CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
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REPORT NO: CTL/P/4991

GLYPHOSATE ACID: L5178Y TK$^{+/-}$ MOUSE LYMPHOMA GENE MUTATION ASSAY

STUDY DETAILS

Sponsor:	Zeneca Agrochemicals
Sponsor Reference:	20160
CTL Test Substance Reference Number:	Y04707/034
CTL Study Number:	VV0123

AUTHOR

[redacted]

DATE OF ISSUE

24 May 1996

STATEMENT OF DATA CONFIDENTIALITY CLAIM

THIS DOCUMENT CONTAINS INFORMATION CONFIDENTIAL AND TRADE SECRET TO ZENECA AGROCHEMICALS.

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 Zeneca Agrochemicals.

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 was conducted in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom GLP Compliance Programme, Department of Health 1989) except for the deviations listed below. These Principles are in accordance with the OECD Principles of Good Laboratory Practice (1982) ISBN 9264 12367 9 (OECD Environment Monograph No 45, OCDE/GD(92)32) and are in conformity with, and implement, the requirements of the European Commission (Directives 87/18/EEC and 88/320/EEC).

These international standards are acceptable to the United States Environmental Protection Agency and this study, therefore, satisfies the requirements of 40 CFR Part 160 and 40 CFR Part 792.

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

(i) the stability, homogeneity and achieved concentration of the test substance in the vehicle used were not determined by analysis

(ii) the stability and achieved concentration of the control substances in the vehicle used were not determined by analysis

(iii) certified purity and stability of the control substances are not available.

[redacted] .. [redacted] .... 24 May 1996

Study Director   Date

This page
may be required
by some
regulatory authorities.

QUALITY ASSURANCE STATEMENT

In accordance with ZENECA 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	Date of QA Report
21 May 96	21 May 96
24 May 96	24 May 96

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

29 Jan 96	29 Jan 96
30 Jan 96	30 Jan 96
05 Mar 96	05 Mar 96
05 Mar 96	05 Mar 96
06 Mar 96	06 Mar 96

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, VV0123.

[redacted] ..... [redacted] ..... 24 May 1996

(CTL Quality Assurance Unit)

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

Name	Title
[redacted]	Study Investigator
	Study Statistician
	Study Reviewer

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TABLES

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

1. SUMMARY

1.1 Study design

To assess the mutagenic potential of glyphosate acid to mammalian cells, L5178Y TK$^{+/-}$ mouse lymphoma cells were treated *in vitro* with various concentrations of test substance, both in the presence and absence of a rat liver derived auxiliary metabolic system (S9-mix). Mutant frequencies were assessed by cell growth in the presence of trifluorothymidine after a 48 hour expression time.

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

1.2 Results

Glyphosate acid was tested up to a maximum concentration of 1000µg/ml in the presence and absence of S9-mix as concentrations in excess of this produced excessive reductions in the pH of the treatment medium. Very little toxicity was seen at the maximum concentration tested. Minimum survival levels, compared to the solvent control cultures, of 90% and 57% were observed in cultures treated with the maximum concentration of glyphosate acid in the presence and absence of S9-mix respectively.

No significant increases in mutant frequency were observed in cultures treated with glyphosate acid in either the presence or absence of S9-mix in either of the independent experiments.

The positive controls induced substantial 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, glyphosate acid is not mutagenic to L5178Y TK+/- cells in the presence or absence of S9-mix.

2. INTRODUCTION

2.1 Purpose

The purpose of this study was to evaluate glyphosate acid 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).

2.2 Regulatory guidelines

The study was conducted according to the following Regulatory Guidelines:

a) OECD guideline reference 476 (1984), Genetic Toxicology, *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 Ninth Adaptation, Commission Directive 87/302/EEC of 18th November 1987, OJ L133 30.5.88. (*In vitro* mammalian cell gene mutation test).

c) US Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation Human and Domestic Animals, Series 84, Mutagenicity, Addendum 9. Publication No. EPA-540/09-91-122 NTIS Report No. PB91-158394 Feb 1991 (as specified in US Environmental Protection Agency Code of Federal Regulations 40CFR Part 798 Health Effects Testing Guidelines, Subpart F - Genetic Toxicity).

2.3 Justification for test system selection

The L5178Y TK$^{+/-}$ mouse lymphoma mutation assay is designed to detect chemically induced forward mutation in cells treated in culture. 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 post-treatment plating efficiency.

2.4 Experimental dates

The experimental work in this study was carried out between 22 November 1995 and 12 March 1996.

2.5 Data storage

An original report and all raw data pertaining to this study are retained in the Archives, Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK.

3. TEST AND CONTROL SUBSTANCES

3.1 Test substance

Name:	Glyphosate acid
Source:	Jealotts Hill
Colour:	White
Physical state:	Solid
Batch reference:	P24
CTL test substance reference number:	Y04707/034
Purity:	95.6%w/w
Storage conditions:	Anti-static bag, ambient temperature

From the information supplied by the Sponsor, the test substance was used within the stated expiry date. 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 and positive controls was dimethylsulphoxide (CTL test substance reference number Y00876/001).

The positive controls were ethyl methanesulphonate (EMS, CTL test substance reference number Y01958/008; Sigma Chemical Company) and N-nitrosodimethylamine (NDMA, CTL test substance reference number Y05348/002; Sigma Chemical Company). EMS is a direct acting mutagen and was tested in the absence of S9-mix at a final concentration of 750µg/ml. NDMA, which requires metabolic activation to its reactive species, was tested in the presence of S9-mix at a final concentration of 600µg/ml.

4. EXPERIMENTAL PROCEDURE

4.1 Dosing preparations

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

The positive control substances were prepared as solutions in 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 200µl/20ml culture.

4.2 Analysis of dosing preparations

In view of the short-term nature of the studies of this type, no analyses of stability, homogeneity 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

A dose-ranging study was performed to determine the concentrations of glyphosate acid to be used in the main mutation assays. Subsequently, 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 glyphosate acid for 4 hours in the presence and absence of S9-mix. After removal of the treatment medium, the cells were counted and a sample diluted to determine the survival immediately after treatment. The remaining 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 (-3.7.2c [TK$^{+/-}$]) cells (ex Dr [redacted] Glaxo-Wellcome, USA) was stored in a liquid nitrogen freezer. The cell stocks have been shown to be free of mycoplasma by enzyme linked immunosorbent assay (ELISA).

Following removal from liquid nitrogen, the cultures were kept at 37°C under an atmosphere of 5% CO_2 in air, either in a gassing incubator or in a hot room in roller bottles rotated on a roller apparatus.

4.5 Metabolic activation system

S9 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-naphthaflavone (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. S9 fraction was added at 5% (1ml S9 added to the 20ml cell culture) and co-factors at 1% (200µl added to the 20ml cell culture).

4.6 Methodology

The medium throughout this study was RPMI 1640 with Hepes, supplemented with 4mM L-glutamine, 200 IU/ml of penicillin, 200µg/ml streptomycin and 10% horse serum. The serum concentration was lowered to 5% during treatment and raised to 20% whenever the cells were dispensed into microwells.

Sterile techniques were used throughout the preparation of materials and on handling the cell cultures.

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 and therefore a bulk culture with a cell density in the range of 1-1.2 x 10^6 cells per ml was prepared prior to each experiment. This culture was then diluted 50:50 v/v with serum free medium to obtain a reduced serum content of 5% at treatment time. Each 20ml treatment culture (5-6 x 10^5 cells per ml) was taken from the bulk 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 250g for 5 minutes, the supernatants removed and the cell pellet resuspended in 50ml of fresh culture medium.

The effect of glyphosate acid 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 increases in mutant frequency (Scott *et al,* 1991).

4.6.3 Survival

To assess survival, a sample of each culture was counted and diluted to give 50ml at 8 cells per ml immediately post treatment. The cell count factor (CCF) was calculated from these cell counts as the cell count for each individual culture/the mean solvent control cell count. The diluted cultures of 50ml at 8 cells/ml constituted the survival cultures and were then dispensed at 200µl per well into two 96 well microwell plates (1.6 cells per well). The plates were then incubated (37°C, 5% CO_2, 98% relative humidity) to allow cell growth.

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 x 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 using a haemocytometer. 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 x 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% CO_2, 98% relative humidity) to allow cell growth.

For the assessment of viability, a sample from each mutation culture (at 1 x 10^4 cells per ml) was diluted to give 50ml at 8 cells per ml before the addition of the TFT. 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 x 10 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, a large colony or no colony (see Section 5.5).

5.1 Calculations

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

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

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

Zero Hour Survival

Figures from the 0 hour survival plates and post-treatment cell counts were used thus:

$$\text{0 hour relative survival} = \frac{\text{CE for a specific treatment}}{\text{CE for solvent control*}} \times 100 \times \text{CCF}$$

* the solvent control CE is based on the total of all the solvent control plates.

The highest concentration assayed was designed to reduce survival to 10%-20% of the solvent control culture value. 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

5.2.1 Cell growth and maintenance

To demonstrate acceptable cell growth and maintenance throughout the course of an experiment, post-expression cloning efficiencies of 50% or greater positive wells should be achieved for the solvent control viability plates.

5.2.2 Spontaneous control data

Based on the historical data for the assay in this laboratory, the spontaneous mutant frequency using TFT as the selective agent, both in the presence and absence of S9-mix, should be within the range of 0.8-6.0 x 10^{-4} mutants per survivor. A spontaneous mutant frequency slightly outside this range may not in itself invalidate the data provided all the other criteria for an acceptable assay are met. However, a second experiment with a spontaneous mutant frequency within the acceptable range should be carried out to ensure that the observed effect is reproducible.

5.2.3 Positive control data

Relevant positive controls are used in the absence and presence of S9-mix i.e., ethylmethanesulphonate and N-nitrosodimethylamine respectively. These must give unequivocal positive responses. Failure of the positive control does not in itself invalidate the data for a test substance that gives a positive effect provided all the other criteria are satisfied. However, this effect should be reproducible in a separate experiment when a satisfactory positive control response is also obtained.

5.3 Criteria for a positive response

A statistically significant dose-related increase in mutant frequency is required, but not only at concentrations eliciting excessive 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 a mutagen 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 levels of excessive 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

Interpretation of data are supported by statistical analysis where considered necessary at the discretion of the Study Director in consultation with the Study Statistician.

6. RESULTS

6.1 Survival data

In a preliminary dose ranging experiment the maximum concentration of glyphosate acid considered appropriate for testing in the mutation experiments was estimated as 1500µg/ml in the presence and absence of S9-mix as a concentration of 2000µg/ml was found to produce an excessive reduction in the pH of the treatment medium. A maximum concentration of

1000µg/ml was however selected for evaluation in both mutation experiments as a concentration of 1500µg/ml was also determined to produce an excessive reduction in the pH of the treatment medium (0.99 units). The reductions in pH seen with 1000µg/ml (0.59 units in the first experiment and 0.40 units in the second, Table 7) were considered acceptable and not to affect the outcome of the study. Very little toxicity was seen at the concentrations tested.

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 glyphosate acid up to 2000µg/ml had no significant effect on the osmolality of the treatment medium (Table 7).

6.2 Mutation data

The Study Director, in consultation with the Study Statistician, decided that a statistical analysis of the data from this study was not necessary.

No significant increases in mutant frequency, compared to the solvent control cultures, were observed in cultures treated with glyphosate acid at any concentration tested in either the presence or absence of S9-mix.

The positive controls, EMS and NDMA, induced substantial 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, glyphosate acid, was assessed in an *in vitro* mammalian cell gene mutation assay in L5178Y TK$^{+/-}$ cells to determine if it had any *in vitro* mammalian cell mutagenic activity. The procedure and experimental design employed complied with the recommendations of OECD guideline 476 (1984), EEC Annex V test methodology (1987) and US EPA guideline (1991).

Glyphosate acid was tested over a range of concentrations, both in the presence and absence of S9-mix in two independent mutation experiments. In each case glyphosate acid was found to reduce the pH of the treatment medium at the higher concentrations tested. The maximum concentration of glyphosate acid evaluated in the mutation assay was 1000µg/ml since concentrations greater than this resulted in an excessive reduction in the pH of the treatment medium. Little toxicity was observed at the concentrations of glyphosate acid tested.

No significant increases in mutant frequency, above the solvent control values, were recorded for any cultures treated with glyphosate acid in either the presence or absence of S9-mix.

The data obtained in this study therefore show that the test sample of glyphosate acid did not induce mutation in L5178Y TK$^{+/-}$ cells following *in vitro* treatment in either the presence or absence of S9-mix.

8. CONCLUSION

It is concluded that, under the conditions of this assay, glyphosate acid is not mutagenic to L5178Y TK$^{+/-}$ cells in the presence or absence of S9-mix.

9. REFERENCES

Kraemer K H, Waters H L and Buchanan J K (1980). Survival of human lymphoblastoid cells after DNA damage measured by growth in microtitre wells, Mutation Research, **72**, 285-294.

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.

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.

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})
GLYPHOSATE ACID		
1500	47	-
1000	57	1.1
667	63	1.0
444	62	0.6
SOLVENT CONTROL		
DMSO (10µl/ml)	93	1.1
POSITIVE CONTROL		
EMS 750	19	12.6

With S9-mix

Concentration (µg/ml)	Mean % Day 0 Relative Survival	Mean Mutant Frequency (x 10^{-4})
GLYPHOSATE ACID		
1500	66	-
1000	90	1.4
667	89	1.2
444	109	0.8
SOLVENT CONTROL		
DMSO (10µl/ml)	96	0.9
POSITIVE CONTROL		
NDMA 600	84	4.3

- = not counted due to excessive change in pH of treatment medium

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})
GLYPHOSATE ACID		
1000	96	2.3
667	93	1.3
444	83	1.2
296	108	1.4
SOLVENT CONTROL		
DMSO (10µl/ml)	100	1.3
POSITIVE CONTROL		
EMS 750	32	12.9

With S9-mix

Concentration (µg/ml)	Mean % Day 0 Relative Survival	Mean Mutant Frequency (x 10^{-4})
GLYPHOSATE ACID		
1000	115	1.8
667	107	1.3
444	118	1.8
296	115	1.5
SOLVENT CONTROL		
DMSO (10µl/ml)	100	1.6
POSITIVE CONTROL		
NDMA 600	103	6.4

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

Conc'n (µg/ml)	Post treatment cell count x10^5/ml	Survival Assay: Plate 1 -ve wells	Survival Assay: Plate 2 -ve wells	Survival Assay: Survival wells plated	Mutation Assay: Plate 1 large colonies +ve wells	Mutation Assay: Plate 2 large colonies +ve wells	Mutation Assay: Plate 1 small colonies +ve wells	Mutation Assay: Plate 2 small colonies +ve wells	Mutation Assay: Mutation wells plated	Viability Assay: Plate 1 -ve wells	Viability Assay: Plate 2 -ve wells	Viability Assay: Viability wells plated	%Day 0 relative survival	Mutant frequency x10^{-6}
Test Substance														
1500	1.1	48	57	192	-	-	-	-	-	-	-	-	40	-
1500	1.9	62	58	192	-	-	-	-	-	-	-	-	53	-
1000	1.8	61	60	192	8	5	18	7	192	25	28	192	50	1.4
1000	1.8	55	52	192	5	1	9	6	192	32	23	192	63	0.7
667	1.3	46	40	192	4	2	8	6	192	38	46	192	62	1.1
667	1.8	51	55	192	6	2	7	6	192	30	30	192	64	0.8
444	1.9	52	54	192	1	0	6	1	192	36	53	192	67	0.4
444	1.9	58	58	192	0	1	6	5	192	44	55	192	57	0.8
Solvent Control														
DMSO 10µl/ml	1.4	41	37	192	4	6	9	11	384	42	31	192	75	1.1
					4	2	2	10						
DMSO 10µl/ml	3.3	56	54	192	7	6	10	11	384	41	16	192	110	1.1
					3	6	8	9						
Positive Control														
EMS 750	1.3	80	68	192	23	16	18	21	192	68	67	192	20	11.8
EMS 750	2.3	83	85	192	14	14	33	33	192	68	60	192	18	13.3

c = contaminated plate
- = not counted due to excessive change in pH of treatment medium

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

Conc'n (µg/ml)	Post treatment cell count x10^5/ml	Survival Assay			Mutation Assay					Viability Assay			%Day 0 relative survival	Mutant frequency x10^{-4}
		Plate 1 -ve wells	Plate 2 -ve wells	Survival wells plated	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		
Test Substance														
1500	0.8	25	28	192	-	-	-	-	-	-	-	-	55	-
1500	1.7	38	45	192	-	-	-	-	-	-	-	-	77	-
1000	2.4	52	37	192	18	12	3	4	192	32	28	192	99	1.5
1000	1.8	45	39	192	22	19	9	5	192	11	9	192	80	1.2
667	1.9	38	48	192	14	10	4	3	192	23	27	192	82	1.0
667	2.1	40	42	192	12	8	5	4	192	40	36	192	96	1.4
444	1.7	28	29	192	2	2	17	10	192	24	25	192	111	1.0
444	1.6	29	27	192	2	0	8	7	192	28	22	192	106	0.6
Solvent Control														
DMSO 10µl/ml	0.9	10	21	192	2	2	10	11	384	27	25	192	88	1.1
					4	1	15	16						
DMSO 10µl/ml	1.8	31	35	192	0	0	5	4	384	31	35	192	103	0.6
					1	4	9	6						
Positive Control														
NDMA 600	1.7	38	30	192	10	15	5	9	192	58	61	192	95	3.8
NDMA 600	1.7	49	38	192	17	13	6	6	192	60	67	192	72	4.8

c = contaminated plate
- = not counted due to excessive change in pH of treatment medium

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

Conc'n (µg/ml)	Post treatment cell count x10^5/ml	Survival Assay			Mutation Assay					Viability Assay			%Day 0 relative survival	Mutant frequency x10^{-4}
		Plate 1 -ve wells	Plate 2 -ve wells	Survival wells plated	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		
Test Substance														
1000	1.3	27	17	192	11	22	34	32	192	14	6	192	93	2.6
1000	1.4	24	21	192	11	17	18	16	192	16	21	192	99	1.9
667	1.6	33	29	192	12	19	13	10	192	16	13	192	88	1.4
667	2.2	45	32	192	8	9	10	9	192	20	25	192	98	1.1
444	2.1	48	43	192	16	10	6	12	192	15	17	192	76	1.2
444	1.5	33	23	192	17	15	10	8	192	14	11	192	90	1.2
296	1.6	18	29	192	15	8	13	6	192	18	21	192	110	1.2
296	2.0	29	36	192	15	14	7	10	192	32	18	192	106	1.6
Solvent Control														
DMSO 10µl/ml	2.3	34	42	192	15	16	8	9	384	16	14	192	104	1.1
					12	9	11	7						
DMSO 10µl/ml	2.0	30	42	192	7	4	12	11	384	30	24	192	96	1.4
					18	11	6	6						
Positive Control														
EMS 750	1.0	61	52	192	44	40	20	29	192	52	44	192	26	13.6
EMS 750	1.5	62	53	192	65	49	21	29	192	27	27	192	38	12.1

c = contaminated plate

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

Conc'n (µg/ml)	Post treatment cell count x10⁵/ml	Survival Assay Plate 1 -ve wells	Survival Assay Plate 2 -ve wells	Survival wells plated	Mutation Assay 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	Viability Assay Plate 1 -ve wells	Viability Assay Plate 2 -ve wells	Viability wells plated	%Day 0 relative survival	Mutant frequency x10⁻⁴
Test Substance														
1000	1.8	29	35	192	12	10	8	16	192	37	31	192	96	2.1
1000	1.7	23	15	192	17	9	4	5	192	30	34	192	134	1.5
667	2.2	40	37	192	8	12	8	8	192	18	29	192	98	1.2
667	2.2	33	32	192	8	18	6	5	192	30	21	192	116	1.3
444	2.2	39	33	192	15	15	10	9	192	18	22	192	105	1.5
444	2.3	31	29	192	18	21	10	9	192	27	23	192	130	2.1
296	1.8	21	27	192	5	16	7	8	192	23	23	192	121	1.2
296	1.9	37	23	192	18	14	13	10	192	18	25	192	108	1.8
Solvent Control														
DMSO 10µl/ml	2.2	35	40	192	12	9	5	7	384	32	21	192	101	1.4
					13	14	7	10						
DMSO 10µl/ml	1.9	33	33	192	16	19	10	11	384	21	19	192	99	1.7
					14	17	16	6						
Positive Control														
NDMA 600	1.8	27	23	192	9	14	18	13	192	62	51	192	118	5.0
NDMA 600	2.1	37	44	192	14	12	12	14	192	67	71	192	88	7.7

c = contaminated plate

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TABLE 7 - OSMOLALITY AND pH DATA

Treatment	pH dose range	pH expt 1	pH expt 2	Osmolality (mmol/kg)
Solvent Control	7.32	7.34	7.28	281
Glyphosate acid 2000µg/ml	6.32			288
Glyphosate acid 1500µg/ml		6.35		*
Glyphosate acid 1000µg/ml	6.83	6.75	6.88	*
Glyphosate acid 667µg/ml		7.01	7.05	*
Glyphosate acid 500µg/ml	7.07			*
Glyphosate acid 444µg/ml		7.34	-	*
Glyphosate acid 296µg/ml			7.19	*
Glyphosate acid 250µg/ml	7.23			*
Glyphosate acid 125µg/ml	7.32			*

- = sample mis-dosed

* = not measured, no significant change in osmolality at concentrations above this.