STD.API/PETRO PUBL TR410-ENGL 1996 ■ 0732290 0564260 968 ■

American Petroleum Institute

Health and Environmental Sciences Department

Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells Exposed to Tertiary Amyl Methyl Ether (TAME)

DECEMBER 1996

TOXICOLOGY REPORT NUMBER 410
CAIS ABSTRACT NO. 43-5239

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STD.API/PETRO PUBL TR410-ENGL 1996 ■ 0732290 0564261 8T4 ■

Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells Exposed to Tertiary Amyl Methyl Ether (TAME)

Health and Environmental Sciences Department

API PUBLICATION NUMBER TR410

PREPARED UNDER CONTRACT BY:

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DECEMBER 1996

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ACKNOWLEDGMENTS

THE FOLLOWING PEOPLE ARE RECOGNIZED FOR THEIR CONTRIBUTIONS OF TIME AND EXPERTISE DURING THIS STUDY AND IN THE PREPARATION OF THIS REPORT:

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FINAL REPORT AMENDMENT I

SPONSOR: **American Petroleum Institute**

TEST ARTICLE I.D.: **Tertiary Amyl Methyl Ether (TAME)**

MA STUDY NO: **G95CA89.330**

DATE OF FINAL REPORT: **06/18/96**

PROTOCOL TITLE: **Chromosome Aberrations in Chinese Hamster Overy (CHO) Cells**

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1. **PART OF FINAL REPORT TO BE AMENDED:** Entire report.

AMENDMENTS:

(a) Change page numbers to accomodate Sponsor's Quality Assurance Report and report format.

(b) Minor grammatical and formatting changes throughout report.

(c) Amend the Table of Contents to include section subheadings.

(d) Add protocol amendment 2 to Appendix B.

REASON FOR THE AMENDMENT: To accomodate changes in report format requested by the Sponsor.

APPROVALS:

Ramadevi Gudi — STUDY DIRECTOR — 11/20/96 — DATE

Claire L Courtemanche — QUALITY ASSURANCE — NOV 22, 1996 — DATE

MICROBIOLOGICAL ASSOCIATES, INC.

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GAI **GOLDMAN ASSOCIATES INTERNATIONAL, INC.**

REPORT OF THE INSPECTION

FOR COMPLIANCE WITH THE

GOOD LABORATORY PRACTICE REGULATIONS

of the

ENVIRONMENTAL PROTECTION AGENCY

Conducted at

MICROBIOLOGICAL ASSOCIATES, INC.
9630 Medical Drive
Rockville, MD

on

May 1 and 9, 1996

Eva K. Zurek
Senior Associate

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5/20/96

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INTRODUCTION

This inspection of Microbiological Associates, Inc. was carried out on behalf of the American Petroleum Institute (API), Washington DC under the Environmental Protection Agency's Testing Consent Order concerning *tert*-amyl methyl ether (TAME). The inspection was to ascertain the facilities' compliance with the Good Laboratory Practice (GLP) regulations issued by the EPA under the Toxic Substances Control Act (TSCA) at 40 CFR 792. The nonclinical laboratory studies audited on this site visit include:

Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells
(MBA Study Number G95CA89.330)

CHO/HGPRT Mutation Assay (MBA Study Number G95CA89.782)

The inspection/audit was performed on May 1 and May 9, 1996, by Eva Zurek, Senior Associate, Goldman Associates International, Inc.

PROCEDURES

This inspection was conducted by a tour of the new genetic tox facility and review of personnel files, QAU procedures, study protocols, relevant standard operating procedures (SOPs), and raw data generated during the conduct of the studies. The studies were in the final draft report stage at the time of the audit.

Claire Courtemanche, Quality Assurance Manager, Toxicology Group, provided all assistance and documents during the inspection. The inspection was initiated at approximately 10 a.m. on May 1, 1996. The inspector returned at 10 a.m. on May 9, 1996 to complete the inspection.

SUMMARY OF FINDINGS

In general, inspection of the facilities, equipment, personnel files, QAU, test article receipt and handling, and SOPs showed management's firm commitment to compliance with the regulations. Thorough documentation of study conduct is enhanced by the use of pre-printed raw data worksheets. However, the following deficiencies were noted with respect to documentation of test system receipt and evaluation for the Mutation Assay:

- There is no documentation that the CHO-K_1-BH_4 cells used for the Mutation Assay were obtained from A. Hsie, Oak Ridge, TN, as stated in the protocol and raw data workbook.

- The earliest record of this cell line is an ampule freeze date of 2/2/81. It was next reconstituted on 1/11/93. There is no documentation to show characterization or karyotypic stability for this or any subsequent freeze lot that was ultimately used for this study.

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- There was no record of testing the freeze lot of cells for mycoplasma contamination , as required by protocol.

- Results of mycoplasma testing for each new frozen lot of cells were not being maintained in the cell line log book, as specified by SOP OPGTO322.R4

- Preparation of the "cleansing" medium (HAT) should be documented. "HAT" should be defined in the raw data.

The findings were discussed with Ms. Courtemanche at the close of the inspection on May 9, 1996. She stated that the study director will be notified, and that he will provide deviation reports, where necessary. The study director will contact appropriate API personnel to determine the best course of action. It may be advisable to perform characterization and mycoplasma testing on another representative sample from the same freeze lot that was used for this testing.

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REPORT OF THE INSPECTION

PERSONNEL

All study personnel had the appropriate training, education, and experience for their assigned tasks. Training records were reviewed for representative technicians involved in the studies audited, and were found to be complete.

MANAGEMENT

Test facility management is supportive of the GLP program as is evidenced by the personnel, facilities, equipment, and methodology provided for the studies that were reviewed. Quality Assurance submits bimonthly written reports to keep management informed of the compliance status of all studies.

STUDY DIRECTOR

Dr. Richard San is the designated study director for the CHO/HGPRT Mutation Assay, and Dr. Patrick Curry is the study director for the Chromosome Aberration study. An SOP has been developed that addresses the replacement of a study director, or the appointment of an alternate study director.

It was noted that Dr. San will have to issue a deviation report with respect to the lack of documentation for the mycoplasma testing for the freeze lot of cells used in the Mutation Assay. Dr. San should provide documentation to support the statement in the final report that " the CHO-K_1-BH_4 cells were obtained from A. Hsie, Oak Ridge National Laboratory, Oak Ridge, TN..'.

QUALITY ASSURANCE

The Quality Assurance responsibilities for genetic toxicology studies are managed by Claire Courtemanche. Records show Ms. Courtemanche to be an experienced and well-qualified Quality Assurance professional. GLP training is provided to all personnel by members of the QAU staff. SOPs are in place and are followed for all QAU activities. All studies (including acutes) are monitored at least once during the in-life phase. QAU has issued the required statements for the two studies audited that state when inspections were conducted and when reports were made to study director and management.

FACILITIES

The Genetic Toxicology laboratory was re-located to a new facility on March 18-20, 1996. The new facilities are clean and spacious and afford the room for complete separation of study activities. A tour of the facility provided by the QAU Manager included the following specialized areas: Test Article Repository; Cold Room (251);

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Tissue Processing Lab; Media Preparation Lab; Ames Lab; Bacterial Counting Lab; Gene Mutation Lab; Counting Room; Microscope Room. A routine maintenance form was located next to each piece of equipment that requires any maintenance.

FACILITIES FOR HANDLING TEST AND CONTROL SUBSTANCES

Specific SOPs have been prepared to control the receipt, storage and distribution of test and control articles at MBA. All test articles are delivered to the loading dock, brought upstairs, opened and unpacked under hoods. All test articles are handled and stored in the Test Article Repository (Room 261), which is a limited access area. Only designated personnel may sign in, open and log in test and control articles. All test articles are logged into a computerized tracking system (TATS), and well-controlled records are maintained for use and distribution. All refrigerators and freezers are alarmed and connected to a central control room and security company. If an alarm sounds, the appropriate personnel are notified.

EQUIPMENT

SOPs are available for each piece of equipment in the genetic toxicology area. Detailed equipment maintenance logs are kept for routine and specialized cleaning, testing, calibration or maintenance operations. Appropriate temperature logs are maintained for all freezers, refrigerators, incubators and water baths. A NIST traceable thermometer is used to calibrate all laboratory thermometers.

STANDARD OPERATING PROCEDURES

All standard operating procedures have been established, as required by the regulations. Relevant SOPs are readily available in the laboratory areas. SOPs are current and well-written. SOPs applicable to these genetic toxicology studies were reviewed. One deviation from an SOP was noted (SOP OPGTO322.R4). This SOP states that "each new frozen lot of cells will be tested for mycoplasma contamination. Results will be maintained in the cell line log book". The cell line log book used to trace the CHO cells for the Mutation Assay did not contain any reported results of mycoplasma testing. If it is more convenient to maintain these reports elsewhere, the SOP should be revised.

ANIMAL AND OTHER TEST SYSTEM CARE

There are SOPs for the handling of the test systems used in the studies that were audited. Good study records were maintained for the preparation of cells for testing. Records show that the CHO-K_1 cell line used for the Chromosome Aberration test was obtained from ATCC, Rockville, MD. There are no records to show that the CHO cells used for the Mutation Assay were received from Dr. Hsie, Oak Ridge, TN, as is stated in the protocol. There are no records for the original receipt of these cells. The earliest record documents an ampule freeze date of 2/2/81. These cells were reconstituted on 1/11/93, and subsequently split, passed and frozen again for a number of times before achieving

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the freeze lot used in the current study. It is suggested that the study director provide some documentation regarding the receipt, characterization or karyotypic stability of this cell line. The preparation of the cleansing medium (HAT) that is added to the cell line prior to freezing should be documented.

TEST, CONTROL, AND REFERENCE SUBSTANCE CHARACTERIZATION

The sponsor has assumed responsibility for all test substance characterization, and this is stated in the study protocol signed by the study director and sponsor.

PROTOCOL

The protocols for the two TAME genetic tox studies were signed by the Study Directors on January 23, 1996. The protocols met all GLP requirements. As mentioned above, Dr. San, the study director for the Mutation Assay , should provide documentation for the receipt of the CHO-K1-BH4 cell line, and prepare a deviation report for the mycoplasma testing for this freeze lot.

CONDUCT OF THE STUDY

Recording and maintenance of raw data is facilitated by the use of pre-printed data sheets which form the Raw Data Workbook for each study. In addition to all of the results , the workbooks contain the study protocols, test article receipt, use and disposition records, identification of study personnel, documentation error codes, test article measuring reports, solubility determinations, pH measurements, Full list of materials and supplies with batch or lot numbers, where needed, equipment identification, and target cell history and preparation. Data sheets were properly completed.

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Abstract

The test article, Tertiary Amyl Methyl Ether (TAME), was tested in the Chromosome Aberration Assay using Chinese hamster ovary (CHO) cells, in both the absence and presence of Aroclor®-induced rat liver S9 (fraction of extracted biotransformation enzymes capable of metabolizing putative promutagens into the ultimate mutagenic agent) at doses of 313 to 5000 µg/ml. All concentrations tested in both the non-activated and the S9-activated test systems were soluble in treatment medium. Toxicity, i.e., cell growth inhibition, was 43% at the 5000 µg/ml dose level in the non-activated test system and 72% at the 5000 µg/ml dose level in the S9-activated test system. No positive response was seen in the non-activated test system. In the S9-activated test system there was a positive response with an increasing concentration of the test article. Under the conditions of this study, the test article, Tertiary Amyl Methyl Ether (TAME), was concluded to be positive in the Chromosome Aberration Assay using CHO cells.

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Appendix A: Historical Control Data A-1
Appendix B: Study Protocol B-1

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AMENDED FINAL REPORT

Study Title

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Test Article

Tertiary Amyl Methyl Ether (TAME)

Author

Patrick T. Curry, Ph.D.

Study Completion Date

June 18, 1996

Performing Laboratory

Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, Maryland 20850

Laboratory Study Number

G95CA89.330

Sponsor Project Number
HES1621-L-00860-MUTAGEN

Sponsor

American Petroleum Institute
1220 L Street, Northwest
Washington, D.C. 20005

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CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

AMENDED FINAL REPORT

Sponsor: **American Petroleum Institute**
1220 L Street, Northwest
Washington, D.C. 20005

Authorized Representative: **Richard Rhoden, Ph.D.**

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **Tertiary Amyl Methyl Ether (TAME)**

Sponsor Project Number: **HES1621-L-00860-MUTAGEN**

MA Study No.: **G95CA89.330**

Test Article Description: **Clear liquid**

Storage Conditions: **Room temperature, protected from exposure to light**

Test Article Receipt: **December 26, 1995**

Study Initiation: **January 23, 1996**

Laboratory Supervisor: Elizabeth H. Schadly 11/20/96
Elizabeth H. Schadly, B.S. Date

Study Director: Ramadevi Gudi 11/20/96
Ramadevi Gudi, Ph.D. Date

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QUALITY ASSURANCE STATEMENT

Study Title: CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Study Number: G95CA89.330

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 23 JAN 96, TO STUDY DIR 23 JAN 96, TO MGMT 23 JAN 96
PHASE: Protocol Review

INSPECT ON 06 FEB 96, TO STUDY DIR 13 FEB 96, TO MGMT 14 FEB 96
PHASE: Test and/or control material administration

INSPECT ON 04 APR 96-05 APR 96, TO STUDY DIR 05 APR 96, TO MGMT 08 APR 96
PHASE: Draft Report

INSPECT ON 09 MAY 96, TO STUDY DIR 09 MAY 96, TO MGMT 09 MAY 96
PHASE: Draft to Revised Draft Report

INSPECT ON 19 JUN 96, TO STUDY DIR 19 JUN 96, TO MGMT 19 JUN 96
PHASE: Revised Draft to Final Report

INSPECT ON 27 AUG 96, TO STUDY DIR 11 NOV 96, TO MGMT 22 NOV 96
PHASE: Amended 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 — NOV 22, 1996

Claire L. Courtemanche
QUALITY ASSURANCE — DATE

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STATEMENT OF COMPLIANCE

Study G95CA89.330 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.

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

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

Ramadevi Gudi

Ramadevi Gudi, Ph.D.
Study Director

11/20/96
Date

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SUMMARY

The test article, Tertiary Amyl Methyl Ether (TAME), was tested in the chromosome aberration assay using Chinese hamster ovary (CHO) cells. The assay was performed in two phases. The first phase, a preliminary toxicity test, was performed to establish the dose range and cell collection times for the chromosome aberration assay. The second phase, the chromosome aberration assay, was used to evaluate the clastogenic potential of the test article. In both phases of the assay the test article was assessed in both the absence and presence of an Aroclor®-induced S9-activation system (fraction of extracted biotransformation enzymes capable of metabolizing putative promutagens into the ultimate mutagenic agent).

Ethanol was determined to be the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in ethanol at a maximum concentration of approximately 500 mg/ml and was soluble in the treatment medium at a concentration of 5000 µg/ml.

In the preliminary toxicity assay, the maximum dose tested was 5000 µg/ml. This dose was achieved using a stock concentration of 500 mg/ml and a 50 µl dosing aliquot. The test article was soluble in solvent at the stock concentration of 500 mg/ml and was soluble in treatment medium at all concentrations tested. Selection of dose levels for the chromosome aberration assay was based on cell growth inhibition relative to the solvent control. Cell harvest times were determined after evaluating the test article effect on the cell cycle kinetics by measuring the average generation time (AGT). Substantial toxicity, i.e., at least 50% cell growth inhibition, was observed at a dose level of 5000 µg/ml in both the non-activated and S9-activated test systems. Based on these findings, the doses chosen for the chromosome aberration assay ranged from 313 to 5000 µg/ml for both the non-activated and the S9-activated systems. The average generation time was delayed from approximately 12 hours to 23.8 hours at the highest dose tested (5000 µg/ml) in the S9-activated study. No cell cycle delay was seen at any of the doses tested in the non-activated study. Based on the cell cycle

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delay seen in the S9-activated portion of the preliminary toxicity assay, the cell harvest time was adjusted to 20 hours. The cell harvest time for the non-activated study was 12 hours.

In the chromosome aberration assay, the test article was soluble in solvent at a stock concentration of 500 mg/ml and was soluble in treatment medium at all concentrations tested. Toxicity (cell growth inhibition) was approximately 43% at the highest dose level evaluated for chromosome aberrations (5000 μg/ml) in the non-activated study. Toxicity was approximately 72% at the highest dose level evaluated for chromosome aberrations (5000 μg/ml) in the S9-activated study. A statistically significant increase in chromosome aberrations was observed at the 2500 μg/ml dose level in the non-activated study ($p \leq 0.05$, Fisher's exact test). However, this response was within the range of the historical control and was not concluded to be biologically significant. Statistically significant increases in the percentage of cells with chromosome aberrations, relative to the solvent control, were observed at the 1250, 2500 and 5000 μg/ml dose levels in the S9-activated test system ($p \leq 0.05$, Fisher's exact test). The Cochran-Armitage test was also positive for a dose responsive trend ($p > 0.05$). Based on the findings of this study, Tertiary Amyl Methyl Ether (TAME) was concluded to be positive for the induction of structural chromosome aberrations in Chinese hamster ovary (CHO) cells.

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INTRODUCTION

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 Chinese hamster ovary (CHO) cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Tertiary Amyl Methyl Ether (TAME), was received by Microbiological Associates, Inc. on December 26, 1995 and was assigned the code number 95CA89. The test article was characterized by the Sponsor as a clear liquid, which should be stored away from flame, sparks, hot surfaces, strong acids or oxidizing materials. No expiration date was provided. Upon receipt, the test article was described as a clear liquid, stored at room temperature, and was protected from exposure to light.

The solvent used to deliver Tertiary Amyl Methyl Ether (TAME) to the test system was ethanol (CAS No. 64-17-5), obtained from Pharmco Products Inc. (Brookfield, CT).

Mitomycin C (MMC, CAS No. 50-07-7), was obtained from the Sigma Chemical Company, (St. Louis, MO) and was dissolved and diluted in sterile distilled water to stock concentrations of 8 and 15 μg/ml for use as the positive control in the non-activated test system. Cyclophosphamide (CP, CAS No. 6055-19-2), was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 1 and 2 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. Complete medium or S9 reaction mixture was used in the untreated control.

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MATERIALS AND METHODS

TEST SYSTEM

Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61) were obtained from American Type Culture Collection, Rockville, MD. To assure the karyotypic stability of the cell line, working cell stocks were not used beyond passage 20. The freeze lot of cells was tested using the Hoechst staining procedure and found to be free of mycoplasma contamination. The use of CHO cells has been demonstrated to be an effective method of detection of chemical clastogens (Preston *et al.*, 1981).

METABOLIC ACTIVATION SYSTEM

Aroclor® 1254-induced rat liver S9 was used as the metabolic activation system. The S9 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 S9 was batch prepared and stored at ≤-70°C until used. Each bulk preparation of S9 was assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 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 S9 per milliliter medium (McCoy's 5A 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 to select the solvent. The test was conducted using the following solvents in the order of preference as listed: purified water, dimethylsulfoxide (DMSO) and ethanol. The test article was tested to determine the solvent, selected in order of preference, that allowed preparation of the most soluble or workable stock concentration, up to 500 mg/ml.

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PRELIMINARY TOXICITY ASSAY

The preliminary toxicity assay was performed for the purpose of selecting dose levels and harvest times for the chromosome aberration assay and consisted of an evaluation of test article effect on cell growth and cell cycle kinetics. CHO cells were seeded for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding the flasks with 5 ml complete medium [McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 units penicillin and 100 µg streptomycin/ml, and 2 mM L-glutamine] for the non-activated study or S9-reaction mixture (4 ml serum free medium plus 1 ml of 5X S9 mix) for the activated study, to which was added 50 µl dosing solution of test article in solvent or solvent alone. The cells were treated for 6 hours without S9 or for 4 hours with S9. Two hours after initiation of treatment, a 50 µl aliquot of 1 mM 5-bromo-2'-deoxyuridine (BrdU) was added to each flask and incubation continued as required. At completion of the exposure period, the treatment medium was removed and the cells were washed with calcium- and magnesium-free phosphate buffered saline (CMF-PBS), refed with 5 ml complete medium containing 0.01 mM BrdU, and returned to the incubator for a total of 24 hours from the initiation of BrdU treatment. Two hours prior to cell harvest, Colcemid® was added to each flask at a final concentration of 0.1 µg/ml. After incubation in Colcemid®, the cells were harvested by trypsinization and counted using a Coulter counter. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the solvent control. Metaphase preparations were made and stained for sister chromatid differentiation using a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). Slides were evaluated for the percentage of first, second and third-plus-subsequent-division metaphase cells for estimation of the test article effect on cell cycle kinetics. The average generation time (AGT) was calculated for each treatment condition.

CHROMOSOME ABERRATION ASSAY

The chromosome aberration assay was performed using standard procedures (Evans, 1976), by exposing duplicate cultures of CHO cells to the test article as well as positive and negative

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controls. For the chromosome aberration assay, CHO cells were seeded at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding duplicate flasks with 5 ml complete medium for the non-activated study or 5 ml S9 reaction mixture for the S9-activated study, to which was added 50 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 to the test article continuously up to cell harvest at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. Two hours prior to the scheduled cell harvest of 12 hours, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/ml and the flasks returned to the incubator until cell collection.

In the S9-activated study, the cells were exposed for 4 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After the exposure period, the treatment medium was removed, the cells were washed with CMF-PBS, refed with complete medium and returned to the incubator. Two hours prior to the scheduled cell harvest of 20 hours, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/ml and the flasks were returned to the incubator until cell collection.

A concurrent toxicity test was conducted in both the non-activated and the S9-activated studies. After cell harvest, an aliquot of the cell suspension was removed from each culture and counted using a Coulter counter. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the solvent control.

COLLECTION OF METAPHASE CELLS

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the non-activated and S9-activated studies by trypsinization. Cells were collected approximately 12

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hours after initiation of treatment in the non-activated portion of the study and approximately 20 hours after initiation of treatment in the S9-activated portion of the study. The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 ml 0.075 M potassium chloride (KCl) and allowed to stand at room temperature for 4-8 minutes. The cells were collected by centrifugation, the supernatant aspirated and the cells fixed with two washes of approximately 2 ml Carnoy's fixative (methanol:glacial acetic acid, 3:1, v/v). The cells were stored overnight or longer in fixative at approximately 2-6°C.

SLIDE PREPARATION

To prepare slides, the fixed cells were centrifuged at approximately 800 rpm for 5 minutes, the supernatant was aspirated, and 1 ml fresh fixative was added. After additional centrifugation (at approximately 800 rpm for 5 minutes) the supernatant fluid was decanted and the cells were resuspended to opalescence in fresh fixative. A sufficient amount of cell suspension was dropped onto the center of a glass slide and allowed to air dry overnight. Slides were identified by the study number, date prepared and the treatment condition. 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. To ensure that a sufficient number of metaphase cells were present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) was determined for each treatment group. Metaphase cells with 20±2 centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations. 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

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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) were also 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.

EVALUATION OF TEST RESULTS

The toxic effects of treatment were based upon cell growth inhibition relative to the solvent-treated control and are presented for the toxicity and aberration studies. The average generation time (AGT), an estimate of the time for one cell cycle, was calculated for each treatment condition in the preliminary toxicity study as: AGT = (24 hours x 100)/ [(number M_1 cells x 1)+(number M_2 cells x 2)+(number M_3 cells x 3)], where M_n is Metaphase. The AGT was used to adjust the cell harvest time in the chromosome aberration assay.

The number and types of aberrations found, the percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined, and the frequency of structural aberrations per cell (mean aberrations per cell) were calculated and reported for each 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 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 measure dose-responsiveness.

All conclusions were founded 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 were increased in a dose-responsive manner with one or more

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concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A significant increase at the high dose only with no dose response was considered suspect. A significant increase at one dose level other than the high dose with no dose response was considered equivocal. Test articles that did not demonstrate a statistically significant increase in aberrations were concluded to be negative.

CRITERIA FOR 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.

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.

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RESULTS AND DISCUSSION

SOLUBILITY TEST

Ethanol was determined to be the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in ethanol at a maximum concentration of approximately 500 mg/ml and was soluble in the treatment medium at a concentration of 5000 µg/ml.

PRELIMINARY TOXICITY ASSAY

Dose levels and post-treatment cell harvest times for the chromosome aberration assay were selected following a preliminary toxicity test based upon a reduction of cell growth (cell growth inhibition) and cell cycle delay after treatment relative to the solvent control. The results of the evaluation of cell growth inhibition are presented in Tables 1 and 2 and the results of the evaluation of cell cycle delay are presented in Tables 3 and 4. CHO cells were exposed to solvent alone and to nine concentrations of test article ranging from 0.5 µg/ml to 5000 µg/ml in the absence and presence of an S9 reaction mixture. The test article was soluble in solvent at a stock concentration of 500 mg/ml, and soluble in treatment medium at all concentrations tested. The osmolality in treatment medium of the highest concentration tested (5000 µg/ml) was 294 mOsm/kg. The osmolality of the solvent was 282 mOsm/kg. The pH of the highest concentration of test article in treatment medium was approximately 7.

Cell growth inhibition relative to the solvent control was 66% at 5000 µg/ml, the highest concentration tested in the non-activated test system, and 64% at 5000 µg/ml, the highest concentration tested in the S9-activated test system. Based on these findings, the doses chosen for the chromosome aberration assay ranged from 313 to 5000 µg/ml for both the non-activated and the S9-activated systems. No cell cycle delay was apparent at any of the doses tested in the non-activated study. The cell cycle was delayed from approximately 12 hours to 23.8 hours at the highest dose tested (5000 µg/ml) in the S9-activated study. Based on the cell cycle kinetics observed in the preliminary toxicity assay, the cell harvest times for the chromosome aberration assay were 12 hours for the non-activated study and 20 hours for the

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S9-activated study. The selected harvest times were used to ensure microscopic evaluation of first-division metaphase cells.

CHROMOSOME ABERRATION ASSAY

The activity of Tertiary Amyl Methyl Ether (TAME) in the induction of chromosome aberrations in CHO cells when treated in the absence of S9-activation is presented by treatment flask in Table 6 and summarized by group in Table 9. The test article was soluble in solvent at a stock concentration of 500 mg/ml, and soluble in treatment medium at all concentrations tested. Toxicity (cell growth inhibition relative to the solvent control) was approximately 43% at 5000 µg/ml, the highest test concentration evaluated for structural chromosome aberrations (Table 5). An additional concentration of 313 µg/ml was tested as a safeguard against excessive toxicity at higher concentrations but was not required for microscopic examination. The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control only at 2500 µg/ml ($p \leq 0.05$, Fisher's exact test). However, the response seen at this dose level was within the range of the historical control and was not concluded to be biologically significant. The percentage of damaged cells in the MMC group was 9.5% ($p \leq 0.01$, Fisher's exact test).

The activity of Tertiary Amyl Methyl Ether (TAME) in the induction of chromosome aberrations in CHO cells when treated in the presence of an S9 reaction mixture is presented by treatment flask in Table 8 and summarized by group in Table 9. Due to contamination of the cultures, this portion of the assay was repeated. Only data collected from the repeat assay are contained in this report. The test article was soluble in solvent at stock concentration (500 mg/ml) and was soluble in treatment medium at all concentrations tested. Toxicity (cell growth inhibition relative to the solvent control) was approximately 72% at 5000 µg/ml, the highest test concentration evaluated for structural chromosome aberrations (Table 7). An additional concentration of 313 µg/ml was tested as a safeguard against excessive toxicity at higher concentrations but was not required for microscopic examination. The percentage of cells with structural aberrations in the test article-treated groups was statistically increased above that of the solvent control at the 1250, 2500 and 5000 µg/ml dose levels ($p \leq 0.05$,

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Fisher's exact test). The Cochran-Armitage test was also positive for a dose response ($p \leq 0.05$). The percentage of damaged cells in the CP group was 32.5% ($p \leq 0.01$, Fisher's exact test).

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CONCLUSION

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

Under the conditions of the assay described in this report, Tertiary Amyl Methyl Ether (TAME) was concluded to be positive in the chromosome aberration assay using Chinese hamster ovary (CHO) cells.

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Table 1. Preliminary Toxicity Test Using Tertiary Amyl Methyl Ether (TAME) in the Absence of Exogenous Metabolic Activation (6 hour treatment, 26 hour harvest)

Treatment[1]	Cell Count ($x10^6$)	Cell Viability[2] (%)	Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Ethanol	2.39	100%	2.39	100%	0%
Tertiary Amyl Methyl Ether (TAME)					
0.5 µg/ml	2.24	99%	2.22	93%	7%
1.5 µg/ml	2.44	98%	2.39	100%	-0%
5 µg/ml	2.53	97%	2.45	103%	-3%
15 µg/ml	2.51	99%	2.48	104%	-4%
50 µg/ml	2.45	99%	2.43	101%	-1%
150 µg/ml	2.31	100%	2.31	97%	3%
500 µg/ml	2.41	100%	2.41	101%	-1%
1500 µg/ml	2.29	99%	2.27	95%	5%
5000 µg/ml	0.81	99%	0.80	34%	66%

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 6 hours at 37±1°C
[2]Viability determined by trypan blue dye exclusion
[3]Viable cells/flask = cell count x % viable cells
[4]Growth index = (cells per flask, treated group/cells per flask, control group), expressed as a percentage
[5]Cell growth inhibition = 100% - % cell growth index

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Table 2. Preliminary Toxicity Test Using Tertiary Amyl Methyl Ether (TAME) in the Presence of Exogenous Metabolic Activation (4 hour treatment, 26 hour harvest)

Treatment[1]	Cell Count ($x10^6$)	Cell Viability[2] (%)	Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Ethanol	1.73	98%	1.70	100%	0%
Tertiary Amyl Methyl Ether (TAME)					
0.5 µg/ml	1.78	100%	1.78	105%	-5%
1.5 µg/ml	2.02	100%	2.02	119%	-19%
5 µg/ml	1.87	99%	1.85	109%	-9%
15 µg/ml	1.95	98%	1.91	113%	-13%
50 µg/ml	1.87	100%	1.87	110%	-10%
150 µg/ml	1.68	98%	1.65	97%	3%
500 µg/ml	1.51	98%	1.48	87%	13%
1500 µg/ml	1.08	99%	1.07	63%	37%
5000 µg/ml	0.68	91%	0.62	36%	64%

[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C
[2]Viability determined by trypan blue dye exclusion
[3]Viable cells/flask = cell count x % viable cells
[4]Growth index = (cells per flask, treated group/cells per flask, control group), expressed as a percentage
[5]Cell growth inhibition = 100% - % cell growth index

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Table 3. Preliminary Toxicity Test Using Tertiary Amyl Methyl Ether (TAME) in the Absence of Exogenous Metabolic Activation

Treatment[1]	Cell Cycle Kinetics Percentage of Cells in M_1	M_2	M_3	Average Generation Time[2] (AGT)
Ethanol	4	96	0	12.2
Tertiary Amyl Methyl Ether (TAME)				
0.5 µg/ml	4	96	0	12.2
1.5 µg/ml	3	97	0	12.2
5 µg/ml	2	98	0	12.1
15 µg/ml	1	99	0	12.1
50 µg/ml	4	96	0	12.2
150 µg/ml	5	95	0	12.3
500 µg/ml	1	99	0	12.1
1500 µg/ml	4	96	0	12.2
5000 µg/ml	13	87	0	12.8

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 6 hours at 37±1°C. Metaphase cells were collected following a 24 hour growth period in BrdU

[2]Average Generation Time:

$$\frac{24 \text{ hours} \times 100}{[(\text{number of } M_1 \text{ cells} \times 1) + (\text{number of } M_2 \text{ cells} \times 2) + (\text{number of } M_3 \text{ cells} \times 3)]}$$

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Table 4. Preliminary Toxicity Test Using Tertiary Amyl Methyl Ether (TAME) in the Presence of Exogenous Metabolic Activation

Treatment[1]	Cell Cycle Kinetics Percentage of Cells in			Average Generation Time[2] (AGT)
	M_1	M_2	M_3	
Ethanol	1	99	0	12.1
Tertiary Amyl Methyl Ether (TAME)				
0.5 µg/ml	6	94	0	12.4
1.5 µg/ml	6	94	0	12.4
5 µg/ml	5	95	0	12.3
15 µg/ml	3	97	0	12.2
50 µg/ml	5	95	0	12.3
150 µg/ml	19	81	0	13.3
500 µg/ml	28	72	0	14.0
1500 µg/ml	91	9	0	22.0
5000 µg/ml	99	1	0	23.8

[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C. Metaphase cells were collected following a 24 hour growth period in BrdU

[2]Average Generation Time:

$$\frac{24 \text{ hours} \times 100}{[(\text{number of } M_1 \text{ cells} \times 1) + (\text{number of } M_2 \text{ cells} \times 2) + (\text{number of } M_3 \text{ cells} \times 3)]}$$

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Table 5. Concurrent Toxicity Test Using Tertiary Amyl Methyl Ether (TAME) in the Absence of Exogenous Metabolic Activation

Treatment[1]	Replicate Flask	Cell Count	Cell Viability[2] (%)	Mean Variable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A B	1.41 1.38	95% 96%	1.33	---	---
Ethanol	A B	1.32 1.41	97% 98%	1.33	100%	0%
Tertiary Amyl Methyl Ether (TAME)						
313 µg/ml	A B	1.32 1.33	95% 99%	1.29	97%	3%
625 µg/ml	A B	1.24 1.30	99% 96%	1.24	93%	7%
1250 µg/ml	A B	1.17 1.13	98% 98%	1.13	85%	15%
2500 µg/ml	A B	1.00 1.08	98% 97%	1.01	76%	24%
5000 µg/ml	A B	0.82 0.75	96% 96%	0.75	57%	43%
MMC* 0.08 µg/ml	A B	1.31 1.21	98% 99%	1.24	93%	7%

*Mitomycin C (MMC, CAS No. 50-07-7)

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 12 hours at 37±1°C

[2]Viability determined by trypan blue dye exclusion

[3]Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B

[4]Growth index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage (Test article concentrations compared to solvent control and positive control compared to untreated control)

[5]Cell growth inhibition = 100% - % cell growth index

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Table 6. Cytogenetic Analysis of CHO Cells Treated with Tertiary Amyl Methyl Ether (TAME) in the Absence of Exogenous Metabolic Activation (12 hour harvest)

Treatment[1]	Flask	Mitotic Index	Cells Scored	Aberrant Cells[2] (%)	Total Number of Structural Aberrations						Severely Damaged Cells[5]	Average Aberrations Per Cell[2,6]
					Chromatid-type[3]			Chromosome-type[4]				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	4.8	100	2	0	2	0	0	0	0	0	0.020
	B	5.8	100	0	0	0	0	0	0	0	0	0.000
Ethanol	A	5.8	100	0	0	0	0	0	0	0	0	0.000
	B	5.6	100	0	3	0	0	0	0	0	0	0.000
Tertiary Amyl Methyl Ether (TAME)[7]												
625 µg/ml	A	4.6	100	1	1	1	0	0	0	0	0	0.010
	B	5.4	100	1	0	1	0	0	0	0	0	0.010
1250 µg/ml	A	5.6	100	0	1	0	0	0	0	0	0	0.000
	B	4.6	100	0	1	0	0	0	0	0	0	0.000
2500 µg/ml	A	3.4	100	5	0	4	0	1	0	0	0	0.050
	B	2.8	100	3	2	3	0	0	0	0	0	0.030
5000 µg/ml	A	4.2	100	1	1	1	0	0	0	0	0	0.010
	B	4.4	100	2	0	2	0	0	0	0	0	0.020
MMC* 0.08 µg/ml	A	2.8	100	11	4	10	1	1	0	0	0	0.120
	B	2.8	100	8	1	6	1	0	0	1	0	0.080

*Mitomycin C (MMC, CAS No. 50-07-7)
[1]CHO cells were treated for 12 hours at 37±1°C in the absence of an exogenous source of metabolic activation
[2]Excluding cells with only gaps
[3]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements
[4]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome
[5]Severely damaged cells include cells with one or more pulverized chromosomes and cells with 10 or more aberrations
[6]Severely damaged cells and pulverizations were counted as 10 aberrations
[7]A dose level of 313 µg/ml was also tested but was not required for analysis

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Table 7. Concurrent Toxicity Test Using Tertiary Amyl Methyl Ether (TAME) in the Presence of Exogenous Metabolic Activation

Treatment[1]	Replicate Flask	Cell Count ($x10^6$)	Cell Viability[2]	Mean Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A B	2.80 3.06	98% 99%	2.89	N/C	N/C
Ethanol	A B	3.17 2.56	98% 100%	2.83	100%	0%
Tertiary Amyl Methyl Ether (TAME)						
313 µg/ml	A B	2.17 2.18	98% 95%	2.10	74%	26%
625 µg/ml	A B	2.08 2.25	98% 97%	2.11	74%	26%
1250 µg/ml	A B	1.83 1.94	100% 99%	1.88	66%	34%
2500 µg/ml	A B	1.30 1.35	97% 98%	1.29	46%	54%
5000 µg/ml	A B	0.80 0.85	98% 97%	0.80	28%	72%
CP* 10 µg/ml	A B	1.96 1.87	97% 100%	1.89	65%	35%

* Cyclophosphamide (CP, CAS No. 6055-19-2)
[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C
[2]Viability determined by trypan blue dye exclusion
[3]Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B
[4]Growth index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage (Test article concentrations compared to solvent control and positive control compared to untreated control)
[5]Cell growth inhibition = 100% - % cell growth index

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Table 8. Cytogenetic Analysis of CHO Cells Treated with Tertiary Amyl Methyl Ether (TAME) in the Presence of Exogenous Metabolic Activation (20 hour harvest)

Treatment[1]	Flask	Mitotic Index	Cells Scored	Aberrant Cells[2] (%)	Total Number of Structural Aberrations						Severely Damaged Cells[5]	Average Aberrations Per Cell[2,6]
					Chromatid-type[3]			Chromosome-type[4]				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	10.6	100	0	0	0	0	0	0	0	0	0.000
	B	9.6	100	0	0	0	0	0	0	0	0	0.000
ETOH	A	6.2	100	1	0	1	0	0	0	0	0	0.010
	B	6.4	100	0	0	0	0	0	0	0	0	0.000
Tertiary Amyl Methyl Ether (TAME)[7]												
625 µg/ml	A	9.4	100	2	0	1	1	0	0	0	0	0.020
	B	11.0	100	2	0	2	0	0	0	0	0	0.020
1250 µg/ml	A	4.6	100	11	2	5	7	1	0	0	0	0.130
	B	6.0	100	9	4	7	4	0	0	0	0	0.110
2500 µg/ml	A	5.0	100	22	6	18	15	0	0	0	0	0.330
	B	4.2	100	20	2	16	9	0	0	0	0	0.250
5000 µg/ml	A	1.2	100	45	4	47	32	9	0	0	3	1.180
	B	1.0	100	34	6	41	15	6	0	0	0	0.620
CP* 10 µg/ml	A	4.0	100	30	1	29	6	1	0	0	2	0.560
	B	2.4	100	35	2	34	14	2	0	0	2	0.700

* Cyclophosphamide (CP, CAS No. 6055-19-2)
[1]CHO cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation
[2]Excluding cells with only gaps
[3]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements
[4]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome
[5]Severely damaged cells include cells with one or more pulverized chromosomes and cells with 10 or more aberrations
[6]Severely damaged cells and pulverizations were counted as 10 aberrations
[7]A dose level of 313 µg/ml was also tested but was not required for analysis

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Table 9. Summary

Treatment	S9 Activation	Harvest Time (hrs)	Mitotic Index	Cells Scored	Aberrations Per Cell[1] (Mean ± SD)	Cells with Aberrations[2] (%)
Untreated	_	12	5.3	200	0.010 ± 0.100	1.0
Ethanol	-	12	5.7	200	0.000 ± 0.000	0.0
Tertiary Amyl Methyl Ether (TAME)						
625 µg/ml	-	12	5.0	200	0.010 ± 0.100	1.0
1250 µg/ml	-	12	5.1	200	0.000 ± 0.000	0.0
2500 µg/ml	-	12	3.1	200	0.040 ± 0.196	4.0**[3]
5000 µg/ml	-	12	4.3	200	0.015 ± 0.122	1.5
MMC† 0.08 µg/ml	-	12	2.8	200	0.100 ± 0.317	9.5**
Untreated	+	20	10.1	200	0.000 ± 0.000	0.0
Ethanol	+	20	6.3	200	0.005 ± 0.071	0.5
Tertiary Amyl Methyl Ether (TAME)						
625 µg/ml	+	20	10.2	200	0.020 ± 0.140	2.0
1250 µg/ml	+	20	5.3	200	0.120 ± 0.395	10.0**
2500 µg/ml	+	20	4.6	200	0.290 ± 0.631	21.0**
5000 µg/ml	+	20	1.1	200	0.900 ± 1.742	39.5**
CP‡ 10 µg/ml	+	20	3.2.	200	0.630 ± 1.528	32.5**

† Mitomycin C (MMC, CAS No. 50-07-7)
‡ Cyclophosphamide (CP, CAS No. 6055-19-2)
[1]Severely damaged cells were counted as 10 aberrations
[2] **, p≤0.01; Fisher's exact test
[3]Statistically significant but within the range of the historical control

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APPENDIX A

Historical Control Data

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IN VITRO MAMMALIAN CYTOGENETIC TEST USING CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES STRUCTURAL ABERRATIONS 1993-1995

NON-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	1.1%	1.2%	34.9%
Standard Deviation	1.0%	1.2%	21.8%
Range	0.0% to 5.5%	0.0% to 6.0%	8% to 100.0%

S9-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	1.6%	1.5%	45.4%
Standard Deviation	1.3%	1.3%	24.8%
Range	0.0% to 5.5%	0.0% to 6.0%	7.5% to 100.0%

[1]Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, PBS, 2% DMSO in saline, CSEP Buffer, citrate buffer, culture medium, 1% human albumin, extraction blanks, human platelets, placebo, 5% dextrose, 0.75% NaCMC, bicarbonate buffered 0.9% saline, sham electrodes.

[2]Positive control for non-activated studies, triethylenemelamine (TEM, 0.25-0.5 μg/ml,) N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 μg/ml), and Mitomycin C (MMC, 0.08-0.15 μg/ml).

[3]Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 μg/ml), and benzo(α)pyrene, (B[α]P, 30 μg/ml).

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APPENDIX B

Study Protocol

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DBM 4-8-96 QA APPROVED

PROTOCOL AMENDMENT I

SPONSOR: **American Petroleum Institute**

TEST ARTICLE I.D.: **Tertiary Amyl Methyl Ether (TAME)**

MA STUDY NO: **G95CA89.330 (Protocol No.: SPGT330)**

SPONSOR PROJECT NO.: **HES1621-L-00860-MUTAGEN**

PROTOCOL TITLE: **Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells**

---

1. **LOCATION:** Page 8 of 9, § 11.1; Records;

AMENDMENT: Change the paragraph to read as follows: "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."

REASON FOR THE AMENDMENT: Relocation of facility.

APPROVALS:

Patrick T. Cummings — STUDY DIRECTOR — 4-5-96 DATE

David A. Gladen — SPONSOR REPRESENTATIVE — 4/10/96 DATE

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QA 11-15-96
APPROVED

PROTOCOL AMENDMENT 2

SPONSOR:	**American Petroleum Institute**
TEST ARTICLE I.D.:	**Tertiary Amyl Methyl Ether (TAME)**
MA STUDY NO:	**G95CA89.330 (Protocol No.: SPGT330)**
SPONSOR PROJECT NO.:	**HES1621-L-00860-MUTAGEN**
PROTOCOL TITLE:	**Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells**

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1. **LOCATION:** Page 2 of 9, § 4.3; Study Director:

   AMENDMENT: Amend the name of the Study Director to Ramadevi Gudi, Ph.D.

   REASON FOR THE AMENDMENT: To reflect a change of Study Director.

2. **LOCATION:** Page 8, § 12.0, Regulatory Requirements/Good Laboratory Practice

   AMENDMENT: Add "yes" in response to the question "Will this study be submitted to a regulatory agency?".

   REASON FOR THE AMENDMENT: To complete the information for the protocol.

APPROVALS:

Ramadevi Gudi — STUDY DIRECTOR — 11/14/96 — DATE

[signature] — SPONSOR REPRESENTATIVE — 11/19/96 — DATE

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QA APPROVED
Received by RA/QA 1-23-95

MA Study Number: G95CA89.330

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1	Name:	**American Petroleum Institute**
2.2	Address:	**1220 L Street, Northwest** **Washington, D.C. 20005**
2.3	Representative:	**Richard Rhoden, Ph.D.**
2.4	Sponsor Project #:	**HES1621-L-00860-MUTAGEN**

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **Tertiary Amyl Methyl Ether (TAME)**

3.2 Controls:

Untreated:	Untreated Cells
Solvent:	Test Article Solvent
Positive:	Mitomycin C (MMC)
	Cyclophosphamide (CP)

3.3 Determination of Strength, Purity, etc.

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

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Genetic Toxicology Division
Microbiological Associates, Inc.

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4.2 Address: 9900 Blackwell Road
Rockville, MD 20850

4.3 Study Director: Patrick T. Curry, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 3-5-96

5.2 Proposed Experimental Completion Date: 3-25-96

5.3 Proposed Report Date: 4-8-96

6.0 TEST SYSTEM

The CHO-K_1 cell line is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. CHO-K_1 cells were obtained from the American Type Culture Collection (repository number CCL 61), Rockville, MD. 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 chromosome aberration assay will be conducted using standard procedures (Evans, 1976), by exposing cultures of CHO cells to a minimum of four concentrations of the test article as well as to positive, untreated, and solvent controls. In the non-activated test system, treatment will be continuous up to the time of cell collection; in the S9 activated test system, exposure will be for 4 hours. The dividing cells will be arrested in metaphase and harvested for microscopic evaluation of chromosome aberrations at approximately 12 hours after the initiation of treatment. In order to ensure evaluation of first-division metaphase cells, the cell collection time may be delayed up to 20 hours in the event of cell cycle delay associated with test article 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.

7.1 Solubility Determination

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

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7.2 Dose Levels

Selection of the dose levels for the cytogenetics assay will be based upon cell growth inhibition relative to the solvent control after treatment. CHO cells will be exposed to solvent alone and to at least nine concentrations of test article. 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 in treatment medium will also be measured. Cells seeded 16-24 hours earlier will be exposed for 6 hours in the absence of S9- and for 4 hours in the presence of S9. Two hours after initiation of exposure, 5-bromo-2'-deoxyuridine (BrdU), will be added to the culture medium at a final concentration of 0.01 mM. The cultures will be grown in medium containing 0.01 mM BrdU for 24 hours. Twenty two hours after BrdU treatment initiation Colcemid® (0.1 μg/ml) will be added to the cultures. Two hours after Colcemid® addition the cells will be harvested by trypsinization and counted using an automatic cell counter and trypan blue dye exclusion. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control. Metaphase preparations will be made and stained for sister chromatid differentiation using a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). Slides will be evaluated for the percentage of first-, second- and third-plus-subsequent-division metaphase cells per 100 cells scored for determination of the test article effect on cell cycle kinetics.

Whenever possible, the high dose for the chromosome aberration assay will be selected to give at least 50% toxicity (cell growth inhibition relative to the solvent). At least three additional dose levels will be included in the chromosome aberration assay. In the event the test article cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, then the highest dose to be tested will be the concentration resulting in minimum precipitation in test medium. Minimum precipitation will be determined by direct visual inspection. In the event the test article demonstrates a dose-responsive increase in toxicity (cell growth inhibition relative to the solvent) at concentrations that exceed solubility in treatment medium, then the highest dose to be tested will be the maximum concentration resulting in a workable suspension that results in at least 50% toxicity. 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. The cell cycle kinetics study will be used to verify the average cell generation time of the target cells and to measure the test article effect on the cell generation time. Post-treatment cell harvest times will be adjusted if there is considerable cell cycle delay.

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7.3 Frequency and Route of Administration

Target cells will be treated for 12 hours in the absence of S9 (or up to 20 hours depending upon cell cycle delay) and for 4 hours in the presence of S9 by incorporation of the test article-solvent mixture into the treatment medium. This technique has demonstrated to be an effective method of detection of chemical clastogens in this test system.

7.4 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 ($MgCl_2$,) 6 mM potassium chloride (KCl), 1mM 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 Control

Untreated cells will be used as the untreated control.

7.5.2 Solvent Control

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

7.5.3 Positive Controls

Mitomycin C will be used at a concentration within 0.05-0.3 μg/ml as the positive control in the non-activated study. Cyclophosphamide will be used at a concentration within 10-50 μg/ml as the positive control in the S9-activated study.

7.6 Preparation of Target Cells

Exponentially growing CHO-K_1 cells will be seeded in complete medium (McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml) for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask. The flasks will be

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incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air for 16-24 hours.

7.7 Identification of Test System

Using a permanent marking pen, the treatment flasks will be identified by the MA study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in duplicate by refeeding the flasks with 5 ml complete medium for the non-activated exposure or 5 ml S9 reaction mixture for the S9-activated exposure, to which will be added 50 µl of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if water, physiological buffer, 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 treated for 12 hours at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. Treatment will be extended to up to 20 hours in those dose levels with considerable cell cycle delay. Treatment will continue until collection of metaphase cells.

In the S9-activated study, the cells will be treated for 4 hours at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. After the treatment period, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator.

A concurrent toxicity test will be conducted in both the non-activated and the S9-activated studies. After cell harvest an aliquot of the cell suspension will be removed from each culture and counted using an automatic cell counter. Cell viability will be determined by trypan blue dye exclusion. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control.

7.9 Collection of Metaphase Cells

Cells will be collected approximately 12 hours after initiation of treatment or at a later time selected to represent the first division metaphase after initiation of treatment if the test article causes considerable cell cycle delay. Cell fixation will not extend beyond 20 hours after initiation of treatment. Two hours prior to harvest, Colcemid® will be added to the cultures at a final concentration of 0.1 µg/ml.

Cells will be harvested by trypsinization, collected by centrifugation and an

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aliquot will be removed for counting using an automatic cell counter and trypan blue dye exclusion. The remainder of the cells will be swollen with 0.075M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 2-6°C. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control. To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. The suspension of fixed cells will be applied dropwise onto a glass microscope slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. The slides will then be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

To ensure that a sufficient number of metaphase cells are present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined for each treatment group. Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20 ± 2 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads from each dose level (100 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations. 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 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 a structural aberration will be recorded using a calibrated microscope stage. 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 chromosome aberrations in either the untreated or solvent control must be no greater than 6%.

8.2 Positive Control

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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 was used.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon cell growth inhibition relative to the solvent-treated control and will be presented for the toxicity and aberration studies. The AGT will be calculated for each treatment condition in the toxicity study as: AGT = (24 hours x 100)/[(number M_1 cells x 1)+(number M_2 cells x 2)+(number M_3 cells x 3)]. The number and types of aberrations found, the percentage of structurally damaged cells in the total population of cells examined, and the mean aberrations per cell will be calculated and 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 percentage of aberrant cells will be performed using the Fisher's exact test. The 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 exact test at any test article dose level, the Cochran-Armitage test will be used to measure dose-responsiveness. All conclusions will be based on sound scientific basis; 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 significant ($p \leq 0.05$). A significant increase at the high dose only with no dose response will be considered suspect. A significant increase at one dose level other than the high dose with no dose response will be considered equivocal. 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 Microbiological Associates, Inc. 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_2 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, untreated, and solvent controls
- number of cell cultures

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- mitotic index and number of metaphases analyzed (method for determination; data given separately for each culture)
- cell growth inhibition relative to the solvent control
- 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

11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, MD, 20850, in accordance with the relevant Good Laboratory Practice Regulations.

11.2 Specimens

All specimens, such as microscope slides, will be held in storage as long as the quality of the preparation affords evaluation or in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was designed to fulfill EPA and OECD test guidelines.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations For Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency?_____

If so, to which agency or agencies?_____

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

13.0 REFERENCES

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, NY.

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Perry, P. and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, Nature, 251: 156-158.

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.

14.0 APPROVAL

Richard A. Rhoden — 1/16/96

SPONSOR REPRESENTATIVE — DATE

RICHARD A. RHODEN

(Print or Type Name)

Patrick T. Lang — 1-23-96

MA STUDY DIRECTOR — DATE

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PROTOCOL AMENDMENT I

QA APPROVED CC 10-31-96

SPONSOR: **American Petroleum Institute**

TEST ARTICLE I.D.: **Tertiary Amyl Methyl Ether (TAME)**

MA STUDY NO: **G95CA89.782**

SPONSOR PROJECT NO.: **HES1621-L-00860-MUTAGEN**

PROTOCOL TITLE: **CHO/HGPRT MUTATION ASSAY**

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1. **LOCATION:** Page 7, § 12.0, Regulatory Requirements/Good Laboratory Practice

   AMENDMENT: Add "yes" in response to the question "Will this study be submitted to a regulatory agency?".

   REASON FOR THE AMENDMENT: To complete the information for the protocol.

2. **LOCATION:** Page 7, § 12.0, Regulatory Requirements/Good Laboratory Practice

   AMENDMENT: Add "U.S. E.P.A." in response to the question "If so, to which agency or agencies?".

   REASON FOR THE AMENDMENT: To complete the information for the protocol.

APPROVALS:

STUDY DIRECTOR	10/31/96 DATE
SPONSOR REPRESENTATIVE	11/8/96 DATE

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