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Mutagenic Potential of Hydroxylamine Hydrochloride (WR 740) in the Mouse Lymphoma Forward Mutation Assay

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DIVISION OF TOXICOLOGY

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The mutagenic potential of hydroxylamine HCl (WR 740) was assessed in the mouse lymphoma thymidine kinase forward mutation assay both with and without metabolic activation by rat liver S-9. In an initial range-finding assay, cells were exposed to test compound concentrations ranging from 5 mg/ml to 0.001 mg/ml. Cytotoxicity was observed at concentrations above 0.3 mg/ml which precluded cloning of these samples for mutation rate determination. Data from this assay were suggestive of a possible mutagenic response (positive responses at 0.06 mg/ml without activation and at 0.3 and 0.06 mg/ml with activation). Two confirmatory assays were conducted with 0.3 mg/ml as the highest concentration of hydroxylamine HCl. Hydroxylamine HCl was not mutagenic in either of the two confirmatory assays. These results indicate hydroxylamine HCl (WR 740) was not mutagenic under the conditions of this study.

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ABSTRACT

The mutagenic potential of hydroxylamine HCl (WR 740) was assessed in the mouse lymphoma thymidine kinase forward mutation assay both with and without metabolic activation by rat liver S-9. In an initial range-finding assay, cells were exposed to test compound concentrations ranging from 5 mg/ml to 0.001 mg/ml. Cytotoxicity was observed at concentrations above 0.3 mg/ml which precluded cloning of these samples for mutation rate determination. Data from this assay were suggestive of a possible mutagenic response (positive responses at 0.06 mg/ml without activation and at 0.3 and 0.06 mg/ml with activation). Two confirmatory assays were conducted with 0.3 mg/ml as the highest concentration of hydroxylamine HCl. Hydroxylamine HCl was not mutagenic in either of the two confirmatory assays. These results indicate hydroxylamine HCl (WR 740) was not mutagenic under the conditions of this study.

Key Words: Mutagenicity, Genetic Toxicology, Mouse Lymphoma Assay, Mutagenesis, *In vitro*, Hydroxylamine hydrochloride, WR 740

PREFACE

TYPE REPORT: Mouse Lymphoma GLP Study Report

TESTING FACILITY:

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PROJECT/WORK UNIT/APC: #3516277A875/380/TLE0

GLP STUDY NUMBER: 85039

STUDY DIRECTOR: MAJ Don W. Korte Jr., PhD, MSC

PRINCIPAL INVESTIGATOR: MAJ John W. Harbell, PhD, MSC

REPORT AND DATA MANAGEMENT:

A copy of the final report, study protocol, retired stability and purity data on the test compound, tissues, and an aliquot of the test compound will be retained in the LAIR Archives.

TEST SUBSTANCE: Hydroxylamine Hydrochloride

OBJECTIVE: The objective of this study was to determine the mutagenic potential of Hydroxylamine hydrochloride (TP046) by using the Mouse Lymphoma Forward Mutation Assay.

ACKNOWLEDGMENTS

SGT Steven K. Sano, SGT Lillie D. Witcher, John Dacey, and Joanne Wong provided research assistance during this study. Colleen S. Kamiyama assisted in preparing this manuscript.

SIGNATURES OF PRINCIPAL SCIENTISTS INVOLVED IN THE STUDY

We, the undersigned, declare that GLP study number 85039 was performed under our supervision, according to the procedures described herein, and that this report is an accurate record of the results obtained.

Don W. Korte 11/00/85

DON W. KORTE, JR. PhD / DATE
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MEMORANDUM FOR RECORD

SUBJECT: GLP Compliance for GLP Study 85039

1. This is to certify that in relation to LAIR GLP Study 85039, the following inspections were made:

29 March 1985	-	Protocol Review
24 April 1985	-	Cell Counts
05 June 1985	-	Cell Counts

2. The institute report entitled "Mutagenic Potential of Hydroxylamine Hydrochloride (WR740) in the Mouse Lymphoma Forward Mutation Assay," Toxicology Series 156, was audited on 21 February 1986 and 15 July 1987.

Carolyn M. Lewis
CAROLYN M. LEWIS
Chief, Quality Assurance

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Mutagenic Potential of Hydroxylamine Hydrochloride (WR 740) in the Mouse Lymphoma Forward Mutation Assay-- Harbell and Korte

INTRODUCTION

Cyanide is a chemical warfare agent which may be employed by hostile forces to influence the outcome of a future conflict. The US Army Medical Research and Development Command has been tasked to prevent the incapacitation, injury, and death that would occur with battlefield exposure of soldiers to agents such as cyanide. Hydroxylamine HCl (WR 740) is being evaluated to ascertain whether it can prevent or significantly reduce the toxicity associated with cyanide exposure. Efficacy studies were sufficiently promising to warrant preliminary toxicological evaluation of hydroxylamine HCl.

Objective of the Study

The objective of this study was to determine the mutagenic potential of Hydroxylamine hydrochloride (TP046) by using the Mouse Lymphoma Forward Mutation Assay.

MATERIALS AND METHODS

Hydroxylamine HCl was evaluated for cytotoxicity and mutagenicity according to LAIR SOP, OP-STX-71 (1).

Test Compound

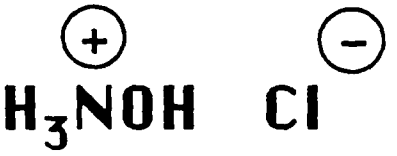
Chemical Name: Hydroxylamine hydrochloride

WRAIR Code No.: WR 740

LAIR Code No.: TP046

Chemical Abstracts Service Registry No.: 5470-11-1

Structural Formula:



Empirical Formula: ClH_4NO

Storage: Hydroxylamine HCl (100g), lot number 3007AK, was obtained from the Aldrich Chemical Co (Milwaukee, WI) in December 1984 and was assigned the LAIR Code number TP046. The test compound was stored in a desiccator at 5°C.

Chemical Properties/Analysis: Data provided by the Aldrich Chemical Company indicated that the test compound was 99% hydroxylamine HCl. Confirmatory analysis of the test material was performed by the Alpha Chemical & Biomedical Laboratories (Petaluma, CA). These data are presented in Appendix A.

Chemical Preparation

Hydroxylamine HCl was dissolved in glass distilled water, titrated to neutrality with NaOH, and filter sterilized. The stock solutions were prepared fresh for each assay immediately before use. So as not to overwhelm the culture medium's buffering capacity, a neutral solution of hydroxylamine was used to allow final exposure concentrations up to 5 mg/ml. For the first assay, the stock concentration was 250 mg/ml while for subsequent assays, the solution was 30 mg/ml.

Positive Controls

Ethyl methanesulfonate (EMS) (Sigma lot no. 83F-0279), added directly to the culture medium so as to provide a final concentration of 0.32 mg/ml, was used as the positive control for the assays conducted without metabolic activation. A stock solution of 5 mg/ml was formed by dissolving 2-acetamide fluorene (2AAF) (Sigma lot no. 113F-3679) in DMSO (Sigma lot no. 113F-0450). One hundred microliters of this stock were used (0.05 mg/ml final concentration) as the positive control for assays conducted with metabolic activation. The final DMSO concentration of the 2AAF-treated cultures did not exceed 1%. Both positive controls were prepared fresh on the day of assay.

Cells

Mouse lymphoma cells L5178Y 3.7.2C $TK^{+/-}$ were provided by Dr. Donald Clive, PhD, Burroughs Wellcome Co, Research Triangle Park, NC 27709. These cells were maintained in antibiotic free Fisher's Medium for Leukemic Cells of Mice (Fisher's Medium) supplemented with 10% horse serum. Six days before each assay began, the cell population was cleared of spontaneous thymidine kinase negative mutants by methotrexate treatment (1) and screened for mycoplasma and other contaminants by using the 3T6 co-culture technique (2). No nonnuclear DNA was detected after four days of co-culture and thus the cell line was presumed to be uncontaminated.

Medium

Powdered Fisher's Medium (basic) was purchased from Sigma Chemical Co (lot no. 113F-4710-1) and prepared in 10 mM HEPES buffered glass distilled water (pH 7.3). The medium was immediately filter sterilized. The sterile medium was supplemented with l-glutamine (2 mM) and sodium pyruvate (1 mM). Sterile horse serum (lot no. 310437) was obtained from Sterile Systems Inc, Logan, Utah, and was heat inactivated (56°C for 30 minutes) before use. Fisher's Medium was supplemented with horse serum at 5%, 10%, or 20% (volume/volume) final concentration. These were designated F_{5P}, F_{10P}, and F_{20P}, respectively, in accordance with standard notation (3).

Metabolic Activation System

The metabolic activation system was composed of Aroclor-induced rat liver 9000 g supernatant fraction (S-9) and an NADPH regenerating system provided by the cofactor mixture. Cofactor mixture, consisting of 2 mg/ml of NADP (Sigma lot no. 100F-7225) and 11.25 mg/ml of sodium isocitrate (Sigma lot no. 64F-3825), was prepared in Fisher's Medium without serum. This solution was prepared immediately before use. When metabolic activation was used, 3 ml of cofactor solution were combined with 6 ml of cell suspension containing the treatment compound. Then 1 ml of S-9 was added to each group. Litton Aroclor-induced rat liver S-9 lot no. RDK120 was used for the initial and first confirmatory assays and lot no. MAR233 was used for the second confirmatory assay. Vials were thawed immediately before use.

Assay Format

Dosing:

Stock cultures of L5178Y 3.7.2C cells were prepared for use by clearing spontaneous mutants and checking for contamination (see "Cells" above). Only cleared and noncontaminated cell populations were used for these assays. L5178Y cells were counted with a Coulter Counter model ZM (Coulter Electronic Inc, Hialeah, Florida) and resuspended in Fisher's Medium with 5% horse serum (F_{5P}) at a concentration of 10^6 cells/ml. After one hour, 6 ml of the cell suspension were pipetted into each culture tube. Stock hydroxylamine HCl and positive controls were added (see Tables 1, 3, and 5 for concentrations). Two negative controls were prepared for both the metabolic activation series and the nonactivation series. One was the first tube in the series while the other was the last. The 100 series (without metabolic activation) received an additional 4 ml of serum-free Fisher's Medium to make the final volume 10 ml. The 200 series (with metabolic activation) received 3 ml of cofactor mixture and 1 ml of freshly thawed S-9 suspension. These additions reduced the serum concentration to 3%. This lower serum concentration was intended to reduce the possible interaction (and inactivation) of test compounds with the serum proteins.

These cultures were maintained at 37°C on a roller drum for 4 hours, washed twice with Fisher's Medium containing 10% horse serum (F_{10P}), resuspended in 20 ml of F_{10P}, and returned to the roller drum. Ten percent serum provided for rapid growth in suspension culture.

Culturing:

Approximately 24 hours after the cultures were first exposed, a sample of each culture was trypsin-treated for 10 minutes to produce a single cell suspension for counting. This suspension was then diluted to the appropriate concentration range and counted (average of three counts). The remaining cells from each culture were then diluted to 3×10^5 cells/ml in 20 ml of F_{10P} and returned to the roller drum. After approximately 48 hours, an aliquot from each culture was again counted. All cultures to be cloned at this point were diluted to 3×10^5 cells/ml in Fisher's Medium with 20% horse serum (F_{20P}). Twenty percent serum was used during cloning to enhance the absolute cloning efficiency.

Cloning

Nonselective:

Soft agar cloning was used to determine the percentage of viable cells (viable count) and thymidine kinase negative mutants (mutant count) in each control and treated culture. To determine the percentage of viable cells, a portion of each freshly diluted culture (3 x 10^5 cells/ml) was further diluted to 600 cells/ml in F_{20P}. One milliliter of this suspension was diluted in 104 ml of F_{20P} containing 0.4% agar (Sigma lot no. 123F-0293) at 37°C. After vigorous mixing, this suspension of 5.7 cells/ml was dispensed into three 100 mm petri dishes (33 ml/dish). The extra 6 ml were provided to compensate for medium which foamed or adhered to the sides and thus could not readily be dispensed into the petri plates. The agar was allowed to harden at room temperature in the laminar flow hood (about 10 minutes).

Selective:

To determine the percentage of thymidine kinase negative mutants, a similar but selective cloning procedure was performed. Ten milliliters of the 3 x 10^5 cells/ml suspension were diluted with 95 ml of F_{20P} with 0.4% agar (final concentration) which contained 1 ug/ml of trifluorothymidine (TFT) (Sigma lot no. 94F-0351). TFT was used to arrest the growth of all cells which contained thymidine kinase. After mixing, 33 ml of this 2.86 x 10^4 cells/ml suspension were placed into each of three 100 mm petri dishes.

After hardening, both the mutant and viable count dishes were incubated for 11 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The number of colonies on each plate was then determined by using a Biotran II Automated Colony Counter (New Brunswick Scientific Co, Edison, New Jersey) with the size setting on zero.

Assay Acceptance Criteria

The following criteria are required according to Brusick (4) for a valid assay.

Plating Efficiency:

The minimum negative control viable count plating efficiency (mean count/mean number of cells plated) should be 70% or greater for the negative control cultures not treated

with the activation mixture. A 100% plating efficiency may be exceeded due to the delay between cell counts and dilutions which allows the cells to continue dividing. However, since the dilutions for the selective and nonselective cloning suspensions are made at the same time, the ratio between the two should not change even with the delay.

Cell Replication:

The cells in the negative control cultures (without S-9 activation) should undergo at least a 15-fold increase in cell number over the two days of suspension culture. Negative control cultures treated with the metabolic activation mixture characteristically show slightly less growth and therefore may not undergo the 15-fold increase.

Positive Control Responses:

A statistically significant mutagenic response (see below) must be induced by the positive controls. Failure to induce a mutagenic response by the positive activation control (2AAF) would invalidate only the activation series provided that the EMS (nonactivation control) induced an appropriate response.

Treatment Concentration:

In the absence of strong mutagenic activity (e.g. possible nonmutagen), cells should be exposed to the test compound concentration to the limits of solubility (usually up to 5 mg/ml) or to the point where suspension growth is reduced by cytotoxicity to 10% of controls.

Cell Replication and Survival

The combined activity of cell replication and survival for each control and treatment group is the product of suspension growth during the two days after exposure and the viable count cloning efficiency (1). Absolute suspension growth (ASG) is measured as a fold increase (usually 15- to 20-fold) in the control cultures over the 48-hour period. For example, the EMS treated culture in the initial assay (Table 1A) grew from 3×10^5 cells/ml to 6.36×10^5 cells/ml, a 2.1-fold increase, during the first 24 hours. The culture was then diluted to 3×10^5 cells/ml and allowed to continue growing for another 24 hours. At that point, the cell concentration was 1.495×10^6 cells/ml, a 4.98-fold increase. Thus the total growth was $2.1 \times 4.98 = 10.5$-fold increase over two days. Relative suspension growth (RSG) compares the

treated groups against the appropriate negative controls. Absolute cloning efficiency (ACE) is the observed number of viable count clones compared to the expected number of 189 per plate (5.7 cells/ml x 33 ml = 189 cells). Relative cloning efficiency (RCE) compares the treated groups with their respective negative controls. Thus, absolute cell survival (ACS) is the product of the suspension growth and absolute cloning efficiency while the relative cell survival (RCS) is the treated ACS compared to the control ACS.

Mutant Frequency

The mutant frequency (MF) is the mean selective plate count divided by the mean nonselective plate count multiplied by the dilution factor (2×10^{-4}). The dilution factor is derived from the ratio of the number of cells plated per ml in the nonselective plates divided by the number of cells plated per ml in the selective plates ($5.7/[2.86 \times 10^4] = 2 \times 10^{-4}$). The induced mutant frequency (IMF) in the treated groups is the observed mutant frequency less the spontaneous mutant frequency of the negative controls. Again, nonactivation and activation series are compared separately. The variance and standard error (SE) of the mutant frequency are calculated by using the mean selective and nonselective plate counts and with an assumed dilution variance of 10% (1,3).

Criteria for a Positive or Negative Response

An individual treatment concentration is considered positive if the assay is valid, the cell survival is at least 10% of controls and the induced mutant frequency is at least three times ($p < 0.01$) the standard error of that mutant frequency. A test compound is considered mutagenic if it yields a correlated positive dose response through several (usually three) treatment concentrations (4,5).

A compound is considered nonmutagenic in this system if a valid assay does not yield a positive response and the limits of compound solubility (up to 5 mg/ml) or 90% reduction in cell survival has been reached. Normally, a determination of mutagenic potential is not made on the basis of only one assay. Both positive and negative assays are confirmed.

Deviations from the Protocol/SOP

None

RESULTS

Hydroxylamine HCl was assayed three times, one initial assay and two confirmatory assays. The compound exposure concentration and resulting data are presented in Tables 1A through 6 while the raw data are contained in Appendix B.

The initial assay (Tables 1A through 2B) covered a dose range of 5 to 0.001 mg/ml. Since concentrations above 0.3 mg/ml were excessively cytotoxic, this concentration was selected as the maximum for future assays. The apparent greater than 100% absolute cloning efficiency was the result of the time delay between cell counting and dilution of these samples. A positive mutagenic response was observed without metabolic activation at 0.06 mg/ml while both 0.3 and 0.06 mg/ml were positive with metabolic activation.

In contrast to the first assay, the second (confirmatory) assay failed to show a positive response (Tables 3A through 4B). There was markedly less cytotoxicity (relative to controls) from the hydroxylamine HCl in the presence of the metabolic activation mixture than without it. Relative suspension growth after two days for doses 0.3, 0.2, and 0.1 mg/ml were 0%, 7%, and 64% of controls without activation and 48%, 58%, and 80% of controls with activation (Table 3A).

In the final confirmatory assay, the activated and nonactivated portions were performed as separate experiments (Tables 5 and 6). Without activation, no statistically significant positive response was observed in any treatment group which had at least 10% survival (0.2 mg/ml or less). The positive response in the 0.25 mg/ml group was observed in a culture with a survival rate of only 4% of controls. With activation, hydroxylamine failed to induce a positive correlated dose response. Although one point, 0.1 mg/ml, was statistically significant, its viable count was depressed compared to the other treated samples while its mutant count was similar to controls. The positive control for this assay, 2AAF, induced a mutation rate which was marginally below the acceptance criteria of three times the standard error (2.8 vs 3).

Table 1A

Cell Survival Data from the Initial Assay Without Activation

Treatment		S-9	Cell Count Day 1 (x 10^3/ml)	Cell Count Day 2 (x 10^3/ml)	ASG[a]	RSG[b]	VC[c]	ACE[d]	RCE[e]	RCS[f]
Control[g]		-	1027	1647	18.96	100	223	118	100	100
EMS	0.3 mg/ml	-	636	1495	10.47	55	169	89	76	42
TP046	5.0 mg/ml	-	9	10	0.03	0	Not Cloned			
TP046	3.0 mg/ml	-	15	30	0.10	1	Not Cloned			
TP046	1.0 mg/ml	-	6	13	0.04	0	Not Cloned			
TP046	0.6 mg/ml	-	14	11	0.04	0	Not Cloned			
TP046	0.3 mg/ml	-	70	280	0.93	5	217	115	98	5
TP046	0.1 mg/ml	-	642	1353	9.47	50	Contaminated			
TP046	0.06 mg/ml	-	799	1355	12.20	64	228	121	102	66
TP046	0.03 mg/ml	-	1185	1599	21.32	112	230	120	103	115
TP046	0.01 mg/ml	-	1068	1727	20.72	109	230	122	103	114
TP046	0.006 mg/ml	-	1075	1563	18.76	99	201	107	90	89
TP046	0.003 mg/ml	-	1161	1839	23.89	126	193	102	86	109
TP046	0.001 mg/ml	-	1004	1803	19.83	105	232	123	104	109

[a] Absolute suspension growth = total fold increase in suspension culture

[b] Relative suspension growth = treated/control x 100

[c] Viable Clone Count = mean of 3 plates

[d] Absolute Cloning Efficiency = viable clone count/189 x 100

[e] Relative Cloning Efficiency = treated/control x 100

[f] Relative Cell Survival = absolute cell survival treated/absolute cell survival control x 100

[g] Mean values from both negative controls

Table 1B

Cell Survival Data from the Initial Assay with Activation

Treatment		S-9	Cell Count Day 1 (x 10^3/ml)	Cell Count Day 2 (x 10^3/ml)	ASG[a]	RSG[b]	VC[c]	ACE[d]	RCE[e]	RCS[f]
Control[g]		+	854	1472	13.74	100	277	147	100	100
2AAF	0.050 mg/ml	+	596	1175	7.84	57	245	129	88	50
TP046	5.0 mg/ml	+	43	47	0.16	1	Not Cloned			
TP046	3.0 mg/ml	+	53	56	0.19	1	Not Cloned			
TP046	1.0 mg/ml	+	30	47	0.16	1	Not Cloned			
TP046	0.6 mg/ml	+	35	33	0.11	1	Not Cloned			
TP046	0.3 mg/ml	+	374	1492	4.97	36	195	103	71	26
TP046	0.1 mg/ml	+	485	1358	7.24	53	227	120	82	43
TP046	0.06 mg/ml	+	739	1299	10.83	79	199	104	72	56
TP046	0.03 mg/ml	+	858	1660	16.05	117	217	115	78	91
TP046	0.01 mg/ml	+	805	1640	14.76	107	230	122	83	90
TP046	0.006 mg/ml	+	803	1531	13.78	100	265	140	96	96
TP046	0.003 mg/ml	+	869	1629	15.75	115	237	126	86	98
TP046	0.001 mg/ml	+	855	1694	15.81	115	Contaminated			

[a] Total fold increase in suspension culture

[b] Relative suspension growth = treated/control x 100

[c] Viable Clone Count = mean of 3 plates

[d] Absolute Cloning Efficiency = viable clone count/189 x 100

[e] Relative Cloning Efficiency = treated/control x 100

[f] Relative Cell Survival = absolute cell survival treated/absolute cell survival control x 100

[g] Mean values from end run negative control only (start run contaminated)

Table 2A

Mutagenesis Data from the Initial Assay Without Activation

Treatment		S-9	RCS[a]	MF[b] ($x10^{-6}$)	IMF[c] ($x10^{-6}$)	SE[d] ($x10^{-6}$)	IMF/SE[e]
Control[f]		-	100	42.8			
EMS	0.3 mg/ml	-	42	366.0	323.2	41.82	7.73
TP046	5.0 mg/ml	-	Not Cloned				
TP046	3.0 mg/ml	-	Not Cloned				
TP046	1.0 mg/ml	-	Not Cloned				
TP046	0.6 mg/ml	-	Not Cloned				
TP046	0.3 mg/ml	-	5	112.6	69.8	13.44	5.19
TP046	0.1 mg/ml	-	Contaminated				
TP046	0.06 mg/ml	-	66	82.0	39.5	10.05	3.95
TP046	0.03 mg/ml	-	115	60.7	18.2	7.69	2.37
TP046	0.01 mg/ml	-	114	27.0	-15.1		
TP046	0.006 mg/ml	-	89	29.9	-12.2		
TP046	0.003 mg/ml	-	109	32.3	-9.8		
TP046	0.001 mg/ml	-	109	42.4	0.4	5.73	0.06

[a] Relative Cell Survival = absolute cell survival treatment/absolute cell survival controls x 100

[b] Mutant Frequency = mutant clone count/viable clone count x dilution factor

[c] Induced Mutant Frequency = treated mutant frequency - control mutant frequency

[d] Standard Error of the mutant frequency calculated only when the IMF>0.

[e] Ratio of the IMF to SE > 3 indicates a positive treatment response

[f] Mean values from both negative controls

Table 2B

Mutagenesis Data from the Initial Assay with Activation

Treatment		S-9	RCS[a]	MF[b] ($x10^{-6}$)	IMF[c] ($x10^{-6}$)	SE[d] ($x10^{-6}$)	IMF/SE[e]
Control[f]		+	100	42.6			
2AAF	0.050 mg/ml	+	50	137.7	95.1	15.91	5.98
TP046	5.0 mg/ml	+	Not Cloned				
TP046	3.0 mg/ml	+	Not Cloned				
TP046	1.0 mg/ml	+	Not Cloned				
TP046	0.6 mg/ml	+	Not Cloned				
TP046	0.3 mg/ml	+	26	76.9	34.3	9.78	3.51
TP046	0.1 mg/ml	+	43	33.6	-9.0		
TP046	0.06 mg/ml	+	56	89.4	46.8	11.11	4.22
TP046	0.03 mg/ml	+	91	43.3	0.7	5.91	0.12
TP046	0.01 mg/ml	+	90	27.8	-14.8		
TP046	0.006 mg/ml	+	96	53.8	11.2	6.79	1.65
TP046	0.003 mg/ml	+	98	55.7	13.1	7.14	1.83
TP046	0.001 mg/ml	+	Contaminated				

[a] Relative Cell Survival = absolute cell survival treatment/absolute cell survival controls x 100

[b] Mutant Frequency = mutant clone count/viable clone count x dilution factor

[c] Induced Mutant Frequency = treated mutant frequency - control mutant frequency

[d] Standard Error of the mutant frequency calculated only when the IMF is >0.

[e] Ratio of the IMF to SE Ratios > 3 indicate a positive treatment response

[f] Mean values from both negative controls

Table 3A

Cell Survival Data from the First Confirmatory Assay Without Activation

Treatment		S-9	Cell Count Day 1 (x 10^3/ml)	Cell Count Day 2 (x 10^3/ml)	ASG[a]	RSG[b]	VC[c]	ACE[d]	RCE[e]	RCS[f]
Control[g]		-	984	1584	17.4	100	140	73	100	100
EMS	0.32 mg/ml	-	559	1334	8.45	49	138	73	99	48
TP046	0.3 mg/ml	-	34	25	0.01	0	Not Cloned			
TP046	0.2 mg/ml	-	178	636	1.27	7	158	84	113	8
TP046	0.1 mg/ml	-	670	1514	11.10	64	155	82	111	70
TP046	0.08 mg/ml	-	781	1520	13.17	76	147	78	105	78
TP046	0.06 mg/ml	-	856	1594	15.41	89	145	77	104	91
TP046	0.03 mg/ml	-	953	1101	11.75	68	199	105	143	96
TP046	0.01 mg/ml	-	987	1387	15.26	88	174	92	124	109
TP046	0.006 mg/ml	-	939	1604	16.58	95	135	71	97	92

[a] Absolute suspension growth = total fold increase in suspension culture

[b] Relative suspension growth = treated/control x 100

[c] Viable Clone Count = mean of 3 plates

[d] Absolute Cloning Efficiency = viable clone count/189 x 100

[e] Relative Cloning Efficiency = treated/control x 100

[f] Relative Cell Survival = absolute cell survival treated/absolute cell survival control x 100

[g] Mean values from both negative controls

Table 3B

Cell Survival Data from the First Confirmatory Assay With Activation

Treatment		S-9	Cell Count Day 1 (x 10^3/ml)	Cell Count Day 2 (x 10^3/ml)	ASG[a]	RSG[b]	VC[c]	ACE[d]	RCE[e]	RCS[f]
Control[g]		+	662	1578	11.05	100	128	68	100	100
2AAF	0.05 mg/ml	+	409	1257	5.87	53	106	56	83	44
TP046	0.3 mg/ml	+	360	1315	5.26	48	144	76	112	54
TP046	0.2 mg/ml	+	470	1208	6.44	58	145	77	113	67
TP046	0.1 mg/ml	+	556	1392	8.82	80	Contaminated			
TP046	0.08 mg/ml	+	595	1545	10.30	93	133	70	104	98
TP046	0.06 mg/ml	+	659	1481	10.86	98	132	70	103	103
TP046	0.03 mg/ml	+	721	1530	12.24	111	142	75	111	124
TP046	0.01 mg/ml	+	669	1509	11.06	100	146	77	114	115
TP046	0.006 mg/ml	+	630	1536	10.75	97	143	76	112	110

[a] Absolute suspension growth = total fold increase in suspension culture

[b] Relative suspension growth = treated/control x 100

[c] Viable Clone Count = mean of 3 plates

[d] Absolute Cloning Efficiency = viable clone count/189 x 100

[e] Relative Cloning Efficiency = treated/control x 100

[f] Relative Cell Survival = absolute cell survival treated/absolute cell survival control x 100

[g] Mean values from both negative controls

Table 4A

Mutagenesis Data from the First Confirmatory Assay Without Activation

Treatment		S-9	RCS[a]	MF[b] ($\times 10^{-6}$)	IMF[c] ($\times 10^{-6}$)	SE[d] ($\times 10^{-6}$)	IMF/SE[e]
Control[f]		-	100	59.9			
EMS	0.32 mg/ml	-	48	313.7	253.8	37.05	6.85
TP046	0.3 mg/ml	-	0	Not Cloned			
TP046	0.2 mg/ml	-	8	65.8	5.9	8.96	0.66
TP046	0.1 mg/ml	-	70	48.1	-11.9		
TP046	0.08 mg/ml	-	78	61.6	1.7	8.65	0.20
TP046	0.06 mg/ml	-	91	83.3	23.4	11.14	2.10
TP046	0.03 mg/ml	-	96	48.2	-11.7		
TP046	0.01 mg/ml	-	109	34.7	-25.3		
TP046	0.006 mg/ml	-	92	71.1	11.2	9.91	1.13

[a] Relative Cell Survival = absolute cell survival treatment/absolute cell survival controls x 100

[b] Mutant Frequency = mutant clone count/viable clone count x dilution factor

[c] Induced Mutant Frequency = treated mutant frequency - control mutant frequency

[d] Standard Error of the mutant frequency calculated only when the IMF>0.

[e] Ratio of the IMF to SE > 3 indicates a positive treatment response

[f] Mean values from both negative controls

Table 4B

Mutagenesis Data from the First Confirmatory Assay With Activation

Treatment		S-9	RCS[a]	MF[b] ($x10^{-6}$)	IMF[c] ($x10^{-6}$)	SE[d] ($x10^{-6}$)	IMF/SE[e]
Control[f]		+	100	78.4			
2AAF	0.05 mg/ml	+	44	267.9	189.6	33.35	5.68
TP046	0.3 mg/ml	+	54	48.6	-29.8	0.00	0.00
TP046	0.2 mg/ml	+	67	96.6	18.2	12.61	1.44
TP046	0.1 mg/ml	+	Contaminated				
TP046	0.08 mg/ml	+	98	72.7	-5.6		
TP046	0.06 mg/ml	+	103	100.0	21.6	13.26	1.63
TP046	0.03 mg/ml	+	124	63.4	-15.0		
TP046	0.01 mg/ml	+	115	80.0	1.6	10.75	0.15
TP046	0.006 mg/ml	+	110	69.9	-8.4		

[a] Relative Cell Survival = absolute cell survival treatment/absolute cell survival controls x 100

[b] Mutant Frequency = mutant clone count/viable clone count x dilution factor

[c] Induced Mutant Frequency = treated mutant frequency - control mutant frequency

[d] Standard Error of the mutant frequency calculated only when the IMF>0.

[e] Ratio of the IMF to SE > 3 indicates a positive treatment response

[f] Mean values from both negative controls

Table 5

Cell Survival Data from the Second Confirmatory Assay

Treatment		S-9	Cell Count Day 1 (x 10^3/ml)	Cell Count Day 2 (x 10^3/ml)	ASG[a]	RSG[b]	VC[c]	ACE[d]	RCE[e]	RCS[f]
Control[g]		-	1039	1584	18.21	100	154	80	100	100
EMS	0.32 mg/ml	-	813	1607	14.47	79	118	62	77	62
TP046	0.30 mg/ml	-	33	80	0.27	Not Cloned				
TP046	0.25 mg/ml	-	122	335	1.12	6	111	59	72	4
TP046	0.20 mg/ml	-	196	724	2.41	13	141	75	92	12
TP046	0.15 mg/ml	-	334	1447	4.82	26	158	84	103	27
TP046	0.10 mg/ml	-	655	1564	11.47	63	151	80	98	62
Control[g]		+	765	1716	14.60	100	173	92	100	100
2AAF	0.05 mg/ml	+	594	1632	10.88	75	123	65	71	53
TP046	0.3 mg/ml	+	409	1271	5.93	41	155	82	90	36
TP046	0.2 mg/ml	+	435	1265	6.33	43	140	74	81	35
TP046	0.1 mg/ml	+	524	1429	8.10	55	80	43	47	26
TP046	0.08 mg/ml	+	490	1418	7.56	52	153	81	89	46
TP046	0.06 mg/ml	+	584	1584	10.03	69	167	89	97	67
TP046	0.03 mg/ml	+	690	1608	12.33	84	170	90	98	84
TP046	0.01 mg/ml	+	794	1636	14.18	97	156	83	90	88
TP046	0.006 mg/ml	+	736	1622	13.52	93	188	100	109	101

[a] Absolute suspension growth = total fold increase in suspension culture
[b] Relative suspension growth = treated/control x 100
[c] Viable Clone Count = mean of 3 plates
[d] Absolute Cloning Efficiency = viable clone count/189 x 100
[e] Relative Cloning Efficiency = treated/control x 100
[f] Relative Cell Survival = absolute cell survival treated/absolute cell survival control x 100
[g] Mean values from both negative controls

Table 6

Mutagenesis Data from the Second Confirmatory Assay

Treatment		S-9	RCS[a]	MF[b] ($x10^{-6}$)	IMF[c] ($x10^{-6}$)	SE[d] ($x10^{-6}$)	IMF/SE[e]
Control[f]		-	100	56.2			
EMS	0.32 mg/ml	-	62	408.7	352.4	49.40	7.13
TP046	0.30 mg/ml	-	0	Not Cloned			
TP046	0.25 mg/ml	-	4	100.0	43.8	13.98	3.13
TP046	0.20 mg/ml	-	12	78.0	9.7	10.59	0.91
TP046	0.15 mg/ml	-	27	73.4	5.1	9.81	0.52
TP046	0.10 mg/ml	-	62	74.2	5.8	10.00	0.58
Control[f]		+	100	47.7			
2AAF	0.05 mg/ml	+	53	67.2	27.3	9.71	2.81
TP046	0.3 mg/ml	+	36	51.6	11.7	7.39	1.59
TP046	0.2 mg/ml	+	35	58.6	18.7	8.39	2.23
TP046	0.1 mg/ml	+	26	85.0	45.1	13.16	3.43
TP046	0.08 mg/ml	+	46	48.4	8.5	7.04	1.20
TP046	0.06 mg/ml	+	67	29.9	-10.0		
TP046	0.03 mg/ml	+	84	59.2	19.3	8.08	2.39
TP046	0.01 mg/ml	+	88	30.8	-9.1		
TP046	0.006 mg/ml	+	101	36.2	-3.7		

[a] Relative Cell Survival = absolute cell survival treatment/absolute cell survival controls x 100

[b] Mutant Frequency = mutant clone count/viable clone count x dilution factor

[c] Induced Mutant Frequency = treated mutant frequency - control mutant frequency

[d] Standard Error of the mutant frequency calculated only when the IMF>0.

[e] Ratio of the IMF to SE > 3 indicates a positive treatment response

[f] Mean values from both negative controls

DISCUSSION

The mutagenesis of hydroxylamine HCl was evaluated in a mouse lymphoma forward mutation study consisting of one initial and two confirmatory assays. The results of this study indicated that hydroxylamine HCl was not mutagenic in the mouse lymphoma test system. While the initial assay was suggestive of a positive response, the subsequent assays failed to confirm such a relationship within the constraints of the acceptance criteria. The 0.1 mg/ml point from the second confirmatory assay with activation was statistically positive though this was attributed to its depressed viable count since doses both above and below it were negative. The data from the last assay, without metabolic activation (Tables 5 and 6), are particularly illustrative. The exposure concentrations all produced marked cytotoxicity (4 to 62% of control survival), but a statistically significant positive response was manifested only at 4% of control survival.

Spontaneous mutation rates were well within published values (3) of 25-115 x 10^{-6} without activation and 25-135 x 10^{-6} with activation. The change in absolute cloning efficiency between the initial assay and the two confirmatory assays was the result of reducing the time between counting the cells and diluting the cells from three hours to one hour. Validation criteria for these assays were within normal limits (absolute cloning efficiency for controls of 70% and significant mutagenicity in positive controls) in all assays except the activation portion of the last assay. In this case, the positive control (2AAF) induced mutagenesis was marginally below three times the standard error (2.8 vs 3). While this assay did not strictly meet the acceptance criteria, it has been included for completeness and because one point was statistically positive.

Hydroxylamine is a strong nucleophile and free radical-generating reagent and thus might be expected to interact with nucleic acids as well as proteins (6). These interactions account for its cytotoxic action and mutagenic action in several cell systems. Hydroxylamine has been used in efforts to induce beneficial mutations in plants (6,7). Treatment performed on the seeds, with doses of up to 3M, resulted in mutations and chromosome damage. High concentrations of hydroxylamine have been used to inactivate and mutate a number of viruses (6,7). More relevant to the present study were the reports of Kao and Puck (8) and Somers and Hsu (9). Kao and Puck (8) reported that hydroxylamine was essentially nonmutagenic although it did induce moderate chromosome damage in a Chinese hamster ovary (CHO) forward

mutation system, while Somers and Hsu reported hydroxylamine induced chromosome breaks in CHO cells. Sano et al (10) have reported that hydroxylamine HCl was not mutagenic in the Ames/*Salmonella* assay. Hydroxylamine HCl was tested over a dose range of 0.4 mg/plate to 0.001 mg/plate both with and without metabolic activation. This Ames/*Salmonella* study used the same sample of hydroxylamine HCl as was used in the present study. Chromosome damage as well as point or frameshift mutations may account for the reported mutagenic action of hydroxylamine. In those studies which showed significant mutagenic activity (6,7), the doses of hydroxylamine used were at least moderately cytotoxic and potentially clastogenic. The Ames/*Salmonella* assay is particularly suited to the study of point and frameshift mutations but must be limited to noncytotoxic doses of test compound. Thus the results of these test systems are not necessarily at odds, but may reflect the different end points of each assay.

Chromosome damage reduces the replication rate of mouse lymphoma cells and thus mutant clones formed by clastogenic action grow more slowly and form smaller colonies (11). The recent modifications in the cloning techniques proposed by Meyer et al (12) or Rudd et al (13) greatly increase the ability to detect small colonies. Thus these modified procedures would be particularly appropriate for the future studies of potential clastogens.

The reduction in hydroxylamine-induced cytotoxicity by the liver S-9 activation system observed *in vitro* is consistent with the role of the liver in the detoxification of hydroxylamine *in vivo*. Mammalian liver contains hydroxylamine reductase which reduces hydroxylamine to ammonia (7).

CONCLUSION

The mutagenic potential of hydroxylamine HCl (WR 740) was evaluated in the mouse lymphoma thymidine kinase forward mutation assay. To be considered a mutagen in this assay, a compound must induce a reproducible, statistically significant mutagenic response which correlates with the dose and occurs at relative cell survival rates of 10% or better. Hydroxylamine HCl did not meet these criteria.

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GLOSSARY

Absolute Cell Survival: The product of the population's total fold increase in growth in suspension culture multiplied by the absolute cloning efficiency under nonselective conditions.

Absolute Cloning Efficiency: The number of colonies counted on the nonselective plates divided by the number of cells originally plated x 100.

Fold Increase in Suspension Growth: The quotient of the cell concentration at the end of the growth period divided by the starting cell concentration.

Induced Mutant Frequency: The mutant frequency of the treated population less the mutant frequency of the control population.

Mutant Frequency: The ratio of the number of colonies on the selective plates divided by the number of colonies on the nonselective plates multiplied by the dilution factor. The dilution factor is the ratio of the number of cells plated on the nonselective plates divided by the number plated on the selective plates.

Relative Cell Survival: The absolute cell survival of the treated population divided by the absolute cell survival of the negative control population x 100.

Relative Cloning Efficiency: The absolute cloning efficiency of the treated population divided by the absolute cloning efficiency of the control population x 100.

Relative Suspension Growth: Total fold increase in cell number during suspension growth of the treated population divided by the total fold increase in cell number during suspension growth of the negative control population x 100.

Total Fold Increase in Suspension Growth: The product of the fold increase for the first day times the fold increase for the second day.

Appendix A: CHEMICAL DATA

Chemists Helping Chemists in Research and Industry

aldrich chemical company, inc.

ANALYTICAL DATA

Date November 16, 1984

Our: 25,588-0 Hydroxylamine hydrochloride, 99%, ACS Reagent

Batch No.: Typical batch

Analytical Results:

Appearance White crystal powder

m.p. 159°C b.p.

n_D^{25} $[\alpha]_D$

Spectral Data:

I.R. Conforms to structure

U.V.

N.M.R.

Assay: Fe - <5ppm
Titratable free acid: <0.25 meq/g

V.P.C. Heavy metals (asPb) <5ppm
Residue after ignition: <0.05%

Titration $KMnO_4$ back titration of $FeSO_4$ - 99% $NH_2OH \cdot HCl$

Other: Conforms to ACS specifications:
Assay: >96.0% $NH_2OH \cdot HCl$ NH_4: To pass test
Insoluble in alcohol: To pass test
Sulfur compounds (as SO_4) >0.005%

A. Napiorkowski

Anna Napiorkowski, Manager
Quality Control/Quality Assurance

LL/sw

P.O. Box 355, Milwaukee, Wisconsin 53201 USA. Telephone (414) 273-3850. Cable Aldrichem TWX 910-262-3052. Telex 26-643

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Appendix A (cont.): CHEMICAL DATA

ALPHA CHEMICAL & BIOMEDICAL LABORATORIES

Joe E. Hodgkins, Ph.D.
Director

September 16, 1986

Letterman Army Institute of Research
Attn: Dr. Conrad Wheeler
Building 1110, Room 0418
Letterman Army Medical Center
Presidio of San Francisco, CA 94129

REPORT
NITROGEN AND CHLORIDE ANALYSIS

Sample Identification:

ACBL Sample #3125 : Hydroxylamine hydrochloride 99%, A.C.S. Reagent, Aldrich. Lot #3007AK

Received in Lab : 8/25/86

Analysis:

Nitrogen determination by elemental analyzer.
Chloride determination by colorimetric assay.

Results:				Calculated Values
#3125	Nitrogen	:	20.12%	20.156%
	Chloride	:	53.15%	51.018%

Note: As expected, the chloride is higher than the calculated amount because of presence of inorganic chloride impurities. The nitrogen values are within the range of high purity.

Joe E. Hodgkins, Ph.D., C.T.
Laboratory Director

telephone report: 9/16/86

Park Plaza Professional Center, 245 Kentucky Street, Petaluma, California 94952 • Tel (707) 778-8607

Appendix B: INDIVIDUAL CULTURE COUNTS

Initial Assay With Metabolic Activation

Treatment			S-9	Cell Counts Day 1	Cell Counts Day 2	Viable Clone Counts	Mutant Clone Counts
Control			+	996	1612	Contaminated	
				1009	1564		
				953	1498		
2AAF	0.050	mg/ml	+	644	1158	266	165
				588	1206	226	160
				555	1162	242	181
TP046	5.0	mg/ml	+	49	59	Not Cloned	
				38	38		
				41	45		
TP046	3.0	mg/ml	+	70	63	Not Cloned	
				45	58		
				44	46		
TP046	1.0	mg/ml	+	30	54	Not Cloned	
				34	42		
				27	45		
TP046	0.6	mg/ml	+	30	34	Not Cloned	
				38	36		
				37	29		
TP046	0.3	mg/ml	+	391	1509	187	74
				386	1472	202	68
				346	1495	197	85
TP046	0.1	mg/ml	+	454	1395	224	35
				492	1311	218	39
				508	1367	238	40
TP046	0.06	mg/ml	+	750	1317	189	86
				726	1301	214	85
				740	1279	195	97
TP046	0.03	mg/ml	+	883	1656	216	41
				846	1664	205	37
				844	1661	230	64

Appendix B (cont.): INDIVIDUAL CULTURE COUNTS

Initial Assay With Metabolic Activation (cont.)

Treatment			S-9	Cell Counts Day 1	Cell Counts Day 2	Viable Clone Counts	Mutant Clone Counts
TP046	0.01	mg/ml	+	776	1576	221	28
				809	1695	240	31
				830	1648	229	38
TP046	0.006	mg/ml	+	812	1575	285	65
				800	1515	248	76
				798	1503	261	73
TP046	0.003	mg/ml	+	887	1645	241	58
				847	1628	242	64
				874	1615	229	78
TP046	0.001	mg/ml	+	844	1629	Contaminated	
				869	1738		
				851	1716		
Control			+	853	1483	272	59
				852	1467	273	58
				856	1466	286	62

Appendix B (cont.): INDIVIDUAL CULTURE COUNTS

Initial Assay Without Metabolic Activation

Treatment			S-9	Cell Counts Day 1	Cell Counts Day 2	Viable Clone Counts	Mutant Clone Counts
Control			-	1073	1711	216	43
				1130	1654	220	40
				1093	1668	205	39
EMS	0.3	mg/ml	-	658	1474	162	290
				628	1520	145	310
				623	1492	199	326
TP046	5.0	mg/ml	-	10	12	Not Cloned	
				8	10		
				8	8		
TP046	3.0	mg/ml	-	22	31	Not Cloned	
				12	36		
				11	24		
TP046	1.0	mg/ml	-	4	12	Not Cloned	
				7	15		
				7	11		
TP046	0.6	mg/ml	-	19	12	Not Cloned	
				10	9		
				14	12		
TP046	0.3	mg/ml	-	85	297	232	113
				60	281	198	124
				64	262	222	130
TP046	0.1	mg/ml	-	637	1340	Contaminated	
				668	1409		
				622	1310		
TP046	0.06	mg/ml	-	767	1359	230	90
				826	1356	218	85
				805	1351	237	106
TP046	0.03	mg/ml	-	1185	1626	238	61
				1239	1628	216	72
				1131	1544	235	76

Appendix B (cont.): INDIVIDUAL CULTURE COUNTS

Initial Assay Without Metabolic Activation (cont.)

Treatment			S-9	Cell Counts Day 1	Cell Counts Day 2	Viable Clone Counts	Mutant Clone Counts
TP046	0.01	mg/ml	-	1053	1693	217	27
				1086	1769	249	34
				1066	1720	225	32
TP046	0.006	mg/ml	-	1022	1557	201	25
				1109	1565	194	36
				1093	1568	209	30
TP046	0.003	mg/ml	-	1177	1854	187	34
				1191	1850	200	29
				1114	1812	191	30
TP046	0.001	mg/ml	-	1026	1741	229	51
				980	1868	234	44
				1007	1800	232	54
Control		mg/ml	-	976	1630	249	66
				940	1642	211	51
				950	1576	236	47

Appendix B (cont.): INDIVIDUAL CULTURE COUNTS
First Confirmatory Assay With Metabolic Activation

Treatment			S-9	Cell Counts Day 1	Cell Counts Day 2	Viable Clone Counts	Mutant Clone Counts
Control			+	682	1638	110	55
				659	1524	104	40
				653	1646	102	58
2AAF	0.05	mg/ml	+	431	1251	89	143
				414	1239	117	139
				381	1282	112	146
TP046	0.3	mg/ml	+	375	1279	147	48
				368	1316	132	30
				338	1350	153	27
TP046	0.2	mg/ml	+	452	1274	144	63
				477	1190	161	72
				480	1160	131	75
TP046	0.1	mg/ml	+	581	1450	Contaminated	
				542	1350		
				546	1377		
TP046	0.08	mg/ml	+	591	1538	136	40
				599	1563	122	58
				594	1533	140	47
TP046	0.06	mg/ml	+	663	1493	134	69
				658	1474	136	63
				655	1475	127	68
TP046	0.03	mg/ml	+	713	1563	132	43
				719	1533	168	55
				730	1495	126	37
TP046	0.01	mg/ml	+	621	1543	138	48
				703	1502	146	66
				683	1481	153	60
TP046	0.006	mg/ml	+	629	1553	137	57
				625	1599	143	57
				636	1456	149	36
Control			+	659	1516	144	39
				694	1573	154	38
				627	1568	15.	59

Appendix B (cont.): INDIVIDUAL CULTURE COUNTS
First Confirmatory Assay Without Metabolic Activation

Treatment			S-9	Cell Counts Day 1	Cell Counts Day 2	Viable Clone Counts	Mutant Clone Counts
Control			-	1012	1558	145	47
				1067	1546	150	37
				1038	1563	149	34
EMS	0.32	mg/ml	-	571	1377	150	212
				526	1340	131	216
				581	1286	134	223
TP046	0.3	mg/ml	-	43	20	Not Cloned	
				28	32		
				31	23		
TP046	0.2	mg/ml	-	177	649	163	52
				172	649	143	46
				185	611	168	59
TP046	0.1	mg/ml	-	634	1571	145	31
				687	1489	155	37
				690	1481	164	43
TP046	0.08	mg/ml	-	791	1511	155	34
				772	1587	135	49
				779	1461	150	54
TP046	0.06	mg/ml	-	871	1623	158	55
				827	1558	143	48
				869	1601	133	78
TP046	0.03	mg/ml	-	940	1116	206	46
				1015	1080	190	51
				905	1108	202	48
TP046	0.01	mg/ml	-	1001	1401	172	31
				1004	1384	187	34
				956	1376	162	27
TP046	0.006	mg/ml	-	950	1601	132	44
				875	1626	130	49
				992	1586	143	53
Control			-	915	1618	130	49
				922	1657	140	39
				951	1562	124	45

Appendix B (cont.): INDIVIDUAL CULTURE COUNTS
Second Confirmatory Assay With Metabolic Activation

Treatment			S-9	Cell Counts Day 1	Cell Counts Day 2	Viable Clone Counts	Mutant Clone Counts
Control			+	765	1795	155	36
				753	1763	150	31
				796	1805	171	40
2AAF	0.05	mg/ml	+	609	1648	126	45
				594	1684	110	39
				578	1565	132	41
TP046	0.3	mg/ml	+	409	1237	160	38
				413	1267	150	39
				405	1310	156	45
TP046	0.2	mg/ml	+	420	1282	126	39
				458	1251	169	44
				427	1263	126	40
TP046	0.1	mg/ml	+	505	1450	74	29
				522	1436	55	33
				544	1401	112	41
TP046	0.08	mg/ml	+	529	1440	150	33
				498	1398	152	40
				442	1416	158	39
TP046	0.06	mg/ml	+	614	1576	172	17
				568	1639	179	22
				569	1537	151	36
TP046	0.03	mg/ml	+	691	1629	174	54
				695	1587	160	43
				683	1609	175	53
TP046	0.01	mg/ml	+	811	1652	159	28
				794	1637	140	21
				777	1619	169	24
TP046	0.006	mg/ml	+	759	1654	198	40
				716	1607	183	37
				732	1606	184	27
Control			+	749	1641	188	36
				769	1648	186	25
				756	1644	186	39

Appendix B (cont.): INDIVIDUAL CULTURE COUNTS
Second Confirmatory Assay Without Metabolic Activation

Treatment			S-9	Cell Counts Day 1	Cell Counts Day 2	Viable Clone Counts	Mutant Clone Counts
Control			-	1045	1624	137	47
				1073	1554	166	50
				1029	1515	141	50
EMS	0.32	mg/ml	-	837	1607	110	228
				782	1594	115	269
				820	1621	128	213
TP046	0.30	mg/ml	-	36	77	Not Cloned	
				32	78		
				30	86		
TP046	0.25	mg/ml	-	129	339	105	59
				117	322	114	62
				121	344	113	73
TP046	0.20	mg/ml	-	211	726	141	53
				180	730	150	58
				196	717	133	55
TP046	0.15	mg/ml	-	347	1426	159	51
				342	1454	155	62
				313	1461	160	62
TP046	0.10	mg/ml	-	662	1601	151	50
				651	1569	130	67
				653	1522	172	52
Control			-	1040	1576	160	56
				1021	1656	164	C
				1026	1579	153	C

C = Contaminated

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