

8EHQ-1003-15265



The Dow Chemical Company  
Midland, Michigan 48674

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



Re: 8EHQ-03-15265 FOLLOW-UP  
7-OXABICYCLO[4.1.0]HEPTANE-3-CARBOXYLIC ACID, MEHTYL ESTER  
CASRN 41088-52-2

Dear Sir/Madam:

The following information is being submitted by The Dow Chemical Company (Dow) pursuant to current guidance issued by EPA indicating EPA's interpretation of Section 8(e) of the Toxic Substances Control Act. While the submitter does not necessarily believe the information indicates a significant risk of injury to health or the environment, EPA guidance seems to indicate that these effects in laboratory animals should be reported to the Agency.

8EHQ-03-15265 described preliminary results from an in-vitro gene mutation study. After the completion of that test, the study was repeated in a different laboratory to address concerns with the first study. The results from the second study found the substance to be mutagenic in the presence and absence of metabolic activation (Aroclor-1254 induced rat liver S9-mix).

A copy of the final report is provided with this letter. For further information, please contact the undersigned.

Sincerely,

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EH&S Product Regulatory Management  
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**STUDY TITLE**

EVALUATION OF CYCLOALIPHATIC EPOXIDE ERL-4140 IN THE MOUSE  
LYMPHOMA (L5178Y TK<sup>+/+</sup>) FORWARD MUTATION ASSAY

**Test Guidelines**

OECD (#476, 1997)  
U.S. EPA (OPPTS 870.5300, 1998)  
EC (2000)

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**Study Completion Date**

24 September 2003

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Peroxymerics Business  
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Midland, Michigan 48674

**Performing Laboratory**

Toxicology & Environmental Research and Consulting  
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Midland, Michigan 48674

**Laboratory Project Study ID**

031080

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## COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Compound: CYCLOALIPHATIC EPOXIDE ERL-4140

Title: EVALUATION OF CYCLOALIPHATIC EPOXIDE ERL-4140 IN THE  
MOUSE LYMPHOMA (L5178Y TK<sup>+/+</sup>) FORWARD MUTATION  
ASSAY

All phases of this study were conducted in compliance with the following Good  
Laboratory Practice Standards:

US Environmental Protection Agency - TSCA GLPs  
Title 40 CFR, Part 792 - Toxic Substances Control Act (TSCA); Good Laboratory  
Practice Standards, Final Rule

Organisation for Economic Co-Operation and Development (OECD)  
OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring,  
Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997)  
ENV/MC/CHEM(98)17

European Community (EC)  
EC Directive 99/11/EC of 8 March 1999 (OJ No. L 77/8-21, 23/3/1999)

Exception: The characterization performed on the test material was NON-GLP.



**SIGNATURE PAGE**

Compound: CYCLOALIPHATIC EPOXIDE ERL-4140

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ASSAY

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## SUMMARY

Cycloaliphatic epoxide ERL-4140 was evaluated in the *in vitro* mouse lymphoma (L5178Y TK<sup>+/+</sup>) forward mutation assay. The genotoxic potential of the test material was assessed in the absence and presence of an externally supplied metabolic activation (S-9) system at concentrations ranging from 100 to 1600 µg/ml. The adequacy of the experimental conditions for detection of induced mutation was confirmed by employing positive control chemicals, methyl methanesulfonate for assays without S-9 and 20-methylcholanthrene for assays with S-9. Negative control cultures were treated with the solvent used to dissolve the test material (*i.e.* dimethylsulfoxide). In the gene mutation assay in the absence of metabolic activation, biologically significant increases in mutant frequencies of up to 7.5-fold were observed in cultures treated with the test material. In the presence of S-9, cycloaliphatic epoxide ERL-4140 induced mutant frequency increases of up to 9.9-fold over concurrent solvent controls. Based upon these results, it was concluded that cycloaliphatic epoxide ERL-4140 induced a mutagenic response in the assay system employed.

## INTRODUCTION

### Principle of the Test System

Thymidine is salvaged by cells from surrounding medium utilizing the enzyme thymidine kinase (TK). This enzyme is essential for the initial phosphorylation of thymidine or thymidine analogs to thymidine monophosphate (TMP). The intracellular concentration of TMP regulates DNA synthesis and cell replication. In normal cells, TMP is synthesized *de novo*, and hence the TK gene is not essential for DNA replication. However, cells lacking a functional TK gene (TK<sup>-</sup> cells) are not capable of utilizing exogenous thymidine or its analogs if the *de novo* synthesis of thymidine is blocked and this leads to the arrest of cell division. In the presence of toxic analogs of thymidine (and under conditions where *de novo* synthesis of TMP is not blocked), normal (*i.e.*, TK competent) cells die because of the incorporation of the toxic nucleotide into DNA whereas cells lacking TK activity can multiply.

The mouse lymphoma forward gene mutation assay utilizes a strain (TK<sup>+/-</sup> -3.7.2C clonal line) of L5178Y mouse lymphoma cells heterozygous at the TK locus. Induced heritable loss of TK activity is presumed to be a mutational event (from TK<sup>+/-</sup> to TK<sup>-/-</sup>) as a result of DNA damage by physical or chemical agents. Mutagen induced or spontaneously occurring TK<sup>-/-</sup> cells can be detected by their inherent resistance to toxic thymidine analogs. Trifluorothymidine (TFT) is such an analog which is incorporated into the DNA of TK-competent (TK<sup>+/-</sup>) cells. Forward mutations at the single functional TK gene (TK<sup>+/-</sup> --> TK<sup>-/-</sup>) result in the loss of the salvage TK enzyme so that TFT is not incorporated into the cellular DNA thereby enabling these cells to grow in the presence of TFT.

The *in vitro* assay using L5178Y mouse lymphoma cells has been developed for the detection of forward gene mutations by chemical or physical agents in a cultured mammalian cell system (see Clive *et al.*, 1973; Clive and Spector, 1975; Clive *et al.*, 1987).

### Objective

The objective of this study was to determine the ability of cycloaliphatic epoxide ERL-4140 to induce mutations at the thymidine kinase (TK) locus of mouse lymphoma cells in culture.

#### Quality Assurance

The study conduct, data, protocol, protocol changes/revisions, and final report were inspected by the Quality Assurance Unit, Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

#### Test Guidelines

OECD Guideline 476 (OECD, 1997), U.S. EPA (1998), and EC (2000).

#### Archiving

The data, protocol, protocol changes/revisions, and final report are archived at Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

### **MATERIALS AND METHODS**

#### Chemical Name

Methyl 3,4-Epoxy cyclohexanecarboxylate

#### IUPAC Name

7-Oxabicyclo(4.1.0)Heptane-3-Carboxylic Acid, Methyl Ester

#### Synonyms

Cycloaliphatic Epoxide Resin, ERL-4140

#### Supplier, City, State (lot, reference number)

The Dow Chemical Company, Midland, Michigan (PK1055R6R1)

#### Purity/Characterization (method of analysis and reference)

99.78% as determined by Gas Chromatography (GC) with Flame Ionization Detection (FID). (Toman, 2003)

#### Characteristics

*Appearance (Physical State, color)*

Liquid

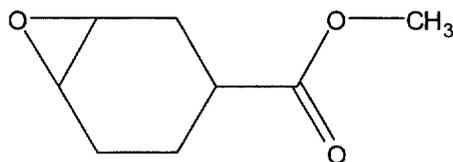
*Molecular Formula*

C<sub>8</sub>H<sub>12</sub>O<sub>3</sub>

*Molecular Weight*

156

*Chemical Structure*



*CAS Number*

41088-52-2

Indicator Cells

The cell line, TK<sup>+/+</sup>-3.7.2C clonal line of L5178Y mouse lymphoma cells originally obtained from Dr. Donald Clive, Burroughs Wellcome Laboratory, Research Triangle Park, North Carolina were used in this study. Stock cultures were stored at approximately -80°C or below. The cultures were periodically checked for mycoplasma contamination (American Type Culture Collection Laboratory, Manassas, Virginia). All laboratory cultures were maintained in logarithmic growth by serial subculturing. Cultures were grown in a shaker incubator at approximately 37°C. To reduce the frequency of spontaneous TK<sup>-/-</sup> mutants prior to use in a mutation assay, cell cultures were cleansed of pre-existing TK<sup>-/-</sup> mutants by exposing them to thymidine, hypoxanthine, methotrexate, and guanine (THMG) for approximately 24 hr. to select against the TK<sup>-/-</sup> phenotype. After 24 hr. treatment with THMG, the cells were placed in growth medium containing THG for an additional 24 hr. period at which time they were returned to normal growth medium for freezing. Prior to use in a mutation assay the frozen cells were returned to growth medium for 4 to 8 days.

Media

The cells were routinely maintained in Fischer's medium (F<sub>10P</sub>) supplemented with an additional 2 mM L-glutamine (Quality Biological Inc., Gaithersburg, Maryland), 10% (V/V) heat-inactivated (56°C, 30 min) horse serum (Hyclone Laboratories, Inc., Logan,

Utah), antibiotics (penicillin G, 20 units/ml; streptomycin sulfate, 20 µg/ml, GIBCO, Grand Island, New York), 0.22 mg/ml sodium pyruvate (Sigma), and 1mg/ml Plurionic F68® (GIBCO). F<sub>0P</sub>, the above medium without serum, was used during treatment. Cloning medium was F<sub>10P</sub> containing 0.28% granulated agar (BBL, Becton Dickinson and Company, Cockeysville, Maryland) to achieve a semi-solid state. The selection medium for the detection of TK<sup>-/-</sup> mutants consisted of the cloning medium supplemented with 1 µg/ml trifluorothymidine (TFT, Sigma). Conditioned medium was F<sub>10P</sub> from which cells had been removed and this medium was used during treatments.

### Controls

Dimethylsulfoxide (DMSO, Sigma), the solvent used to dissolve the test material, was used as the negative control treatment. Methyl methanesulfonate (MMS, Sigma, CAS # 66-27-3) was used at concentrations of 10 and 15 µg/ml of culture medium in the nonactivation assay while 20-methylcholanthrene (20-MCA, Sigma, CAS # 56-49-5) was used at concentrations of 2.5 and 5.0 µg/ml of culture medium in the presence of the metabolic activation system.

### Treatment Procedure

Cells from logarithmically growing stock cultures (maintained in an orbital shaker at 37 °C) were treated in individual 50 ml culture tubes. Each tube consisted of 6 ml of a cell suspension containing 1x10<sup>6</sup> cells/ml in a 1:1 mix of F<sub>0P</sub> and conditioned medium. To these cells, 4 ml of either S-9 mix (see below) or F<sub>0P</sub> were added along with the test material. The treatment consisted of duplicate cultures per dose level with the exception of positive controls where only one replicate was used. Following the addition of the test compounds, the tubes were incubated for approximately 4 hr. at 37°C in a roller drum (approximately 25-35 orbits/minute). At the end of the incubation period, the cells were pelleted, rinsed with F<sub>10P</sub> and resuspended in 20 ml F<sub>10P</sub>. The tubes were returned to the roller drum and maintained at 37°C during a standard expression period of two days.

### Identification of the Test System

All test cultures were identified using self adhesive labels containing a code system that identifies the test material, experiment number, treatment, and replicate.

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### Preparation of the Treatment Solutions

Stock solutions of the test article and positive control chemicals were prepared fresh just prior to treatment. Due to the limited aqueous solubility, the test material and 20-MCA were first dissolved in DMSO and further diluted in the culture medium to obtain the desired concentrations in treatment medium. MMS was dissolved in phosphate buffered saline and further diluted in treatment medium to obtain the desired concentrations.

During initial solubility testing, the pH and osmolalities of the negative control treatment solution and solutions containing 1630 µg/ml (limit dose of 10 mM is 1560 µg/ml) of the test material were determined with a Denver Basic pH meter (Denver Instrument Co, Arvada, Colorado) and Osmette A freezing point Osmometer (Precision Systems, Inc., Natick, Massachusetts), respectively. Alterations in the pH of the culture medium have been shown to induce false positive responses in *in vitro* genotoxicity assays (Thilagar *et al.*, 1984; Galloway *et al.*, 1985; Cifone, 1985). There was no appreciable change in either the pH or the osmolality of the culture medium following the addition of the test material as compared to the culture medium with solvent alone (culture medium with the test material, pH = 7.46; osmolality = 433 mOsm/kgH<sub>2</sub>O; culture medium with 1% DMSO, pH = 7.44, osmolality 439 mOsm/kgH<sub>2</sub>O).

### Analytical Verification of Dosing Solutions

The selected concentrations of the test material in the treatment solutions used for the treatment were verified by the Analytical Chemistry Laboratory, Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan. Samples were analyzed by HPLC with UV detection.

### *In Vitro* Metabolic Activation

S-9 liver homogenates prepared from Aroclor 1254-induced male Sprague-Dawley rats were purchased from a commercial source and stored at approximately -80°C or below. Thawed S-9 was reconstituted at a final concentration of approximately 10% (v/v) in a "mix" (O'Neill *et al.*, 1982). The mix consisted of the following co-factors: 10 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 5 mM glucose-6-phosphate, 4 mM nicotinamide adenine dinucleotide phosphate, 10 mM CaCl<sub>2</sub>, 30 mM KCl, and 50 mM sodium phosphate (pH 8.0). The reconstituted mix was added to the culture medium to obtain the desired final concentration of S-9 in the culture (*i.e.*, 2% v/v). Hence, the final concentrations of the co-factors in the culture medium were 1/5 of the concentrations stated above.

### Toxicity Assay

This assay was conducted for selecting concentrations of the test material to be used in the gene mutation assay. The cells were treated with various concentrations of the test material in the presence and absence of S-9 factor. After termination of treatment, the cells were rinsed to remove the test material and incubated in F<sub>10P</sub>. At approximately 24 hr. after treatment (Day 1), the test cultures were counted and diluted to a concentration of approximately  $3 \times 10^5$  cells/ml with fresh F<sub>10P</sub>. If the treated cells failed to multiply to a density of  $4 \times 10^5$  on the first day following treatment, the culture was returned to the incubator without any dilution. On day 2, cultures were again counted for cell density. From these cell counts, the following indices were calculated:

- Day 1 SG = Suspension Growth over first day  
= 
$$\frac{\text{Number of cells per ml on Day 1}}{3 \times 10^5 \text{ cells/ml}}$$
- Day 2 SG = Suspension Growth during second day  
= 
$$\frac{\text{Number of cells per ml on Day 2}}{3 \times 10^5 \text{ cells/ml (or the previous day cell number if no adjustment was made on the previous day)}}$$
- CSG = Cumulative Suspension Growth during first 2 days  
= Day 1 SG x Day 2 SG

$$\begin{aligned} \text{Day 2 RSG (\%)} &= \text{Cumulative relative suspension growth over first two days} \\ &= \frac{\text{Cumulative suspension growth of treated culture during first 2 days} \times 100}{\text{Average cumulative suspension growth of negative cultures during first 2 days}} \end{aligned}$$

### Gene Mutation Assay

Each dose level was set up in duplicate from the time of treatment until the completion of the assay. At the end of treatment, cells were returned to the incubator for phenotypic expression. At 24 hr. following treatment (day 1), the test cultures were counted and diluted to a concentration of approximately  $3 \times 10^5$  cells/ml with fresh F<sub>10P</sub>. If the treated cells failed to multiply to a density of  $4 \times 10^5$  cells/ml on the first day following treatment, the culture was returned to the incubator without any dilution. On day 2 (48 hr. following treatment) cultures were again counted and treatment levels with desired levels of toxicity were selected for cloning. Cultures with < 10% day 2 RSG

were not cloned (with one exception, see the Results and Discussion section below). A total sample size of  $3 \times 10^6$  cells from each culture was suspended in cloning medium with trifluorothymidine (TFT) and plated into three petri dishes (100 mm), allowed to gel for approximately 15 min. at 0-6°C, and returned to the incubator for approximately 12 days to allow for mutant colony formation. The cloning efficiency was determined by serially diluting the sample in cloning medium without TFT and then plating the cells into 3 petri dishes (100 mm) at a concentration of approximately 200 cells per dish. The dishes were returned to the incubator for approximately 12 days before counting the number of colonies per dish.

An image analyzer (LAI High-Resolution Colony Counting System, Loats Associates, Inc., Westminster, Maryland) was used to count and size colonies. The separation of small and large colonies was determined by inspection of colony sizing histograms of each culture. Mutant colonies form a bimodal distribution and the cutoff between the two distributions was set manually.

The parameter relative total growth (RTG) was used to determine the cytotoxicity of various treatments. Calculations for RTG are described below:

- $\% \text{ RTG} = \text{Day 2 RSG} \times \text{relative cloning efficiency (RCE)} \times 100$
- $\text{RCE} = \frac{\text{Cloning efficiency of the treated at the time of mutant selection}}{\text{Average cloning efficiency of the control at the time of mutant selection}}$
- $\text{Cloning Efficiency (CE)} = \frac{\text{Average \# of colonies in the TFT-free plates}}{\text{\# of cells seeded per plate}}$
- The mutant frequency per  $10^6$  clonable cells was calculated as below:

$$\frac{\text{Average no. of mutants per TFT plate}}{\text{Cloning Efficiency}}$$

- Mutant Index was calculated as below:

$$\frac{\text{Mutant frequency of the treated}}{\text{Average mutant frequency of the control}}$$

Initially, plates for cloning efficiency were counted and the RTG values calculated. Only those cultures with RTG values  $\geq 10\%$  were counted for mutants.

### Statistics

The activation and non-activation assays were considered independent assays with their own negative and positive controls. An assay was considered acceptable if: (1) the mutation frequency of positive controls was significantly higher than the negative controls, and (2) the mutant frequency of the negative controls was within  $35\text{-}140 \times 10^{-6}$ . The negative controls must have an average absolute cloning efficiency between 65-120% and a cumulative suspension growth greater than 8.

Mutant frequencies were evaluated based upon biological significance criteria. The test chemical was considered positive when the conditions listed below were met:

- a) The average mutant frequency in at least one dose level of the treated cultures (resulting in  $\geq 10\%$  relative total growth) is  $95 \times 10^{-6}$  above the average of the concurrent solvent controls (assuming these to be in the range of  $35\text{-}140 \times 10^{-6}$ ).
- b) There is a positive dose related linear trend. This was tested using a linear trend test at  $\alpha = 0.05$ , provided the above criteria is met.

The test material was considered negative in this assay if the following condition was met:

- a) There was no evidence of increase in mutant frequency at RTG values  $\geq 10\%$ .

The test material was considered equivocal in this assay if the following conditions were met:

- a.) There was a significant increase in mutant frequency only at RTG values  $> 10\%$  and  $< 20\%$ .
- b.) There was no evidence of increase in mutant frequency at RTG values  $\geq 20\%$ .

## RESULTS AND DISCUSSION

### Assay A1 – Preliminary Toxicity Assay

In a preliminary toxicity assay (A1), the test material was evaluated at concentrations ranging from 6.1 to 1560 µg/ml (Tables 1A and 1B) in the absence and presence of an externally supplied metabolic activation system (S-9). The highest concentration represented the limit dose of 10 mM. There was a concentration related increase in cytotoxicity as assessed by day 2 relative suspension growth (RSG); cultures with S-9 displayed slightly higher levels of cytotoxicity as compared to those without S-9. Based upon the results of this assay, concentrations in the range of 100 to 1600 µg/ml were selected for the gene mutation assay (Assay B1) without and with S-9. The highest concentration selected was slightly above the limit dose of 10 mM (*i.e.*, 1560 µg/ml).

The frequencies of mutants in cultures treated with the test material in the absence of S-9 are shown in Tables 2A and 2B. The mutation frequencies in cultures treated with the test material in the presence of S-9 are shown in Tables 2C and 2D. The results of the concentration checks on the stock solutions used for the mutation assay is shown in Table 3. The laboratory historical background values of TFT resistant (TFT<sup>r</sup>)(TK<sup>-/-</sup>) mutant frequency in the cell line used for this study are shown in Tables 4 and 5.

### Assay B1 – Mutagenicity Assay

In Assay B1 without metabolic activation (Tables 2A and 2B), cultures treated with cycloaliphatic epoxide ERL-4140 showed moderate to no toxicity as assessed by day 2 RSG. Cultures treated with the 3 lowest concentrations, *i.e.*, 100, 200, and 400 µg/ml, did not display evidence of toxicity and were not selected for cloning as they were considered to be superfluous for estimating the mutagenicity of the test material. The concentrations selected for cloning were 600, 800, 1000, 1200, 1400, and 1600 µg/ml.

The relative total growth (RTG) in the test material treated cultures varied from 26 to 95%. Increases in mutant frequencies that satisfied the criteria for a positive response were observed at treatment levels of 1000, 1200, 1400, and 1600 µg/ml. At the highest concentration, there was up to a 7.5-fold increase in the mutant frequency over the solvent controls. Statistical analysis indicated a significant (alpha = 0.05) linear trend in the mutant frequency with increasing dose. The test material induced a preponderance of small colony mutants over large colony mutants. Cultures treated with the positive control chemical had significantly higher mutant frequencies compared to the solvent

control. The solvent control values were within the range of the laboratory historical data (Tables 4 and 5). All criteria for a valid assay were satisfied.

In Assay B1 with metabolic activation (Tables 2C and 2D), cultures treated with cycloaliphatic epoxide ERL-4140 displayed excessive toxicity at concentrations of 800, 1000, 1200, 1400, and 1600  $\mu\text{g/ml}$  as determined by day 2 RSG. Therefore, these cultures were not selected for cloning with one exception. One replicate of 800  $\mu\text{g/ml}$  was cloned due to a technical error.

RTG estimates indicated excessive toxicity ( $< 10\%$  RTG) at treatment levels of 800 and 600  $\mu\text{g/ml}$ ; therefore the mutant plates were not enumerated for these dose levels. The remaining concentration levels, *i.e.*, 100, 200, and 400  $\mu\text{g/ml}$ , had RTG values ranging from 14 to 112%. Biologically significant increases in mutant frequencies were observed at concentration levels of 200 and 400  $\mu\text{g/ml}$ . These increases approached approximately 10-fold over the solvent controls at the 400  $\mu\text{g/ml}$  dose level. Statistical analysis identified a significant linear trend ( $\alpha = 0.05$ ) in mutant frequency with increasing dose level. Colony sizing data indicated a preferential increase in small colony mutants over the large colonies. The positive control treatments induced significant increases in mutant frequencies. The negative control values were within the range of the laboratory historical data (Tables 4 and 5). All criteria for a valid assay were satisfied in this trial.

#### Analytical Verification of Dosing Solutions

The analytically observed concentrations of the test material in the stock solutions in Assay B1 ranged from 106 to 120 % of target (Table 3).

### **CONCLUSION**

Under the experimental conditions used, cycloaliphatic epoxide ERL-4140 was considered to be mutagenic in the absence and presence of S-9 activation in this mouse lymphoma forward mutation assay.

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Table 1A. Survival of Cells Treated with Cycloaliphatic Epoxide ERL-4140 in the Absence of S-9 Activation – Assay A1

Conc. (ug/ml)	Day 1 Growth			Day 2 Growth			
	Cells/ml (x10 <sup>6</sup> )	SG	RSG	Cells/ml (x10 <sup>6</sup> )	SG	CSG	RSG
0 <sup>a</sup>	1600000	5.3	100	1360000	4.5	24.2	100
6.1	1500000	5.0	94	1700000	5.7	28.3	117
12.19	1600000	5.3	100	1660000	5.5	29.5	122
24.38	1600000	5.3	100	1600000	5.3	28.4	118
48.75	1460000	4.9	91	1200000	4.0	19.5	81
97.5	1260000	4.2	79	1500000	5.0	21.0	87
195	1220000	4.1	76	1680000	5.6	22.8	94
390	940000	3.1	59	1600000	5.3	16.7	69
780	1240000	4.1	78	820000	2.7	11.3	47
1560	780000	2.6	49	780000	2.6	6.8	28

<sup>a</sup> Solvent Control is 1% DMSO

Table 1B. Survival of Cells Treated with Cycloaliphatic Epoxide ERL-4140 in the Presence of S-9 Activation – Assay A1

Conc. (ug/ml)	Day 1 Growth			Day 2 Growth			
	Cells/ml (x10 <sup>6</sup> )	SG	RSG	Cells/ml (x10 <sup>6</sup> )	SG	CSG	RSG
0 <sup>a</sup>	1260000	4.2	100	1600000	5.3	22.4	100
6.1	1300000	4.3	103	1220000	4.1	17.6	79
12.19	1140000	3.8	90	1660000	5.5	21.0	94
24.38	1420000	4.7	113	1620000	5.4	25.6	114
48.75	1300000	4.3	103	1540000	5.1	22.2	99
97.5	1220000	4.1	97	1640000	5.5	22.2	99
195	1360000	4.5	108	1620000	5.4	24.5	109
390	920000	3.1	73	1300000	4.3	13.3	59
780	600000	2.0	48	1040000	3.5	6.9	31
1560	260000	0.9	21	400000	1.5	1.3	6

<sup>a</sup> Solvent Control is 1% DMSO

Table 2A. Summary of Cycloaliphatic Epoxide ERL-4140 Results in the Absence of S-9 Activation – Assay B1

Conc. (ug/ml)	Cum. RSG (%)		Plating Efficiency		RTG		Mutant Frequency (x10 <sup>6</sup> )		Mutant Index		
	Day 1	Day 2	Abs %	Rel %	Total	Small	Large	Total	Small	Large	
0 <sup>a</sup>	114	117	86	98	114	83	59	24	0.91	0.87	1.02
0 <sup>a</sup>	86	83	90	102	85	99	76	24	1.09	1.13	0.98
100	124	126	□	□	□	□	□	□	□	□	□
100	105	131	□	□	□	□	□	□	□	□	□
200	86	115	□	□	□	□	□	□	□	□	□
200	110	101	□	□	□	□	□	□	□	□	□
400	105	108	□	□	□	□	□	□	□	□	□
400	82	98	□	□	□	□	□	□	□	□	□
600	95	106	78	89	94	148	116	31	1.62	1.73	1.31
600	80	97	83	95	92	156	110	46	1.71	1.64	1.92
800	90	86	97	111	95	173	138	35	1.90	2.06	1.44
800	78	90	87	99	89	172	126	46	1.89	1.87	1.93
1000	90	102	64	72	74	245 <sup>b</sup>	199	46	2.69	2.96	1.93
1000	101	86	72	81	70	277 <sup>b</sup>	213	65	3.04	3.17	2.70
1200	86	78	72	82	64	323 <sup>b</sup>	274	50	3.55	4.07	2.07
1200	76	71	71	81	57	354 <sup>b</sup>	307	47	3.89	4.57	1.96
1400	72	73	48	55	40	521 <sup>b</sup>	450	71	5.72	6.70	2.96
1400	76	50	83	94	47	415 <sup>b</sup>	346	69	4.55	5.15	2.86
1600	57	56	50	57	32	633 <sup>b</sup>	533	100	6.94	7.94	4.16
1600	65	40	56	64	26	687 <sup>b</sup>	569	118	7.54	8.47	4.94
10 <sup>c</sup>	95	80	61	70	56	652 <sup>b</sup>	562	90	7.15	8.37	3.74
15 <sup>c</sup>	82	82	35	40	33	989 <sup>b</sup>	864	125	10.85	12.86	5.23

<sup>a</sup> Solvent Control is 1% DMSO

<sup>b</sup> Biologically significantly higher than the concurrent negative control with a positive linear trend test, alpha = 0.05

<sup>c</sup> Positive Control is MMS

□ Not plated as they were considered redundant

Table 2B. Summary of Cycloaliphatic Epoxide ERL-4140 Results in the Absence of S-9 Activation Cell and Colony Counts -- Assay B1

Conc. (µg/ml)	Day 1 Growth		Day 2 Growth		No. of Colonies on Viable Count Plates			No. of Colonies on Mutant Plates				
	Conc (x10 <sup>6</sup> ) <sup>a</sup>	Growth	Conc (x10 <sup>6</sup> ) <sup>a</sup>	Growth	#A	#B	#C	Total	#A	#B	#C	Total
0 <sup>b</sup>	1.20	4.0	1.34	4.5	136	191	191	517	66	83	67	215
0 <sup>b</sup>	0.90	3.0	1.28	4.3	170	196	171	538	101	82	84	267
100	1.30	4.3	1.34	4.5	□	□	□	□	□	□	□	□
100	1.10	3.7	1.64	5.5	□	□	□	□	□	□	□	□
200	0.90	3.0	1.76	5.9	□	□	□	□	□	□	□	□
200	1.16	3.9	1.20	4.0	□	□	□	□	□	□	□	□
400	1.10	3.7	1.36	4.5	□	□	□	□	□	□	□	□
400	0.86	2.9	1.58	5.3	□	□	□	□	□	□	□	□
600	1.00	3.3	1.46	4.9	153	151	164	468	125	114	107	346
600	0.84	2.8	1.60	5.3	162	171	167	500	140	121	129	389
800	0.94	3.1	1.26	4.2	194	194	196	585	182	163	161	505
800	0.82	2.7	1.52	5.1	170	174	178	522	167	144	138	448
1000	0.94	3.1	1.50	5.0	129	125	128	381	171	144	153	468
1000	1.06	3.5	1.12	3.7	116	170	†	429	227	191	177	595
1200	0.90	3.0	1.20	4.0	124	175	132	431	230	227	239	696
1200	0.80	2.7	1.22	4.1	133	146	146	425	277	264	211	752
1400	0.76	2.5	1.32	4.4	86	108	94	288	275	248	229	751
1400	0.80	2.7	0.86	2.9	145	175	176	495	373	309	345	1027
1600	0.60	2.0	1.28	4.3	113	95	94	302	334	294	327	956
1600	0.68	2.3	0.82	2.7	109	118	110	338	419	362	379	1160
10 <sup>c</sup>	1.00	3.33	1.10	3.7	155	95	118	369	449	392	361	1202
15 <sup>c</sup>	0.86	2.87	1.32	4.4	83	67	60	209	381	316	337	1034

<sup>a</sup> Cell concentration per ml

<sup>b</sup> Solvent Control is 1% DMSO

<sup>c</sup> Positive Control is MMS

□ Not plated as they were considered redundant

† Lost to contamination

Table 2C. Summary of Cycloaliphatic Epoxide ERL-4140 Results in the Presence of S-9 Activation – Assay B1

Conc. (ug/ml)	Cum. RSG (%)		Plating Efficiency		RTG (% Surv)	Mutant Frequency (x10 <sup>-6</sup> )		Mutant Index			
	Day 1	Day 2	Abs %	Rel %		Total	Small	Large	Total	Small	Large
0 <sup>a</sup>	101	97	80	102	99	106	82	25	1.00	1.06	0.85
0 <sup>a</sup>	99	103	77	98	101	106	73	33	1.00	0.94	1.15
100	99	98	89	114	112	117	85	33	1.11	1.10	1.13
100	110	104	74	94	98	131	94	37	1.24	1.21	1.30
200	106	94	71	91	86	324 <sup>b</sup>	293	31	3.06	3.79	1.09
200	92	68	95	121	82	329 <sup>b</sup>	286	43	3.11	3.71	1.49
400	90	28	38	49	14	886 <sup>b</sup>	776	109	8.36	10.04	3.81
400	87	36	33	42	15	1052 <sup>b</sup>	928	124	9.93	12.01	4.33
600	44	17	9	11	2	§	§	§	§	§	§
600	55	23	9	12	3	§	§	§	§	§	§
800	28	7	3	4	0	§	§	§	§	§	§
800	25	7	*	*	*	*	*	*	*	*	*
1000	17	4	*	*	*	*	*	*	*	*	*
1000	17	4	*	*	*	*	*	*	*	*	*
1200	15	1	*	*	*	*	*	*	*	*	*
1200	18	1	*	*	*	*	*	*	*	*	*
1400	14	1	*	*	*	*	*	*	*	*	*
1400	19	2	*	*	*	*	*	*	*	*	*
1600	3	0	*	*	*	*	*	*	*	*	*
1600	3	0	*	*	*	*	*	*	*	*	*
2.5 <sup>c</sup>	76	57	55	70	40	829 <sup>b</sup>	648	181	7.82	8.38	6.30
5.0 <sup>c</sup>	60	28	16	20	6	§	§	§	§	§	§

<sup>a</sup> Solvent Control is 1% DMSO  
<sup>b</sup> Biologically significantly higher than the concurrent negative control with a positive linear trend test, alpha = 0.05  
<sup>c</sup> Positive Control is MCA  
 § Mutant plates are not counted when RTG values are <10%  
 \* Not plated due to excessive toxicity as determined by Day 2 RSG

Table 2D. Summary of Cycloaliphatic Epoxide ERL-4140 Results in the Presence of S-9 Activation Cell and Colony Counts – Assay BI

Conc. ( $\mu\text{g/ml}$ )	Day 1 Growth		Day 2 Growth		No. of Colonies on Viable Count Plates			No. of Colonies on Mutant Plates			Total	
	Conc ( $\times 10^6$ ) <sup>a</sup>	Growth	Conc ( $\times 10^6$ ) <sup>a</sup>	Growth	#A	#B	#C	Total	#A	#B		#C
0 <sup>b</sup>	0.880	2.93	1.400	4.67	160	172	146	478	97	84	74	254
0 <sup>b</sup>	0.860	2.87	1.520	5.07	161	136	164	461	101	80	62	244
100	0.860	2.87	1.440	4.80	145	211	180	536	126	102	86	315
100	0.960	3.20	1.380	4.60	149	144	149	442	105	100	85	290
200	0.920	3.07	1.300	4.33	121	157	149	427	284	217	192	693
200	0.800	2.67	1.080	3.60	188	191	†	569	355	298	284	936
400	0.780	2.60	0.460	1.53	85	67	79	231	354	317	352	1022
400	0.760	2.53	0.600	2.00	60	80	57	198	363	335	341	1040
600	0.380	1.27	0.720	1.89	13	26	14	53	□	□	□	□
600	0.480	1.60	0.600	2.00	21	13	23	56	□	□	□	□
800	0.240	0.80	0.300	1.25	13	5	3	21	□	□	□	□
800	0.220	0.73	0.300	1.36	*	*	*	*	*	*	*	*
1000	0.146	0.49	0.160	1.10	*	*	*	*	*	*	*	*
1000	0.144	0.48	0.180	1.25	*	*	*	*	*	*	*	*
1200	0.132	0.44	0.050	0.38	*	*	*	*	*	*	*	*
1200	0.154	0.51	0.060	0.39	*	*	*	*	*	*	*	*
1400	0.126	0.42	0.030	0.24	*	*	*	*	*	*	*	*
1400	0.168	0.56	0.064	0.38	*	*	*	*	*	*	*	*
1600	0.024	0.08	0.014	0.58	*	*	*	*	*	*	*	*
1600	0.026	0.09	0.012	0.46	*	*	*	*	*	*	*	*
2.5 <sup>c</sup>	0.660	2.20	1.100	3.67	92	122	114	327	522	401	434	1357
5.0 <sup>c</sup>	0.520	1.73	0.680	2.27	26	35	35	95	□	□	□	□

† Lost to contamination  
 □ Mutant plates are not counted when RTG values are <10%  
 \* Not plated due to excessive toxicity as determined by Day 2 RSG

<sup>a</sup> Cell concentration per ml  
<sup>b</sup> Solvent Control is 1% DMSO  
<sup>c</sup> Positive Control is MCA



Table 4. Recent Historical Laboratory Background Values of Trifluorothymidine (TFT) Resistant (TFT<sup>r</sup>)(TK<sup>-/-</sup>) Mutant Frequency in Mouse L5178Y Lymphoma Cells

Year	S-9 (- or +)	FREQUENCIES <sup>a</sup>				
		Mean	Std. Dev.	Minimum	Maximum	N <sup>b</sup>
1996	-	104.8	35.99	53.0	184.0	29
	+	93.9	36.82	44.0	168.0	13
1998	-	75.0	47.22	30.0	153.0	10
	+	60.8	17.35	47.0	84.0	4
1999	-	90.8	15.57	55.0	115.0	15
	+	109.6	19.13	83.0	144.0	16
2000	-	56.3	10.31	39.0	74.0	12
	+	112.3	36.66	50.0	174.0	12
2001	-	78.3	18.32	49.0	120.0	39
	+	100.7	27.90	60.0	194.0	40
2002	-	90.5	16.59	70.0	123.0	8
	+	109.5	45.48	79.0	206.0	8
2003	-	74.6	19.81	47.0	104.0	8
	+	105.7	31.73	76.0	146.0	6

<sup>a</sup> Frequencies TG<sup>r</sup> mutants per 10<sup>6</sup> cells

<sup>b</sup> Number of experiments

Table 5. Recent Historical Laboratory Individual Background Values of Trifluorothymidine (TFT) Resistant (TFT<sup>r</sup>)(TK<sup>-/-</sup>) Mutant Frequency in Mouse L5178Y Lymphoma Cells

Year	S-9 + or -	No. of TFT <sup>r</sup> Mutants Per 10 <sup>6</sup> Clonable Cells
1996	-	129, 127, 131, 149, 115, 100, 117, 103, 85, 71, 99, 86, 128, 124, 79, 68, 87, 76, 67, 70, 55, 64, 53, 143, 180, 106, 83, 184, 159
	+	110, 79, 44, 60, 86, 67, 62, 78, 86, 119, 107, 168, 155
1998	-	122, 115, 36, 30, 40, 34, 123, 153, 46, 51
	+	47, 48, 64, 84
1999	-	55, 67, 86, 95, 87, 99, 101, 104, 95, 115, 94, 109, 93, 82, 80
	+	83, 83, 100, 103, 112, 120, 107, 105, 134, 135, 117, 144, 119, 120, 88, 84
2000	-	55, 57, 41, 39, 54, 61, 45, 74, 59, 64, 61, 65
	+	50, 55, 88, 97, 134, 120, 123, 120, 121, 107, 174, 159
2001	-	63, 50, 113, 120, 120, 76, 53, 81, 71, 78, 74, 88, 106, 82, 96, 65, 84, 88, 94, 57, 64, 69, 61, 66, 67, 108, 97, 59, 67, 81, 75, 75, 72, 77, 94, 72, 80, 61, 49
	+	119, 142, 194, 86, 112, 128, 96, 75, 84, 79, 72, 78, 115, 141, 121, 88, 97, 66, 60, 79, 90, 89, 96, 94, 86, 107, 124, 88, 182, 112, 69, 85, 89, 92, 94, 97, 107, 112, 97, 84
2002	-	81, 78, 95, 100, 70, 82, 95, 123
	+	80, 110, 206, 147, 79, 93, 82, 79
2003	-	69, 61, 62, 72, 47, 81, 101, 104
	+	146, 146, 85, 76, 88, 93