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CHEMICAL MANUFACTURERS ASSOCIATION

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FYI-



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Dr. Lynn Goldman  
Assistant Administrator  
Office of Prevention, Pesticides and Toxic Substances TS-7101  
Environmental Protection Agency  
401 M Street, SW, Room 637, East Tower  
Washington, DC 20460

**Contains No CBI**

Dear Dr. Goldman: \_\_\_\_\_

The Chemical Manufacturers Association makes available to the public and appropriate government agencies final reports of environmental, health and safety research that it manages. In keeping with this policy, the following recently completed reports are enclosed:

PENTABROMODIPHENYL OXIDE: Chromosome Aberrations In Human Peripheral Blood Lymphocytes

This report does not include confidential information.

If you have any questions, please call Has Shah of my staff at 703-741-5637.

Sincerely,

Enclosure



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**FINAL REPORT**

Study Title

**CHROMOSOME ABERRATIONS IN  
HUMAN PERIPHERAL BLOOD LYMPHOCYTES**

Test Article

Pentabromodiphenyl Oxide

Authors

Ramadevi Gudi, Ph.D.  
Elizabeth H. Schadly, B.S.

Study Completion Date

December 12, 1996

Performing Laboratory

Microbiological Associates, Inc.  
9630 Medical Center Drive  
Rockville, Maryland 20850

Laboratory Study Number

G96AO63.342

Sponsor

Chemical Manufacturers Association  
1300 Wilson Boulevard  
Arlington, VA 22209

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## STATEMENT OF COMPLIANCE

Study, G96AO63.342 was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.

The stability of the test or control article under the test conditions was not determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

Ramadevi Gudi  
Ramadevi Gudi, Ph.D.  
Study Director

12/12/96  
Date

QUALITY ASSURANCE STATEMENT

Study Title: CHROMOSOME ABERRATIONS IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES  
Study Number: G96A063.342  
Study Director: Ramadevi Gudi, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07 JUN 96, TO STUDY DIR 07 JUN 96, TO MGMT 07 JUN 96  
PHASE: Protocol Review

INSPECT ON 11 JUN 96, TO STUDY DIR 11 JUN 96, TO MGMT 24 JUN 96  
PHASE: Preparation of cell cultures

INSPECT ON 12 SEP 96, TO STUDY DIR 12 SEP 96, TO MGMT 24 SEP 96  
PHASE: Draft Report

INSPECT ON 06 NOV 96, TO STUDY DIR 06 NOV 96, TO MGMT 11 NOV 96  
PHASE: Draft to Revised Draft Report

INSPECT ON 12 DEC 96, TO STUDY DIR 12 DEC 96, TO MGMT 12 DEC 96  
PHASE: Revised Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Claire L. Courtemanche  
Claire L. Courtemanche  
QUALITY ASSURANCE

Dec 12, 1996

DATE

**CHROMOSOME ABERRATIONS IN  
HUMAN PERIPHERAL BLOOD LYMPHOCYTES**

**FINAL REPORT**

**Sponsor: Chemical Manufacturers Association  
1300 Wilson Boulevard  
Arlington, VA 22209**

**Authorized Representative: Dr. Hasmukh C. Shah**

**Performing Laboratory: Microbiological Associates, Inc. (MA)  
9630 Medical Center Drive  
Rockville, Maryland 20850**

**Test Article I.D.: Pentabromodiphenyl Oxide**

**MA Study No.: G96AO63.342**

**Test Article Description: pale yellow sticky liquid**

**Storage Conditions: room temperature, protected from light**

**Test Article Receipt: May 21, 1996 (sample 1), August 21, 1996 (sample 2)**

**Study Initiation: June 5, 1996**

**Laboratory Supervisor: Elizabeth H. Schadly, B.S.**

**Study Director:** *Ramadevi Gudi* 12/12/96  
Ramadevi Gudi, Ph.D. Date

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## SUMMARY

The test article, Pentabromodiphenyl Oxide, was tested in the *in vitro* mammalian cytogenetic test using human peripheral blood lymphocytes (HPBL) both in the absence and presence of metabolic activation. The assay was performed in two phases. The first phase, the initial chromosome aberration assay, was conducted to establish the dose range for testing and to evaluate the clastogenic potential of the test article. The second phase, the independent repeat chromosome aberration assay, was performed to confirm the test system response to the test article seen in the initial assay.

Dimethylsulfoxide (DMSO) was the solvent of choice based on information provided by the Sponsor, the solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at approximately 500 mg/ml, the maximum concentration tested.

In the initial chromosome aberration assay, the maximum dose tested was 2500 µg/ml. This dose was achieved using a stock concentration of 250 mg/ml, and a 100 µl dosing aliquot added to 10 ml fresh complete medium or S9 reaction mix. Test article concentrations greater than 2500 µg/ml were insoluble in treatment medium and were not used in the test system. Visible precipitate was observed in treatment medium at dose levels 750 and 2500 µg/ml and the test article was soluble but cloudy in treatment medium (no visible precipitate) at dose level 250 µg/ml. The test article was soluble in treatment medium at all other dose levels tested. In the non-activated portion of the initial assay HPBL cells were exposed to the test article continuously for 20 hours; in the S9-activated portion of the initial chromosome aberration assay, HPBL cells were exposed to the test article for 4 hours. Metaphase cells were collected for microscopic evaluation at 20 hours after the initiation of treatment. Toxicity (mitotic inhibition) of approximately 47% was observed at the highest dose level (250 µg/ml) evaluated for chromosome aberrations, in the non-activated study. In the S9-activated study, no toxicity was observed at the highest dose level (2500 µg/ml) evaluated for chromosome aberrations. No statistically significant increases in chromosome aberrations were observed in the non-activated test system relative to the solvent control group ( $p > 0.05$ , Fisher's exact test). A statistically significant increase in chromosome aberrations was observed in the S9-activated test system relative to the solvent control group at the high dose level only, 2500 µg/ml ( $p < 0.05$ , Fisher's exact test). The Cochran-Armitage test was positive for a dose-response trend ( $p \leq 0.5$ ).

Based on the results of the initial assay, an independent repeat chromosome aberration assay was conducted in the absence of a metabolic activation system at dose levels of 32, 63, 125, 250, and 500 µg/ml and in the presence of an Aroclor-induced S9 metabolic activation system at dose levels of 313, 625, 1250, 2500, and 3750 µg/ml. The test article was soluble but cloudy in treatment medium at dose levels 250, 313, 500, and 625 µg/ml, was workable in treatment medium at dose levels 1250 and 2500 µg/ml, and was insoluble in treatment medium at dose level 3750 µg/ml. The test article was soluble in treatment medium at all other concentrations tested. In the independent repeat assay, HPBL cells were exposed to the test article continuously for 20 or 44 hours in the non-activated test

system and for 4 hours in the S9-activated test system. Metaphase cells were collected for microscopic evaluation in both the non-activated and S9-activated studies at 20 and 44 hours after the initiation of treatment. Toxicity, measured by mitotic inhibition, was approximately 50% and 79% at the 20 and 44 hour harvests, respectively, at the highest dose level (500 µg/ml) evaluated for chromosome aberrations in the non-activated studies. In the S9-activated studies, toxicity was approximately 44% and 85% at the 20 and 44 hour harvests, respectively, at the highest dose levels (1250 and 3750 µg/ml) evaluated for chromosome aberrations. Dose levels 2500 and 3750 µg/ml in the 20 hour harvest were not analyzed due to no scorable metaphase cells. Therefore only three test article dose levels were scored in the S9 activated 20 hour harvest. This constitutes a deviation from the protocol. This deviation was documented in the raw data with a deviation report and was concluded by the Study Director to have no significant effect on the integrity of the study. No statistically significant increases in structural chromosome aberrations were observed in either the non-activated or S9-activated studies, regardless of dose level or harvest time ( $p > 0.05$ , Fisher's exact test). No statistically significant increases in numerical chromosome aberrations were observed in either the non-activated or S9-activated studies at the 44 hour harvest time, regardless of dose level ( $p > 0.05$ , Fisher's exact test).

A weak positive response was observed in the initial assay at the highest dose level only (2500 µg/ml) in the presence of S9. The response of 4% aberrant cells was barely outside the range of the historical solvent control (0-3%) and within the criteria for the determination of a valid test for the untreated and solvent controls (6%). The 1% increase over the historical solvent control range is considered to have no biological significance. In addition, in the independent repeat assay, although no scorable metaphases were observed at this dose level at the 20 hour harvest time, there was no significant response seen at this dose level and at the next highest dose level at the 44 hour harvest. Based on these findings of the study, Pentabromodiphenyl Oxide was concluded to be negative for the induction of structural and numerical chromosome aberrations in human peripheral blood lymphocytes.

## PURPOSE

The purpose of this study was to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in human peripheral lymphocytes.

## CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Pentabromodiphenyl Oxide, was received by Microbiological Associates, Inc. on May 21, 1996 (sample 1) and on August 21, 1996 (sample 2) and was assigned the code number 96AO63. Samples 1 and 2 were composites that were supplied by Wildlife International. The test article was characterized by the Sponsor as a clear, amber, dense, very viscous liquid. No expiration date was provided. Upon receipt, the test article was described as a pale yellow sticky liquid and was stored at room temperature, protected from exposure to light. The solvent used to deliver Pentabromodiphenyl Oxide to the test system was DMSO (CAS No.: 67-68-5), supplied by the Fisher Scientific Company.

Mitomycin C (MMC; CAS No.:50-07-7), was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 12.5 and 25 µg/ml for use as the positive control in the non-activated test system. Cyclophosphamide (CP; CAS No.:6055-19-2), was obtained from Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 2.5 and 5 mg/ml for use as the positive control in the S9-activated test system. For each positive control one dose with sufficient scorable metaphase cells was selected for analysis. The solvent for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. RPMI-1640 complete medium (containing 15% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin and 100 µg streptomycin/ml) supplemented with 1% PHA, or S9 reaction mixture was used in the untreated control.

## MATERIALS AND METHODS

### Test System

Peripheral blood lymphocytes were obtained from healthy 39 and 40 year old adult females with no recent history of either radiotherapy, viral infection or the administration of drugs. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston, et al., 1981).

### Activation System

Aroclor 1254-induced rat liver S-9 was used as the metabolic activation system. The S-9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg. five days prior to sacrifice. The S-9 was batch prepared and stored at  $\leq -70^{\circ}\text{C}$  until used. Each bulk preparation of S-9 was assayed for

its ability to metabolize 2-aminoanthracene and 7,12-dimethyl-benz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S-9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 μl S-9 per milliliter medium (RPMI 1640 serum-free medium supplemented with 100 units penicillin and 100 μg streptomycin/ml, and 2 mM L-glutamine).

### Solubility Test

A solubility test was conducted using dimethylsulfoxide (DMSO) which was selected by the Sponsor as the solvent of choice. The test was performed to determine the level of solubility of the test article in DMSO, which permitted preparation of the highest soluble or workable stock concentration, up to 500 mg/ml.

### Chromosome Aberration Assays (Initial and Independent Repeat)

The chromosome aberration assay was performed using standard procedures (Evans, 1976), by exposing duplicate cultures of HPBL to nine concentrations of the test article as well as positive, solvent and untreated controls. In the initial assay, the dividing cells were harvested at approximately 20 hours from the initiation of treatment.

The independent repeat chromosome aberration assay was performed by exposing duplicate cultures of HPBL to at least four concentrations of the test article as well as positive, solvent and untreated controls. The concentrations tested were selected based on the findings of the initial assay. For the independent repeat assay, the dividing cells were harvested at two time points, 20 and 44 hours after the initiation of treatment.

For the chromosome aberration assays, 0.6 ml heparinized blood was inoculated into centrifuge tubes containing 9.4 ml complete medium supplemented with 1% PHA. The tubes were incubated at 37±1°C in a humidified atmosphere of 5±1% CO<sub>2</sub> in air for approximately 44-48 hours. Treatment was carried out by refeeding with approximately 10 ml fresh complete medium or S9 reaction mixture to which was added 100 μl of dosing solution of test or control article in solvent or solvent alone. An untreated control, consisting of cells in complete medium or S9 reaction mixture was also included.

In the non-activated study, the cells were exposed for 20 or 44 hours at 37±1°C in a humidified atmosphere of 5±1% CO<sub>2</sub> in air. In the S9-activated studies, the cells were exposed for 4 hours at 37±1°C in a humidified atmosphere of 5±1% CO<sub>2</sub> in air. After the exposure period, the treatment medium was removed, the cells washed with calcium and magnesium free-phosphate buffered saline (CMF-PBS), refeed with complete medium containing 1% PHA and returned to the incubator for an additional 16 hours for the first harvests or 40 hours for the delayed harvest. For all treatment groups, two hours prior to the scheduled cell harvests at 20 or 44 hours after treatment initiation, Colcemid® was added to the cultures at a final concentration of 0.1 μg/ml.

### Collection of Metaphase Cells

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the activated and non-activated studies by centrifugation. The cells were collected by centrifugation at approximately 1200 rpm for about 5 minutes. The cell pellet was resuspended in 5 ml 0.075 M KCl and incubated at  $37\pm 1^\circ\text{C}$  for 20 minutes. At the end of the KCl treatment and immediately prior to centrifuging, the cells were gently mixed and approximately 0.5 ml of fixative (methanol:glacial acetic acid, 3:1 v/v) was added to each tube. The cells were collected by centrifugation, the supernatant aspirated, and the cells were fixed with two washes with approximately 3-5 ml fixative and stored in fixative overnight or longer at approximately 2-6°C.

### Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 1200 rpm for 5 minutes, the supernatant fluid was aspirated, and the cells were resuspended in 1 ml cold fresh fixative. The cells were collected by centrifugation and the supernatant aspirated, leaving 0.1 to 0.3 ml fixative above the cell pellet. An aliquot of cell suspension was dropped onto a glass slide and allowed to air dry overnight. Slides were identified by the study number, dose level, activation condition, rest time, replicate tube designation and date prepared. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

### Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 46 centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate treatment condition) were examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (>10 aberrations) also were recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted. In the delayed harvests, the percent polyploid cells was recorded per 100 metaphase cells.

## Controls

Mitomycin C was used as the positive control in the non-activated study at final concentrations of 0.13 and 0.25  $\mu\text{g/ml}$ . Cyclophosphamide was used as the positive control in the S-9 activated study at final concentrations of 25 and 50  $\mu\text{g/ml}$ . For both positive controls one dose with sufficient scorable metaphase cells was selected for analysis. The solvent vehicle for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Growth medium or S9 reaction mixture was used in the untreated control.

## Evaluation of Test Results

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and are presented for both the initial and the independent repeat chromosome aberration assays. The number and types of aberrations, the percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined, the percentage of numerically damaged cells in the total population of cells examined, and the frequency of structural aberrations per cell (mean aberrations per cell) was reported for each treatment group. The mean and standard deviation are presented in the data tables and included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage test was used to determine dose-responsiveness.

All conclusions were based on sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentages of cells with aberrations are increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ( $p \leq 0.05$ ). A reproducible and significant increase at a single dose level will be considered positive. The test article was concluded to be negative if no statistically significant increase was observed relative to the solvent control.

## Criteria for Determination of a Valid Test

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ( $p \leq 0.05$ , Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

## Record and Specimen Archives

Upon completion of the final report, all raw data, reports, and stained and coded slides are maintained in the archives of Microbiological Associates, Inc. located in Rockville, Maryland.

## RESULTS AND DISCUSSION

### Solubility Test

Dimethylsulfoxide (DMSO) was the solvent of choice based on information provided by the Sponsor, the solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at approximately 500 mg/ml, the maximum concentration tested.

### Initial Assay

In the initial chromosome aberration assay HPBL were exposed to nine concentrations of test article ranging from 0.25 µg/ml to 2500 µg/ml. The test article was soluble but cloudy in treatment medium at dose level 250 µg/ml and was workable in treatment medium at dose levels 750 µg/ml and higher. Test article concentrations greater than 2500 µg/ml were insoluble in treatment medium and were not used in the test system. The test article was soluble in treatment medium at all other dose levels tested. The osmolality and pH of the highest concentration tested, 2500 µg/ml, were 365 mmol/kg and approximately 7.5, respectively. The osmolality of the solvent, DMSO, was 384 mmol/kg. Metaphase cells were collected for microscopic evaluation at 20 hours after the initiation of treatment.

The activity of Pentabromodiphenyl Oxide in the induction of chromosome aberrations in HPBL was tested following a 20 hour exposure period in the absence of an exogenous source of metabolic activation. The findings of the cytogenetic analysis are presented by treatment culture in Table 1 and summarized by group in Table 3. Concentrations of 7.5, 25, 75 and 250 µg/ml were evaluated microscopically for structural chromosome aberrations. At the highest test concentration evaluated (250 µg/ml) the mitotic index was reduced 47% relative to the solvent control. The lower concentrations 0.25, 0.75 and 2.5 µg/ml were tested as a guard against excessive toxicity at higher dose levels but were not required for analysis. The higher concentrations 750 and 2500 µg/ml were not selected for analysis due to an insufficient number of scorable cells. The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control at any dose level ( $p > 0.05$ , Fisher's exact test). The percentage of aberrant cells in the MMC group was 6% ( $p \leq 0.01$ , Fisher's exact test).

The activity of Pentabromodiphenyl Oxide in the induction of chromosome aberrations in HPBL cells was tested using a 4 hour exposure and a 16 hour recovery period in the presence of an exogenous source of metabolic activation. The findings of the cytogenetic analysis are presented by treatment flask in Table 2 and summarized by

group in Table 3. Concentrations of 75, 250, 750 and 2500 µg/ml were evaluated microscopically for structural chromosome aberrations. At the highest test concentration evaluated (2500 µg/ml) the mitotic index was reduced 14% relative to the solvent control. The lower concentrations (0.25, 0.75, 2.5, 7.5 and 25 µg/ml) were tested as a guard against excessive toxicity at higher dose levels but were not required for analysis. The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control at the high dose level only, 2500 µg/ml ( $p \leq 0.05$ , Fisher's exact test). The Cochran-Armitage test was positive for a dose-response trend ( $p \leq 0.05$ ). The percentage of aberrant cells in the CP group was 12.5% ( $p \leq 0.01$ , Fisher's exact test).

### Independent Repeat Assay

Based on the results of the initial assay, concentrations of 32, 63, 125, 250, and 500 µg/ml were selected for further study in the non-activated portion of the independent repeat assay with 20 and 44 hour cell harvest times. In the S9-activated study, concentrations of 313, 625, 1250, 2500, and 3750 µg/ml were selected for further study in the independent repeat assay using a 4 hour exposure period with 20 and 44 hour harvest times. The test article was soluble but cloudy in treatment medium at concentrations 250, 313, 500 and 625 µg/ml, was workable in treatment medium at concentrations of 1250 and 2500 µg/ml, and was insoluble in treatment medium at concentration 3750 µg/ml. The test article was soluble in treatment medium at all other concentrations tested. The osmolality of the highest concentration (3750 µg/ml) tested was 387 mmol/kg. The osmolality of the solvent, DMSO, was 412 mmol/kg. The pH of the highest concentration tested was approximately 7.

The findings of the cytogenetic analysis for groups tested in the absence of an exogenous source of metabolic activation are presented by treatment culture in Tables 4 and 5 and summarized by group in Table 8. Concentrations of 63, 125, 250 and 500 µg/ml were evaluated microscopically for structural chromosome aberrations. At the highest test concentration evaluated, 500 µg/ml, the mitotic indices were reduced 50% and 79% relative to the solvent control at the 20 hour and 44 hour harvests, respectively. The lower concentration 32 µg/ml was tested as a guard against excessive toxicity at higher dose levels but was not required for analysis. The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control at any dose level or harvest time ( $p > 0.05$ , Fisher's exact test). The percentage of cells with numerical aberrations in the test article-treated groups was not significantly increased above that of the solvent control at the 44 hour harvest time, regardless of dose level ( $p > 0.05$ , Fisher's exact test). The percentages of aberrant cells in the MMC groups were 13% and 40.5% ( $p \leq 0.01$ , Fisher's exact test) for the 20 and 44 hour harvest times, respectively.

The findings of the cytogenetic analysis for groups tested in the presence of an exogenous source of metabolic activation are presented by treatment flask in Tables 6 and 7 and summarized by group in Table 8. Concentrations of 313, 625 and 1250 µg/ml at the 20 hour harvest and 625, 1250, 2500 and 3750 µg/ml at the 44 hour harvest were evaluated microscopically for structural chromosome aberrations. The lower concentration

313 µg/ml was tested as a guard against excessive toxicity at higher dose levels but was not required for analysis. At the highest test concentrations evaluated for chromosome aberrations, 1250 and 3750 µg/ml, in the 20 and 44 hour harvests, respectively, the mitotic indices were reduced 44% and 85% relative to the solvent control. Concentrations 2500 and 3750 µg/ml in the 20 hour harvest were not analyzed due to the absence of scorable metaphase cells. Therefore only three test article concentrations were scored in the S9 activated 20 hour harvest. This constitutes a deviation from the protocol. This deviation was documented in the raw data with a deviation report and was concluded by the Study Director to have no significant effect on the integrity of the study. The percentage of cells with structural aberrations in the test article-treated groups was not statistically increased above that of the solvent control at any dose level or harvest time ( $p > 0.05$ , Fisher's exact test). The percentage of cells with numerical aberrations in the test article-treated groups was not statistically increased above that of the solvent control at the 44 hour harvest time, regardless of dose level ( $p > 0.05$ , Fisher's exact test). The percentages of aberrant cells in the CP groups were 14.5% and 17% ( $p \leq 0.01$ , Fisher's exact test) for the 20 and 44 hour harvests, respectively.

#### CONCLUSION

The positive and negative controls fulfilled the requirements for a valid test.

A weak positive response was observed in the initial assay at the highest dose level only (2500 µg/ml) in the presence of S9. The response of 4% aberrant cells was barely outside the range of the historical solvent control (0-3%) and within the criteria for the determination of a valid test for the untreated and solvent controls (6%) and also within the historical solvent control range for non-activated system. The 1% increase over the historical solvent control range is considered to have no biological significance. In addition, in the independent repeat assay, although no scorable metaphases were observed at this dose level at the 20 hour harvest time, there was no significant response seen at this dose level and at the next highest dose level at the 44 hour harvest. Based on these findings of the study, Pentabromodiphenyl Oxide was concluded to be negative for the induction of structural and numerical chromosome aberrations in human peripheral blood lymphocytes.

## REFERENCES

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- Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), *Chemical Mutagens, Principles and Methods for their Detection*, vol 4. Plenum Press, New York.
- Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the Gene-Tox Program, *Mutation Research*, 87:143-188.
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TABLE 1  
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED  
WITH PENTABROMODIPHENYL OXIDE IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INITIAL ASSAY: 20 HOUR HARVEST

Treatment <sup>1,8</sup>	Culture	Mitotic Index <sup>2</sup>	Cells Scored	Aberrant Cells <sup>3</sup> (%)	Total Number of Structural Aberrations						Severely Damaged Cells <sup>7</sup>	Average Aberrations Per Cell <sup>3</sup>
					Chromatid-type			Chromosome-type				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	0.8	100	0	0	0	0	0	0	0	0	0.000
	B	2.0	100	0	0	0	0	0	0	0	0	0.000
DMSO	A	2.2	100	0	0	0	0	0	0	0	0	0.000
	B	1.2	100	0	0	0	0	0	0	0	0	0.000
Pentabromodiphenyl Oxide												
0.25 µg/ml <sup>8</sup>												
0.75 µg/ml <sup>8</sup>												
2.5 µg/ml <sup>8</sup>												
7.5 µg/ml	A	2.4	100	0	0	0	0	0	0	0	0	0.000
	B	2.6	100	0	0	0	0	0	0	0	0	0.000
25 µg/ml	A	0.4	100	1	1	1	0	0	0	0	0	0.010
	B	0.8	100	1	0	1	0	0	0	0	0	0.010
75 µg/ml	A	1.4	100	2	0	2	0	0	0	0	0	0.020
	B	1.4	100	0	0	0	0	0	0	0	0	0.000
250 µg/ml	A	0.6	100	0	0	0	0	0	0	0	0	0.000
	B	1.2	100	0	0	0	0	0	0	0	0	0.000
750 µg/ml <sup>8</sup>												
2500 µg/ml <sup>8</sup>												
MMC, 0.25 µg/ml	A	0.8	100	6	2	3	3	1	0	0	0	0.070
	B	0.4	100	6	3	0	6	0	0	0	0	0.060

<sup>1</sup>HPBL were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.

<sup>2</sup>Mitotic index = number mitotic figures x 100/500 cells counted.

<sup>3</sup>Excluding cells with only gaps.

<sup>4</sup>Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

<sup>5</sup>Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

<sup>6</sup>Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations. Severely damaged cells and pulverizations were counted as 10 aberrations.

<sup>8</sup>The lower dose levels were also tested as a guard against excessive toxicity at higher dose levels but were not required for analysis. Dose levels 750 and 2500 µg/ml were not evaluated due to an insufficient number of scorable cells.

TABLE 2  
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED  
WITH Pentabromodiphenyl Oxide IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INITIAL ASSAY: 20 HOUR HARVEST

Treatment <sup>1</sup>	Culture	Mitotic Index <sup>2</sup>	Cells Scored	Aberrant Cells <sup>3</sup> (%)	Total Number of Structural Aberrations						Severely Damaged Cells <sup>5</sup>	Average Aberrations Per Cell <sup>6</sup>
					Chromatid-type <sup>4</sup>			Chromosome-type <sup>4</sup>				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	2.8	100	0	0	0	0	0	0	0	0	0.000
	B	2.8	100	0	0	0	0	0	0	0	0	0.000
DMSO	A	1.2	100	1	0	0	0	0	1	0	0	0.010
	B	1.6	100	0	3	0	0	0	0	0	0	0.000
Pentabromodiphenyl Oxide												
0.25 µg/ml <sup>7</sup>												
0.75 µg/ml <sup>7</sup>												
2.5 µg/ml <sup>7</sup>												
7.5 µg/ml <sup>7</sup>												
25 µg/ml <sup>7</sup>												
75 µg/ml	A	0.4	100	0	0	0	0	0	0	0	0	0.000
	B	1.8	100	0	0	0	0	0	0	0	0	0.000
250 µg/ml	A	0.4	100	2	1	1	0	1	0	0	0	0.020
	B	1.0	100	0	0	0	0	0	0	0	0	0.000
750 µg/ml	A	0.6	100	2	1	0	0	1	1	0	0	0.020
	B	1.2	100	1	1	0	1	0	0	0	0	0.010
2500 µg/ml	A	1.4	100	5	2	2	2	0	0	0	1	0.140
	B	1.0	100	3	1	3	0	0	0	0	0	0.030
CP, 25 µg/ml	A	0.7	100	0	4	12	4	0	0	0	0	0.160
	B	0.4	100	10	5	10	3	0	0	0	0	0.130

<sup>1</sup>HPBL were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.

<sup>2</sup>Mitotic index = number mitotic figures x 100/500 cells counted.

<sup>3</sup>Excluding cells with only gaps.

<sup>4</sup>Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

<sup>5</sup>Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

<sup>6</sup>Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations. severely damaged cells and pulverizations were counted as 10 aberrations.

<sup>7</sup>Lower dose levels were tested as a guard against excessive toxicity at higher dose levels but were not required for analysis.

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TABLE 3  
SUMMARY  
INITIAL ASSAY

Treatment	S9 Activation	Treatment/ Harvest Time (Hrs.)	Mitotic Index (MI) (Mean)	% Change <sup>1</sup> from Control HI	Cells Scored	Aberrations Per Cell <sup>2</sup> (Mean ± SD)	Cells With Aberrations <sup>3</sup> (%)
Untreated	-	20/20	1.4	-	200	0.000 ± 0.000	0.0
DMSO	-	20/20	1.7	-	200	0.000 ± 0.000	0.0
Pentabromodiphenyl Oxide							
7.5 µg/ml	-	20/20	2.5	-47	200	0.000 ± 0.000	0.0
25 µg/ml	-	20/20	0.6	65	200	0.010 ± 0.100	1.0
75 µg/ml	-	20/20	1.4	18	200	0.010 ± 0.100	1.0
250 µg/ml	-	20/20	0.9	47	200	0.000 ± 0.000	0.0
MHC, 0.25 µg/ml	-	20/20	0.6	57	200	0.065 ± 0.267	6.0**
Untreated	+	4/20	2.8	-	200	0.000 ± 0.000	0.0
DMSO	+	4/20	1.4	-	200	0.005 ± 0.071	0.5
Pentabromodiphenyl Oxide							
75 µg/ml	+	4/20	1.1	21	200	0.000 ± 0.000	0.0
250 µg/ml	+	4/20	0.7	50	200	0.010 ± 0.100	1.0
750 µg/ml	+	4/20	0.9	36	200	0.015 ± 0.122	1.5
2500 µg/ml	+	4/20	1.2	14	200	0.085 ± 0.728	4.0*
CP, 25 µg/ml	+	4/20	0.3	89	200	0.145 ± 0.406	12.5**

<sup>1</sup>% reduction in mitotic index relative to negative (solvent or untreated) control. Test article concentrations are compared to the solvent control and the positive control is compared to the untreated control.

<sup>2</sup>severely damaged cells were counted as 10 aberrations.

<sup>3</sup>\*, p<0.05; \*\*, p<0.01; Fisher's exact test.

TABLE 4  
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED  
WITH Pentabromodiphenyl Oxide IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 20 HOUR HARVEST

Treatment	Culture	Mitotic Index	Cells Scored	Aberrant Cells (%)	Total Number of Structural Aberrations						Severely Damaged Cells <sup>5</sup>	Average Aberrations Per Cell <sup>6</sup>
					Chromatid-type			Chromosome-type				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	1.0	100	0	0	0	0	0	0	0	0	0.000
	B	1.6	100	2	0	2	0	0	0	0	0	0.020
DMSO	A	0.8	100	0	0	0	0	0	0	0	0	0.000
	B	2.8	100	1	0	0	0	3	0	0	0	0.030
Pentabromodiphenyl Oxide												
32 µg/ml												
63 µg/ml	A	1.2	100	3	0	2	0	1	0	0	0	0.030
	B	2.2	100	1	0	1	0	0	0	0	0	0.010
125 µg/ml	A	1.6	100	0	0	0	0	0	0	0	0	0.000
	B	1.0	100	0	0	0	0	0	0	0	0	0.000
250 µg/ml	A	1.8	100	1	0	2	0	0	0	0	0	0.020
	B	1.0	100	1	0	0	0	1	0	0	0	0.010
500 µg/ml	A	1.2	100	0	0	0	0	0	0	0	0	0.000
	B	0.6	100	0	0	0	0	0	0	0	0	0.000
MMC 0.25 µg/ml	A	0.4	100	13	2	13	1	3	0	0	0	0.170
	B	0.2	100	13	9	15	4	5	0	0	0	0.240

<sup>1</sup>HPBL were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.

<sup>2</sup>Mitotic index = number mitotic figures x 100/500 cells counted.

<sup>3</sup>Excluding cells with only gaps.

<sup>4</sup>Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

<sup>5</sup>Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

<sup>6</sup>Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

<sup>7</sup>Severely damaged cells and pulverizations were counted as 10 aberrations.

<sup>8</sup>Lower dose level was tested as a guard against excessive toxicity at higher dose levels but was not required for analysis.

TABLE 5  
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED  
WITH Pentabromodiphenyl Oxide IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 44 HOUR HARVEST

Treatment <sup>1</sup>	Flask	Mitotic Index <sup>2</sup>	Cells Scored	Cells with Aberrations <sup>3</sup>		Number of Structural Aberrations						Severely Damaged Cells <sup>5</sup>	Average Structural Aberrations Per Cell <sup>3</sup>
				(%)		Chromatid-type			Chromosome-type				
				Numerical	Structural	Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	4.6	100	0	1	0	1	0	0	0	0	0	0.010
	B	2.2	100	0	1	0	1	0	0	0	0	0	0.010
DMSO	A	6.2	100	0	0	0	0	0	0	0	0	0	0.000
	B	5.0	100	0	1	0	1	0	0	0	0	0	0.010
Pentabromodiphenyl Oxide													
32 µg/ml <sup>6</sup>													
63 µg/ml	A	5.4	100	0	0	0	0	0	0	0	0	0	0.000
	B	4.0	100	0	0	0	0	0	0	0	0	0	0.000
125 µg/ml	A	3.4	100	0	1	0	0	1	0	0	0	0	0.010
	B	5.4	100	0	0	0	0	0	0	0	0	0	0.000
250 µg/ml	A	2.6	100	2	2	0	1	0	1	0	0	0	0.020
	B	4.2	100	0	0	0	0	0	0	0	0	0	0.000
500 µg/ml	A	0.6	100	0	1	0	1	0	0	0	0	0	0.010
	B	1.8	100	0	1	1	1	0	0	0	0	0	0.010
MMC 0.13 µg/ml	A	2.8	100	0	42	5	53	23	0	0	1	1	0.870
	B	1.2	100	0	39	9	39	25	0	0	0	0	0.640

<sup>1</sup>HPBL were treated for 44 hours at 37±1°C in the absence of an exogenous source of metabolic activation.

<sup>2</sup>Mitotic index = number mitotic figures x 100/500 cells counted.

<sup>3</sup>Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.

<sup>4</sup>Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

<sup>5</sup>Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

<sup>6</sup>Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

Severely damaged cells and pulverizations were counted as 10 aberrations.

<sup>7</sup>Lower dose level was tested as a guard against excessive toxicity at higher dose levels but was not required for analysis.

TABLE 6  
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED  
WITH Pentabromodiphenyl Oxide IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 20 HOUR HARVEST

Treatment <sup>1</sup>	Culture	Mitotic Index	Cells Scored	Aberrant Cells (%)	Total Number of Structural Aberrations						Severely Damaged Cells <sup>5</sup>	Average Aberrations Per Cell <sup>6</sup>
					Chromatid-type			Chromosome-type				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	0.8	100	0	1	0	0	0	0	0	0	0.000
	B	1.8	100	2	0	1	0	1	0	0	0	0.020
DMSO	A	1.6	100	0	0	0	0	0	0	0	0	0.000
	B	1.6	100	0	0	0	0	0	0	0	0	0.000
Pentabromodiphenyl Oxide												
313 µg/ml	A	1.0	100	1	0	1	0	0	0	0	0	0.010
	B	0.8	100	0	1	0	0	0	0	0	0	0.000
625 µg/ml	A	0.2	100	3	1	2	0	1	0	0	0	0.030
	B	0.2	100	0	0	0	0	0	0	0	0	0.000
1250 µg/ml	A	0.2	100	0	0	0	0	0	0	0	0	0.000
	B	1.6	100	1	1	1	0	0	0	0	0	0.010
2500 µg/ml <sup>2</sup>												
3750 µg/ml <sup>2</sup>												
CP 25 µg/ml	A	0.2	100	18	3	22	3	2	0	0	0	0.270
	B	0.2	100	11	4	14	2	1	0	0	0	0.170

<sup>1</sup>HPBL were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.

<sup>2</sup>Mitotic index = number mitotic figures x 100/500 cells counted.

<sup>3</sup>Excluding cells with only gaps.

<sup>4</sup>Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.

<sup>5</sup>Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

<sup>6</sup>Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

Severely damaged cells and pulverizations were counted as 10 aberrations.

<sup>7</sup>Higher dose levels were not analyzed due to absence of scorable cells.

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TABLE 7  
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED  
WITH PENTABROMODIPHENYL OXIDE IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 44 HOUR HARVEST

Treatment <sup>1,5</sup>	Flask	Mitotic Index <sup>2</sup>	Cells Scored	Cells with Aberrations <sup>3</sup>		Number of Structural Aberrations						Severely Damaged Cells <sup>4</sup>	Average Structural Aberrations Per Cell <sup>3</sup>
				Numerical (%)	Structural (%)	Chromatid-type Gaps	Chromatid-type Breaks	Chromosome-type Exchange	Chromosome-type Breaks	Chromosome-type Dic	Chromosome-type Ring		
Untreated cells	A	5.6	100	0	0	0	0	0	0	0	0	0	0.000
	B	7.0	100	1	1	0	0	0	0	C	1	0	0.010
DMSO	A	11.2	100	1	0	0	0	0	0	0	0	0	0.000
	B	7.4	100	0	0	0	0	0	0	0	0	0	0.000
Pentabromodiphenyl Oxide 313 µg/ml <sup>5</sup>													
625 µg/ml	A	2.2	100	1	0	0	0	0	0	0	0	0	0.000
	B	1.2	100	0	1	0	1	0	0	0	0	0	0.010
1250 µg/ml	A	3.2	100	0	0	0	0	0	0	0	0	0	0.000
	B	2.4	100	0	0	0	0	0	0	0	0	0	0.000
2500 µg/ml	A	1.2	100	0	1	0	1	0	0	0	0	0	0.010
	B	0.8	100	0	0	0	0	0	0	0	0	0	0.000
3750 µg/ml	A	1.8	100	0	2	1	2	0	0	0	0	0	0.020
	B	1.0	100	0	0	1	0	0	0	0	0	0	0.000
CP 25 µg/ml	A	2.0	100	0	22	1	22	5	2	0	0	1	0.390
	B	3.4	100	0	12	2	15	2	0	1	0	0	0.180

<sup>1</sup>HPBL were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.

<sup>2</sup>Mitotic index = number mitotic figures x 100/500 cells counted.

<sup>3</sup>Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.

<sup>4</sup>Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.

<sup>5</sup>Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

<sup>6</sup>Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

<sup>7</sup>Severely damaged cells and pulverizations were counted as 10 aberrations.

<sup>8</sup>Lower dose level tested as a guard against excessive toxicity at higher dose levels but was not required for analysis.

TABLE 8  
SUMMARY

INDEPENDENT REPEAT ASSAY

Treatment	S9 Activation	Treatment/ Harvest Time (Hrs.)	Mitotic Index (MI) (Mean)	% Change from Control MI	Total Cells Scored	Aberrations Per Cell <sup>1</sup> (Mean ± SD)	Cells With Aberrations (%)	
							Numerical	Structural
Untreated	-	20/20	1.3	-	200	0.010 ± 0.100		1.0
DMSO	-	20/20	1.8	-	200	0.015 ± 0.212		0.5
Pentabromodiphenyl Oxide								
63 µg/ml	-	20/20	1.7	6	200	0.020 ± 0.140		2.0
125 µg/ml	-	20/20	1.3	28	200	0.000 ± 0.000		0.0
250 µg/ml	-	20/20	1.4	22	200	0.015 ± 0.158		1.0
500 µg/ml	-	20/20	0.9	50	200	0.000 ± 0.000		0.0
MMC 0.25 µg/ml	-	20/20	0.3	77	200	0.205 ± 0.578		13.0**
Untreated								
DMSO	-	44/44	3.4	-	200	0.010 ± 0.100	0.0	1.0
DMSO	-	44/44	5.6	-	200	0.005 ± 0.071	0.0	0.5
Pentabromodiphenyl Oxide								
63 µg/ml	-	44/44	4.7	16	200	0.000 ± 0.000	0.0	0.0
125 µg/ml	-	44/44	4.4	21	200	0.005 ± 0.071	0.0	0.5
250 µg/ml	-	44/44	3.4	39	200	0.010 ± 0.100	1.0	1.0
500 µg/ml	-	44/44	1.7	79	200	0.010 ± 0.100	0.0	1.0
MMC 0.13 µg/ml	-	44/44	2.0	41	200	0.755 ± 1.222	0.0	40.5**
Untreated								
DMSO	+	4/20	1.3	-	200	0.010 ± 0.100		1.0
DMSO	+	4/20	1.6	-	200	0.000 ± 0.000		0.0
Pentabromodiphenyl Oxide								
313 µg/ml	+	4/20	0.9	44	200	0.005 ± 0.071		0.5
625 µg/ml	+	4/20	0.2	88	200	0.015 ± 0.122		1.5
1250 µg/ml	+	4/20	0.9	44	200	0.005 ± 0.071		0.5
CP 25 µg/ml	+	4/20	0.2	85	200	0.220 ± 0.651		14.5**
Untreated								
DMSO	+	4/44	6.3	-	200	0.005 ± 0.071	0.5	0.5
DMSO	+	4/44	9.3	-	200	0.000 ± 0.000	0.5	0.0
Pentabromodiphenyl Oxide								
625 µg/ml	+	4/44	1.7	82	200	0.005 ± 0.071	0.5	0.5
1250 µg/ml	+	4/44	2.8	70	200	0.000 ± 0.000	0.0	0.0
2500 µg/ml	+	4/44	1.0	89	200	0.005 ± 0.071	0.0	0.5
3750 µg/ml	+	4/44	1.4	85	200	0.010 ± 0.100	0.0	1.0
CP 25 µg/ml	+	4/44	2.7	57	200	0.285 ± 0.904	0.0	17.0**

<sup>1</sup>% reduction in mitotic index relative to negative (solvent or untreated) control. Test article concentrations are compared to the solvent control and the positive control is compared to the untreated control.

<sup>2</sup>Severely damaged cells were counted as 10 aberrations.

\*\* p < 0.01; Fisher's exact test.

<sup>3</sup>Data not collected for 20 hour harvest time.

**APPENDIX I**  
**Historical Control Data**

IN VITRO MAMMALIAN CYTOGENETIC TEST USING  
HUMAN PERIPHERAL LYMPHOCYTES

HISTORICAL CONTROL VALUES  
1993-1995

NON-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control <sup>1</sup>	Positive Control <sup>2</sup>
Mean	0.2%	0.3%	19.9%
Standard Deviation	0.5%	0.6%	13.9%
Range	0.0% to 2.5%	0.0% to 4.5%	6.5% to 87.5%

S-9 ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control <sup>1</sup>	Positive Control <sup>3</sup>
Mean	0.3%	0.3%	15.0%
Standard Deviation	0.5%	0.5%	5.8%
Range	0.0% to 2.0%	0.0% to 3.0%	7.0% to 34.0%

<sup>1</sup>Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, lactic acid buffer, placebo, 0.105N HCl, AD284 diluent, and 45% hydroxypropyl- $\beta$ -cyclodextrin

<sup>2</sup>Positive control for non-activated studies, mitomycin C. (MMC)

<sup>3</sup>Positive control for S-9 activated studies, cyclophosphamide, (CP)

**APPENDIX II**  
**Study Protocol**

QA  
6-7-96  
**APPROVED**

Received by RA/OA 6-7-96

MA Study Number: G96AO63.342

**Chromosome Aberrations in Human Peripheral Blood  
Lymphocytes**

1.0 PURPOSE

The purpose of this study is to test the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in human peripheral blood lymphocytes (HPBL).

2.0 SPONSOR

- 2.1 Name: Chemical Manufacturers Association
- 2.2 Address: 1300 Wilson Boulevard  
Arlington, VA 22209
- 2.3 Representative: Dr. Hasmukh C. Shah

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

- 3.1 Test Article: **Pentabromodiphenyl Oxide**
- 3.2 Controls: Untreated: Untreated cells  
Solvent: Test Article Solvent  
Positive: Mitomycin C (MMC), Cyclophosphamide (CP)

3.3 Determination of Purity

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article.

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Microbiological Associates, Inc.

4.0 TESTING FACILITY AND KEY PERSONNEL

- 4.1 Name: Genetic Toxicology Division  
Microbiological Associates, Inc.
- 4.2 Address: 9630 Medical Center Drive  
Rockville, MD 20850
- 4.3 Study Director: Ramadevi Gudi, Ph.D.

Protocol No. SPGT342 01/03/96

1 of 8

 **MICROBIOLOGICAL  
ASSOCIATES, INC.**

MA Study No. G96AO63.342

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4.4 Associate Study Director: Donald Putman, Ph.D.

## 5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 6/11/96

5.2 Proposed Experimental Completion Date: 8/30/96

5.3 Proposed Report Date: 9/13/96

## 6.0 TEST SYSTEM

Peripheral blood lymphocytes will be obtained from healthy adults without six months exposure to either radiotherapy, viral infections or the administration of drugs. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston et al., 1981).

## 7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The initial assay will be performed by exposing lymphocytes to a minimum of nine concentrations of the test article as well as positive, untreated and solvent controls in duplicate cultures (Evans and O'Riordan, 1975). In the non-activated test system, exposure will be continuous up to the time of cell collection; in the S9-activated system, exposure will be for 4 hours. The dividing cells will be harvested at a single time point, approximately 20 hours from the initiation of treatment. The highest scorable dose level with at least 50% mitotic inhibition and three lower doses will be evaluated microscopically for structural chromosome aberrations. In the independent repeat assay, a minimum of four concentrations will be selected based on the findings of the initial assay. The exposure conditions will remain the same but two harvests will be carried out, approximately 20 hours after the initiation of treatment and 24 hours later (44 hours after initiation of treatment). The clastogenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group. The test article will also be assessed for its ability to induce numerical chromosome aberrations.

### 7.1 Solubility Determination

Unless the Sponsor has indicated the test article solvent, a solubility determination will be conducted to determine solvent and the maximum soluble concentration up to a maximum of 500 mg/ml. Vehicles compatible with this test system, in order of preference, include but are not limited to distilled water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5), and acetone (CAS 67-64-1). The vehicle will be the solvent, selected in order of preference, that permits preparation of the highest stock concentration, up to 500 mg/ml.

## 7.2 Dose Levels

For the initial cytogenetics assay, HPBL cells will be exposed to at least nine concentrations of test article at half-log dilutions. The highest concentration tested will be 5 mg/ml for freely soluble test articles, or the maximum concentration resulting in a workable suspension for poorly soluble test articles. The pH will be measured at the highest test article treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest treatment condition will also be measured. If excessive precipitation of the test article-solvent solution occurs upon addition to treatment medium, or if the osmolality of the treatment medium is excessive, the Sponsor will be consulted.

For the independent repeat assay, HPBL cells will be exposed to at least four concentrations of test article at no greater than half-fold dilutions, the highest concentration being that which is estimated to cause at least 50% mitotic inhibition (regardless of solubility) relative to the solvent control. In the absence of at least 50% mitotic inhibition the concentration resulting in minimum precipitation in test medium will be the highest concentration tested. Minimum precipitation will be determined by direct visual inspection. If excessive precipitation of the test article-solvent solution occurs upon addition to treatment medium, or if the osmolality of the test article-solvent solution in treatment medium exceeds the osmolality of the treatment medium-solvent solution by approximately 100 mOs/kg or greater, the Sponsor will be consulted. The pH and osmolality will be monitored as in the initial assay.

## 7.3 Frequency and Route of Administration

Target cells will be treated for approximately 20 or 44 hours in the non-activated system, depending upon harvest time, and for 4 hours in the S9-activated system. Treatment will be achieved by incorporation of the test article-solvent mixture into the medium. This technique has demonstrated to be an effective method of detection of chemical clastogens in this test system (Evans, 1976).

## 7.4 Metabolic Activation System

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S9 will be thawed and mixed with cofactors to contain 2 mM magnesium chloride (MgCl<sub>2</sub>), 6 mM potassium chloride (KCl), 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S9 per ml serum-free medium.

## 7.5 Controls

### 7.5.1 Untreated Controls

Untreated cells will be used as the untreated control.

### 7.5.2 Solvent Control

The solvent vehicle for the test article will be used as the solvent control. For solvents other than water or medium, the final concentration in treatment medium will not exceed 1%.

### 7.5.3 Positive Controls

Mitomycin C (MMC) will be used at a concentration within 0.10-0.25 µg/ml as the positive control in the non-activated study. Cyclophosphamide (CP) will be used at a concentration within 25-100 µg/ml as the positive control in the S9-activated study.

## 7.6 Preparation of Target Cells

Peripheral blood cells will be cultured in complete medium (RPMI-1640 containing 15% fetal bovine serum, 2mM L-glutamine, 100 units penicillin and 100 µg streptomycin/ml) containing 1% phytohemagglutinin (PHA) by adding 0.6 ml heparinized blood to a centrifuge tube containing 9.4 ml complete medium with 1% PHA. The tubes will be incubated upright at 37±1°C in a humidified atmosphere of 5±1% CO<sub>2</sub> in air for 44-48 hours.

## 7.7 Identification of Test System

Using a permanent marking pen, the culture flasks/test tubes, etc. used in the test system will be identified by the study number, the treatment condition and test phase.

## 7.8 Treatment of Target Cells

All treatments will be carried out in duplicate. About 44-48 hours after culture initiation, tubes will be refed (centrifugation at approximately 1200 rpm for approximately 5 minutes) with 10 ml of complete medium for the non-activated exposure or 10 ml of S9 reaction mixture for the activated exposure to which will be added 100 µl of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if water or medium is used as the solvent. An untreated control consisting of cells in complete medium or S9 reaction mixture will also be included.

In the non-activated study, the cells will be exposed for 20 hours (harvest 1) or for 44 hours (harvest 2) at  $37 \pm 1^\circ\text{C}$  in a humidified atmosphere of  $5 \pm 1\%$   $\text{CO}_2$  in air. Exposure will continue until collection of metaphase cells.

In the S9-activated study, the cells will be exposed for 4 hours at  $37 \pm 1^\circ\text{C}$  in a humidified atmosphere of  $5 \pm 1\%$   $\text{CO}_2$  in air. After the exposure period, the cells will be centrifuged at approximately 1200 rpm for approximately 5 minutes, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium containing 1% PHA, and returned to the incubator for an additional 16 hours (harvest 1) or 40 hours (harvest 2).

#### 7.9 Collection of Metaphase Cells

Cells will be collected approximately 20 hours and 44 hours after initiation of treatment. These times are selected to assure analysis of first division metaphase cells after initiation of test article treatment as well as to allow for analysis of delayed cells. Two hours prior to harvest, Colcemid® will be added to the cultures at a final concentration of  $0.1 \mu\text{g/ml}$ . The cells will be collected by centrifugation (approximately 1200 rpm for 5 minutes), treated with  $0.075\text{M}$  KCl, washed with two changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately  $2-6^\circ\text{C}$ . To prepare slides, the cells will be collected by centrifugation, resuspended in fresh fixative. One to two drops of fixed cells will be dropped onto a wet slide and air-dried. The slide will be identified by the MA study number, treatment condition and date. At least two slides will be prepared from each treatment tube. The slides will be stained with Giemsa and permanently mounted.

#### 7.10 Scoring for Metaphase Aberrations

Prior to analysis, slides from the initial and independent repeat studies will be scanned for scorable metaphases. In each study, whenever possible, the high dose will be selected to yield approximately 50% or greater mitotic inhibition. Slides from the highest scorable dose level and three lower dose levels will be selected for analysis.

Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 46 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentric and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be

considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells ( $\geq 10$  aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations will be recorded using a calibrated microscope stage. In the delayed harvest, the percent polyploid cells will be evaluated per 100 metaphase cells. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted.

## 8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

### 8.1 Untreated and Solvent Controls

The frequency of cells with structural and numerical chromosome aberrations in the untreated or solvent control must be no greater than 6%.

### 8.2 Positive Controls

The percentage of cells with aberrations must be statistically increased ( $p \leq 0.05$ , Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water is used.

## 9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and will be presented for both the initial and the independent repeat chromosome aberration assays. The number and types of aberrations, the percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined, the percentage of numerically damaged cells in the total population of cells examined, and the frequency of structural aberrations per cell (mean aberrations per cell) will be reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells will be performed using the Fisher's exact test. Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage trend test will be used to measure dose-responsiveness.

All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ( $p \leq 0.05$ ). A reproducible and significant increase at a single dose level will be considered positive. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

## 10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data.

Results presented will include, but not be limited to:

- cells used
- test conditions: composition of medium, CO<sub>2</sub> concentration, incubation time, concentration of test article and rationale for selection of concentration, duration of treatment, duration of treatment with and concentration of Colcemid®, type of metabolic activation system used, positive, solvent and untreated controls
- number of cell cultures
- number of metaphases analyzed (data given separately for each culture)
- mitotic index
- criteria for scoring aberrations
- type and number of aberrations, given separately for each treated and control culture
- historical control data

## 11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Quality Assurance Unit of Microbiological Associates, Inc., Rockville, MD, in accordance with the relevant Good Laboratory Practice Regulations.

## 12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with EU Legislation L 383 a/143, B.10 Mutagenicity (*In Vitro* Mammalian Cytogenetic Test), December, 1992; OECD Guideline 473 (Genetic Toxicology: *In Vitro* Mammalian Cytogenetic Test), May, 1983; and EPA Health Effects Testing Guidelines, Subpart 798.5375 (*In Vitro* Mammalian Cytogenetics), Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for EPA TSCA, Regulations, 40 CFR 792 and OECD ISBN 922-84-12367-9.

Will this study be submitted to a regulatory agency? Yes

If so, to which agency or agencies? EU and EPA-TSCA

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Evans, H.J. and M.L. O'Riordan. 1975. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. *Mutation Res.* 31:135-148.

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Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff, J.S. Wassom. 1981. Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the Gene-Tox Program. *Mutation Res.* 87:143-188.

Scott, D., N.D. Sanford, B.J. Dean and D.J. Kirkland. 1990. Metaphase Chromosome Aberration Assays In Vitro. In: *Basic Mutagenicity Tests: UKEMS Recommended Procedures*. D.J Kirkland (ed). Cambridge University Press, New York, NY.

14.0 APPROVAL

Hamukh Shah 5-29-96  
SPONSOR REPRESENTATIVE DATE

\_\_\_\_\_  
(Print or Type Name)

Ramadevi Rudi 6/5/96  
MA STUDY DIRECTOR DATE

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