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June 3, 2004

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Attention: TSCA Section 8(e) Coordinator
Office of Pollution Prevention and Toxics
Environmental Protection Agency
1200 Pennsylvania Avenue
Washington, DC 20460-0001

Dear Sir or Madam:

This information is submitted under the provisions of the Toxic Substances Control Act 90 Stat. 2029, 15 USC 2607, Section 8(e). Hercules Incorporated recently completed a series of in vitro mutagenicity assays on allyl alcohol (CASRN: 107-18-6). Attached are three Regulatory Reports prepared by our contractor, Central Toxicology Laboratory, Alderley Park, England. The reports are dated May 4, 10, and 13, 2004, mailed on May 7 and 17, 2004. We had possession of all the reports by May 20, 2004.

The Regulatory Reports are titled:

- 1) Bacterial Mutation Assay in *S. typhimurium* (Ames test strains) and *E. coli*
- 2) L5178 TK+/- Mouse Lymphoma Mutagen Assay
- 3) *In Vitro* Cytogenetics Assay in Human Lymphocytes



The Bacterial Mutation Assay in *S. typhimurium* and *E. coli* report indicated that allyl alcohol gave a negative response, i.e., non-mutagenic both in the presence and absence of S9-mix.

However, allyl alcohol did induce a cleat positive response in both the mouse lymphoma model (in the presence of S9-mix) and in the human lymphocyte model (both in the presence and absence of S9-mix).

We are aware of the results of the recent NTP mutagen studies, though there are only data summaries (no reports) available on the NTP website. We plan to share these new results with NTP. Likewise, we will share the results with the manufacturers of allyl alcohol by sending copies of our reports to Lyondell, who volunteered to sponsor allyl alcohol in the HPV Challenge program.

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June 3, 2004

Hercules Incorporated is not a manufacturer of allyl alcohol. However, we use allyl alcohol in the manufacture of other substances. As such, we may be a 'processor' as defined under TSCA.

Sincerely,



Gary L. McCallister
Director, Regulatory Affairs

GLM:cj
Enclosures

2004letters.doc/22-23

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MR 276199

**CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK**

CTL/YV6638/REGULATORY/REPORT

**ALLYL ALCOHOL: BACTERIAL MUTATION ASSAY IN
S.TYPHIMURIUM AND *E.COLI***

**CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK**

CTL/YV6638/REGULATORY/REPORT
ALLYL ALCOHOL: BACTERIAL MUTATION ASSAY IN
S.TYPHIMURIUM AND E.COLI

STUDY DETAILS

Sponsor: Hercules Incorporated
Hercules Research Centre
500 Hercules Road
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USA

Sponsor Reference: C03310
CTL Test Substance Reference Number: Y04185/002
CTL Study Number: YV6638
Document Number: CTL/YV6638/REG/REPT

AUTHOR

R D Callander

DATE OF ISSUE

13 May 2004

STATEMENT OF DATA CONFIDENTIALITY CLAIM

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It should not be disclosed in any form to an outside party, nor should information contained herein be used by a registration authority to support registration of this product or any other product without the written permission of Hercules Incorporated.

STATEMENT OF GLP COMPLIANCE AND AUTHENTICATION

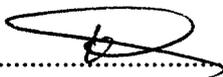
I, the undersigned, declare that the objectives laid down in the protocol were achieved and that the data generated are valid. The report fully and accurately reflects the procedures used and the raw data generated in the above study.

The study (YV6638) was conducted in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom GLP Regulations 1999, Statutory Instrument No. 3106) except for the deviations listed below. These Principles are in accordance with the OECD Principles of Good Laboratory Practice, revised 1997 (ENV/MC/CHEM(98)17).

The following GLP deviations are considered not to affect the integrity of the study or the validity of the conclusions drawn:

- (i) the stability of the test substance has not been reported
- (ii) the stability and achieved concentration of the test substance in the vehicle used were not determined by analysis.

R D Callander
Study Director

.....

13 May 2004
Date

This page
may be required
by some
regulatory authorities.

QUALITY ASSURANCE STATEMENT

In accordance with CTL policy and QA procedures for Good Laboratory Practice, this report has been audited and the conduct of this study has been inspected as follows:

Date	Audit/Inspection	Date of QA Report
29 Apr 2004	Draft report	05 May 2004
13 May 2004	Final report review	13 May 2004

In addition, inspections associated with this type of study were made as follows:

08 Dec 2003	Protocol	08 Dec 2003
09 Dec 2003	Dilutions, plating	09 Dec 2003
19 Dec 2003	Dose preparation	19 Dec 2003
16 Jan 2004	Counting	16 Jan 2004

Facilities and process based procedures associated with this type of study were inspected in accordance with QA Standard Operating Procedures.

So far as can be reasonably established, the methods described and the results given in the final report accurately reflect the raw data produced during the study, YV6638.

C Rooney

(CTL Quality Assurance Unit)

.......... 13 May 2004

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

Name	Title
R D Callander	Study Director, CTL
B M Elliott	Study Reviewer

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This page is provided for the Regulatory Authority Reviewer's notes.

1. SUMMARY

1.1 Study design

Allyl Alcohol was evaluated in a bacterial mutagenicity assay (based on Maron and Ames (1983)) over a range of concentrations using four strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and one strain of *Escherichia coli* (WP2 *uvrA* (pKM101)) in the presence and absence of a rat liver - derived metabolic activation system (S9-mix).

1.2 Results

In at least two separate experiments with each tester strain, the test substance did not induce any significant, reproducible increases in the observed numbers of revertant colonies, either in the presence or absence of S9-mix.

The sensitivity of the test system, and the metabolic activity of the S9-mix, were clearly demonstrated by the increases in the numbers of revertant colonies induced by positive control substances.

1.3 Conclusion

It is concluded that, under the conditions of this assay, Allyl Alcohol gave a negative, i.e. non-mutagenic, response in *S.typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E.coli* strain WP2 *uvrA* (pKM101) in both the presence and absence of S9-mix.

2. INTRODUCTION

2.1 Purpose

The purpose of this study was to investigate whether the test substance induced gene mutation in certain strains of the bacteria *Salmonella typhimurium* and *Escherichia coli*.

2.2 Regulatory guidelines

The study was conducted according to the following Regulatory Guidelines:

- a) Annex V to Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances, published in the 26th Adaptation, Commission Directive 2000/32/EEC of 19 May 2000, OJ L136 8.6.2000 (title amended in the 28th Adaptation, Commission Directive 2001/59/EEC of 6 August 2001, OJ L225 21.8.2001). B.13/14, Mutagenicity - reverse mutation test using bacteria.
- b) ICH Harmonised Tripartite Guidelines S2A: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (adopted at Step 4 of the ICH process 19 July 1995) and S2B: Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals (adopted at Step 4 of the ICH process 16 July 1997).
- c) OECD guideline reference 471 (1997). Bacterial Reverse Mutation Assay.
- d) USEPA Health Effects Test Guideline OPPTS 870.5100 (1998). Bacterial Reverse Mutation Test.

2.3 Justification for test system selection

The bacterial mutation assay was used as it is a well-established assay, designed to detect reversion to amino acid independence (*his*⁻ to *his*⁺ for *S.typhimurium*, *trp*⁻ to *trp*⁺ for *E.coli* strains) induced by chemicals which cause base changes or frameshift mutations in the genome of these organisms. The use of strains TA1535, TA1537, TA98, TA100 and WP2 *uvrA* (pKM101) is in accordance with the current scientific recommendations for the conduct of this assay.

2.4 Study dates

The study was initiated on 17 December 2003. The experimental phase was started on 13 January 2004 and was completed on 06 February 2004.

2.5 Data storage

An original report, the study protocol and all raw data pertaining to this study will be retained in the CTL Archives, Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK for a minimum of 5 years from the date of issue of the final report. At the end of this period the Sponsor will be contacted regarding the further retention, return to the Sponsor or destruction of the archived materials.

3. TEST AND CONTROL SUBSTANCES

3.1 Test substance

Name:	Allyl Alcohol
Source:	Sigma-Aldrich
Colour:	Colourless
Physical state:	Liquid
Batch reference:	407813/1 13102
CTL test substance reference number:	Y04185/002
Purity:	99.5%
Storage conditions:	Ambient temperature in the dark
Expiry date:	Not specified

The test substance characterisation was the responsibility of the Sponsor.

In all cases where the concentration of test substance is quoted, the concentration refers to the concentration of test substance not corrected for purity.

3.2 Control substances

The control substance and solvent for the test was dried dimethylsulphoxide (DMSO: CTL test substance reference number Y00876/011). The positive control substances are detailed in the following table:

Chemical	Supplier	CTL Ref	Solvent
Acridine Mutagen ICR191	Sigma	Y03243/003	DMSO
2-Aminoanthracene (2AA)	Sigma	Y01142/006	DMSO
Benzo [<i>a</i>] pyrene (BP)	Lancaster Synthesis	Y00111/005	DMSO
Daunomycin HCl (DR)	Sigma	Y01165/002	DMSO
N-Ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	Sigma	Y02125/001	DMSO
Sodium Azide (NaZ)	Sigma	Y06019/001	H ₂ O

4. EXPERIMENTAL PROCEDURE

4.1 Dosing preparations

Individual stock solutions of the test substance were prepared at an appropriate concentration for each experiment in DMSO and serial dilutions were carried out as required in each case.

The positive control substances were prepared as solutions in the solvents detailed in the table above.

All test and positive control substance dosing preparations were prepared as close to the time of culture treatment as possible and were dosed at a dosing volume of 100µl/plate (apart from in the pre-incubation experiment, where the volume was reduced to 20µl/plate).

4.2 Analysis of dosing preparations

In view of the short-term nature of the studies of this type, no analyses of stability or achieved concentrations were carried out on the preparations of the test substance, or of homogeneity, stability or achieved concentrations on the preparations of the positive control substances, either prior to or after addition to the bacteria.

4.3 Experimental design

The principle of the assay was as described by Maron and Ames (1983) and the procedure employed followed the Ames protocol modified in accordance with the procedures outlined in OECD guideline 471.

The four *Salmonella typhimurium* tester strains (TA1535, TA1537, TA98 and TA100) and the one *Escherichia coli* strain (WP2 *uvrA* (pKM101)) are fully described in Ames *et al* (1975) and references therein, and in Venitt and Crofton-Sleigh (1979).

Allyl Alcohol was initially assayed using the standard plate incorporation protocol over a dose range of 5000 to 100µg per plate, both in the presence and absence of S9-mix prepared from phenobarbital/β-naphthoflavone-induced Sprague-Dawley (SD) rats (see Section 4.5). Due to the level of toxicity observed in this experiment, the test substance was re-tested in strains TA1535, TA1537, TA98 and TA100 over the lower dose ranges of 200 to 5.0µg per plate (+S9-mix) and 100 to 2.0µg per plate (-S9-mix). Allyl Alcohol was subsequently re-tested in all five strains over the dose ranges 5000 to 100µg per plate (WP2 *uvrA* (pKM101) -S9-mix), 2500 to 50µg per plate (WP2 *uvrA* (pKM101) +S9-mix) and 200 to 5.0µg per plate (TA1535, TA1537, TA98 and TA100 ±S9-mix); the +S9-mix phase of this final assay was conducted using a pre-incubation protocol. The incubation period for each experiment was 3 days (at 37°C).

For each experiment, positive control substances were tested to validate the bacterial strains and to confirm the activity of the S9-mix used.

The experimental design for each strain was as follows:

a) Allyl Alcohol	6 concentrations per strain as shown in Tables 1-6 3 plates per concentration
b) Solvent Control: DMSO (100µl)	5 plates
c) <u>Positive Controls</u>	
+S9 : 2AA (all Salmonella strains) BP (WP2 <i>uvrA</i> (pKM101))	1 concentration per strain as shown in Tables 1-6
-S9 : DR (TA98) ENNG (WP2 <i>uvrA</i> (pKM101)) ICR 191 (TA1537) NaZ (TA1535 and TA100)	3 plates per concentration

4.4 Bacterial cultures

Stock cultures of each strain were prepared and maintained in liquid nitrogen as described by Maron and Ames (1983). The routine source of inocula for overnight cultures was one set of frozen stocks, used and discarded on an approximately weekly basis. The overnight culture from each new frozen culture was screened for the deep-rough characters, DNA repair deficiency and Ampicillin resistance as previously described (Maron and Ames (1983) with one modification:-

The presence of the *uvrB* deletion (Salmonella) and the *uvrA* mutation (*E. coli*) was confirmed by testing the sensitivity of each culture to mitomycin C (10µl of a 10µg/ml solution) in the same manner as sensitivity to crystal violet was tested. Damage to DNA caused by mitomycin C is repaired in normal bacteria by the *uvr* excision repair pathway, and is thus toxic to strains deficient at either the *uvrA* or *uvrB* loci.

When fresh frozen stocks were prepared, the strains were tested for amino acid requirement and for reversion properties using diagnostic mutagens as described by Ames *et al* (1975) and Maron and Ames (1983), except that the mutagens were incorporated in the top agar layer as in a standard experiment (see Section 4.6.1), rather than spot tested as described by Ames *et al*.

4.5 Metabolic activation system

This work was conducted with and without S9-mix incorporated in the top-agar. The S9-mix was prepared as required (on each day of experimentation) as follows:

	Volume per 30ml S9-mix
S9 fraction	3ml
Sucrose-Tris-EDTA buffer (S9 buffer)	7ml
Cofactor solution	20ml

In tests without metabolic activation, the S9 fraction and cofactor solution were replaced by an equivalent volume of S9 buffer. Both the S9-mix and the S9 buffer were kept on ice until used.

S9 was purchased from MolTox Inc., and was prepared from male Sprague Dawley rats, dosed once daily (by oral gavage) for 3 days with a combined phenobarbital (80mg/kg bodyweight) and β -naphthoflavone (100mg/kg) corn oil solution. The treated animals were sacrificed on the day following the third dose. A 25% w/v homogenate (the S9 fraction) was prepared according to the method given in Callander *et al* (1995).

The cofactor solution was prepared as a single stock solution of $\text{Na}_2\text{HP0}_4$, KCl, glucose-6-phosphate, NADP (Na salt) and MgCl_2 (150:49.5:7.5:6:12 mM) in sterile deionised water, and adjusted to a final pH of 7.4.

4.6 Methodology

0.1ml aliquots of an overnight culture (10-12 hours) of each bacterial strain were dispensed into the required number of appropriate containers, and stored at room temperature until required.

Top agar consisting of 0.6% w/v agar and 0.5% w/v sodium chloride in deionised water was melted by brief autoclaving and stored at approximately 50°C until required.

Prior to testing the molten top agar was prepared by adding sterile 0.5mM histidine/0.5mM biotin stock solution (10ml solution:100ml agar) for *Salmonella* work, and by adding sterile tryptophan solution (10ml 0.5mM stock:100ml agar) for *E.coli* work. Separate agars were prepared for each species.

4.6.1 Plate incorporation protocol

0.5ml S9-mix (or S9 buffer) was then added to the number of aliquots of one strain required for one concentration, followed by 0.1ml of the appropriate concentration of test substance preparation. Finally, 2.0ml top agar was then added to each aliquot, and the resulting mixture poured rapidly onto the surface of a prepared pre-labelled Vogel Bonner plate (9cm diameter vented Petri-dish prepared with 25ml Vogel Bonner minimal medium and containing 1.5% w/v agar and 2% w/v glucose) and allowed to gel. Plates were then incubated inverted at 37°C for 3 days in the dark.

Following the total incubation period the plates were examined for the lack of microbial contamination and evidence that the test was valid: i.e. there was a background lawn on the solvent control plates and on the plates for (at least) the lower concentrations of test substance, and that the positive controls had responded as expected. All plates were counted using an automated colony counter adjusted appropriately to permit the optimal counting of mutant colonies. Plates that were obviously contaminated were recorded as such without counting.

4.6.2 Pre-incubation protocol

The assay procedure was as for the plate-incorporation protocol described above, except that

- a) each compound/solvent dose was added in 0.02ml volumes, with the total volume made up to 0.1ml with phosphate buffered saline;
- b) before adding the top agar, each compound/strain group of bijoux were placed on an orbital shaker (at approximately 140rpm) for approximately 60 minutes (at 37°C).

5. DATA EVALUATION

Test data from individual experiments are considered valid if:

- a) the concurrent solvent control data are acceptable;
- b) the positive control data show acceptable increases;

Failure of one or more tester strain/S9 combinations does not invalidate the data for the remainder of a concurrent experiment.

A positive response in a (valid) individual experiment is achieved when one or both of the following criteria are met:

- a) a significant, dose-related increase in the mean number of revertants is observed;
- b) a two-fold or greater increase in the mean number of revertant colonies (over that observed for the concurrent solvent control plates) is observed at one or more concentrations

A negative result in a (valid) individual experiment is achieved when:

- a) there is no significant dose-related increase in the mean number of revertant colonies per plate observed for the test substance; and
- b) in the absence of any such dose response, no increase in colony numbers is observed (at any test concentration) which exceeds 2x the concurrent solvent control.

For a positive response in an individual experiment to be considered indicative of an unequivocal positive, i.e. mutagenic, result for that strain/S9 combination, then the observed effect(s) must be consistently reproducible.

All derived calculations (i.e. mean colony count/plate; standard deviation, etc.) shown in the results tables were carried out by computer. Counts from contaminated plates are not included in these calculations.

6. RESULTS

The numbers of revertant colonies per plate observed in the four *Salmonella typhimurium* tester strains (TA1535, TA1537, TA98 and TA100) and one *Escherichia coli* strain (WP2 *uvrA* (pKM101)), when exposed to Allyl Alcohol and to the various positive and solvent control substances are shown in Tables 1-6. All derived values in the results tables are expressed, where appropriate, to one decimal place: values of 0.05+ have been rounded up to 0.1.

In at least two separate assays with each tester strain, the test substance did not induce any significant, reproducible increases in the observed number of revertant colonies, either in the presence or absence of S9-mix (Tables 1-6).

The positive controls for each experiment induced the expected responses (Tables 1-6), indicating the strains were responding satisfactorily in each case.

7. CONCLUSIONS

It is concluded that, under the conditions of this assay, Allyl Alcohol gave a negative, i.e. non-mutagenic, response in *S.typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E.coli* strain WP2 *uvrA* (pKM101) in both the presence and absence of S9-mix.

8. REFERENCES

Ames B N, McCann J and Yamasaki E (1975). Methods of detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Res* **31**, 347-364.

Callander R D, Mackay J M, Clay P, Elcombe C R and Elliott B M (1995). Evaluation of phenobarbital/ β -naphthoflavone as an alternative S9-induction regime to AROCLOR 1254 in the rat for use in *in vitro* genotoxicity assays. *Mutagenesis* **10**, 517-522.

Maron D M and Ames B N (1983). Revised methods for the Salmonella mutagenicity test. *Mutation Res* **113**, 173-215.

Venitt S and Crofton-Sleigh C (1979). Bacterial mutagenicity tests of phenazine, methosulphate and three tetrazolium salts. *Mutation Res* **68**, 107-116.

TABLE 1 - DATA FOR EXPERIMENTAL PHASE 1 (plate incorporation +S9)

Experiment: YV6638 Phase 1+

Date Plated: 12/01/2004
Date Counted: 15/01/2004

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	Y04185/002	5000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		2500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		1000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		500µg	0.0	0.0	0.0	0 S, 0 S, 0 S
		200µg	53.3	46.2	0.4	0 S, 82, 78
		100µg	100.7	19.7	0.7	111, 78, 113
	DMSO	139.8	19.8		142, 171, 119, 127, 140	
TA1535	Y04185/002	5000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		2500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		1000µg	2.0	2.6	0.1	1 S, 0 S, 5 S
		500µg	4.3	2.5	0.2	7 S, 2 S, 4 S
		200µg	9.3	2.1	0.4	11, 10, 7
		100µg	35.0	6.9	1.4	31, 43, 31
	DMSO	24.2	3.5		28, 21, 26, 26, 20	
TA1537	Y04185/002	5000µg	11.7	0.6	0.9	12 S, 12 S, 11 S
		2500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		1000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		500µg	0.3	0.6	0.0	1 S, 0 S, 0 S
		200µg	8.0	5.6	0.6	2, 9, 13
		100µg	10.3	2.3	0.8	13, 9, 9
	DMSO	13.2	3.3		15, 18, 11, 10, 12	
TA98	Y04185/002	5000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		2500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		1000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		500µg	0.0	0.0	0.0	0 S, 0 S, 0 S
		200µg	8.7	6.7	0.3	12, 1, 13
		100µg	26.0	5.6	1.0	27, 31, 20
	DMSO	27.0	4.3		22, 28, 23, 31, 31	
WP2 <i>uvrA</i> (pKM101)	Y04185/002	5000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		2500µg	3.7	6.4	0.0	11 A, 0 A, 0 A
		1000µg	182.0	13.0	1.2	169, 182, 195
		500µg	166.7	28.3	1.1	197, 141, 162
		200µg	167.3	25.8	1.1	163, 144, 195
		100µg	150.3	7.2	1.0	142, 155, 154
	DMSO	151.8	10.8		164, 152, 159, 136, 148	
TA100	2AA	1µg	834.7	97.7	6.0	848, 731, 925
TA1535	2AA	2µg	151.3	50.1	6.3	137, 110, 207
TA1537	2AA	2µg	134.0	26.9	10.2	129, 110, 163
TA98	2AA	1µg	1943.7	569.0	72.0	2026, 1338, 2467
WP2 <i>uvrA</i> (pKM101)	BP	5µg	1121.7	82.9	7.4	1027, 1157, 1181

Key to Positive Controls

2AA 2-Aminoanthracene
BP Benzo[a]pyrene

Key to Plate Postfix Codes

A Lawn absent
S Sparse/Incomplete lawn

TABLE 2 - DATA FOR EXPERIMENTAL PHASE 1 (plate incorporation -S9)

Experiment: YV6638 Phase 1-

Date Plated: 13/01/2004
Date Counted: 16/01/2004

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	Y04185/002	5000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		2500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		1000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		200µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		100µg	0.0	0.0	0.0	0 A, 0 A, 0 A
	DMSO		61.0	4.7		56, 58, 67, 59, 65
TA1535	Y04185/002	5000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		2500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		1000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		200µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		100µg	0.0	0.0	0.0	0 A, 0 A, 0 A
	DMSO		8.4	2.9		10, 4, 10, 11, 7
TA1537	Y04185/002	5000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		2500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		1000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		200µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		100µg	0.0	0.0	0.0	0 A, 0 A, 0 A
	DMSO		4.4	1.5		4, 6, 2, 5, 5
TA98	Y04185/002	5000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		2500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		1000µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		500µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		200µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		100µg	0.0	0.0	0.0	0 A, 0 A, 0 A
	DMSO		13.6	4.5		11, 18, 16, 7, 16
WP2 uvrA (pKM101)	Y04185/002	5000µg	95.3	3.5	0.9	99, 95, 92
		2500µg	109.0	10.1	1.0	111, 98, 118
		1000µg	101.3	2.1	0.9	103, 99, 102
		500µg	121.3	18.5	1.1	131, 100, 133
		200µg	124.7	14.2	1.1	115, 118, 141
		100µg	96.7	7.2	0.9	93, 92, 105
	DMSO		111.8	31.3		82, 119, 141, 76, 141
TA100	NaZ	2µg	461.3	133.1	7.6	547, 308, 529
TA1535	NaZ	2µg	540.0	52.1	64.3	566, 480, 574
TA1537	ICR	2µg	124.7	58.0	28.3	164, 58, 152
TA98	DR	1µg	413.7	51.9	30.4	459, 425, 357
WP2 uvrA (pKM101)	ENNG	1µg	476.7	50.0	4.3	504, 419, 507

Key to Positive Controls

NaZ	Sodium Azide
ICR	Acridine Mutagen ICR191
DR	Daunomycin Hydrochloride
ENNG	N-Ethyl-N'-nitro-N-nitrosoguanidine

Key to Plate Postfix Codes

A	Lawn absent
---	-------------

TABLE 3 - DATA FOR EXPERIMENTAL PHASE 2 (plate incorporation +S9)

Experiment: YV6638: Phase 2

Date Plated: 26/01/2004
Date Counted: 29/01/2004

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	Y04185/002	200µg	0.7	1.2	0.0	0 A, 0 A, 2 A
		100µg	109.0	10.0	0.7	109, 99, 119
		50µg	126.7	18.6	0.8	142, 106, 132
		20µg	122.0	13.9	0.8	131, 129, 106
		10µg	124.7	22.7	0.8	133, 99, 142
		5.0µg	130.3	18.7	0.8	138, 144, 109
		DMSO	156.4	10.7		159, 152, 149, 174, 148
TA1535	Y04185/002	200µg	0.3	0.6	0.0	0 A, 1 A, 0 A
		100µg	7.0	3.0	0.8	7, 4, 10
		50µg	13.0	4.4	1.5	18, 11, 10
		20µg	12.3	3.1	1.4	9, 15, 13
		10µg	12.0	4.6	1.4	7, 13, 16
		5.0µg	11.0	4.6	1.3	7, 16, 10
		DMSO	8.8	2.5		5, 12, 9, 9, 9
TA1537	Y04185/002	200µg	12.3	8.5	0.6	22 A, 6 A, 9 A
		100µg	16.7	1.5	0.8	17, 18, 15
		50µg	18.7	4.6	0.8	16, 24, 16
		20µg	20.7	4.6	0.9	18, 26, 18
		10µg	23.3	3.5	1.1	27, 20, 23
		5.0µg	24.0	5.3	1.1	28, 18, 26
		DMSO	22.0	1.4		24, 21, 23, 21, 21
TA98	Y04185/002	200µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		100µg	13.0	0.0	0.3	13, 13, 13
		50µg	14.7	2.5	0.4	12, 15, 17
		20µg	22.3	4.5	0.6	18, 27, 22
		10µg	31.0	7.9	0.8	34, 37, 22
		5.0µg	32.0	3.0	0.8	35, 32, 29
		DMSO	38.2	8.1		49, 32, 43, 38, 29
TA100	2AA	1µg	882.3	129.7	5.6	736, 983, 928
TA1535	2AA	2µg	411.7	74.9	46.8	353, 496, 386
TA1537	2AA	2µg	336.7	87.4	15.3	307, 435, 268
TA98	2AA	1µg	1640.0	379.0	42.9	1337, 2065, 1518

Key to Positive Controls

2AA 2-Aminoanthracene

Key to Plate Postfix Codes

A Lawn absent

TABLE 4 - DATA FOR EXPERIMENTAL PHASE 2 (plate incorporation -S9)

Experiment: YV6638: Phase 2

Date Plated: 26/01/2004

Date Counted: 29/01/2004

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	Y04185/002	100µg	81.7	13.3	0.6	97 S, 75 S, 73 S
		50µg	102.3	13.3	0.7	109, 87, 111
		20µg	97.0	3.6	0.7	100, 93, 98
		10µg	123.0	16.6	0.9	130, 104, 135
		5.0µg	143.7	15.2	1.0	141, 160, 130
		2.0µg	138.7	17.8	1.0	131, 126, 159
		DMSO	139.2	11.9		126, 148, 151, 144, 127
TA1535	Y04185/002	100µg	1.3	0.6	0.2	1 S, 1 S, 2 S
		50µg	6.0	0.0	0.8	6, 6, 6
		20µg	11.7	5.8	1.5	5, 15, 15
		10µg	11.0	2.0	1.4	13, 9, 11
		5.0µg	9.7	2.5	1.2	12, 10, 7
		2.0µg	12.7	0.6	1.6	13, 12, 13
		DMSO	8.0	2.9		5, 10, 12, 7, 6
TA1537	Y04185/002	100µg	18.3	3.5	1.2	18 S, 15 S, 22 S
		50µg	16.3	1.5	1.0	15, 18, 16
		20µg	16.3	5.1	1.0	22, 15, 12
		10µg	17.3	7.2	1.1	22, 9, 21
		5.0µg	22.3	1.2	1.4	23, 23, 21
		2.0µg	19.7	1.5	1.2	20, 18, 21
		DMSO	15.8	2.6		13, 13, 18, 18, 17
TA98	Y04185/002	100µg	4.3	3.8	0.2	6 S, 7 S, 0 S
		50µg	18.0	2.0	0.9	16, 18, 20
		20µg	26.3	2.1	1.3	28, 24, 27
		10µg	21.3	1.2	1.0	22, 20, 22
		5.0µg	23.7	2.5	1.1	26, 21, 24
		2.0µg	24.0	4.4	1.2	22, 21, 29
		DMSO	20.6	5.1		20, 23, 16, 16, 28
TA100	NaZ	2µg	765.3	102.0	5.5	847, 798, 651
TA1535	NaZ	2µg	672.3	78.6	84.0	760, 608, 649
TA1537	ICR	2µg	371.7	60.9	23.5	398, 415, 302
TA98	DR	1µg	616.7	67.9	29.9	665, 646, 539

Key to Positive Controls

NaZ Sodium Azide
 ICR Acridine Mutagen ICR191
 DR Daunomycin Hydrochloride

Key to Plate Postfix Codes

S Sparse/Incomplete lawn

TABLE 5 - DATA FOR EXPERIMENTAL PHASE 3 (pre-incubation +S9)

Experiment: YV6638 Phase 3+

Date Plated: 03/02/2004
Date Counted: 06/02/2004

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	Y04185/002	200µg	0.3	0.6	0.0	1 A, 0 A, 0 A
		100µg	63.3	76.0	0.4	149, 4 S, 37 S
		50µg	132.7	24.4	0.9	106, 154, 138
		20µg	156.3	34.8	1.1	196, 142, 131
		10µg	124.7	22.9	0.9	114, 109, 151
		5.0µg	139.3	3.2	1.0	143, 137, 138
		DMSO	142.6	10.8		132, 152, 152, 147, 130
TA1535	Y04185/002	200µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		100µg	1.3	2.3	0.1	4 S, 0 S, 0 S
		50µg	8.7	1.5	0.6	10, 9, 7
		20µg	9.7	4.6	0.7	7, 15, 7
		10µg	10.3	2.3	0.7	13, 9, 9
		5.0µg	8.7	2.5	0.6	9, 11, 6
		DMSO	14.0	6.7		21, 12, 21, 6, 10
TA1537	Y04185/002	200µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		100µg	1.3	2.3	0.1	4 S, 0 S, 0 S
		50µg	9.3	2.5	0.5	9, 12, 7
		20µg	12.3	3.5	0.6	16, 9, 12
		10µg	21.3	5.7	1.1	26, 15, 23
		5.0µg	21.7	2.5	1.1	22, 24, 19
		DMSO	19.4	5.5		18, 20, 18, 28, 13
TA98	Y04185/002	200µg	0.0	0.0	0.0	0 S, 0 S, 0 S
		100µg	1.7	0.6	0.0	2 S, 2 S, 1 S
		50µg	7.3	3.2	0.2	6, 5, 11
		20µg	17.7	3.8	0.5	16, 15, 22
		10µg	28.0	3.5	0.8	26, 32, 26
		5.0µg	36.0	7.5	1.0	37, 28, 43
		DMSO	34.5	6.0		43, C, 29, 32, 34
WP2 uvrA (pKM101)	Y04185/002	2500µg	31.7	45.7	0.1	0 S, 11 S, 84 S
		1000µg	223.0	64.5	0.8	294, 168, 207
		500µg	331.7	37.3	1.2	343, 290, 362
		200µg	413.3	36.1	1.5	393, 392, 455
		100µg	410.0	33.2	1.5	395, 387, 448
		50µg	388.0	81.7	1.4	321, 364, 479
		DMSO	272.4	17.3		250, 282, 272, 263, 295
TA100	2AA	1µg	2136.0	996.3	15.0	3276, 1432, 1700
TA1535	2AA	2µg	287.3	13.6	20.5	273, 300, 289
TA1537	2AA	2µg	318.3	22.3	16.4	307, 344, 304
TA98	2AA	1µg	2765.7	337.2	80.2	2573, 2569, 3155
WP2 uvrA (pKM101)	BP	5µg	725.7	61.6	2.7	700, 796, 681

Key to Positive Controls

2AA 2-Aminoanthracene
BP Benzo[a]pyrene

Key to Plate Postfix Codes

A Lawn absent
S Sparse/Incomplete lawn
C Contaminated

TABLE 6 - DATA FOR EXPERIMENTAL PHASE 3 (plate incorporation -S9)

Experiment: YV6638 Phase 3-

Date Plated: 30/01/2004

Date Counted: 02/02/2004

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	Y04185/002	200µg	1.7	0.6	0.0	2 A, 2 A, 1 A
		100µg	83.3	9.3	0.7	91, 86, 73
		50µg	76.3	1.5	0.7	78, 75, 76
		20µg	98.3	13.3	0.9	106, 106, 83
		10µg	86.3	7.6	0.7	83, 81, 95
		5.0µg	105.3	5.0	0.9	100, 110, 106
	DMSO		115.4	15.2		109, 140, 99, 116, 113
TA1535	Y04185/002	200µg	0.0	0.0	0.0	0 A, 0 A, 0 A
		100µg	4.3	2.1	0.4	5, 2, 6
		50µg	3.0	1.7	0.3	5, 2, 2
		20µg	9.3	3.5	0.9	13, 6, 9
		10µg	8.0	5.6	0.8	9, 13, 2
		5.0µg	13.7	3.2	1.3	10, 15, 16
	DMSO		10.2	3.3		11, 15, 10, 6, 9
TA1537	Y04185/002	200µg	8.0	1.7	0.6	9 S, 9 S, 6 S
		100µg	16.0	8.7	1.1	21, 21, 6
		50µg	13.7	4.2	1.0	17, 15, 9
		20µg	15.0	4.4	1.1	12, 20, 13
		10µg	15.7	4.0	1.1	11, 18, 18
		5.0µg	10.7	6.0	0.8	5, 10, 17
	DMSO		14.0	4.7		15, 20, 13, 15, 7
TA98	Y04185/002	200µg	0.0	0.0	0.0	0 S, 0 S, 0 S
		100µg	15.0	5.3	0.5	11, 13, 21
		50µg	20.0	3.6	0.7	21, 16, 23
		20µg	23.3	7.6	0.8	18, 20, 32
		10µg	19.7	2.5	0.7	22, 17, 20
		5.0µg	32.3	9.3	1.1	22, 35, 40
	DMSO		29.0	4.3		24, 27, 34, 33, 27
WP2 uvrA (pKM101)	Y04185/002	5000µg	301.0	51.2	1.5	268, 360, 275
		2500µg	262.7	0.6	1.3	262, 263, 263
		1000µg	294.3	23.0	1.5	293, 272, 318
		500µg	217.3	19.9	1.1	203, 209, 240
		200µg	235.7	20.0	1.2	237, 255, 215
		100µg	212.3	10.1	1.1	207, 224, 206
	DMSO		200.6	19.3		204, 200, 197, 228, 174
TA100	NaZ	2µg	1004.0	108.3	8.7	1107, 1014, 891
TA1535	NaZ	2µg	707.3	63.0	69.3	770, 708, 644
TA1537	ICR	2µg	382.7	80.8	27.3	402, 452, 294
TA98	DR	1µg	997.3	62.6	34.4	1018, 927, 1047
WP2 uvrA (pKM101)	ENNG	1µg	884.7	221.5	4.4	644, 930, 1080

Key to Positive Controls

NaZ	Sodium Azide
ICR	Acridine Mutagen ICR191
DR	Daunomycin Hydrochloride
ENNG	N-Ethyl-N'-nitro-N-nitrosoguanidine

Key to Plate Postfix Codes

A	Lawn absent
S	Sparse/Incomplete lawn

APPENDIX 1 - HISTORICAL CONTROL DATA

	+S9 Plate Incorporation		+S9 Pre-Incubation		-S9	
TA 100	Solvent Controls	1.0µg 2AA	Solvent Controls	1.0µg 2AA	Solvent Controls	2.0µg NaZ
Mean ± SD	130 ± 38	1222 ± 647	132 ± 35	1563 ± 850	123 ± 36	914 ± 372
N	649	255	609	244	1228	488
Minimum	41	365	60	348	55	310
Maximum	245	3720	228	4430	232	2632
TA 1535	Solvent Controls	2.0µg 2AA	Solvent Controls	2.0µg 2AA	Solvent Controls	2.0µg NaZ
Mean ± SD	13 ± 7	211 ± 74	13 ± 6	233 ± 80	12 ± 7	552 ± 193
N	695	281	619	251	1259	505
Minimum	3	69	3	83	2	35
Maximum	46	402	37	482	45	1323
WP2 <i>uvrA</i> (pKM101)	Solvent Controls	5.0µg BP	Solvent Controls	5.0µg BP	Solvent Controls	1.0µg ENNG
Mean ± SD	180 ± 31	944 ± 333	190 ± 32	856 ± 172	158 ± 35	1130 ± 544
N	654	158	610	142	1225	493
Minimum	101	75	107	332	50	249
Maximum	287	3041	297	1261	282	2924
TA 98	Solvent Controls	1.0µg 2AA	Solvent Controls	1.0µg 2AA	Solvent Controls	1.0µg DR
Mean ± SD	29 ± 9	1553 ± 689	31 ± 10	2505 ± 1125	23 ± 8	960 ± 625
N	667	275	613	247	1225	493
Minimum	8	153	5	568	4	50
Maximum	65	4019	71	4965	61	2716
TA 1537	Solvent Controls	2.0µg 2AA	Solvent Controls	2.0µg 2AA	Solvent Controls	2.0µg ICR
Mean ± SD	12 ± 8	233 ± 155	14 ± 8	386 ± 381	11 ± 7	121 ± 117
N	675	275	640	256	1249	505
Minimum	1	26	1	33	1	13
Maximum	34	692	39	4517	43	631

MZ 276 199

**CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK**

CTL/VV0306/REGULATORY/REPORT

**ALLYL ALCOHOL: L5178Y TK⁺/- MOUSE LYMPHOMA
MUTATION ASSAY**

**CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK**

**CTL/VV0306/REGULATORY/REPORT
ALLYL ALCOHOL: L5178Y TK^{+/-} MOUSE LYMPHOMA
MUTATION ASSAY**

STUDY DETAILS

Sponsor: Hercules Incorporated
Hercules Research Centre
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Wilmington
DE 19808-1599
USA

Sponsor Reference: CO3310
CTL Test Substance Reference Number: Y04185/002
CTL Study Number: VV0306
Document Number: CTL/VV0306/REG/REPT

AUTHOR

P Clay

DATE OF ISSUE

04 May 2004

STATEMENT OF DATA CONFIDENTIALITY CLAIM

THIS DOCUMENT CONTAINS INFORMATION CONFIDENTIAL AND TRADE SECRET TO HERCULES INCORPORATED.

It should not be disclosed in any form to an outside party, nor should information contained herein be used by a registration authority to support registration of this product or any other product without the written permission of Hercules Incorporated.

STATEMENT OF GLP COMPLIANCE AND AUTHENTICATION

I, the undersigned, declare that the objectives laid down in the protocol were achieved and that the data generated are valid. The report fully and accurately reflects the procedures used and the raw data generated in the above study.

The study (VV0306) was conducted in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom GLP Regulations 1999, Statutory Instrument No. 3106) except for the deviations listed below. These Principles are in accordance with the OECD Principles of Good Laboratory Practice, revised 1997 (ENV/MC/CHEM(98)17).

The OECD international standards are acceptable to the United States Environmental Protection Agency and Japan Ministry of International Trade and Industry and this study, therefore, satisfies the requirements of EPA 40 CFR Part 160 (FIFRA) Part 792 (TSCA) and JMITI Kikyoku No.85.

The following GLP deviations are considered not to affect the integrity of the study or the validity of the conclusions drawn:

- (i) the stability of the test substance has not been reported
- (ii) the stability and achieved concentration of the test substance in the vehicle used were not determined by analysis.

P Clay
Study Director



.....

04 May 2004
Date

This page
may be required
by some
regulatory authorities.

QUALITY ASSURANCE STATEMENT

In accordance with CTL policy and QA procedures for Good Laboratory Practice, this report has been audited and the conduct of this study has been inspected as follows:

Date	Audit/Inspection	Date of QA Report
05 Apr 2004	Draft report	07 Apr 2004
30 Apr 2004	Final report review	04 May 2004

In addition, inspections associated with this type of study were made as follows:

24 Nov 2003	Counting	28 Nov 2003
29 Jan 2004	Protocol	29 Jan 2004
10 Feb 2004	Dilutions	10 Feb 2004
10 Feb 2004	Dose administration	10 Feb 2004
12 Feb 2004	Dilutions	13 Feb 2004
24 Mar 2004	Dose preparation	24 Mar 2004
26 Mar 2004	Addition of TFT, plating	26 Mar 2004

Facilities and process based procedures associated with this type of study were inspected in accordance with QA Standard Operating Procedures.

So far as can be reasonably established, the methods described and the results given in the final report accurately reflect the raw data produced during the study, VV0306.

A R Tarry


.....

04 May 2004

(CTL Quality Assurance Unit)

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

Name	Title
P Clay	Study Director, CTL
B M Elliott	Study Reviewer
I Pate	Study Statistician

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This page is provided for the Regulatory Authority Reviewer's notes.

1. SUMMARY

1.1 Study design

To assess the potential of allyl alcohol to cause gene mutation or clastogenic effects in mammalian cells, L5178Y TK^{+/+} mouse lymphoma cells were treated *in vitro* with various concentrations of the test substance, both in the presence and absence of a rat liver derived auxiliary metabolic system (S9-mix). Large and small mutant colonies were scored for all cultures in each experiment. Mutant frequencies were assessed by cell growth in the presence of trifluorothymidine after a 48 hour expression time.

Allyl alcohol was tested both in the presence and absence of S9-mix in two independent experiments.

1.2 Results

Allyl alcohol was tested up to maximum concentrations of 30µg/ml and 581µg/ml in the presence and absence of S9-mix respectively, the latter concentration being approximately equivalent to 10mM, the limit concentration for this assay. Minimum survival levels, compared to the solvent control cultures, of 11% and 103% were observed in cultures treated with the maximum concentrations of allyl alcohol in the presence and absence of S9-mix respectively.

No significant increases in mutant frequency were observed in cultures treated with allyl alcohol in the absence of S9-mix in either of the independent experiments. In the presence of S9-mix, dose related, statistically significant increases in mutant frequency were observed in both experiments.

The positive controls induced appropriate increases in mutant frequency in all mutation experiments thus demonstrating the activity of the S9-mix and that the assay was performing satisfactorily in being capable of detecting known mutagens.

1.3 Conclusion

It is concluded that, under the conditions of this assay, allyl alcohol is mutagenic in L5178Y TK^{+/+} cells treated *in vitro* in the presence of S9-mix.

2. INTRODUCTION

2.1 Purpose

The purpose of this study was to evaluate allyl alcohol for its ability to induce forward mutation in L5178Y TK^{+/+} mouse lymphoma cells as monitored by cell growth in medium containing the anti-metabolite trifluorothymidine (TFT). The scoring of both large and small mutant colonies allows an evaluation for both gene mutation and clastogenic events in the L5178Y TK^{+/+} cells.

2.2 Regulatory guidelines

The study was conducted according to the following Regulatory Guidelines:

- a) OECD guideline reference 476 (1997), *In Vitro* Mammalian Cell Gene Mutation Tests.
- b) Annex V to Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances, published in the 26th Adaptation, Commission Directive 2000/32/EC of 19 May 2000, OJ L136 8.6.2000. B17: *In vitro* mammalian cell gene mutation test.
- c) Health Effects Guidelines, OPPTS 870.5300, In Vitro Mammalian Cell Gene Mutation Test.

2.3 Justification for test system selection

The L5178Y TK^{+/+} mouse lymphoma mutation assay is designed to detect chemically induced gene mutation and/or clastogenic effects in cells treated in culture by measuring forward mutation. The cells are an established cell line and are exposed to various concentrations of the test substance, grown for the expression time and plated into microwells in the presence and absence of TFT to estimate the number of mutant cells per viable cell (the mutant frequency). Since forward mutation can also be seen in solvent control cultures, the assay is based on the observation of an increased mutant frequency over and above that seen in the

solvent control cultures. The cytotoxicity of the test substance is assessed by relative total growth.

2.4 Study dates

The study was initiated on 17 December 2003. The experimental phase started on 7 January 2004 and was completed on 2 March 2004.

2.5 Data storage

An original report, the study protocol and all raw data pertaining to this study will be retained in the CTL Archives, Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK for a minimum of 5 years from the date of issue of the final report. At the end of this period the Sponsor will be contacted regarding the further retention, return to the Sponsor or destruction of the archived materials.

3. TEST AND CONTROL SUBSTANCES

3.1 Test substance

Name:	Allyl alcohol
Source:	Sigma-Aldrich
Product number:	05788
Colour:	Colourless
Physical state:	Liquid
Lot number:	407813/1
CTL test substance reference number:	Y04185/002
Purity:	99.9% w/w (specification $\geq 99.5\%$)
Storage conditions:	Flammables cabinet at ambient temperature in the dark

A Sigma-Aldrich Certificate of Analysis has been retained with the study data. The characterisation of the test substance is the responsibility of the Sponsor.

In all cases where the concentration of test substance is quoted, this concentration refers to the concentration of test substance not corrected for purity.

3.2 Control substances

3.2.1 Control substance and vehicle for the test substance

Name:	Dried dimethylsulphoxide (DMSO)
Source:	BDH, UK
CTL test substance reference number:	Y00876/011

3.2.2 Positive control substance (in the absence of S9-mix)

Name:	Ethylmethanesulphonate (EMS)
Source:	Sigma, Poole, Dorset, UK
CTL test substance reference number:	Y01958/008
Final concentration:	500µg/ml

3.2.3 Positive control substance (in the presence of S9-mix)

Name:	Benzo[<i>a</i>]pyrene (BP)
Source:	Sigma, Poole, Dorset, UK
CTL test substance reference number:	Y00111/045
Final concentration:	1µg/ml

3.2.4 Vehicle for positive control substances

Name:	Dried dimethylsulphoxide (DMSO)
Source:	BDH, UK
CTL test substance reference number:	Y00876/011

4. EXPERIMENTAL PROCEDURE

4.1 Dosing preparations

An individual stock of the test substance was prepared for each experiment in dried dimethylsulphoxide and dilutions were carried out as required in each case.

The positive control substances were prepared as solutions in dried dimethylsulphoxide.

All test and positive control substance dosing preparations were prepared as close to the time of culture treatment as possible and were dosed at 10µl/ml culture.

4.2 Analysis of dosing preparations

In view of the short-term nature of the studies of this type, no analyses of stability or achieved concentrations were carried out on the preparations of the test or positive control substances either prior to or after addition to the cell cultures.

4.3 Experimental design

Two series of exponentially growing suspension cultures of L5178Y cells were treated in duplicate with the solvent control, positive controls or a range of concentrations of Allyl Alcohol for 4 hours in the presence and absence of S9-mix. The cells were then cultured to allow any induced mutants to be expressed. During this expression time the growth rate was monitored and, where appropriate, the cells subcultured daily. At the end of the 48 hour expression time, samples were grown in both selective and non selective medium, and the results obtained used to determine the mutant frequency per viable cell.

4.4 Cell maintenance

A bank of L5178Y TK^{+/+} 3.7.2.c cells (ex Dr J Cole, MRC, Sussex) was stored in a liquid nitrogen freezer. The cell stocks have been shown to be free of mycoplasma.

Following removal from liquid nitrogen, the cultures were kept at 37°C under an atmosphere of 5% CO₂ in air in a gassing incubator, or in a hot room in roller bottles rotated on a roller apparatus.

4.5 Metabolic activation system

S9 was purchased from Moltox, USA and was prepared from male Sprague Dawley rats dosed once daily (by oral gavage) for 3 days with a combined phenobarbital (80mg/kg bodyweight) and β-naphthoflavone (100mg/kg bodyweight) corn oil preparation. The treated animals were sacrificed on the day following the third dose. A 25% w/v homogenate (the S9 fraction) was prepared according to the method given in Callander *et al* (1995).

The co-factor solution was prepared as a stock solution of 75mM NADP (disodium salt) and 1200mM glucose-6-phosphate (monosodium salt) in RPMI 1640 with a final pH adjusted to 7.5. Both the S9 fraction and the co-factors were added at 1% (200µl of each added to the 20ml cell culture).

4.6 Methodology

The tissue culture medium was prepared under sterile conditions by addition of donor horse serum (50ml), L-glutamine (5ml of 200mM) and penicillin/streptomycin (5ml of 5,000 IU penicillin/ml; 5,000µg streptomycin/ml) to RPMI-1640 culture medium (500ml, with Hepes). The volume of donor horse serum added was increased to 100ml whenever the cells were dispensed into microwells and reduced to 25ml for the treatment period.

4.6.1 Cell preparation

A fresh sample of cells was brought up from liquid nitrogen storage for each experiment. A minimum of 10^7 cells in exponential growth were required per treatment, therefore a bulk culture was prepared prior to each experiment and diluted with serum free medium to obtain a reduced serum content of 5% at treatment time. Each 20ml treatment culture (5×10^5 cells per ml) was taken from this culture.

4.6.2 Culture treatment

Just prior to treatment, thawed samples of S9 fraction and co-factors (S9-mix) were added to the appropriate cell cultures.

Aliquots of the test substance, solvent control or positive controls were administered to duplicate cultures as appropriate to the experimental design. The cultures were treated for 4 hours. During this period the treated cell cultures were rotated on a roller apparatus in a 37°C hot room. At the end of the treatment period the cultures were centrifuged at 250xg for 5 minutes, the supernatants removed and the cell pellet resuspended in 50ml of fresh culture medium.

The effect of allyl alcohol on the pH and osmolality of the treatment medium was investigated as changes in pH and increases in osmolality have been reported to result in artefactual responses in genotoxicity assays (Scott *et al*, 1991).

4.6.3 Survival

Survival was measured by relative total growth (RTG). RTG is a measure of growth of test cultures both during the two-day expression and cloning phases of the assay, relative to the vehicle control.

4.6.4 Expression time

The post-treatment cultures were returned to the roller apparatus in the 37°C hot room for a 48 hour expression period. To maintain exponential growth during the expression time, each culture was counted and, where appropriate, diluted daily to give approximately 2×10^5 cells per ml in 50ml, thereby ensuring approximately 10^7 cells at each subculture.

4.6.5 Mutation assay

After the 48 hour expression period, the mutation assay was performed. The cell density of each culture was determined and the cultures were then divided into two series of dilutions. The first was to form the cultures for assessment of mutants by TFT selection; the second was to assess the viability of the cultures (in the absence of TFT).

For the assessment of mutants, a sample of each of the post-expression cultures was diluted to give 50ml at 1×10^4 cells per ml. TFT was then added to the mutation cultures to give a final concentration of 4µg/ml. Each TFT treated culture was then dispensed at 200µl per well into 2 x 96 well microwell plates (2000 cells per well). These plates were then incubated (37°C, 5% CO₂, 98% relative humidity) to allow cell growth.

For the assessment of viability, a sample from each mutation culture (at 1×10^4 /ml) was diluted to give 50ml at 8 cells per ml. No TFT was added to these cultures. Each viability culture was then dispensed and incubated as for the mutation cultures.

5. DATA EVALUATION

Cell growth in individual microwell plates was assessed after 10-13 days using a dissecting microscope. The survival plates and viability plates were scored for the number of wells containing no cell growth (negative wells). The mutation plates were scored so that each well contained either a small colony (considered to be associated with clastogenic effects), a large colony (considered to be associated with gene mutation effects) or no colony.

5.1 Calculations

Calculations were based on $P(0)$, the proportion of wells in which a colony had not grown (Kraemer *et al*, 1980).

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

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

Relative total growth (RTG) is a measure of growth of test cultures both during the two-day expression and cloning phases of the assay, relative to the vehicle control. The relative suspension growth (RSG) of each test culture was multiplied by the relative cloning efficiency (RCE) of the test culture at the time of mutant selection:

$$\text{RTG} = \text{RSG} \times \text{RCE} / 100$$

Relative Suspension Growth (RSG) is given by: $\text{SG}_{(\text{test})} / \text{SG}_{(\text{control})} \times 100$

where SG (suspension growth) is calculated as follows:

$$\text{SG} = \frac{[\text{Day 1 cell count}]}{[2 \times 10^5*]} \times \frac{[\text{Day 2 cell count}]}{[2 \times 10^5*]}$$

* Or appropriate cell concentration if lower

The relative cloning efficiency (RCE) is given by: $\text{CE}_{(\text{test})} / \text{CE}_{(\text{control})} \times 100$

The highest concentration assayed was designed to reduce RTG to 10%-20% of the solvent control culture value unless limited by solubility, pH or osmolality effects or a limit concentration of 5 µl/ml, 5000 µg/ml or 10mM (whichever is the lowest). No mutation assay plates were quantitated if survival fell below 10%.

Mutant Frequency (M.F.)

The mutant frequency for each culture was then calculated

$$\text{M.F.} = \frac{\text{CE in selective medium (mutation)}}{\text{CE in non-selective medium (viability)}}$$

The mutant frequency calculations were based on the total mutant colony counts.

5.2 Criteria for an acceptable assay

Failure of any one experiment to satisfy any one of the acceptance criteria below would not in itself invalidate the data from that experiment provided all other criteria for an acceptable experiment were met. However, a further experiment would be carried out to ensure that the observed effect was reproducible.

5.2.1 Cell growth and maintenance

To demonstrate acceptable cell growth and maintenance throughout the course of an experiment, adequate post-expression cloning efficiencies should be achieved for the solvent control viability plates. These should generally be in the range of 50%-100% throughout the study.

5.2.2 Spontaneous control data

The spontaneous mutant frequency using TFT as the selective agent, both in the presence and absence of S9-mix, should be no less than 0.6×10^{-4} mutants per survivor (Clive *et al*, 1995).

5.2.3 Positive control data

Relevant positive controls are used in the absence and presence of S9-mix i.e., ethylmethanesulphonate and benzo[*a*]pyrene respectively. These must give appropriate positive responses.

5.3 Criteria for a positive response

A statistically significant dose-related increase in mutant frequency is required, but not only at concentrations eliciting high levels of toxicity. An associated absolute increase in mutant number above the solvent control values is a further requirement. Such a response must be reproducible in an independent experiment for the test substance to be described as positive in this assay.

5.4 Criteria for a negative response

A negative response is obtained when there is no reproducible statistically significant dose-related increase in mutant frequency.

When reproducible significant increases in mutant frequency are seen only at high levels of toxicity, or when such increases are not accompanied by an increase in absolute numbers of

mutants over solvent control values, consideration should be given to such factors as statistical significance of the difference between treated and control cultures, and dose response relationships in order to clarify the response. Failing this, results from an independent experiment should be obtained to attempt to clarify the result.

5.5 Criteria for scoring mutation plates

Each well of the mutation plates (those containing TFT) was scored as containing either a small colony, a large colony or no colony according to the following criteria:

Small Colony - a small colony was one whose average diameter was less than 25% of the diameter of the well and was usually around 15% of the diameter of the well. A small colony should also have shown a dense clonal morphology.

Large Colony - a large colony was one whose average diameter was greater than 25% of the diameter of the well. A large colony should also have shown less densely packed cells, especially around the edges of the colony.

Any well which contained more than one small colony was scored as a small colony. Any well which contained more than one large colony was scored as a large colony. Any well which contained a combination of large and small colonies was scored as a large colony.

An empty well was one which contained no cell growth.

5.6 Statistics

The data were considered by logit regression, using a complimentary log-log link function. The dependent variable was the number of empty wells. This procedure provided maximum likelihood estimates of log mutant frequencies. Variances were inflated by the between duplicate heterogeneity factor.

Intergroup comparisons of log mutant frequency comparing each treated group with the solvent control were performed within each experiment.

All tests were one-sided. Similar analyses were carried out separately for the positive controls.

6. RESULTS

6.1 Survival data

The maximum concentration tested in the absence of S9-mix was 581µg/ml. This concentration is approximately equivalent to 10mM and as such is the limit concentration for this assay. This concentration resulted in survival levels relative to the solvent control of 103% and 113% in the first and second experiments respectively. In the presence of S9-mix, the maximum concentrations evaluated for mutant frequency were 20µg/ml and 30µg/ml giving survival levels of 40% and 11% in the first and second experiments respectively.

Summaries of the data are presented in Tables 1-2, with the full data shown in Tables 3-6.

Treatment of the culture medium with concentrations of the test substance used in this study had no significant effect on osmolality or pH (Table 7).

6.2 Mutation data

No statistically or biologically significant increases in mutant frequency, compared to the solvent control cultures, were observed in cultures treated with allyl alcohol at any concentration tested in the absence of S9-mix. In the presence of S9-mix, dose related increases in mutant frequency were observed in both experiments. These increases achieved statistical significance at the higher concentrations in both experiments and were generally associated with increases in mutant numbers.

The positive controls, EMS and BP, induced appropriate increases in mutant frequency in all mutation experiments, demonstrating the activity of the S9-mix and that the assay was performing satisfactorily in being capable of detecting known mutagens.

7. DISCUSSION

The test substance, allyl alcohol, was assessed in an *in vitro* mammalian cell mutation assay in L5178Y TK^{+/+} cells to determine if it had the potential to cause gene mutation or clastogenic effects. The procedure and experimental design employed complied with the

recommendations of OECD guideline 476 (1997), EEC Annex V test methodology (2000) and Health Effects Guidelines (OPPTS 870.5300).

Allyl alcohol was tested over a range of concentrations, both in the presence and absence of S9-mix in two independent mutation experiments. In the presence of S9-mix, allyl alcohol was found to be biologically active in the test system, causing concentration related reductions in survival down to a value of 11%. In the absence of S9-mix, allyl alcohol was tested up to a 10mM limit concentration (581µg/ml), and no significant reductions in survival were observed.

No significant increases in mutant frequency were observed in cultures treated with allyl alcohol in the absence of S9-mix in either of the independent experiments. In the presence of S9-mix, dose related, statistically significant increases in mutant frequency were observed in both experiments.

The data obtained in this study therefore show that the test sample of allyl alcohol is mutagenic in L5178Y TK^{+/+} cells following *in vitro* treatment in the presence of S9-mix.

8. CONCLUSION

It is concluded that, under the conditions of this assay, allyl alcohol is mutagenic in L5178Y TK^{+/+} cells treated *in vitro* in the presence of S9-mix.

9. REFERENCES

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- Callander R D, Mackay J M, Clay P, Elcombe C R and Elliott B M (1995). Evaluation of phenobarbital/ β -naphthoflavone as an alternative S9-induction regime to Aroclor 1254 in the rat for use in *in vitro* genotoxicity assays, *Mutagenesis*, **10**, 517-522.
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TABLE 1 - SUMMARY OF DATA FOR EXPERIMENTAL PHASE 1

Without S9-mix

Concentration (µg/ml)	Mean % Day 0 Relative Survival	Mean Mutant Frequency (x 10 ⁻⁴)
ALLYL ALCOHOL		
581	103	1.3
400	147	0.9
200	136	0.9
100	140	1.0
50	154	1.0
25	122	0.9
SOLVENT CONTROL		
DMSO (10µl/ml)	100	1.0
POSITIVE CONTROL		
EMS 500	57	7.6**

With S9-mix

Concentration (µg/ml)	Mean % Day 0 Relative Survival	Mean Mutant Frequency (x 10 ⁻⁴)
ALLYL ALCOHOL		
50	0	b
40	3	b
30	1	b
20	40	2.9**
10	60	0.7
5	101	1.1
SOLVENT CONTROL		
DMSO (10µl/ml)	100	0.6
POSITIVE CONTROL		
BP 1	4	14.7**

Key to Statistical Significance: ** = P < 0.01

b = not counted due to excessive toxicity

TABLE 2 - SUMMARY OF DATA FOR EXPERIMENTAL PHASE 2

Without S9-mix

Concentration (µg/ml)	Mean % Day 0 Relative Survival	Mean Mutant Frequency (x 10 ⁻⁴)
ALLYL ALCOHOL		
581	113	1.8
400	74	1.7
200	132	1.1
100	134	1.2
50	120	1.4
SOLVENT CONTROL		
DMSO (10µl/ml)	100	1.3
POSITIVE CONTROL		
EMS 500	51	12.0**

With S9-mix

Concentration (µg/ml)	Mean % Day 0 Relative Survival	Mean Mutant Frequency (x 10 ⁻⁴)
ALLYL ALCOHOL		
40	9	b
30	11	6.9**
25	26	3.7**
20	25	3.2**
15	43	2.0*
10	66	1.2
5	59	1.8*
SOLVENT CONTROL		
DMSO (10µl/ml)	100	1.2
POSITIVE CONTROL		
BP 1	39	6.1**

Key to Statistical Significance: * = P < 0.05
 ** = P < 0.01

b = not counted due to excessive toxicity

ALLYL ALCOHOL: L5178Y TK^{+/+} MOUSE LYMPHOMA MUTATION ASSAY

TABLE 3 - TEST DATA FOR EXPERIMENTAL PHASE 1 (-S9)

Conc'h (µg/ml)	Suspension Growth				Mutation Assay						Viability Assay				Mutant frequency x10 ⁻⁴	
	Start cell concentration x10 ⁷ /ml	24 hour cell count x10 ⁷ /ml	Post 24 hour dilution cell count x10 ⁷ /ml	48 hour cell count x10 ⁷ /ml	Plate 1		Plate 2		Plate 1		Plate 2		Viability			
					large colonies +ve wells	large colonies +ve wells	large colonies +ve wells	large colonies +ve wells	small colonies +ve wells	small colonies +ve wells	small colonies +ve wells	small colonies +ve wells	-ve wells	-ve wells		wells plated
Test Substance																
581		6.2		8.3	9	9	15	4	4	4	4	4	22	22	153	1.0
581		5.6		8.0	9	7	7	3	3	3	3	3	51	56	53	1.7
400		6.1		8.7	16	14	14	2	4	4	4	4	12	23	183	1.0
400		6.2		8.5	5	9	9	1	3	3	3	3	31	37	111	0.8
200		6.5		8.4	9	6	6	5	2	2	2	2	24	35	130	0.8
200		6.0		8.4	13	13	13	1	3	3	3	3	23	25	141	1.0
100		6.1		8.2	15	9	9	2	5	5	5	5	24	21	147	1.0
100		5.7		8.5	15	13	13	3	2	2	2	2	26	23	134	1.1
50		5.8		7.2	14	16	16	2	3	3	3	3	18	13	154	0.9
50		5.8		8.5	21	13	13	1	4	4	4	4	19	22	154	1.2
25		6.0		7.6	12	17	17	3	3	3	3	3	29	22	122	1.2
25		5.4		7.3	4	10	10	3	3	3	3	3	26	16	121	0.6
Solvent Control																
Solvent		4.9		8.1	9	9	15	4	4	4	4	4	23	31	102	1.1
control					10	8	8	6	6	6	6	6				
Solvent		4.6		8.7	13	15	15	2	5	5	5	5	26	31	98	1.0
control					10	7	7	2	1	1	1	1				
Positive Control																
EMS 500		4.2		6.1	49	52	52	12	11	11	11	11	29	34	58	7.5
EMS 500		4.0		7.0	46	51	51	12	10	10	10	10	35	36	56	7.8

TABLE 4 - TEST DATA FOR EXPERIMENTAL PHASE 1 (+S9)

Conch (µg/ml)	Suspension Growth				Mutation Assay						Viability Assay				Mutant frequency x10 ⁻⁴	
	Start cell concentration x10 ⁷ /ml	24 hour cell count x10 ⁷ /ml	Post 24 hour dilution cell count x10 ⁷ /ml	48 hour cell count x10 ⁷ /ml	Plate 1 large colonies +ve wells	Plate 2 large colonies +ve wells	Plate 1 small colonies +ve wells	Plate 2 small colonies +ve wells	Mutation wells plated	Plate 1 -ve wells	Plate 2 -ve wells	Viability wells plated	% relative total growth			
					Test Substance											
50		1.5		1.5	b	b	b	b	b	b	b	b	96	95	0	b
50		1.5		1.6	b	b	b	b	b	b	b	b	96	96	0	b
40		1.3		1.4	b	b	b	b	b	b	b	b	96	95	0	b
40		1.8		2.7	b	b	b	b	b	b	b	b	81	81	6	b
30		1.2		1.4	b	b	b	b	b	b	b	b	83	86	2	b
30		1.3		1.3	b	b	b	b	b	b	b	b	93	92	1	b
20		2.0		4.5	6	7	12	12	12	12	12	12	49	46	40	2.4
20		2.5		5.1	2	7	13	14	14	14	14	14	57	59	40	3.3
10		2.8		6.9	3	2	1	0	0	0	0	0	66	64	47	0.7
10		3.5		5.1	4	4	2	0	0	0	0	0	52	49	72	0.7
5		4.0		6.5	4	8	2	5	5	5	5	5	49	52	105	1.3
5		3.8		5.9	3	7	2	2	2	2	2	2	43	53	98	0.9
					Solvent Control											
Solvent control		4.0		7.3	1	6	0	0	0	0	0	0	54	58	99	0.8
Solvent control		4.5		7.6	1	2	1	0	0	0	0	0	60	60	101	0.4
					Positive Control											
BP 1		1.6		2.8	4	3	13	12	12	12	12	12	85	85	4	12.0
BP 1		1.6		2.7	7	3	13	14	14	14	14	14	86	88	3	17.4

b = not counted due to excessive toxicity

ALLYL ALCOHOL: L5178Y TK⁺ MOUSE LYMPHOMA MUTATION ASSAY

TABLE 5 - TEST DATA FOR EXPERIMENTAL PHASE 2 (-S9)

Conc'n (µg/ml)	Suspension Growth				Mutation Assay						Viability Assay				Mutant frequency x10 ⁻⁴	
	Start cell concentration x10 ⁷ /ml	24 hour cell count x10 ⁷ /ml	Post 24 hour dilution cell count x10 ⁷ /ml	48 hour cell count x10 ⁷ /ml	Plate 1		Plate 2		Plate 1		Plate 2		Viability			% relative total growth
					large colonies +ve wells	large colonies +ve wells	small colonies +ve wells	small colonies +ve wells	large colonies +ve wells	large colonies +ve wells	small colonies +ve wells	small colonies +ve wells	-ve wells	-ve wells		
Test Substance																
581		3.4		3.0	48	51	9	6	1	1					155	1.6
581		2.7		3.9	31	25	10	9	8	18					70	2.0
400		2.7		3.3	31	24	10	9	1	9					88	1.3
400		2.4		4.5	24	25	12	7	6	30					60	2.1
200		2.6		3.7	20	30	12	12	4	4					102	1.2
200		3.9		4.6	23	19	7	7	6	7					161	1.0
100		3.8		3.3	23	18	13	11	8	4					116	1.2
100		3.8		3.9	19	25	11	14	5	4					151	1.2
50		4.1		3.8	21	24	9	10	5	10					133	1.3
50		4.1		4.2	21	20	7	7	7	23					107	1.5
Solvent Control																
Solvent control		3.5		4.3	24	25	7	11	15	19					87	1.4
Solvent control		4.4		4.3	11	14	12	5	18	14					113	1.1
Positive Control																
EMS 500		3.1		3.0	68	66	16	22	10	22					56	10.1
EMS 500		3.5		3.6	69	67	16	12	26	37					47	13.8

ALLYL ALCOHOL: L5178Y TK⁺/- MOUSE LYMPHOMA MUTATION ASSAY

TABLE 6 - TEST DATA FOR EXPERIMENTAL PHASE 2 (+S9)

Concn (µg/ml)	Suspension Growth				Mutation Assay						Viability Assay				Mutant Frequency x10 ⁻⁴		
	Start cell concentration x10 ⁵ /ml	24 hour cell count x10 ⁵ /ml	Post 24 hour dilution cell count x10 ⁵ /ml	48 hour cell count x10 ⁵ /ml	Plate 1 large colonies +ve wells	Plate 2 large colonies +ve wells	Plate 1 small colonies +ve wells	Plate 2 small colonies +ve wells	Mutation wells plated	Plate 1 -ve wells	Plate 2 -ve wells	Viability wells plated	% relative total growth				
Test Substance																	
40		1.5		2.8	b	b	b	b	b				39	41		8	b
40		1.6		2.6	b	b	b	b	b				27	32		10	b
30		1.9		3.9	26	21	24	26	26				27	42		12	5.5
30		1.8		3.4	18	29	41	26	26				39	41		9	8.2
25		2.5		4.9	12	15	33	31	31				24	33		23	4.2
25		2.5		4.7	17	15	27	28	28				17	23		29	3.1
20		3.1		4.8	21	23	22	16	16				30	25		29	3.6
20		3.4		4.1	10	9	19	19	19				31	41		21	2.9
15		3.7		5.1	20	15	14	11	11				18	31		40	2.2
15		3.5		5.0	14	16	18	13	13				18	18		46	1.8
10		4.2		5.9	8	20	10	7	7				27	7		67	1.2
10		4.0		5.3	15	15	13	7	7				13	15		64	1.3
5		4.1		5.5	15	19	4	10	10				13	22		60	1.4
5		4.2		5.2	28	24	10	10	10				16	19		58	2.2
Solvent Control																	
Solvent control		5.4		6.3	18	19	7	2	2				16	18		92	1.3
Solvent control		5.9		5.5	18	13	6	11	11				8	15		108	1.1
Positive Control																	
BP 1		3.7		4.5	35	30	27	36	36				24	21		38	6.1
BP 1		3.7		5.1	34	27	32	29	29				23	29		39	6.2

b = not counted due to excessive toxicity

TABLE 7 - OSMOLALITY AND pH DATA

Treatment		pH	Osmolality (mmol/kg)
Solvent Control	10µl/ml	7.22	440
Allyl alcohol			
	25µg/ml	7.21	435
	50µg/ml	7.18	425
	100µg/ml	7.19	428
	200µg/ml	7.19	420
	400µg/ml	7.20	413
	581µg/ml	7.21	417

TABLE 8 - HISTORICAL CONTROL DATA

	-S9-mix		+S9-mix	
	Vehicle control	Positive control	Vehicle control	Positive control
Mean [standard deviation] (mutant frequency x10 ⁻⁴)	1.4 [0.5]	11.2 [8.4]	1.4 [0.6]	12.6 [11.3]
Range (mutant frequency x10 ⁻⁴)	0.7-3.3	3.8-69.2	0.5-3.9	2.8-65.1
N	73	73	64	64

N = number of observations

MR 276199

**CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK**

CTL/SV1223/REGULATORY/REPORT

**ALLYL ALCOHOL: *IN VITRO* CYTOGENETIC ASSAY
IN HUMAN LYMPHOCYTES**

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**CTL/SV1223/REGULATORY/REPORT
ALLYL ALCOHOL: *IN VITRO* CYTOGENETIC ASSAY
IN HUMAN LYMPHOCYTES**

STUDY DETAILS

Sponsor: Hercules Incorporated
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Wilmington
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USA

Sponsor Reference: CO3310
CTL Test Substance Reference Number: Y04185/002
CTL Study Number: SV1223
Document Number: CTL/SV1223/REG/REPT

AUTHOR

V Fox

DATE OF ISSUE

10 May 2004

STATEMENT OF DATA CONFIDENTIALITY CLAIM

THIS DOCUMENT CONTAINS INFORMATION CONFIDENTIAL AND TRADE SECRET TO HERCULES INCORPORATED.

It should not be disclosed in any form to an outside party, nor should information contained herein be used by a registration authority to support registration of this product or any other product without the written permission of Hercules Incorporated.

STATEMENT OF GLP COMPLIANCE AND AUTHENTICATION

I, the undersigned, declare that the objectives laid down in the protocol were achieved and that the data generated are valid. The report fully and accurately reflects the procedures used and the raw data generated in the above study.

The study (SV1223) was conducted in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom GLP Regulations 1999, Statutory Instrument No. 3106) except for the deviations listed below. These Principles are in accordance with the OECD Principles of Good Laboratory Practice, revised 1997 (ENV/MC/CHEM(98)17).

The OECD international standards are acceptable to the United States Environmental Protection Agency and Japan Ministry of International Trade and Industry and this study, therefore, satisfies the requirements of EPA 40 CFR Part 160 (FIFRA) Part 792 (TSCA) and JMITI Kikyoku No.85.

The following GLP deviations are considered not to affect the integrity of the study or the validity of the conclusions drawn:

- (i) the stability of the test substance has not been reported
- (ii) the stability and achieved concentration of the test substance in the vehicle used were not determined by analysis.

V Fox
Study Director


.....

10 May 2004
Date

This page may be
Required by some
Regulatory Authorities

QUALITY ASSURANCE STATEMENT

In accordance with CTL policy and QA procedures for Good Laboratory Practice, this report has been audited and the conduct of this study has been inspected as follows:

Date	Audit/Inspection	Date of QA Report
21 Jan 2004	Slide coding	22 Jan 2004
22 Jan 2004	Slide scoring	22 Jan 2004
31 Mar 2004	Draft report	01 Apr 2004
07 May 2004	Final report review	07 May 2004

In addition, inspections associated with this type of study were made as follows:

15 Jan 2004	Protocol	15 Jan 2004
30 Jan 2004	Transfer to centrifuge tubes	30 Jan 2004
12 Feb 2004	Dilutions	13 Feb 2004
04 Mar 2004	Dose administration	04 Mar 2004
05 Mar 2004	Slide preparation	09 Mar 2004
18 Mar 2004	Dose preparation	18 Mar 2004
18 Mar 2004	Addition of S9	18 Mar 2004

Facilities and process based procedures associated with this type of study were inspected in accordance with QA Standard Operating Procedures.

So far as can be reasonably established, the methods described and the results given in the final report accurately reflect the raw data produced during the study, SV1223.

V L Wright

(CTL Quality Assurance Unit)

.....

10 May 2004

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

Name	Title
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1. SUMMARY

1.1 Study design

Allyl alcohol was evaluated for its clastogenic potential in an *in vitro* cytogenetic assay using human lymphocytes in two separate experiments treated in the presence and absence of a rat liver-derived metabolic activation system (S9-mix). In both experiments cultures were treated for a period of 3 hours both in the presence and absence of S9-mix. All cultures were harvested 68 hours after culture initiation.

Cultures treated with allyl alcohol at the following concentrations were selected for chromosomal aberration analysis along with the appropriate solvent and positive control cultures.

Experiment 1		Experiment 2	
+S9-mix	-S9-mix	+S9-mix	-S9-mix
3 hour treatment	3 hour treatment	3 hour treatment	3 hour treatment
581µg/ml	581µg/ml	200µg/ml	581µg/ml
400µg/ml	400µg/ml	100µg/ml	400µg/ml
100g/ml	100g/ml	25µg/ml	100µg/ml

1.2 Results

The highest concentrations selected for chromosomal aberration analysis were the limit concentration for the assay or limited by cytotoxic effects on the chromosomes.

Statistically and biologically significant increases in the percentage of aberrant cells, compared to the solvent control values, were recorded in cultures from Experiment 1 in the presence of S9-mix and in cultures from Experiment 2 in the presence and absence of S9-mix.

The sensitivity of the test system, and the metabolic activity of the S9-mix employed, were clearly demonstrated by the increases in the percentage of aberrant cells induced by the positive control agents, mitomycin C and cyclophosphamide.

1.3 Conclusion

It is concluded that, under the conditions of this assay, allyl alcohol is clastogenic to cultured human lymphocytes treated *in vitro* in the presence and absence of S9-mix.

2. INTRODUCTION

2.1 Purpose

The purpose of this study was to investigate whether the test substance induced chromosomal damage *in vitro* in the human peripheral blood lymphocyte cytogenetic assay.

2.2 Regulatory guidelines

The study was conducted according to the following Regulatory Guidelines.

- a) OECD guideline reference 473 (1997). *In Vitro* Mammalian Chromosome Aberration Test.
- b) Annex V to Council Directive 67/548/EEC on the approximation of law, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances, published in the 26th Adaptation, Commission Directive 2000/32/EC of 19 May 2000, OJ L136 8.6.2000. B10: In Vitro Mammalian Chromosomal Aberration Test.
- c) ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Adopted at Step 4 of the ICH process 19 July 1995.
- d) ICH Harmonised Tripartite Guideline S2B. Genotoxicity: A Standard Battery for Genotoxicity Testing for Pharmaceuticals. Adopted at Step 4 of the ICH process 16 July 1997.

2.3 Justification for test system selection

The *in vitro* human lymphocyte cytogenetic assay is designed to detect chemically induced changes in metaphase chromosomes of cells treated in culture. The human peripheral lymphocyte used is a sensitive target cell for the induction of *in vitro* chromosomal damage when stimulated to provide large numbers of rapidly dividing cells in culture (Scott *et al*, 1990). When the cells are in exponential growth they are exposed to various concentrations of the test substance. The cells are harvested, metaphase spreads are prepared and analysed for the presence or absence of chromosomal damage. Since chromosomal damage can also

be seen in solvent control cultures, the assay is based on the observation of an increased incidence of chromosomal damage over and above that seen in the solvent control cultures.

2.4 Study dates

The study was initiated on 17 December 2003. The experimental phase started on 6 January 2004 and was completed on 16 March 2004.

2.5 Data storage

An original report, the study protocol and all raw data, samples and specimens pertaining to this study are retained in the CTL Archives, Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK for a minimum of 5 years from the date of issue of the final report. At the end of this period the Sponsor will be contacted regarding the further retention, return to the Sponsor or destruction of the archived materials.

3. TEST AND CONTROL SUBSTANCES

3.1 Test substance

Name:	Allyl alcohol
Source:	Sigma-Aldrich
Product number:	05788
Colour:	Colourless
Physical state:	Liquid
Lot number:	407813/1
CTL test substance reference number:	Y04185/002
Purity:	99.9% w/w (specification $\geq 99.5\%$)
Storage conditions:	Flammables cabinet at ambient temperature in the dark

A Sigma-Aldrich Certificate of Analysis has been retained with the study data. The characterisation of the test substance is the responsibility of the Sponsor.

In all cases where the concentration of test substance is quoted, this concentration refers to the concentration of test substance not corrected for purity.

3.2 Control substances

The control substance and solvent for the test sample was dried dimethylsulphoxide (CTL test substance reference number Y00876/011).

The positive control substances, mitomycin C (CTL test substance reference number Y02201/004) and cyclophosphamide (CTL test substance reference number Y01259/042) were obtained from Sigma Chemical Company.

4. EXPERIMENTAL PROCEDURES

4.1 Dosing preparations

An individual stock solution of the test substance was prepared for each experiment in dried dimethylsulphoxide and serial dilutions were carried out as required in each case.

Both positive control substances were prepared as solutions in sterile double deionised water (CTL test substance reference number Y04517/035).

All test and positive control substance dosing preparations were prepared as close to the time of culture treatment as possible and were dosed at 100µl/10ml culture.

4.2 Analysis of dosing preparations

In view of the short term nature of studies of this type, no analyses of stability, homogeneity or achieved concentration were carried out on the preparations of the test or positive control substances either prior to or after addition to the human peripheral blood lymphocyte cultures.

4.3 Experimental design

Duplicate human peripheral blood cultures were exposed to the solvent, test substance or positive control substances at appropriate concentrations in the following experiments:

- a) A cytogenetic experiment was conducted using a sample of pooled blood. Cells were exposed to the test substance and control substances for a period of 3 hours, both in the

presence and absence of S9-mix. Solvent, untreated and positive control cultures were included.

- b) A second independent cytogenetic experiment was conducted using a sample of pooled blood. Cells were exposed to the test substance and control substances for a period of 3 hours in the presence and absence of S9-mix. Solvent, untreated and positive control cultures were included.

Treatment of the cultures started approximately 48 hours after culture initiation. A single sampling time, 20 hours after the start of treatment (68 hours after culture initiation), was used. The OECD guideline for this assay recommend a period equivalent to about 1.5 cell cycles between the start of treatment and sampling. The sampling time of 20 hours after the start of treatment, used in this study, was based on a measured mean cell cycle time for cultured human peripheral blood lymphocytes of 13.5 hours in this Laboratory (September 1992 and November 1993; CTL/T/2856).

In both experiments a range of concentrations of allyl alcohol was used in order to define suitable concentrations for chromosomal aberrations analysis.

4.4 Culture establishment

Human blood samples were obtained by venepuncture in lithium heparin tubes on the days of culture initiation from healthy, non-smoking donors. Equal volumes of blood from 2 female and 2 male donors were pooled together for Experiment 1 and Experiment 2 respectively. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.

At 0 hours, cultures (10ml) were established by the addition of 0.5ml of whole blood to RPMI-1640 (Dutch modification) tissue culture medium supplemented with approximately 10% foetal bovine serum (FBS), 1.0IU/ml heparin, L-glutamine (2mM), 100IU/ml penicillin and 100µg/ml streptomycin. The lymphocytes were stimulated to enter cell division by addition of phytohaemagglutinin (PHA; at 5% v/v) and the cultures were maintained at approximately 37°C for 48 hours with gentle daily mixing where possible.

4.5 Metabolic activation system

The metabolic activation system (S9-mix) used in this study was prepared as required (on each day of culture treatment) as a 1:1 mixture of S9 fraction and cofactor solution.

S9 was purchased from MolTox Inc., and was prepared from male Sprague Dawley rats, dosed once daily (by oral gavage) for 3 days with a combined phenobarbital (80mg/kg bodyweight) and β -naphthoflavone (100mg/kg bodyweight) corn oil preparation. The treated animals were sacrificed on the day following the third dose. A 25% w/v homogenate (the S9 fraction) was prepared according to the method given in Callander *et al* (1995).

The cofactor solution was prepared as a single stock solution of Na_2HPO_4 , KCl, glucose-6-phosphate, NADP (Na salt) and MgCl_2 (150 : 49.5 : 7.5 : 6 : 12mM) in sterile double deionised water and adjusted to a final pH of 7.4.

4.6 Culture treatment

Approximately 48 hours after culture establishment, aliquots of the test substance, solvent control or positive controls were administered to duplicate cultures as appropriate to the experiment design. In addition, 200 μ l of a 1:1 mix of S9 and co-factor solution was added to each culture to be treated in the presence of S9-mix. Cultures from both experiments were treated for a period of 3 hours at 37°C, after which the culture medium was removed following centrifugation and replaced with fresh supplemented RPMI-1640 culture medium. The cultures were re-incubated at 37°C for the remainder of the 68 hour growth period.

The effect of allyl alcohol on the pH and osmolality of the culture medium was investigated, using single cultures containing medium only, as changes in pH and increases in osmolality have been reported to result in the production of chromosomal aberrations (Scott *et al*, 1991). The solubility of the test substance in the treated blood cultures and in media only cultures was assessed immediately after treatment and at the end of the treatment period.

4.7 Culture harvesting

Approximately 2 hours prior to harvesting, the cultures were treated with colcemid at a final concentration of 0.4 μ g/ml. Sixty-eight hours after culture establishment, the cultures were centrifuged, the supernatant was removed and the cells were re-suspended in approximately 10ml of 0.075M KCl at room temperature for approximately 10 minutes. The cultures were

centrifuged, the supernatant was removed and the remaining cells were fixed in freshly prepared methanol/glacial acetic acid fixative (3:1 v/v) added dropwise and made up to a volume of approximately 10ml. The fixative was removed following centrifugation and replaced with freshly prepared fixative. After at least two subsequent changes of fixative, slides were made by dropping the cell suspension on to clean, moist labelled microscope slides. The slides were air dried, stained in filtered Giemsa stain (10% Gurr's R66 in buffered [pH 6.8] double deionised water) for 7 minutes, rinsed in water, air-dried and mounted with coverslips in DPX.

4.8 Slide analysis

Slides were examined to determine that they were of suitable quality and, where appropriate, the mitotic index was determined by examining 1000 lymphocytes per culture and calculating the percentage of cells in metaphase.

For each experiment, both in the presence and absence of S9-mix, duplicate cultures treated with allyl alcohol at three concentrations were selected for chromosomal aberration analysis along with the appropriate solvent and positive control cultures.

The slides were coded prior to analysis and one hundred cells in metaphase, where possible, were analysed from each selected culture for the incidence of structural chromosomal damage, according to the principles of the criteria recommended by Scott *et al* (1990).

5. DATA EVALUATION

The percentages of aberrant metaphases and the number of aberrations per cell were calculated for each treatment scored, both including and excluding cells with only gap-type aberrations.

The Fisher Exact Probability Test (one-sided) was used to evaluate statistically the percentage of metaphases showing aberrations (excluding cells with only gap-type aberrations). Data from each treatment group, in the presence and absence of S9-mix, was compared with the respective solvent control group value. The data have been interpreted as follows:

- a) No statistically significant increase in the percentage of aberrant cells (at any concentration) above concurrent solvent control values - **NEGATIVE**.
- b) A statistically significant increase in the percentage of aberrant cells above concurrent solvent control values, which falls within the laboratory solvent control range - **NEGATIVE**.
- c) An increase in the percentage of aberrant cells, at least at one concentration, which is substantially greater than the laboratory historical solvent control values - **POSITIVE**.
- d) A statistically significant increase in the percentage of aberrant cells which is above concurrent solvent values and which is above the historical solvent control range upper value but below that described in (c) may require further evaluation.

6. RESULTS

6.1 Determination of mitotic indices and selection of concentrations

Two independent cytogenetic experiments were carried out using ranges of allyl alcohol concentrations as detailed in Tables 1 and 2 which also detail the individual culture and mean mitotic indices for the solvent controls and the allyl alcohol treated cultures.

The highest concentrations selected for chromosomal aberration analysis were the limit concentration for the assay (581 µg/ml; 10mM) or limited by cytotoxic effects on the chromosomes (200 µg/ml). Concentrations above this were not suitable for analysis due to excessive cytotoxic effects on the chromosomes.

Reductions in mean mitotic activity, compared to the solvent control values, were observed in cultures from Experiment 1 (37% +S9-mix) and Experiment 2 (23% +S9-mix; 16% -S9-mix) treated with the highest concentrations of allyl alcohol selected for chromosomal aberration analysis (Tables 1 and 2). No reduction in mitotic activity was observed in Experiment 1 in the absence of S9-mix.

Treatment of the culture medium with allyl alcohol up to 581 µg/ml (10mM) had no significant effect on osmolality or pH (Table 5).

6.2 Results of chromosomal aberration analyses

From the results detailed in Section 6.1, cultures treated with following concentrations of allyl alcohol were selected for chromosomal aberration analysis.

Experiment 1		Experiment 2	
+S9-mix 3 hour treatment	-S9-mix 3 hour treatment	+S9-mix 3 hour treatment	-S9-mix 3 hour treatment
581 µg/ml	581 µg/ml	200 µg/ml	581 µg/ml
400 µg/ml	400 µg/ml	100 µg/ml	400 µg/ml
100 µg/ml	100 µg/ml	25 µg/ml	100 µg/ml

The results of the chromosomal aberration analysis are shown in Tables 3 and 4 expressed as the mean percentage of aberrant cells (excluding cells with only gap-type aberrations) at each concentration. Individual data from each culture are given in Tables 6 and 7.

Statistically and biologically significant increases in the percentage of aberrant cells, compared to the solvent control values, were recorded in cultures from Experiment 1 in the presence of S9-mix and treated in cultures from Experiment 2 in the presence and absence of S9-mix.

The positive control materials, mitomycin C and cyclophosphamide induced statistically and biologically significant increases in the percentage of aberrant cells, compared to the solvent control cultures.

7. DISCUSSION

The test substance, allyl alcohol, was assessed in an *in vitro* cytogenetic assay in cultured human peripheral blood lymphocytes to determine if it had any *in vitro* clastogenic activity. The procedures and experimental design employed complied with the recommendations of

the OECD Guideline 473 (1997), EEC Annex V B10 (2000), ICH (1995 and 1997) and the UKEMS Recommended Procedures for Basic Mutagenicity Tests (Scott, 1990).

Allyl alcohol was tested over a range of concentrations, both in the presence and absence of S9-mix, in two independent cytogenetic tests.

Statistically and biologically significant increases in the percentage of aberrant cells, compared to the solvent control values, were recorded in cultures from Experiment 1 in the presence of S9-mix and treated in cultures from Experiment 2 in the presence and absence of S9-mix.

The sensitivity of the test system, and the metabolic activity of the S9-mix employed, were clearly demonstrated by the increases in the frequencies of aberrant cells induced by the positive control agents, mitomycin C and cyclophosphamide.

The data obtained in this study therefore show that the test sample of allyl alcohol induced chromosomal damage in human peripheral blood lymphocytes following *in vitro* treatment in the presence of S9-mix following a 3 hour treatment period and in the absence of S9-mix when treated for a 20 hour treatment period.

8. CONCLUSION

It is concluded that, under the conditions of this assay, allyl alcohol is clastogenic to cultured human lymphocytes treated *in vitro* in the presence and absence of S9-mix.

9. REFERENCES

Callander R D, Mackay J M, Clay P, Elcombe C R and Elliott B M (1995). "Evaluation of phenobarbital/ β -naphthoflavone as an alternative S9- induction regime to AROCLOR 1254 in the rat for use in *in vitro* genotoxicity assays". *Mutagenesis* **10**, 517-522.

Scott D, Danford N D, Dean B J and Kirkland D J (1990). Metaphase Chromosome Aberration Assays *In Vitro*. In: Kirkland D J (Ed) "Basic Mutagenicity Tests : UKEMS Recommended Procedures". Cambridge University Press, Cambridge, pp 62-86.

Scott D, Galloway S M, Marshall R R, Ishidate M Jr, Brusick D, Ashby J and Myhr B C (1991). "Genotoxicity Under Extreme Culture Conditions". *Mutation Research*, **257**, 147-204.

**TABLE 1 - MITOTIC INDICES IN THE ABSENCE OF METABOLIC ACTIVATION
(S9-MIX)**

EXPERIMENT 1			EXPERIMENT 2		
Treatment	Mitotic Index %	Mean % Mitotic Index	Treatment	Mitotic Index %	Mean % Mitotic Index
Solvent Control (10µl/ml)	8.7 13.4	11.1	Solvent Control (10µl/ml)	12.8 14.6	13.7
Allyl alcohol (µg/ml)			Allyl alcohol (µg/ml)		
581	14.6 11.9	13.3	581	13.0 9.9	11.5
400	10.2 13.2	11.7	400	8.5 11.0	9.8
200	a a		200	a a	
100	15.6 14.8	15.2	100	13.0 10.7	11.9
50	a a		50	a a	
25	a a		25	a a	
10	a a		10	a a	
5	a a		5	a a	

a - Mitotic index not required for selection of concentrations for chromosomal aberration analysis

TABLE 2 - MITOTIC INDICES IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1			EXPERIMENT 2		
Treatment	Mitotic Index %	Mean % Mitotic Index	Treatment	Mitotic Index %	Mean % Mitotic Index
Solvent Control (10µl/ml)	9.7 12.3	11.0	Solvent Control (10µl/ml)	11.4 12.4	11.9
Allyl alcohol (µg/ml)			Allyl alcohol (µg/ml)		
581	5.7 8.1	6.9	581	b b	
400	8.9 9.7	9.3	400	b b	
200	a a		200	7.4 11.0	9.2
100	12.4 12.3	12.4	100	13.1 12.9	13.0
50	a a		50	a a	
25	a a		25	15.3 16.8	16.1
10	a a		10	a a	
5	a a		5	a a	

a - Mitotic index not required for selection of concentrations for chromosomal aberration analysis

b - Not suitable for chromosomal aberration analysis due to excessive cytotoxic effects on the chromosomes

TABLE 3 - MEAN CHROMOSOMAL ABERRATIONS AND MITOTIC INDICES IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

Treatment		Mean % Aberrant Cells Excluding Gaps	Mean % Mitotic Index
Experiment 1			
Solvent Control	10µl/ml	0.00	11.1
Mitomycin C	0.5µg/ml	23.00**	10.9Δ
Allyl alcohol			
	581µg/ml	1.00	13.3
	400µg/ml	1.00	11.7
	100µg/ml	1.00	15.2
Experiment 2			
Solvent Control	10µl/ml	4.00	13.7
Mitomycin C	0.5µg/ml	40.00**	6.8Δ
Allyl alcohol			
	581µg/ml	12.50**	11.5
	400µg/ml	16.80**	9.8
	100µg/ml	7.50	11.9

** Statistically significant increase in the percentage of aberrant cells at p<0.01 using Fisher's Exact Test (one-sided).

Δ Positive control mitotic index and % aberrant cells are determined from a single culture.

TABLE 4 - MEAN CHROMOSOMAL ABERRATIONS AND MITOTIC INDICES IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

Treatment		Mean % Aberrant Cells Excluding Gaps	Mean % Mitotic Index
Experiment 1			
Solvent Control	10µl/ml	2.00	11.0
Cyclophosphamide	50µg/ml	32.00**	5.1Δ
Allyl alcohol			
	581µg/ml	10.50**	6.9
	400µg/ml	3.50	9.3
	100µg/ml	3.50	12.4
Experiment 2			
Solvent Control	10µl/ml	2.00	11.9
Cyclophosphamide	50µg/ml	48.00**	9.2Δ
Allyl alcohol			
	200µg/ml	18.00**	9.2
	100µg/ml	7.00*	13.0
	25µg/ml	3.00	16.1

* Statistically significant increase in the percentage of aberrant cells at p<0.05 using Fisher's Exact Test (one-sided).

** Statistically significant increase in the percentage of aberrant cells at p<0.01 using Fisher's Exact Test (one-sided).

Δ Positive control mitotic index and % aberrant cells are determined from a single culture.

TABLE 5 - OSMOLALITY AND pH DATA

Treatment		pH	Osmolality (mmol/kg)
Solvent Control	10µl/ml	7.47	407
Allyl alcohol	581µg/ml	7.37	398
	400µg/ml	7.36	410
	200µg/ml	7.35	423
	100µg/ml	7.36	412
	50µg/ml	7.37	409
	25µg/ml	7.32	407
	10µg/ml	7.33	410
	5µg/ml	7.36	419

TABLE 6 - INDIVIDUAL CULTURE DATA IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1

CONCENTRATION	TREATMENT	CULTURE	NO OF CELLS EXAMINED	NO OF CELLS WITH GIVEN ABERRATIONS				MITOTIC INDEX
				GAPS	BRKS	FRAGS-MINS	MULT	
Dimethylsulphoxide	A		100					8.700
	B		100					13.400
Mitomycin C	A		100	4	11	4	9	10.900
	B		100					14.600
Allyl Alcohol	A		100					11.900
	B		100					10.200
400 ug/ml	A		100					13.200
	B		100	1	1	1	1	15.600
100 ug/ml	A		100					14.800
	B		100					

Key:

- Brks
- Frags-Mins
- Mult
- Intc
- Oth
- breaks
- fragments and minutes
- multiple damage
- interchanges
- others, rearrangements

TABLE 6 - INDIVIDUAL CULTURE DATA IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 2

CONCENTRATION	TREATMENT	CULTURE	NO OF CELLS EXAMINED	GAPS	BRKS	FRAGS-MINS	MULT	INTC	OTH	MITOTIC INDEX
Dimethylsulphoxide	10 ul/ml	A	100	3	2	3	3			12.800
		B	100	2	2	3	3			14.600
Mitomycin C	0.2 ug/ml	A	25		10	1	1			6.800
	581 ug/ml	A	100	1	9	1	1			13.000
Allyl alcohol	400 ug/ml	B	100		5	13	13			9.900
		A	75	6	10	1	1			8.500
	100 ug/ml	B	50	5	10					11.000
		A	100	1	8					13.000
		B	100		7					10.700

Key:

- Brks - breaks
- Frag-Mins - fragments and minutes
- Mult - multiple damage
- Intc - interchanges
- Oth - others, rearrangements

TABLE 7 - INDIVIDUAL CULTURE DATA IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1

CONCENTRATION	TREATMENT	CULTURE	NO OF CELLS EXAMINED	GAPS	NO OF CELLS WITH GIVEN ABERRATIONS			MITOTIC INDEX	
					BRKS	FRAGS-MINS	MULT		
Dimethylsulphoxide	10 ul/ml	A	100	4	2			9.700	
		B	100	1	2			12.300	
Cyclophosphamide	50 ug/ml	A	50	8	13	1	2	1	5.100
		B	100	4	12	1		1	5.700
Allyl Alcohol	581 ug/ml	A	100	3	5	3			8.100
		B	100	3	2	1	1		8.900
100 ug/ml		A	100	4	3	2			9.700
		B	100	4	3	2			12.400
		A	100	4	3	2			12.300

Key:

- Brks - breaks
- Frag-Mins - fragments and minutes
- Mult - multiple damage
- Intc - interchanges
- Oth - others, rearrangements

TABLE 7 - INDIVIDUAL CULTURE DATA IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 2

CONCENTRATION	TREATMENT	CULTURE	NO OF CELLS EXAMINED	GAPS	BRKS	FRAGS-MINS	MULT	INTC	OTH	NO OF CELLS WITH GIVEN ABERRATIONS		MITOTIC INDEX
										FRAGS-MINS	MULT	
Dimethylsulphoxide	10 ul/ml	A	100		3							11.400
		B	100	1						1		12.400
Cyclophosphamide	50 ug/ml	A	25	2	9			1				9.200
	200 ug/ml	A	25	2	9							7.400
Allyl alcohol	200 ug/ml	B	75	2	7		2				1	11.000
	100 ug/ml	A	100		2			10				13.100
		B	100		2							12.900
	25 ug/ml	A	100	3	4							15.300
		B	100	1	2							16.800

- Key:
- Brks - breaks
 - Frag-Mins - fragments and minutes
 - Mult - multiple damage
 - Intc - interchanges
 - Oth - others, rearrangements

APPENDIX 1 - HISTORICAL CONTROL DATA

April 1995 – December 2002

Solvent controls

	68 hour sampling time	
	+S9-mix	-S9-mix
Mean % aberrant cells excluding gaps	1.0 ± 0.9 n = 241	1.0 ± 0.9 n = 267
Range (% aberrant cells excluding gaps)	0.0 – 5.5	0.0 – 4.0

Positive controls

	68 hour sampling time	
	+S9-mix	-S9-mix
Positive control substance	Cyclophosphamide	Mitomycin C
Mean % aberrant cells excluding gaps	29.3 ± 10.8 n = 241	36.5 ± 11.2 n = 267
Range (% aberrant cells excluding gaps)	7.0 - 60.0	9.0 - 64.0