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LP1845 Liquid Gun Propellant Dermal Toxicity Studies

AN ASSESSMENT OF THE CLASTOGENIC POTENTIAL OF LP1846 UTILIZING THE MAMMALIAN CELL CYTOGENETICS ASSAY WITH CHINESE HAMSTER OVARY (CHO) CELLS

Final Report

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FOREWORD

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Daniel C Blair 4/3/91

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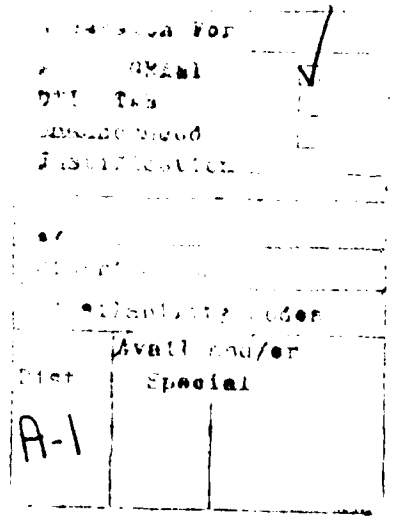

COMPLIANCE STATEMENT

To the best of my knowledge, the study "AN ASSESSMENT OF THE CLASTOGENIC POTENTIAL OF LP1846 UTILIZING THE MAMMALIAN CELL CYTOGENETICS ASSAY WITH CHO CELLS (LSC-7662-4)" was conducted in general conformance with the applicable Environmental Protection Agency Good Laboratory Practice standards with the following exceptions:

1. Test substance characterization and stability data were not developed at SRI International.

2. Assays to verify concentration, stability, and homogeneity of the test substance in the carrier vehicle were not performed.

These deviations should not affect the results or conclusions of this study.

The duties of SRI's Quality Assurance Unit included verifying the calculations, inspecting laboratory techniques at critical phases of the study, certifying proper identification and notebook entry of samples, and reviewing the Final Report. A quality assurance statement will accompany the Final Report.

After the study was initiated, modifications of the protocol--by either the Testing Laboratory or the Sponsor--were submitted in writing to the other party. All agreed-upon modifications were in the form of Protocol Amendments, which stated the specific modifications and the reasons for the modifications and were signed and dated by the Sponsor's Representative and the Testing Laboratory's Study Director. Protocol Amendment 1 was executed on February 21 1990. Each Party retained an original, signed copy of each Protocol Amendment.

Daniel C. Blachman, Study Director

11/8/91
Date

QUALITY ASSURANCE UNIT
Final Report and
Conflict of Interest Statement

The Quality Assurance Unit of SRI International assures that An Assessment of the Clastogenic Potential of LP1846 Utilizing the Mammalian Cell Cytogenetics Assay with Chinese Hamster Ovary (CHO) Cells, conducted for the U. S. Army Medical Research Acquisition and Activity, was performed in compliance with Good Laboratory Practices as set forth by Toxic Substances Control Act, Environmental Protection Agency.

An inspection was performed on October 19, 1989 during chemical dilution and cell exposure phases. The Study Director was informed of any findings following inspection and SRI Management was informed on October 19, 1989.

Audit of the raw data generated during the study and Draft Final Report Verification were completed February 21, 1990. The Study Director and SRI Management were notified of results on February 22, 1990. The Final Report was reviewed on November 28, 1990 and reaudit was deemed unnecessary. The Final Report accurately describes the methods and Standard Operating Procedures (SOPs) utilized during the study and does reflect the raw data that was generated during the conduct of the study. Any deviations from the protocol or SOPs were made with proper documentation.

This statement certifies that the personnel listed below participated in the inspection and audit of this study. These personnel have not been involved in the generation or evaluation of the data. Participation by the individuals listed below poses no conflict of interest.

Jill E. Kovach

I verify that the above is true to the best of my knowledge.

Jill E Kovach
Quality Assurance Unit

28 November 1990
Date

ACKNOWLEDGMENTS

Key Personnel

The following individuals participated in the conduct of this study:

Daniel C. Blachman	Study Director
Glenn D. Cunningham	Biological Technician
Kathleen E. Garin	Cell Biologist
Kathryn D. Suing	Cell Biologist

The following outside scientist was used in this study:

Barbara E. Stewart	Cytogenetic Technician

Barbara E. Stewart performed cytogenetic analysis on prepared CHO cell slides.

SUMMARY

SRI International assessed the ability of LP1846 to induce chromosomal aberrations (CA) in Chinese hamster ovary (CHO) cells both in the presence and absence of rat S-9 metabolic activation enzymes (MA). In the definitive studies in the absence of MA, CHO cells exposed to LP1846 at concentrations of 62.5, 125.0 and 250.0 µg/ml with an 8- to 10-hour harvest were evaluated microscopically for mitotic indices and for chromosomal aberrations. In the presence of MA, CHO cells exposed to LP1846 concentrations of 625.0, 1250.0, and 2500.0 µg/ml with an 8- to 10- hour harvest were evaluated microscopically for mitotic indices and for chromosomal aberrations. Vehicle and positive controls were included in the study. On the basis of our criteria for interpretation, we conclude that LP1846 induces chromosomal aberrations in the presence but not in the absence of MA under the conditions used in this study.

CONTENTS

INTRODUCTION

Mammalian cell cultures are valuable tools for assessing the clastogenic potential of chemicals (Preston et al., 1981). In such studies, cells are exposed to chemicals with and without rat S-9 metabolic activation enzymes (MA) and are later examined for changes in chromosome structure (Galloway, et al., 1985). Because human chromosomal abnormalities are associated with spontaneous abortion, congenital malformations, neoplasia, and infertility (Hamerton, 1971), and because approximately 0.5% of live human births bear a chromosomal abnormality (Hamerton, 1971; Hook and Hamerton, 1977), the results of chromosomal aberration studies are relevant to the health of present and future generations.

The purpose of this study was to provide data relating to the test article's health or environmental effects that would be of suitable quality and integrity for submission pursuant to section 4 (a) of the Toxic Substances Control Act. This study therefore was conducted in compliance with Environmental Protection Agency 40 CFR Part 792 Good Laboratory Practice Standards.

Testing of LP1846 began on 28 September 1989 (exposure of CHO cell cultures to test article) and was completed on 15 January 1990 (completion of microscopic analysis). Protocol Amendment Number 1 was executed on February 21 1990. Copies of the final report and the raw data will be stored in SRI's Records Center for at least 10 years. The prepared microscope slides will be retained for at least 10 years in SRI's Tissue Storage Facility.

This study was initiated upon execution of the protocol by the study director (signed 24 August 1989) and will be complete upon the study director's signature of the compliance statement of the final report.

MATERIALS

Test Article

- Name: LP1846
- Lot: 1846-03
- Supplier: United States Army
- Date material received at SRI International: 3 July 1989
- Quantity of material received from supplier: 20-ml bottle
- Description: Clear, colorless liquid
- Purity and stability: To be documented by U.S. Army Ballistic Research Laboratory
- Storage conditions: Stored at room temperature in original and secondary containers

Vehicle Control Article

- Name: Sterile, purified deionized water
- CAS No.: 7732-18-5
- Source: *Millipore Super-Q System* (No.123365), located in Building M, Room 212, SRI International.
- Description: Clear, colorless liquid
- Storage conditions: Stored in clear glass bottle at room temperatu

Positive Control Article for the Assay with MA

- Name: Cyclophosphamide (CP; CAS No. 50-18-0)
- Lot: 114F-0393
- Supplier: Sigma Chemical Company
- Date material received at SRI International: 1 April 1986
- Quantity of material received from supplier: 5 g
- Description: White powder
- Purity: 98%
- Stability: Stable when stored as recommended by the manufacturer
- Storage conditions: Stored at approximately 2°C in original and secondary lightproof containers, as recommended by the manufacturer

Positive Control Article for the Assay Without MA

- Name: Methylmethanesulfonate (MMS; CAS No. 66-27-3)
- Lot No.: 102387
- Supplier: Aldrich Chemical Company
- Date material received at SRI International: 29 January 1980
- Quantity of material received from supplier: 25 g
- Description: Clear, colorless liquid
- Purity: 97%
- Stability: Stable when stored as recommended by the manufacturer
- Storage conditions: Stored at approximately 4°C in original and secondary lightproof containers, as recommended by the manufacturer

Cell Culture

- Type of cells: Chinese hamster ovary (CHO) cells, ATCC CCL 61, CHO-K1, proline-requiring
- Source of cells: American Type Culture Collection
  12301 Parklawn Drive
  Rockville, MD 20852

S-9 Preparation

- Animal Type: Rat, Male, Fischer-344, 180-275 g
- Source: Simonsen Laboratories
  1180C Day Road
  Gilroy, CA 95020
- S-9 Lot Number: IR-45

METHODS

Cell Culture

Chinese hamster ovary (CHO) cells were used for *in vitro* cytogenetic testing. The cells were grown in an atmosphere of 5% CO_2 at 37°C in McCoy's 5a medium with 15% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin solution to maintain exponential growth. This medium was also used during exposure of the cells to the test article without metabolic activation. McCoy's 5a medium with 2.5% FBS (containing L-glutamine and penicillin-streptomycin in the above concentrations) was used during the exposure of cells to the test article with MA. Cell cultures were maintained and cell exposures performed in T-75 flasks. Cell cultures used in experiments were labeled with the date that cultures were inoculated, initials of the person who inoculated them, cell concentration at time of inoculation, experiment number, and flask number.

Metabolic Activation

An Aroclor 1254-induced rat liver homogenate preparation (S-9) was the MA system. Liver enzymes were induced by injecting adult male Fischer-344 rats with Aroclor 1254 (500 mg/kg) 5 days before they were sacrificed. The S-9 consisted of 9000 x g supernatant of liver homogenized in sucrose-phosphate buffer (1 g wet weight of liver to 3 ml of sucrose-phosphate buffer). The S-9 was prepared in large lots and stored frozen in liquid nitrogen until used. The MA mixture consisted of one part S-9 to nine parts McCoy's 5a medium containing 2.5% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin solution. Cofactors were added at concentrations of 24 mg NADP and 45 mg sodium isocitrate per milliliter S-9. The MA

mixture was freshly prepared and kept on ice before use. The batch of S-9 used for these studies was designated IR-45.

Dilution of Test Article

Immediately before each assay, the test article was diluted in water to form a series of concentrations that, when diluted in the exposure medium, yielded the appropriate set of test concentrations. The maximum concentration of vehicle (water) added to the exposure medium was 1% (v/v).

Cytotoxicity Study

Preliminary cytotoxicity studies were conducted to determine (a) the most appropriate concentrations of the test article to use in the definitive experiments and (b) the optimal cell fixation time. A series of five concentrations of the test article and a vehicle control were used to assess cytotoxicity, with duplicate flasks being used for each treatment. These studies were conducted both with and without MA at LP1846 concentrations of 8.0, 40.0, 200.0, 1000.0, and 5000.0 μg/ml (Table 1).

The cytotoxicity studies were designed to assess the effects of the test article on cell proliferation (mitotic index, MI) as well as on the kinetics of the cell cycle. The MI was assessed by determining the proportion of cells on a slide that were in metaphase. Cell-cycle kinetics were assessed by determining the number of replications of DNA that occurred after treatment with the test article, using bromodeoxyuridine (BrdU) to differentially label chromatids. In the absence of MA, the test article and 0.01 mM BrdU were added to the cells and the cells were incubated in the dark at 37°C for 8 hours. After exposure, the cells were washed and

incubated in fresh medium containing 0.01 mM BrdU for 13.5 hours. In the presence of MA, the cells were exposed to the test article in the MA mixture for 2 hours at 37°C. After exposure, the cells were washed and incubated in fresh medium containing 0.01 mM BrdU for 21.5 hours.

After incubation in the presence of BrdU, the cells were washed, fresh medium containing 0.4 µg/ml colchicine was added, and the cultures were incubated for an additional 2.5 hours at 37°C. The cells were then harvested as described below (see "Definitive Study"), placed on prepared slides, and stained using a fluorescence-plus-Giemsa technique. For each flask, an overall mitotic index was determined based on at least 1000 cells per flask, and 26 cells in metaphase per flask were classified as M1 or M2 cells based on whether they had progressed through one (M1) or two (M2) cycles of DNA synthesis during the incubation period after exposure to the test article. Under normal circumstances, 80% or more of cells exposed to various vehicle control articles in this laboratory (e.g., Water,DMSO, Ethanol) are M2 cells when using this protocol.

The mitotic index data were analyzed by chi-square methods to assess effects of the test article on overall cell proliferation, with differences being considered significant at the $P < 0.05$ level. Cell-cycle data (i.e., the numbers of M1 and M2 cells) were also analyzed by chi-square methods, again with significance at $P < 0.05$.

Dose Selection for Definitive Study

In the absence of any cytotoxicity, the highest concentration of test article to be used in the definitive assay was 5 mg/ml or less, depending on solubility. The cell cultures in this case were harvested 8 to 10 hours after initiation of exposure to the test

article. If, in the cytotoxicity study, the test article had a significant effect on the overall mitotic index but not on cell-cycle kinetics (e.g., lysis of cells), the highest concentration to be used in the definitive assay was one within a factor of 2 of that which resulted in a significant effect on the mitotic index. The cell cultures in this case were harvested 8 to 10 hours after initiation of exposure to the test article. If the test article had a significant effect on cell-cycle kinetics (i.e., a significantly reduced number of M2 cells and a corresponding increase in M1 cells compared with the vehicle control), the highest concentration used in the definitive assay was one within a factor of 2 of that producing a significant effect on the cell cycle, and the cell culture harvest time was delayed to 24 to 26 hours after initiation of exposure to the test article. If it was not known whether the test article would induce cell-cycle delay sufficient to preclude analysis of cells for chromosomal aberrations, two sets of duplicate cultures were exposed to the test article, with one set (two cultures) being harvested at the earlier time and one set at the later time. The cultures harvested at 8 to 10 hours were cytogenetically analyzed in preference to the 24- to 26-hour cultures, if sufficient metaphases for analysis were available from the cultures harvested at the earlier time. If the harvest time needed to be delayed, extra cultures exposed to the vehicle (i.e., vehicle controls) were included and harvested at the later time.

The times and temperatures reported above (cytotoxicity studies) are approximate.

Definitive Study

For the definitive study, the test article was assayed with and without MA, and duplicate flasks were used for each of ten concentrations of the test article as well as for the positive and

vehicle controls (see study design, Table 2). Cells were harvested at 8 to 10 hours after initiation of treatment in the absence of effects on cell-cycle kinetics or 24 to 26 hours after initiation of treatment if the test article induced cell-cycle delay.

For the experiment performed without MA, the test and control articles were added to fresh complete McCoy's 5a medium and the cells were grown at 37°C for 8 hours. The medium containing the test article was then aspirated, and the cells were washed three times with phosphate-buffered saline (PBS) at 37°C. Fresh complete McCoy's 5a medium containing 0.4 µg/ml colchicine was added to the cultures to be harvested at 8 to 10 hours. For the cultures to be harvested at 24 hours, fresh complete McCoy's medium was added to the cultures for an additional 13.5 hours of incubation at 37°C. Colchicine was then added to a final concentration of 0.4 µg/ml.

For the experiment performed with MA, the cell cultures were exposed to the test article in the MA mixture for 2 hours. This shorter exposure time was used to prevent cytotoxic effects from the MA preparation. After exposure, the medium containing the test article and the MA mixture was aspirated, the cells were washed three times with PBS, and fresh complete McCoy's 5a medium was added. The cultures to be harvested at 8 hours were then incubated for an additional 6 to 8 hours. Cultures to be harvested at 24 hours were incubated for 22 to 24 hours. After the incubation period colchicine was added to a final concentration of 0.4 µg/ml.

After 2.5 hours in colchicine, the cells were harvested. At harvest, the medium, which may have contained dividing cells, was removed and saved. The cultures were rinsed with 2 ml of PBS, and the PBS was pooled with the culture medium. The cultures were then incubated at 37°C with 2 ml of trypsin until the cells detached from the flasks, and the cells in trypsin were pooled with the cells in culture medium and PBS. A final rinse with 1 ml of PBS was

performed, and the rinse solution was added to the pooled cell suspension in culture medium, PBS, and trypsin. This cell suspension was then centrifuged, the supernatant was aspirated, and 4 ml of a hypotonic solution of 0.075 M KCl was added to the cells for 15 minutes at 37°C. The cells were then centrifuged, the supernatant was aspirated and the cells were suspended in a fixative of absolute methanol:glacial acetic acid (3:1). After three changes of fixative, air-dried slides were prepared from all flasks. The slides were stained in 3% Giemsa (Gurr's R66 in M/15 Sorensen's buffer, pH 6.8) for 20 minutes, rinsed in deionized water, and passed through xylene; coverslips were mounted with Permount.

The times and temperatures reported above (definitive studies) are approximate.

Cytogenetic Analysis

Slides prepared from the vehicle controls, the positive controls, and three concentrations of the test article from each activation condition were coded by an individual not involved in the microscopic evaluation. The highest concentration chosen for scoring was one that was within a factor of 2 of that which resulted in significant toxicity; if no toxicity was seen, the highest concentration was 5 mg/ml (solubility permitting). The slides were decoded only after all slides in each group had been analyzed completely.

Two individuals each analyzed separate, coded slides prepared from each flask (2 flasks per experimental point). Slides were evaluated for mitotic index, based on at least 1000 cells/flask, and 50 cells per flask (25 per scorer) were evaluated for chromosomal

aberrations, resulting in a total of 100 cells evaluated per experimental point.

For analysis of the slides, score sheets were used to record the cytogeneticist's initials, date, microscope used, coded slide number, project number, quality of the slide, vernier settings, MI, and numbers of various categories of chromatid-type and chromosome-type aberrations for each cell scored. SRI's classification of aberrations is based on definitions given by Savage (1975). After analysis was completed, the slides were decoded and the score sheets were summarized.

Statistical Analysis

Data from the definitive studies in the presence and in the absence of metabolic activation were analyzed separately.

The following statistics were calculated for each treatment: MI, the total number of chromosomal aberrations and the frequency of chromosomal aberrations per cell, the frequency of aberrant cells, the number and frequency of aberrations in each category, the number and frequency of cells with structurally aberrant chromosomes, the number and frequency of polyploid cells, and the number and frequency of severely damaged cells (i.e., cells with ≥ 5 chromosome aberrations). The number of cells with structural chromosomal damage and the total number of structural chromosomal aberrations observed in the test article and positive control treatment groups were statistically compared with those in the vehicle control, using Pearson chi-square with a significance level of $\alpha = 0.05$. SRI proprietary software and software purchased from the SAS Institute Inc. (Cary, NC) were used for statistical analysis.

A valid chromosomal aberration assay was defined as one in which (1) the chromosomal aberration frequencies in the positive control were significantly ($P < 0.05$) elevated above those in the vehicle control and (2) the highest concentration evaluated in the definitive study was within a factor of 2 of the level that resulted in a significant effect on the cell cycle or the mitotic index.

Criteria for Interpretation

Positive. A test article was considered to have elicited a positive response in the in vitro CHO cytogenetic assay if the frequency of cells with structural chromosomal damage or the frequency of structural chromosomal aberrations per cell was significantly greater ($p < 0.05$) in the test-article-treated cells than in the vehicle control cells. A positive response also required a dose-related increase in chromosomal abnormalities.

Negative. A test article was considered to have elicited a negative response if neither criterion for a positive response was met.

Inconclusive. The results of this assay were considered inconclusive if there was reason to believe that the concentrations of the test article selected for evaluation were inappropriate (i.e., lack of or excessive cytotoxicity) or if there was an elevation in chromosomal abnormalities that was statistically significant but not dose-related.

RESULTS AND DISCUSSION

Cytotoxicity Studies

Data from the cytotoxicity studies of LP1846 are summarized in Table 1. In the experiment conducted in the both the absence and presence of MA, the media became yellow upon the addition of the test article. With increased dose the yellow color became more intense, as in the 1,000 and 5,000 μg/ml cultures, which were pale-yellow and medium-yellow, respectively, when compared to the reddish color of the untreated media. This indicates that the test article had acidified the media, (changing the color of the phenol indicator in the media). The acidification apparently increased with increasing test article dose, as indicated by the color change of the pH indicator.

In the cytotoxicity study conducted in the absence of MA, a significant decrease in MI was seen at a concentration of 1000.0 μg/ml LP1846 (MI of 0.0%, compared with 4.8% for the vehicle control). No cells were present in cultures treated with a concentration of 5000.0 μg/ml LP1846 in the absence of metabolic activation. Retardation of the cell cycle was noted in cultures treated with LP1846 at concentrations of 200.0 and 1000.0 μg/ml (these doses had 98.1% and 75.0% metaphase cells in M1 metaphase, compared with 3.8% in the concurrent vehicle control cultures). On the basis of these findings, LP1846 concentrations of 31.3, 62.5, 125.0, 250.0, and 500.0 μg/ml were chosen for the definitive study without MA with an 8- to 10-hour harvest, and concentrations of 250.0 and 500.0 μg/ml were chosen for the definitive study without MA with a 24- to 26-hour harvest.

In the cytotoxicity study conducted in the presence of MA, a significant decrease in MI was seen at 5000.0 μg/ml. Retardation of the cell cycle was also noted in cultures treated with 5000.0 μg/ml

LP1846 (67.9% M1 metaphase cells seen, compared with 0.0% in the concurrent vehicle control cultures). Based on these findings, LP1846 concentrations of 312.5, 625.0, 1250.0 and 2500.0 µg/ml were chosen for the definitive study with MA at an 8- to 10-hour harvest, and concentrations of 1250.0, 2500.0 and 5000.0 µg/ml were chosen for the definitive study with MA at a 24- to 26-hour harvest. The experimental design for the definitive study is summarized in Table 2.

Definitive Studies

Data from the definitive cytogenetic studies of LP1846 are summarized in Tables 3 and 4. Two measures of cytogenetic damage were evaluated statistically: the percentage of structurally aberrant cells and the frequency of structural aberrations per cell. When it was not known at what exact dose level the cytogenetic evaluation of cultures would be prevented due to cell cycle delay, two sets of cultures were used, with one set harvested at 8 hours and one set at 24 hours. The cultures harvested at 8 hours were cytogenetically evaluated in preference to those harvested at 24 hours if both were available for analysis.

In the absence of MA, the 500 µg/ml dose group (24 hour harvest) was not acceptable for analysis due to complete toxicity. The three highest concentrations of LP1846 acceptable for analysis were 62.5, 125.0, and 250.0 µg/ml (8- to 10-hr harvest). A large decrease in MI was seen at 125.0 and 250.0 µg/ml relative to the concurrent (8 hr) vehicle control (2.2% and 0.6%, respectively vs 5.7%), indicating significant toxicity. Chi-square analysis revealed no significant differences among the vehicle-control- and LP1846-treated dose groups in the percentage of structurally aberrant cells (Table 3). Chi-square analysis similarly revealed no significant differences among the vehicle-control- and LP1846-

treated dose groups in the frequency of structural aberrations per cell (Table 3).

In the presence of MA, the 5,000 µg/ml dose group (24 hour harvest) was not acceptable for analysis due to complete toxicity. In the 625.0, 1250.0 and 2500.0 µg/ml dose groups, the MA mixture became yellow upon the addition of the test article. This indicates that the test article had acidified the MA mixture, (changing the color of the phenol indicator in the media). The three highest concentrations of LP1846 acceptable for analysis were 625.0, 1250.0 and 2500.0 µg/ml (8- to 10-hr harvest). Some decrease in MI was seen in the 625.0 and 1250.0 µg/ml dose groups (5.5% and 5.1% MI compared to 7.9% in the concurrent vehicle control). A large decrease in MI was seen at 2500 ug/ml LP1846 relative to the 8-hr vehicle control (0.9% vs 7.9%, respectively), indicating toxicity. Chi-square analysis revealed a significant difference between the vehicle-control-treated cells and those treated with 2500 µg/ml LP1846 in the percentage of structurally aberrant cells and the frequency of structural aberrations per cell (Table 4).

The positive control articles in both the presence and the absence of MA induced statistically significant increases in chromosomal damage as compared with the vehicle controls.

Based on our criteria for interpretation, as specified in the protocol, we conclude that under the conditions used in this study, LP1846 induces chromosomal aberrations in CHO cells in the presence but not in the absence of MA.

The dose-response of LP1846 in the chromosomal aberration assay was not defined, due to the limited number of doses available for analysis in the cytogenetic assay. The increase in chromosomal aberrations seen in the 2500 µg/ml dose group is considered to be a dose-related, statistically significant increase in chromosomal

aberrations. However, the increase in chromosomal aberrations was seen only at the highest dose analyzed in the presence of MA, a dose that showed significant toxicity as measured by mitotic index. This dose also showed an acidification of the MA mixture, as indicated by the color change upon addition of the test article. It has been reported in the literature that extremes of pH and osmolarity can cause chromosomal aberrations (Brusick, D. J. ,1987; Galloway, S. M., et.al, 1987). Additionally, the clastogenic activity of the metabolic activation mixture may have been enhanced by the lower pH in the LP1846 treatment groups (Brusick, D. J. ,1987). Further studies to define the dose response, along with studies to examine the osmolarity and pH effects of the test article on the test system would help to clarify the response seen in this study.

Table 1

CYTOTOXICITY STUDIES OF LP1846 IN CHO CELLS
WITH AND WITHOUT METABOLIC ACTIVATION

Treatment[a]	Conc. (µg/ml)	Time (hr)	MA	Mean Mitotic Index (%)	Mean % Metaphases in M1	Mean % Metaphases in M2
Without MA						
Vehicle Control (1% WATER)	0.0	24	-	4.8	3.8	96.2
LP1846	8.0	24	-	4.9	0.0	100.0
LP1846	40.0	24	-	3.5	1.9	98.1
LP1846	200.0	24	-	8.0	98.1	1.9
LP1846	1000.0	24	-	0.0[b,c]	75.0[b,c]	25.0[b,c]
LP1846	5000.0	24	-	0.0	---	---
With MA						
Vehicle Control (1% WATER)	0.0	24	+	6.7	0.0	100.0
LP1846	8.0	24	+	5.4	0.0	100.0
LP1846	40.0	24	+	5.0	1.9	98.1
LP1846	200.0	24	+	5.1	3.8	96.2
LP1846	1000.0	24	+	4.7	3.8	96.2
LP1846	5000.0	24	+	2.2[c]	67.9[c]	32.1[c]

[a] Two flasks used per concentration of LP1846 and for vehicle controls.
[b] Fewer than 10 metaphase cells available for cell cycle analysis.
[c] Significantly less than concurrent vehicle control ($P < 0.05$).

Table 2

EXPERIMENTAL DESIGN OF THE DEFINITIVE CYTOGENETIC ASSAY OF LP1846 PERFORMED IN THE PRESENCE AND ABSENCE OF EXOGENOUS METABOLIC ACTIVATION (8-10 & 24-26 hr HARVEST)

Treatment	Harvest Time (hr)	Flasks/ Treatment	Cells Analyzed/ Flask	Total Cells Analyzed
LP1846 (μg/ml)				
-Without MA				
31.3	8-10	2		
62.5*	8-10	2	50 for each	100 for each
125.0*	8-10	2	of the marked	of the marked
250.0*	8-10	2	(*) doses	(*) doses
500.0	8-10	2		
250.0	24-26	2		
500.0	24-26	2		
-With MA				
312.5	8-10	2		
625.0*	8-10	2	50 for each	100 for each
1250.0*	8-10	2	of the marked	of the marked
2500.0*	8-10	2	(*) doses	(*) doses
1250.0	24-26	2		
2500.0	24-26	2		
5000.0	24-26	2		
Vehicle Control				
-Without MA	8-10	2	50	100
(1% water)	24-16	2		
-With MA	8-10	2	50	100
(1% water)	24-26	2		
Positive Control				
-Without MA				
(110.0 μg/ml MMS)	8-10	2	50	100
-With MA				
(110.0 μg/ml CP)	8-10	2	50	100

TABLE 3

CYTOGENETIC EVALUATION OF CHO CELLS EXPOSED TO LP1846
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION (8- to 10-hr harvest)

Treatment Concentration	Solvent Control 1% Water	LP1846 62.50 µg/ml	LP1846 125.00 µg/ml	LP1846 250.00 µg/ml	Positive Control 110.00 µg/ml MMS
Mitotic index (%)	5.7	4.7	2.2	0.6	4.2
Number of cells analyzed	100	100	100	100	100
Number (%) aberrant cells	5(5.0)	1(1.0)	6(6.0)	5(5.0)	16(16.0)
Number (%) cells with* structurally abnormal chromosomes	5(5.0)	1(1.0)	6(6.0)	5(5.0)	16(16.0)+
Number (%) cells with:					
Chromosome deletions$^{\Phi}$	2(2.0)	1(1.0)	1(1.0)	5(5.0)	2(2.0)
Chromosome exchanges$^{\Phi}$	0	0	0	0	0
Chromatid deletions$^{\Phi}$	3(3.0)	0	6(6.0)	2(2.0)	13(13.0)
Chromatid exchanges$^{\Phi}$	0	0	0	0	3(3.0)
Polyploidy$^{\Phi}$	0	0	0	0	0
Severe Damage	0	0	0	0	0
Number (frequency/cell) of aberrations:					
Overall aberrations	5(0.05)	1(0.01)	8(0.08)	8(0.08)	19(0.19)
Structural aberrations*	5(0.05)	1(0.01)	8(0.08)	8(0.08)	19(0.19)+
Chromosome deletions$^{\Phi}$	2(0.02)	1(0.01)	1(0.01)	6(0.06)	2(0.02)
Chromosome exchanges$^{\Phi}$	0	0	0	0	0
Chromatid deletions$^{\Phi}$	3(0.03)	0	7(0.07)	2(0.02)	14(0.14)
Chromatid exchanges$^{\Phi}$	0	0	0	0	3(0.03)
Number (%) cells with:					
Chromatid gaps$^{\Phi}$	0	2(2.0)	4(4.0)	3(3.0)	8(8.0)
Isochromatid gaps$^{\Phi}$	0	1(1.0)	1(1.0)	0	0

* No significant differences were noted among the solvent control and LP1846 treated cells ($p > 0.05$).
\+ The positive control was significantly higher than the concurrent solvent control ($p < 0.05$)
$^{\Phi}$ The denominator used in calculating these statistics was the total number of cells analyzed minus the number of cells with severe damage.

TABLE 4

CYTOGENETIC EVALUATION OF CHO CELLS EXPOSED TO LP1846
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION (8- to 10-hr harvest)

Treatment Concentration	Solvent Control 1% Water	LP1846 625.00 µg/ml	LP1846 1250.00 µg/ml	LP1846 2500.00 µg/ml	Positive Control 110.00 µg/ml CP
Mitotic index (%)	7.9	5.5	5.1	0.9	0.6
Number of cells analyzed	100	100	100	100	100
Number (%) aberrant cells	1(1.0)	2(2.0)	1(1.0)	18(18.0)	32(32.0)
Number (%) cells with structurally abnormal chromosomes	1(1.0)	2(2.0)	1(1.0)	18(18.0)*	32(32.0)+
Number (%) cells with:					
Chromosome deletions$^{\Phi}$	0	0	0	7(7.0)	6(6.3)
Chromosome exchanges$^{\Phi}$	0	1(1.0)	0	0	1(1.0)
Chromatid deletions$^{\Phi}$	1(1.0)	1(1.0)	1(1.0)	12(12.0)	14(14.6)
Chromatid exchanges$^{\Phi}$	0	0	0	0	11(11.5)
Polyploidy$^{\Phi}$	0	0	0	0	0
Severe Damage	0	0	0	0	4(4.0)
Number (frequency/cell) of aberrations:					
Overall aberrations	1(0.01)	2(0.02)	2(0.02)	19(0.19)	55(0.55)
Structural aberrations	1(0.01)	2(0.02)	2(0.02)	19(0.19)*	55(0.55)+
Chromosome deletions$^{\Phi}$	0	0	0	7(0.07)	6(0.06)
Chromosome exchanges$^{\Phi}$	0	1(0.01)	0	0	1(0.01)
Chromatid deletions$^{\Phi}$	1(0.01)	1(0.01)	2(0.02)	12(0.12)	16(0.17)
Chromatid exchanges$^{\Phi}$	0	0	0	0	12(0.13)
Number (%) cells with:					
Chromatid gaps$^{\Phi}$	1(1.0)	2(2.0)	2(2.0)	9(9.0)	8(8.3)
Isochromatid gaps$^{\Phi}$	0	1(1.0)	0	2(2.0)	3(3.1)

*Significant differences were noted between the solvent control and LP1846-treated cells ($p < 0.001$).
+ The positive control was significantly higher than the concurrent solvent control ($p < 0.001$).
$^{\Phi}$ The denominator used in calculating these statistics was the total number of cells analyzed minus the number of cells with severe damage.

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