pH/ Osmolality (mMol.kg^{-1})	Post- Treatment Cell Count	Day 1 Survival		Total Wells Scored	Cell Count Factor (CCF)	Cloning Efficiency	Survival	Day 1 Relative Survival %
				Plate 1 Empty Wells	Plate 2 Empty Wells					
DMSO	(200 μl added)	6.91/388	13.0	26	19	192	1.00	0.91	0.91	100.0
HOAT	0.5	-	13.0	25	18	192	1.00	0.94	0.94	103.1
	1.5	-	13.4	17	22	192	1.03	1.00	1.03	113.2
	5.0	-	15.6	26	20	192	1.20	0.89	1.07	118.2
	15.0	-	14.6	32	33	192	1.12	0.68	0.76	83.8
	50.0	-	14.0	28	30	192	1.08	0.75	0.81	88.9
	150.0	-	12.6	27	27	192	0.97	0.79	0.77	84.7
	500.0	7.04/393	10.8	34	37	192	0.83	0.62	0.52	57.0
	1500.0	6.88/389	2.9	54	47	192	0.22	0.40	0.09	9.9
	p4167.0	4.54/376	3.9	96	96	192	0.30	0.00	0.00	0.0

p= Precipitation

TABLE 4

Mouse Lymphoma Mutation Test in the Absence of S9 Mix: 4 h Exposure
Data Summary
(Assay 1)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 0		Day 2			Statistical Comparison
		Cloning Efficiency %	Relative Survival	Cloning Efficiency %	Mutant Fraction ($\times 10^{-6}$)	Ratio of Small to Large Colonies	
Vehicle Control Mean	→	88	-	83	80	1.35	-
R0P	(4 ml added)	94	99	65	117	1.45	-
		96	96	87	81	1.08	
		91	107	70	71	1.25	
		71	99	112	52	1.63	
EMS	250	130	109	89	651	0.23	*
		83	90	87	477	0.48	
MMS	15	69	64	29	1013	2.70	*
		95	85	33	1000	3.04	
HOAT	156	114	134	NPS	NPS	-	-
		118	117	NPS	NPS	-	
	313	130	153	NPS	NPS	-	-
		94	113	NPS	NPS	-	
	625	88	115	106	52	1.86	-
		108	101	68	77	1.38	
	1250	114	110	69	68	1.83	-
		118	128	69	55	2.50	

NPS = Not plated - surplus

* = Significant difference in log mutant fraction compared with vehicle control ($P < 0.05$)

TABLE 4 (continued)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 0		Day 2			Statistical Comparison
		Cloning Efficiency %	Relative Survival	Cloning Efficiency %	Mutant Fraction ($\times 10^{-6}$)	Ratio of Small to Large Colonies	
HOAT	2500	103	102	62	127	1.33	-
		135	126	59	114	2.00	
	5000	98	88	61	293	2.17	*
		87	99	60	302	1.76	

* = Significant difference in log mutant fraction compared with vehicle control ($P < 0.05$)
 Test for linear trend of mutant fraction with concentration of HOAT = significant ($P < 0.001$)

TABLE 5
Mouse Lymphoma Mutation Test in the Presence of S9 Mix
Data Summary
(Assay 2)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 0		Day 2			Statistical Comparison
		Cloning Efficiency %	Relative Survival	Cloning Efficiency %	Mutant Fraction ($\times 10^{-6}$)	Ratio of Small to Large Colonies	
Vehicle Control Mean	→	78	-	85	92	1.83	-
ROP	(4 ml added)	98	120	78	82	1.88	-
		70	93	78	105	1.23	
		75	97	85	96	2.22	
		70	90	100	85	2.00	
3-MC	2.5	84	80	57	990	1.71	*
		80	71	45	1442	2.68	
HOAT	156	106	162	NPS	NPS	-	-
		103	145	NPS	NPS	-	
	313	114	170	NPS	NPS	-	-
		108	132	NPS	NPS	-	
	625	141	162	77	110	2.75	-
		114	156	87	102	1.21	
	1250	95	127	74	141	1.77	-
		105	136	66	124	1.64	

NPS = Not plated - surplus

* = Significant difference in log mutant fraction compared with vehicle control ($P < 0.05$)

TABLE 5 (continued)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 0		Day 2			Statistical Comparison
		Cloning Efficiency %	Relative Survival	Cloning Efficiency %	Mutant Fraction ($\times 10^{-8}$)	Ratio of Small to Large Colonies	
HOAT	2500	83	104	59	193	2.90	*
		79	112	56	225	2.07	
	5000	84	96	62	326	1.37	*
		78	89	69	209	1.18	

* = Significant difference in log mutant fraction compared with vehicle control ($P < 0.05$)
Test for linear trend of mutant fraction with concentration of HOAT = significant ($P < 0.001$)

TABLE 6

Mouse Lymphoma Mutation Test in the Absence of S9 Mix: 24 h Exposure
Data Summary
(Assay 3)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 1		Day 3			Statistical Comparison
		Cloning Efficiency %	Relative Survival	Cloning Efficiency %	Mutant Fraction ($\times 10^{-6}$)	Ratio of Small to Large Colonies	
Vehicle Control Mean	→	71	-	101	88	1.16	-
R0P	(8 ml added)	72	109	92	76	1.50	-
		73	102	112	79	0.72	
		67	88	100	76	1.70	
		72	100	100	121	0.71	
EMS	150	51	52	58	1870	0.38	*
		60	68	82	1604	0.32	
MMS	5	41	40	64	1152	1.90	*
		37	42	47	1301	1.55	
HOAT	63	NPS	-	NPS	NPS	-	-
		NPS	-	NPS	NPS	-	
	125	NPS	-	NPS	NPS	-	-
		NPS	-	NPS	NPS	-	
	250	80	104	NPS	NPS	-	-
		92	122	NPS	NPS	-	
	500	75	86	NPS	NPS	-	-
		79	95	NPS	NPS	-	

NPS = Not plated - surplus

* = Significant difference in log mutant fraction compared with vehicle control ($P < 0.05$)

TABLE 6 (continued)

Chemical	Concentration (µg.ml ⁻¹)	Day 1		Day 3			Statistical Comparison
		Cloning Efficiency %	Relative Survival	Cloning Efficiency %	Mutant Fraction (x 10 ⁻⁶)	Ratio of Small to Large Colonies	
HOAT	1000	71	88	98	53	1.71	-
		80	100	100	82	1.90	
	1500	87	99	88	66	1.10	-
		85	100	125	115	1.53	
	2000	79	61	74	209	0.89	*
		106	68	100	151	1.78	
	2500	91	60	82	283	1.29	*
		101	62	114	221	1.71	

* = Significant difference in log mutant fraction compared with vehicle control (P < 0.05)
 Test for linear trend of mutant fraction with concentration of HOAT = significant (P = 0.004)

TABLE 7

Mouse Lymphoma Mutation Test in the Presence of S9 Mix
Data Summary
(Assay 4)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 0		Day 2			Statistical Comparison
		Cloning Efficiency %	Relative Survival	Cloning Efficiency %	Mutant Fraction ($\times 10^{-4}$)	Ratio of Small to Large Colonies	
Vehicle Control Mean	→	89	-	96	109	0.54	-
ROP	(4 ml added)	94	109	84	94	0.40	-
		91	102	116	84	0.55	
		77	85	79	139	0.58	
		95	104	103	120	0.62	
3-MC	2.5	116	97	80	645	1.30	*
		83	86	88	671	1.15	
HOAT	625	98	127	80	61	1.00	-
		120	152	120	94	0.39	
	1250	120	120	94	143	0.96	-
		100	113	112	104	0.60	
	2500	103	113	88	133	0.48	-
		96	112	88	118	2.27	
	5000	100	109	92	152	1.76	-
		95	98	96	121	0.90	

* = Significant difference in log mutant fraction compared with vehicle control ($P < 0.05$)
Test for linear trend of mutant fraction with concentration of HOAT = not significant ($P = 0.20$)

APPENDIX 1

Cell Cultures

CELL STORAGE

Cells in logarithmic phase of growth were collected by centrifugation (1000 rpm x 5 min (200 g)) and resuspended in freezing medium to a final cell density of ca 5×10^6 cells.ml⁻¹. Samples (2 ml) were frozen slowly before being stored in liquid nitrogen. New cultures are established from frozen stocks every 6 months.

Initially, a new culture was seeded into a T-75 flask containing 20 ml culture medium, gassed with 5% CO₂ in air. The cell suspension was gently layered on the bottom of the flask without disturbing the cells and incubated for ca 24 h at 37°C. When the cells were determined to be growing well (3-5 generations in 24 h) a stock culture was established by seeding 3×10^5 cells.ml⁻¹ in fresh culture medium into a polypropylene centrifuge tube (Corning 250 ml), where it was maintained on a 10 rpm roller at 37°C. Cultures were examined periodically for mycoplasma infection.

CELL DILUTION

With a generation time of 10-11 h, there is a 5-fold increase in cell number in 24 h and 25-fold in 48 h. Cultures were subcultured daily (except at weekends) to a density of approximately 3×10^5 cells.ml⁻¹ to maintain the cells in logarithmic growth. For weekend maintenance, the cultures were diluted back to 1×10^4 cells.ml⁻¹.

CELL 'CLEANSING'

The tk⁺tk⁻ heterozygote cells grown in suspension spontaneously mutate to tk⁻tk⁻ at a rate of 2×10^{-6} mutations/generation. These homozygous mutants were removed before testing began.

On Wednesday, THGM stock solution (1 ml) was added to a sub-population of the stock cell suspension (100 ml). The following day the culture was pelleted and resuspended in culture medium supplemented with THG (1%). This treatment of the cells was carried out once only before their use in an experiment. The culture was ready for use on Monday in a mutation experiment. Unused cells were disposed of by Wednesday of the same week.

Mechanism: Methotrexate (THGM) blocks folate metabolism by binding strongly to dihydrofolate reductase, hence thymidylate synthetase-mediated thymidylate (TMP) production stops. TMP production can be maintained by TK phosphorylation of exogenous thymidine (THG). Purine metabolism is similarly affected and this block is surmounted by added hypoxanthine (THG). Glycine (THG) is added to provide a single carbon source to replace the blocked folate metabolic pathway. Hence tk⁺tk⁻ cells can survive the methotrexate while tk⁻tk⁻ cells die.

APPENDIX 2

In vitro Activation Preparation Form
Activation Batch: FLI 087

PREPARATION Operator: Wanda Zajac Animal: Rat Sex: Male Strain: Fischer 344 Supplier: Harlan Olac Limited Organ: Liver Total grams of organ: 226 Details of spin: 9,000 g supernatant Total volume of preparation (ml): 678 Storage temperature: -196°C		Date Prepared: 9 June 1998 Date of Expiry: 9 December 1998 Number of animals: 20 Average animal weight (g): 244 Date induced: 4 June 1998 Inducer: Aroclor 1254 Supplier: Monsanto (UK) Limited Preparation solution: 0.15 M KCl Number of vials prepared: 97 x 5 ml Sterility check: Sterile			
METABOLIC ACTIVATION Operator: Pam Cattanach Strain: <i>S. typhimurium</i> TA 1538 Date plated: 9 June 1998		Culture batch: F088 Batch No. (plates): 000346 Date counted: 11 June 1998			
Substance	Quantity per Plate	Revertant Colonies per Plate			
					Mean
Dimethylsulphoxide	100 µl	17	10	10	12
2-Aminoanthracene	0.5 µg	612	616	404	544
2-Acetylaminofluorene	10 µg	484	519	374	459
4-Acetylaminofluorene	1 mg	77	68	63	69
Benzo(a)pyrene	5 µg	191	206	179	192
Dimethylaminoazobenzene	100 µg	72	68	64	68
QUALITY ASSURANCE <p style="text-align: right;">Auditor: Gillian Birnie Date Audited: 1 July 1998</p>					

APPENDIX 3

Mouse Lymphoma Toxicity Test in the Absence of S9 Mix: 4 h exposure
Suspension Growth

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Daily Suspension Count		Total Suspension Growth	Relative Suspension Growth
		Day 1	Day 2		
DMSO	(100 μl added)	11.0	15.9	19.4	100.0
HOAT	0.5	10.0	16.1	17.9	92.1
	1.5	12.0	14.9	19.9	102.2
	5.0	12.2	13.4	18.2	93.5
	15.0	12.0	13.2	17.6	90.6
	50.0	11.8	13.0	17.0	87.7
	150.0	11.4	11.9	15.1	77.6
	500.0	13.0	12.3	17.8	91.4
	1500.0	12.0	16.7	22.3	114.6
	4167.0	3.0	2.9	1.0	5.0

APPENDIX 4

Mouse Lymphoma Toxicity Test in the Presence of S9 Mix
Suspension Growth

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Daily Suspension Count		Total Suspension Growth	Relative Suspension Growth
		Day 1	Day 2		
DMSO	(100 μl added)	10.0	11.1	12.3	100.0
HOAT	0.5	8.4	13.4	12.5	101.4
	1.5	8.4	14.9	13.9	112.8
	5.0	9.0	13.6	13.6	110.3
	15.0	9.8	13.6	14.8	120.1
	50.0	11.4	14.7	18.6	151.0
	150.0	10.8	12.1	14.5	117.7
	500.0	10.8	11.6	13.9	112.9
	1500.0	10.4	14.5	16.8	135.9
	4167.0	3.7	4.4	1.8	14.7

APPENDIX 5

Mouse Lymphoma Toxicity Test in the Absence of S9 Mix: 24 h exposure
Suspension Growth

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Daily Suspension Count			Total Suspension Growth	Relative Suspension Growth
		Day 1	Day 2	Day 3		
DMSO	(200 μl added)	13.0	9.0	14.8	64.1	100.0
HOAT	0.5	13.0	9.5	14.8	67.7	105.6
	1.5	13.4	9.0	18.6	83.1	129.5
	5.0	15.6	7.9	12.3	56.1	87.5
	15.0	14.6	6.9	13.6	50.7	79.1
	50.0	14.0	7.6	13.6	53.6	83.6
	150.0	12.6	10.3	12.8	61.5	95.9
	500.0	10.8	7.1	11.2	31.8	49.6
	1500.0	2.9	4.1	7.7	3.5	5.5
	4167.0	3.9	1.9	3.7	1.6	2.5

APPENDIX 6

Mouse Lymphoma Mutation Test In the Absence of S9 Mix: 4 h Exposure
Day 0 Relative Survival (Assay 1)

Chemical	Concentration ($\mu\text{g ml}^{-1}$)	Osmolality (mMol.kg ⁻¹)	Post- Treatment Cell Count	Day 0 Cloning Efficiency		Total Wells Scored	Cell Count Factor (CCF)	Cloning Efficiency	Survival	Day 0 Relative Survival %
				Plate 1 Empty Wells	Plate 2 Empty Wells					
Vehicle Control Mean	→	-	3.7	-	-	-	-	0.88	0.87	-
ROP (4 ml added)		323	3.4	19	24	192	0.91	0.94	0.85	99
			3.2	17	24	192	0.86	0.96	0.83	96
			3.8	17	28	192	1.02	0.91	0.93	107
			4.5	32	30	192	1.21	0.71	0.85	99
			2.7	10	14	192	0.72	1.30	0.94	109
EMS		-	3.5	23	28	192	0.94	0.83	0.78	90
			3.0	32	32	192	0.81	0.69	0.55	64
			2.9	21	21	192	0.78	0.95	0.74	85
MMS		-	3.8	20	11	192	1.02	1.14	1.16	134
			3.2	17	12	192	0.86	1.18	1.01	117
			3.8	15	9	192	1.02	1.30	1.33	153
HOAT		-	3.9	21	22	192	1.05	0.94	0.98	113
			3.8	15	9	192	1.02	1.30	1.33	153
			3.2	17	12	192	0.86	1.18	1.01	117

APPENDIX 6 (continued)

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Osmolality ($\text{mMol}\cdot\text{kg}^{-1}$)	Post- Treatment Cell Count	Day 0 Cloning Efficiency Plates		Total Wells Scored	Cell Count Factor (CCF)	Cloning Efficiency	Survival	Day 0 Relative Survival %	
				Plate 1 Empty Wells	Plate 2 Empty Wells						
HOAT	625	-	4.2	21	26	192	1.13	0.88	0.99	115	
				16	18	192	0.81	1.08	0.87	101	
				3.0							
	1250	-	-	3.1	20	11	192	0.83	1.14	0.95	110
					13	16	192	0.94	1.18	1.11	128
					3.5						
	2500	327	-	3.2	16	21	192	0.86	1.03	0.88	102
					10	12	192	0.81	1.35	1.09	126
					3.0						
	5000	352	-	2.9	21	19	192	0.78	0.98	0.76	88
					18	30	192	0.99	0.87	0.86	99
					3.7						

APPENDIX 7
 Mouse Lymphoma Mutation Test in the Presence of S9 Mix
 Day 0 Relative Survival
 (Assay 2)

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Osmolality ($\text{mMol}\cdot\text{kg}^{-1}$)	Post- Treatment Cell Count	Day 0 Cloning Efficiency Plates		Total Wells Scored	Cell Count Factor (CCF)	Cloning Efficiency	Survival	Day 0 Relative Survival %
				Plate 1 Empty Wells	Plate 2 Empty Wells					
R0P	(4 ml added)	305	3.4	-	-	-	-	0.78	0.78	-
			3.2	20	20	192	0.95	0.98	0.93	120
			3.5	33	30	192	1.04	0.70	0.72	93
			3.4	26	32	192	1.01	0.75	0.75	97
3-MC	2.5	-	3.4	29	34	192	1.01	0.70	0.70	90
			2.5	24	26	192	0.74	0.84	0.62	80
			2.3	24	29	192	0.68	0.80	0.55	71
HOAT	156	-	4.0	21	14	192	1.19	1.06	1.26	162
			3.7	19	18	192	1.10	1.03	1.13	145
			3.9	16	15	192	1.16	1.14	1.32	170
			3.2	15	19	192	0.95	1.08	1.03	132

APPENDIX 8

Mouse Lymphoma Mutation Test in the Absence of S9 Mix: 24 h Exposure
Day 1 Relative Survival (Assay 3)

Chemical	Concentration ($\mu\text{g}\cdot\text{m}^{-1}$)	Osmolality ($\text{mMol}\cdot\text{kg}^{-1}$)	Post- Treatment Cell Count	Day 1 Cloning Efficiency Plates		Total Wells Scored	Cell Count Factor (CCF)	Cloning Efficiency	Survival	Day 1 Relative Survival %
				Plate 1 Empty Wells	Plate 2 Empty Wells					
Vehicle Control Mean ROP	8 ml added)	-	11.1	-	-	-	-	0.71	0.71	-
			12.0	36	25	192	1.08	0.72	0.77	109
			11.0	33	27	192	0.99	0.73	0.72	102
			10.4	32	34	192	0.94	0.67	0.63	88
EMS	150	-	11.0	36	25	192	0.99	0.72	0.71	100
			8.0	44	41	192	0.72	0.51	0.37	52
			8.8	32	41	192	0.79	0.60	0.48	68
MMS	5	-	7.5	47	52	192	0.68	0.41	0.28	40
			9.0	54	53	192	0.81	0.37	0.30	42
			HOAT	63	-	-	0	-	-	-
			NPS	-	-	0	-	-	-	-
			NPS	-	-	0	-	-	-	-
			NPS	-	-	0	-	-	-	-
	125	-	NPS	-	-	0	-	-	-	-
			NPS	-	-	0	-	-	-	-
			NPS	-	-	0	-	-	-	-

NPS = Not plated - surplus

APPENDIX 8 (continued)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Osmolality (mMol.kg^{-1})	Post- Treatment Cell Count	Day 1 Cloning Efficiency Plates		Total Wells Scored	Cell Count Factor (CCF)	Cloning Efficiency	Survival	Day 0 Relative Survival %	
				Plate 1 Empty Wells	Plate 2 Empty Wells						
HOAT	250	-	10.2	25	28	192	0.92	0.80	0.74	104	
				20	24	192	0.94	0.92	0.86	122	
				10.4							
	500	-	-	9.0	27	31	192	0.81	0.75	0.61	86
					15	39	192	0.85	0.79	0.67	95
					9.4						
	1000	-	-	9.8	31	31	192	0.88	0.71	0.62	88
					25	28	192	0.88	0.80	0.71	100
					9.8						
	1500	-	-	9.0	27	21	192	0.81	0.87	0.70	99
					27	22	192	0.83	0.85	0.71	100
					9.2						
2000	-	-	6.0	26	28	192	0.54	0.79	0.43	61	
				17	18	192	0.45	1.06	0.48	68	
				5.0							
2500	-	-	5.2	25	20	192	0.47	0.91	0.42	60	
				15	23	192	0.43	1.01	0.44	62	
				4.8							

APPENDIX 9 (continued)

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Osmolality ($\text{mMol}\cdot\text{kg}^{-1}$)	Post- Treatment Cell Count	Day 0 Cloning Efficiency		Total Wells Scored	Cell Count Factor (CCF)	Cloning Efficiency	Survival	Day 0 Relative Survival %
				Plate 1 Empty Wells	Plate 2 Empty Wells					
HOAT	2500	-	3.4	15	22	192	0.98	1.03	1.01	113
			3.6	20	21	192	1.04	0.96	1.00	112
			3.4	24	15	192	0.98	1.00	0.97	109
	5000	-	3.2	23	19	192	0.92	0.95	0.87	98

APPENDIX 10
 Mouse Lymphoma Mutation Test in the Absence of S9 Mix: 4 h exposure
 Suspension Growth
 (Assay 1)

Chemical	Concentration (µg.ml ⁻¹)	Daily Suspension Count		Total Suspension Growth	Relative Suspension Growth
		Day 1	Day 2		
Vehicle Control Mean	→	-	-	17.0	-
R0P (4 ml added)	11.0	16.0	19.6	114.7	88.7
	10.0	13.6	15.1	94.9	101.7
	11.2	13.0	16.2	65.7	65.7
	10.4	15.0	17.3	65.7	45.6
EMS	250	8.4	12.0	39.9	65.7
	15	6.0	10.2	65.7	65.7
MMS	15	7.0	10.0	39.9	65.7
	156	12.0	NPS	45.6	65.7
HOAT	156	10.6	NPS	45.6	65.7
	313	13.6	NPS	45.6	65.7
	625	11.8	12.4	45.6	65.7
	1250	10.0	15.4	45.6	65.7
		9.2	15.4	92.4	92.4

NPS = Not plated - surplus

APPENDIX 10 (continued)

Chemical	HOAT	Concentration ($\mu\text{g}\cdot\text{m}^{-3}$)	Daily Suspension Count		Total Suspension Growth	Relative Suspension Growth
			Day 1	Day 2		
		2500	10.8	13.6	16.3	95.7
		5000	10.4	13.8	15.9	93.6
			8.4	15.2	14.2	83.2
			9.2	16.8	17.2	100.8

Mouse Lymphoma Mutation Test in the Presence of S9 Mix
 Suspension Growth
 (Assay 2)

APPENDIX 11

Chemical	Concentration (µg.ml ⁻¹)	Daily Suspension Count		Total Suspension Growth	Relative Suspension Growth
		Day 1	Day 2		
Vehicle Control Mean	→	-	-	13.8	-
R0P (4 ml added)	9.0	14.2	14.2	14.2	102.8
3-MC	9.0	15.4	15.4	15.4	111.5
	8.0	14.0	14.0	12.4	90.1
	9.0	13.2	13.2	13.2	95.6
	2.5	4.0	12.8	5.7	41.2
HOAT	4.2	13.6	13.6	6.3	46.0
	156	11.6	NPS	-	-
313	11.8	NPS	NPS	-	-
	12.6	NPS	NPS	-	-
	11.2	NPS	NPS	-	-
	625	11.0	15.6	19.1	138.1
1250	11.4	16.6	21.0	152.2	131.6
	11.2	14.6	18.2	18.3	132.2
	10.4	15.8	18.3	18.3	132.2

NPS = Not plated - surplus

Chemical	HOAT	Concentration ($\mu\text{g}\cdot\text{m}^{-3}$)	Daily Suspension Count		Total Suspension Growth	Relative Suspension Growth
			Day 1	Day 2		
		2500	9.2	15.0	15.3	111.0
		2500	9.0	15.0	15.0	108.6
		5000	8.2	14.4	13.1	95.0
			8.0	16.0	14.2	103.0

APPENDIX 11 (continued)

APPENDIX 12
 Mouse Lymphoma Mutation Test in the Absence of S9 Mix: 24 h exposure
 Suspension Growth
 (Assay 3)

Chemical	Concentration (µg.ml ⁻¹)	Daily Suspension Count			Total Suspension Growth	Relative Suspension Growth
		Day 1	Day 2	Day 3		
Vehicle Control Mean	←	-	-	-	32.5	-
ROP	(8 ml added)	12.0	6.4	11.0	31.3	96.2
EMS	150	11.0	7.3	11.4	33.9	104.2
		10.4	7.2	11.6	32.2	98.9
		11.0	6.0	13.4	32.8	100.7
		8.0	7.4	9.0	19.7	60.7
MMS	5	8.8	5.7	8.8	16.3	50.3
		7.5	5.2	10.0	14.4	44.4
HOAT	500	9.0	5.0	13.0	21.7	66.6
		9.4	9.0	8.6	-	-
HOAT	250	10.4	7.1	NPS	-	-
		10.2	7.9	NPS	-	-
		NPS	-	-	-	-
		NPS	-	-	-	-
HOAT	125	NPS	-	-	-	-
		NPS	-	-	-	-
HOAT	63	NPS	-	-	-	-
		NPS	-	-	-	-

NPS = Not plated - surplus

APPENDIX 12 (continued)

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Daily Suspension Count			Total Suspension Growth	Relative Suspension Growth
		Day 1	Day 2	Day 3		
HOAT	1000	9.8	11.0	14.0	55.9	171.8
		9.8	9.0	15.4	50.3	154.6
	1500	9.0	10.4	13.2	45.8	140.7
		9.2	9.5	12.0	38.8	119.4
	2000	6.0	10.4	12.8	29.6	90.9
		5.0	9.4	13.8	24.0	73.8
	2500	5.2	6.6	12.4	15.8	48.5
		4.8	7.0	12.0	14.9	45.9

APPENDIX 13

Mouse Lymphoma Mutation Test in the Presence of S9 Mix
Suspension Growth
(Assay 4)

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Daily Suspension Count		Total Suspension Growth	Relative Suspension Growth
		Day 1	Day 2		
<i>Vehicle Control Mean</i>	\rightarrow	-	-	17.2	-
R0P	(4 ml added)	10.4	16.0	18.5	107.3
		10.6	14.0	16.5	95.7
		10.6	15.4	18.1	105.3
		10.0	14.2	15.8	91.6
3-MC	2.5	6.2	11.6	8.0	46.4
		6.0	15.0	10.0	58.1
HOAT	625	14.0	16.6	25.8	149.9
		12.0	15.4	20.5	119.2
	1250	13.2	13.4	19.7	114.1
		11.8	15.6	20.5	118.8
	2500	12.0	14.8	19.7	114.6
		12.0	15.6	20.8	120.8
	5000	9.2	15.0	15.3	89.0
		8.4	16.4	15.3	88.9

APPENDIX 14

Mouse Lymphoma Mutation Test in the Absence of S9 Mix: 4 h exposure
 Day 2 Cloning Efficiency (non-mutants)
 (Assay 1)

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Day 2 Cloning Efficiency Plates		Total Wells Scored	Cloning Efficiency (non- mutants)
		Plate 1 Empty Wells	Plate 2 Empty Wells		
<i>Vehicle Control Mean</i>	\rightarrow	-	-	-	0.83
R0P	(4 ml added)	35	33	192	0.65
		23	25	192	0.87
		38	25	192	0.70
		13	19	192	1.12
EMS	250	26	20	192	0.89
		29	19	192	0.87
MMS	15	57	64	192	0.29
		55	58	192	0.33
HOAT	156	NPS	NPS	0	-
		NPS	NPS	0	-
	313	NPS	NPS	0	-
		NPS	NPS	0	-
	625	18	17	192	1.06
		34	31	192	0.68
	1250	31	33	192	0.69
		35	29	192	0.69

NPS = Not plated - surplus

APPENDIX 14 (continued)

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Day 2 Cloning Efficiency Plates		Total Wells Scored	Cloning Efficiency (non- mutants)
		Plate 1 Empty Wells	Plate 2 Empty Wells		
HOAT	2500	32	39	192	0.62
		41	34	192	0.59
	5000	36	36	192	0.61
		36	38	192	0.60

APPENDIX 15

Mouse Lymphoma Mutation Test in the Presence of S9 Mix
 Day 2 Cloning Efficiency (non-mutants)
 (Assay 2)

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Day 2 Cloning Efficiency Plates		Total Wells Scored	Cloning Efficiency (non- mutants)
		Plate 1 Empty Wells	Plate 2 Empty Wells		
Vehicle Control Mean	→	-	-	-	0.85
R0P	(4 ml added)	24	31	192	0.78
		30	25	192	0.78
		27	22	192	0.85
		20	19	192	1.00
3-MC	2.5	42	35	192	0.57
		47	46	192	0.45
HOAT	156	NPS	NPS	0	-
		NPS	NPS	0	-
	313	NPS	NPS	0	-
		NPS	NPS	0	-
	625	26	30	192	0.77
		24	24	192	0.87
	1250	35	24	192	0.74
		32	35	192	0.66

NPS = Not plated - surplus

APPENDIX 15 (continued)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 2 Cloning Efficiency Plates		Total Wells Scored	Cloning Efficiency (non- mutants)
		Plate 1 Empty Wells	Plate 2 Empty Wells		
HOAT	2500	36	39	192	0.59
		35	43	192	0.56
	5000	36	35	192	0.62
		32	32	192	0.69

APPENDIX 16

Mouse Lymphoma Mutation Test in the Absence of S9 Mix: 24 h exposure
 Day 3 Cloning Efficiency (non-mutants)
 (Assay 3)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 3 Cloning Efficiency Plates		Total Wells Scored	Cloning Efficiency (non- mutants)
		Plate 1 Empty Wells	Plate 2 Empty Wells		
<i>Vehicle Control Mean</i>	\rightarrow	-	-	-	1.01
ROP	(8 ml added)	20	24	192	0.92
		11	21	192	1.12
		20	19	192	1.00
		15	24	192	1.00
EMS	150	39	37	192	0.58
		27	25	192	0.82
MMS	5	32	37	192	0.64
		47	44	192	0.47
HOAT	63	NPS	NPS	0	-
		NPS	NPS	0	-
	125	NPS	NPS	0	-
		NPS	NPS	0	-
	250	NPS	NPS	0	-
		NPS	NPS	0	-
	500	NPS	NPS	0	-
		NPS	NPS	0	-

NPS = Not plated - surplus

APPENDIX 16 (continued)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 3 Cloning Efficiency Plates		Total Wells Scored	Cloning Efficiency (non- mutants)
		Plate 1 Empty Wells	Plate 2 Empty Wells		
HOAT	1000	21	19	192	0.98
		17	22	192	1.00
	1500	25	22	192	0.88
		14	12	192	1.25
	2000	37	22	192	0.74
		16	23	192	1.00
	2500	23	29	192	0.82
		18	13	192	1.14

APPENDIX 17

Mouse Lymphoma Mutation Test in the Presence of S9 Mix
 Day 2 Cloning Efficiency (non-mutants)
 (Assay 4)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 2 Cloning Efficiency Plates		Total Wells Scored	Cloning Efficiency (non- mutants)
		Plate 1 Empty Wells	Plate 2 Empty Wells		
<i>Vehicle Control Mean</i>	\rightarrow	-	-	-	0.96
ROP	(4 ml added)	27	23	192	0.84
		17	13	192	1.16
		25	29	192	0.79
		18	19	192	1.03
3-MC	2.5	27	26	192	0.80
		22	25	192	0.88
HOAT	625	24	29	192	0.80
		14	14	192	1.20
	1250	21	22	192	0.94
		22	10	192	1.12
	2500	24	23	192	0.88
		22	25	192	0.88
	5000	25	19	192	0.92
		24	17	192	0.96

APPENDIX 18

Mouse Lymphoma Mutation Test in the Absence of S9 Mix: 4 h exposure
Day 2 Mutant Selection
(Assay 1)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 2 Mutant Colonies						Ratio of small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($\times 10^{-6}$)	Mutant Fraction ($\times 10^{-6}$)
		Plate 1		Plate 2		Small Colony Wells						
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells							
Vehicle Control Mean	→	-	-	-	-	-	-	-	-	-	80	
R0P	(4 ml added)	6	9	5	7		1.45	192	165	75.77	117	
		4	9	8	4		1.08	192	167	69.75	81	
		6	5	2	5		1.25	192	174	49.22	71	
		1	9	7	4		1.63	192	171	57.92	52	
EMS	250	53	12	54	13		0.23	192	60	581.58	651	
		38	21	35	14		0.48	192	84	413.34	477	
MMS	15	11	34	12	28		2.70	192	107	292.33	1013	
		11	29	12	41		3.04	192	99	331.19	1000	
HOAT	156	NPS	NPS	NPS	NPS		-	0	-	-	-	
		NPS	NPS	NPS	NPS		-	0	-	-	-	
		NPS	NPS	NPS	NPS		-	0	-	-	-	
		NPS	NPS	NPS	NPS		-	0	-	-	-	

NPS = Not plated - surplus

APPENDIX 18 (continued)

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Day 2 Mutant Colonies						Ratio of small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($\times 10^{-6}$)	Mutant Fraction ($\times 10^{-6}$)
		Plate 1		Plate 2		Small Colony Wells						
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells							
HOAT	625	5	6	2	7	1.86	192	172	55.00	52		
		3	6	5	5						1.38	192
	1250	4	2	2	9	1.83	192	175	46.35	68		
		1	5	3	5						2.50	192
	2500	4	10	8	6	1.33	192	164	78.81	127		
		2	7	6	9						2.00	192
	5000	7	17	11	22	2.17	189	132	179.47	293		
		13	13	8	24						1.76	192

APPENDIX 19

Mouse Lymphoma Mutation Test in the Presence of S9 Mix
Day 2 Mutant Selection
(Assay 2)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 2 Mutant Colonies						Ratio of small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($\times 10^{-6}$)	Mutant Fraction ($\times 10^{-6}$)
		Plate 1		Plate 2		Large Colony Wells	Small Colony Wells					
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells							
Vehicle Control Mean	→	-	-	-	-	-	-	-	-	-	92	
R0P	(4 ml added)	4	10	4	5			1.88	192	169	63.80	82
		5	10	8	6			1.23	192	163	81.87	105
		6	9	3	11			2.22	192	163	81.87	96
		6	6	4	14			2.00	192	162	84.95	85
3-MC	2.5	23	38	25	44			1.71	192	62	565.18	990
		20	50	18	52			2.68	192	52	653.13	1442
HOAT	156	NPS	NPS	NPS	NPS			-	0	-	-	-
		NPS	NPS	NPS	NPS			-	0	-	-	-
		NPS	NPS	NPS	NPS			-	0	-	-	-
		NPS	NPS	NPS	NPS			-	0	-	-	-

NPS = Not plated - surplus

APPENDIX 19 (continued)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 2 Mutant Colonies						Ratio of small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($\times 10^{-6}$)	Mutant Fraction ($\times 10^{-6}$)
		Plate 1		Plate 2		Small Colony Wells						
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells							
HOAT	625	4	17	4	5	192	162	84.95	110			
		4	11	10	6	192	161	88.05	102			
	1250	8	11	5	12	192	156	103.82	141			
		7	8	4	10	192	163	81.87	124			
	2500	4	17	6	12	192	153	113.53	193			
		6	13	8	16	192	149	126.77	225			
	5000	15	19	12	18	192	128	202.73	326			
		14	10	8	16	192	144	143.84	209			

APPENDIX 20

Mouse Lymphoma Mutation Test in the Absence of S9 Mix: 24 h exposure
Day 3 Mutant Selection (Assay 3)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 3 Mutant Colonies						Ratio of small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($\times 10^{-6}$)	Mutant Fraction ($\times 10^{-6}$)
		Plate 1		Plate 2		Small Colony Wells						
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells							
Vehicle Control Mean	→	-	-	-	-	-	-	-	-	-	88	
ROP	(8 ml added)	6	8	4	7	7	1.50	192	167	69.75	76	
		10	6	8	7	7	0.72	192	161	88.05	79	
		6	8	4	9	9	1.70	192	165	75.77	76	
		11	10	13	7	7	0.71	192	151	120.11	121	
EMS	150	61	25	62	22	22	0.38	192	22	1083.23	1870	
		67	24	68	19	19	0.32	192	14	1309.22	1604	
MMS	5	23	47	28	50	50	1.90	192	44	736.65	1152	
		32	38	21	44	44	1.55	192	57	607.22	1301	
HOAT	63	NPS	NPS	NPS	NPS	NPS	-	0	-	-	-	
		NPS	NPS	NPS	NPS	NPS	-	0	-	-	-	
		NPS	NPS	NPS	NPS	NPS	-	0	-	-	-	
		NPS	NPS	NPS	NPS	NPS	-	0	-	-	-	

NPS = Not plated - surplus

APPENDIX 20 (continued)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 3 Mutant Colonies						Ratio of small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($\times 10^{-6}$)	Mutant Fraction ($\times 10^{-6}$)
		Plate 1		Plate 2		Large Colony Wells	Small Colony Wells					
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells							
HOAT	250	NPS	NPS	NPS	NPS	NPS	NPS	0	-	-	-	
		NPS	NPS	NPS	NPS	NPS	NPS	0	-	-	-	
	500	NPS	NPS	NPS	NPS	NPS	NPS	0	-	-	-	
		NPS	NPS	NPS	NPS	NPS	NPS	0	-	-	-	
	1000	5	6	2	6	6	6	192	173	52.10	53	
		6	6	4	13	4	13	192	163	81.87	82	
	1500	3	6	7	5	7	5	192	171	57.92	66	
		7	17	12	12	12	12	192	144	143.84	115	
	2000	14	12	13	12	13	12	192	141	154.37	209	
		10	19	8	13	8	13	192	142	150.83	151	
	2500	17	15	14	25	14	25	192	121	230.85	283	
		13	25	15	23	15	23	192	116	251.95	221	

NPS = Not plated - surplus

APPENDIX 21

Mouse Lymphoma Mutation Test in the Presence of S9 Mix
Day 2 Mutant Selection
(Assay 4)

Chemical	Concentration ($\mu\text{g.ml}^{-1}$)	Day 2 Mutant Colonies						Ratio of small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($\times 10^{-6}$)	Mutant Fraction ($\times 10^{-6}$)
		Plate 1		Plate 2		Large Colony Wells	Small Colony Wells					
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells							
Vehicle Control Mean	→	-	-	-	-	-	-	-	-	-	109	
ROP	(4 ml added)	12	4	8	4			0.40	192	164	78.81	94
		14	5	8	7			0.55	192	158	97.45	84
		15	8	9	6			0.58	192	154	110.27	139
		18	8	8	8			0.62	192	150	123.43	120
3-MC	2.5	23	34	31	36			1.30	192	68	518.99	645
		35	34	27	37			1.15	192	59	589.98	671
HOAT	625	4	5	5	4			1.00	192	174	49.22	61
		13	7	15	4			0.39	192	153	113.53	94
		11	11	12	11			0.96	192	147	133.53	143
	1250	14	10	11	5			0.60	192	152	116.81	104

APPENDIX 21 (continued)

Chemical	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Day 2 Mutant Colonies						Ratio of small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($\times 10^6$)	Mutant Fraction ($\times 10^6$)
		Plate 1			Plate 2							
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells							
HOAT	2500	15	8	12	5	192	0.48	152	116.81	133		
		7	12	4	13	192	2.27	156	103.82	118		
	5000	12	20	5	10	192	1.76	145	140.38	152		
		13	7	8	12	192	0.90	152	116.81	121		

APPENDIX 22

The calculations involved in the result analysis for the first methyl methane-sulphonate-treated culture in Assay 1 are described below. The calculations may contain rounding errors.

Method of calculating the Day 0 Relative Survival (Appendix 6).

This figure represents the cloning efficiency of the culture immediately after treatment; adjusted to account for any cells lost to toxicity during treatment.

The cloning efficiency (CE) is calculated from the zero term of the Poisson distribution using the formula:

$$CE = \frac{-\ln(P(0))}{\text{number of cells per well}}$$

$$\text{where } P(0) = \frac{\text{empty wells}}{\text{total wells}}$$

In this instance,

$$P(0) = \frac{32 + 32}{192} = 0.33$$

$$CE = \frac{-\ln(0.33)}{1.6} = 0.69$$

At the outset of the experiment, the cells in each culture are at a density of $3 \times 10^5 \text{ ml}^{-1}$. Any divergence from this density by the end of the exposure period is accounted for by the cell count factor (CCF).

$$CCF = \frac{\textit{individual treated post - treatment cell count}}{\textit{mean vehicle control post - treatment cell count}}$$

In this instance,

$$CCF = \frac{3.0}{3.7} = 0.81$$

Survival (S) is expressed as the cloning efficiency multiplied by the cell count factor:

$$S = CE \times CCF$$

In this instance,

$$S = 0.69 \times 0.81 = 0.55$$

Relative Survival (RS) is expressed as:

$$RS = \frac{\textit{individual survival value}}{\textit{mean vehicle control survival value}}$$

In this instance,

$$RS = \frac{0.55}{0.87} = 0.64$$

Thus, 64% of the cells treated with MMS were clonable after 4 h exposure.

On Day 2, the CE of the non-mutants is calculated as above (Appendix 14).

In this instance, the value is 0.29.

Method of calculating the Relative Suspension Growth (Appendix 10).

This figure represents the potential of the culture to multiply itself over the 2 day expression period (or 3 day, in the case of experiments with 24 h exposure periods), by accounting for the concentration to which the cells are diluted after counting:

On Day 1, after counting, the cells were diluted to $3.0 \times 10^5 \text{.ml}^{-1}$. On Day 2, the cells had reached $10.2 \times 10^5 \text{.ml}^{-1}$.

The calculation for suspension growth from Day 1 to Day 2 would be:

$$\frac{10.2}{3.0} = 3.4$$

The cells therefore had multiplied 3.4-fold between Day 1 and Day 2.

The calculation for the full period (Total Suspension Growth), therefore, is:

$$\frac{\text{Day 1 Count}}{\text{Final Concentration on Day 0}} \times \frac{\text{Day 2 Count}}{\text{Final Concentration on Day 1}}$$

In this instance,

$$\frac{6.0}{3.0} \times \frac{10.2}{3.0} = 6.8$$

The cells therefore had multiplied 6.8-fold over the 2 day expression period.

This figure is then expressed as a percentage of the mean vehicle control value to give the Relative Suspension Growth.

In this instance,

$$\frac{6.8}{17.0} \times \frac{100}{1} = 39.9\%$$

Method of calculating the Mutant Fraction (Appendix 18).

This figure gives the number of mutant cells per million clonable cells. The number of empty wells is obtained by subtracting the small colony wells and the large colony wells from the total wells plated.

In this instance,

$$192 - (11 + 34 + 12 + 28) = 107$$

The CE of the mutant cells is calculated as for the non-mutants.

In this instance,

$$P(0) = \frac{107}{192} = 0.56$$

$$CE = \frac{-\ln(0.56)}{2000} = 292.33 \times 10^{-6}$$

This figure is finally adjusted to compensate for the CE of the whole population, thus:

$$\text{Mutant fraction per viable cell} = \frac{\text{CE of mutants}}{\text{CE of non - mutants}}$$

In this instance,

$$\text{Mutant fraction per viable cell} = \frac{292.33}{0.29} = 1013 \times 10^{-6}$$

APPENDIX 23

Control Data from Recent Experiments (1998-Present)
 Growth Medium: RPMI 1640 Medium with Heat-inactivated Horse Serum
 (Results from Present Study Not Included)

Control	Concentration ($\mu\text{g.ml}^{-1}$)	S9	Exposure Time	n*	Mutant Fraction. 10^{-6}			% Survival			Mean Colony Size Ratio (Small/Large)
					Mean	Standard Deviation	Range	Mean	Standard Deviation	Range	
Vehicle (all vehicles, pooled)	-	Without	4 h	4	92	13	80-104	-	-	-	1.35
Ethyl methanesulphonate (EMS)	250	Without	4 h	4	651	79	547-728	93	12	79-107	0.49
Methyl methanesulphonate (MMS)	15	Without	4 h	4	1143	326	822-1573	79	7	68-84	2.27
Vehicle (all vehicles, pooled)	-	Without	24 h	5	96	28	67-128	-	-	-	1.24
Ethyl methanesulphonate (EMS)	100/150	Without	24 h	4	1390	245	1023-1525	71	22	43-95	0.46
Methyl methanesulphonate (MMS)	5	Without	24 h	4	1546	44	1500-1605	47	9	38-59	1.80
Vehicle (all vehicles, pooled)	-	With	4 h	7	110	24	67-150	-	-	-	1.05
3-Methylcholanthrene (3-MC)	2.5	With	4 h	7	1190	345	596-1569	72	16	50-92	1.46

* = Each vehicle control value is the mean of 4 replicate cultures
 Each positive control value is the mean of 2 replicate cultures

Data audited by: G Farrell (Quality Assurance)
 20 October 1998

APPENDIX 23 (continued)

Control Data from Recent Experiments (1993-1998)
 Growth Medium: Fischer's Medium with Donor Horse Serum

Control	S9	n*	Mutant Fraction. 10 ⁻⁶			% Survival			Mean Colony Size Ratio S/L† (n = Variable)
			Mean	Standard Deviation	Range	Mean	Standard Deviation	Range	
Vehicle (all vehicles, pooled)	Without	153	58	22	17-124	-	-	-	1.10
Ethyl methanesulphonate (EMS)	Without	153	319	72	172-631	62	12	35-106	0.33
Methyl methanesulphonate (MMS)†	Without	98	307	78	126-636	23	9	7-56	1.78
Vehicle (all vehicles, pooled)	With	157	53	18	27-111	-	-	-	1.10
3-Methylcholanthrene (3-MC)	With	157	405	153	147-1065	36	13	9-82	1.05

* = Each vehicle control value is the mean of 4 replicate cultures
 Each positive control value is the mean of 2 replicate cultures

† = Started in 1995. (Dose levels are variable: 10 or 15 µg.ml⁻¹)

+ = Started in 1995 (S/L = Small/Large colonies)

Data audited by: G. Birnie(Quality Assurance)
 16 March 1998