American
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Health and Environmental
Sciences Department

Chinese Hamster Ovary (CHO)/HGPRT Mutation Assay of Tertiary Amyl Methyl Ether (TAME)

DECEMBER 1996

TOXICOLOGY REPORT NUMBER 411
CAIS ABSTRACT NO. 43-5240

Chinese Hamster Ovary (CHO)/HGPRT Mutation Assay of Tertiary Amyl Methyl Ether (TAME)

Health and Environmental Sciences Department

API PUBLICATION NUMBER TR411

PREPARED UNDER CONTRACT BY:

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DECEMBER 1996

American Petroleum Institute

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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: **G95CA89.782**

DATE OF FINAL REPORT: **06/19/96**

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

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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 1 to Appendix B.

(e) Add memo on cell history record to Appendix B.

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

APPROVALS:

Richard [illegible] 11/22/96

STUDY DIRECTOR — DATE

Claire L Courtemanche Nov 22, 1996

QUALITY ASSURANCE — DATE

MICROBIOLOGICAL ASSOCIATES, INC.

The Quality Assurance Report for the American Petroleum Institute contained on these pages was performed for two laboratory studies. This publication, TR411, corresponds to MBA Study G95CA89.782. The remaining MBA study (G95CA89.330) is published under API publication TR410.

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

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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.

ABSTRACT

The test article, Tertiary Amyl Methyl Ether (TAME), was tested in the CHO/HGPRT Mutation Assay in the absence of Aroclor®-induced rat liver S9 at doses of 1000 to 5000 µg/ml and in the presence of Aroclor®-induced rat liver S9 at doses of 500 to 5000 µg/ml. No positive responses, i.e., treated cultures with mutant frequencies >40 mutants per 10^6 clonable cells, were observed. All concentrations were soluble in treatment medium. Toxicity, i.e., cloning efficiency ≤50% of the solvent control, was observed at doses of 4000 and 5000 µg/ml with S9 activation. Under the conditions of this study, test article Tertiary Amyl Methyl Ether (TAME) was concluded to be negative in the CHO/HGPRT Mutation Assay.

TABLE OF CONTENTS

AMENDED FINAL REPORT

Study Title

CHO/HGPRT MUTATION ASSAY

Test Article

Tertiary Amyl Methyl Ether (TAME)

Authors

Richard H. C. San, Ph.D.

Jane J. Clarke, B.A.

Study Completion Date

06/19/96

Performing Laboratory

Microbiological Associates, Inc.

9900 Blackwell Road

Rockville, MD 20850

Laboratory Study Number

G95CA89.782

Sponsor Project Number

HES1621-L-00860-MUTAGEN

Sponsor

American Petroleum Institute

1220 L Street, Northwest

Washington, DC 20005

CHO/HGPRT MUTATION ASSAY
AMENDED FINAL REPORT

Sponsor: **American Petroleum Institute**
1220 L Street, Northwest
Washington, DC 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)**

Test Article specific gravity : **0.77 g/ml (Provided by Sponsor)**

Sponsor Project No.: **HES1621-L-00860-MUTAGEN**

MA Study No.: **G95CA89.782**

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 Manager: _____ 11/22/96
Jane J. Clarke, B.A. Date

Study Director: _____ 11/22/96
Richard H. C. San, Ph.D. Date

QUALITY ASSURANCE STATEMENT

Study Title: CHO/HGPRT MUTATION ASSAY

Study Number: G95CA89.782

Study Director: Richard H. C. San, 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 25 JAN 96, TO STUDY DIR 25 JAN 96, TO MGMT 25 JAN 96
PHASE: Protocol Review

INSPECT ON 14 FEB 96, TO STUDY DIR 14 FEB 96, TO MGMT 20 FEB 96
PHASE: Scoring toxicity plates

INSPECT ON 27 MAR 96, TO STUDY DIR 27 MAR 96, TO MGMT 08 APR 96
PHASE: Draft Report

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

INSPECT ON 27 AUG 96, TO STUDY DIR 27 AUG 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

STATEMENT OF COMPLIANCE

Study No. G95CA89.782, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. 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 have not been 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.

Richard H. C. San, Ph.D.
Study Director

11/22/96
Date

SUMMARY

The test article, Tertiary Amyl Methyl Ether (TAME), was tested in the CHO/HGPRT Mutation Assay in the absence and presence of Aroclor®-induced rat liver S9. The assay was performed in two phases. The first phase, the preliminary toxicity assay, was used to establish the dose range for the mutagenesis assay. The second phase, the mutagenesis assay, was used to evaluate the mutagenic potential of the test article.

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 concentration of 500 mg/ml, the maximum concentration tested.

In the preliminary toxicity assay, the maximum concentration of Tertiary Amyl Methyl Ether (TAME) tested was 5000 μg/ml. Treatment medium had a film on the surface but no visible precipitate at 5000 μg/ml. Concentrations of ≤1500 μg/ml were soluble in treatment medium. Selection of dose levels for the mutagenesis assay was based on the cloning efficiency relative to the solvent control. Substantial toxicity, i.e., cloning efficiency ≤50% of the solvent control, was observed at no doses without activation and at 5000 μg/ml with S9 activation. Based on these findings, the doses chosen for the mutagenesis assay ranged from 1000 to 5000 μg/ml for the non-activated cultures and from 500 to 5000 μg/ml for the S9-activated cultures.

In the mutagenesis assay, no positive responses, i.e., treated cultures with mutant frequencies >40 mutants per 10^6 clonable cells, were observed. All concentrations were soluble in treatment medium. Toxicity, i.e., cloning efficiency ≤50% of the solvent control, was observed at doses of 4000 and 5000 μg/ml with S9 activation.

Under the conditions of this study, test article Tertiary Amyl Methyl Ether (TAME) was concluded to be negative in the CHO/HGPRT Mutation Assay.

INTRODUCTION

The purpose of this study was to evaluate the mutagenic potential of the test article based on quantitation of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

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. Upon receipt, the test article was described as a clear liquid and was stored at room temperature, protected from exposure to light.

The vehicle used to deliver Tertiary Amyl Methyl Ether (TAME) was ethanol (CAS 64-17-15) obtained from Pharmco Products Inc. (Brookfield, CT).

Ethyl methanesulfonate (EMS), CAS 62-50-0, was obtained from Eastman Laboratory Chemicals (Rochester, NY) and was used at a stock concentration of 20 µl/ml as the positive control for the non-activated test system. Benzo(a)pyrene (B(a)P), CAS 50-32-8 was obtained from Sigma Chemical Company (St. Louis, MO) and was used at a stock concentration of 400 µg/ml as the positive control for the S9-activated test system.

TEST SYSTEM

CHO-K_1-BH_4 cells were obtained from Dr. Abraham Hsie, Oak Ridge National Laboratories, Oak Ridge, TN. CHO cells were cleansed in medium supplemented with hypoxanthine, aminopterin and thymidine (HAT), then frozen. In deviation from the protocol, there was no record of testing the freeze lot of cells for mycoplasma contamination. However, a freeze lot of cells derived from this freeze lot was confirmed to be mycoplasma-free. Therefore, this

deviation did not adversely impact the intergrity and conclusions of the study. Cells used in the mutation assay were within four subpassages from frozen stock in order to assure karyotypic stability. Consistency in the negative and positive control values obtained in this laboratory over the years is indicative of the stability of this cell line.

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-dimethyl-benz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 reaction mixture was prepared by mixing S9 and 10 mM calcium chloride ($CaCl_2$) with a filter-sterilized cofactor pool to contain 100 µl S9/ml cofactor pool, 4 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 mM glucose-6-phosphate, 30 mM potassium chloride (KCl), 10 mM magnesium chloride ($MgCl_2$), and 50 mM sodium phosphate buffer, pH 8.0. The S9 reaction mixture was stored on ice until used.

SOLUBILITY TEST

A solubility test was conducted to select the vehicle. The test was conducted using one or more of the following solvents in the order of preference as listed: distilled water, dimethylsulfoxide, ethanol and acetone. The test article was tested to determine the vehicle, selected in order of preference, that permitted preparation of the highest soluble or workable stock concentration, up to 500 mg/ml.

PRELIMINARY TOXICITY ASSAY

The preliminary toxicity assay was used to establish the optimal dose levels for the mutagenesis assay and consisted of evaluation of test article effect on colony-forming

efficiency. CHO cells were exposed for 5 hours at 37±1°C to the vehicle alone or one of nine concentrations of test article ranging from 0.5 to 5000 µg/ml in both the absence and presence of S9-activation.

MUTAGENESIS ASSAY

The mutagenesis assay was used to evaluate the mutagenic potential of the test article. CHO cells were exposed for 5 hours at 37±1°C to the vehicle alone, appropriate positive controls or one of five concentrations of test article in duplicate in both the absence and presence of S9.

TREATMENT OF THE TARGET CELLS

The mutagenesis assay was performed according to a protocol developed from published methodologies (Hsie *et al.*, 1981; and O'Neill *et al.*, 1977). Exponentially growing CHO-K_1-BH_4 cells were seeded in F12FBS5-Hx at a density of $5x10^5$ cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 18-24 hours. F12FBS5-Hx is Ham's F12 medium without hypoxanthine supplemented with 5% dialyzed FBS, 100 units penicillin/ml, 100 µg streptomycin/ml and 2mM L-glutamine/ml.

The time of initiation of chemical treatment was designated as day 0. Treatment was carried out by refeeding the treatment flasks with 5 ml F12FBS5-Hx/25 cm^2 flask for the non-activated study and 4 ml F12FBS5-Hx and 1 ml S9 reaction mixture/25 cm^2 flask for the S9-activated study, to which was added either 50 µl dosing solution of test or control article in vehicle or vehicle alone. Duplicate flasks of cells were exposed to one of at least five concentrations of the test article for 5 hours at 37±1°C. After the treatment period, all media were aspirated, the cells washed with Ca^{++}- and Mg^{++}-free Hanks' balanced salt solution (CMF-HBSS) and cultured in F12FBS5-Hx for an additional 18-24 hours at 37±1°C. At this time, the cells were subcultured to assess cytotoxicity and to initiate the phenotypic expression period.

EVALUATION OF CYTOTOXICITY

For evaluation of cytotoxicity, the replicates from each treatment condition were detached using trypsin and subcultured independently in F12FBS5-Hx, in triplicate, at a density of 100 cells/60 mm dish. After 7-10 days incubation, the colonies were rinsed with HBSS, fixed

cells/60 mm dish. After 7-10 days of incubation, the colonies were rinsed with HBSS, fixed with methanol, stained with 10% aqueous Giemsa and counted; cloning efficiency was then determined.

EXPRESSION OF THE MUTANT PHENOTYPE

For expression of the mutant phenotype, the replicates from each treatment condition were trypsinized and subcultured independently in F12FBS5-Hx, in duplicate, at a density no greater than 10^6 cells/100 mm dish. Subculturing by trypsinizing at 2-3 day intervals was employed for the 7-9 day expression period. At the end of the expression period, selection for the mutant phenotype was performed.

SELECTION OF THE MUTANT PHENOTYPE

For selection of the TG-resistant phenotype, the replicates from each treatment condition were trypsinized and replated, in quintuplicate, at a density of $2x10^5$ cells/100 mm dish in F12FBS5-Hx containing 10 μM 6-thioguanine (TG, 2-amino-6-mercaptopurine). For cloning efficiency determinations at the time of selection, 100 cells/60 mm dish were plated in triplicate. After 7-10 days of incubation, the colonies were fixed, stained and counted for both cloning efficiency and mutant selection.

EVALUATION OF TEST RESULTS

The cytotoxic effects of each treatment condition were expressed relative to the solvent-treated control (relative cloning efficiency). The mutant frequency (MF) for each treatment condition was calculated by dividing the total number of mutant colonies by the number of cells selected (usually $2x10^6$ cells: 10 plates at $2x10^5$ cells/plate), corrected for the cloning efficiency of cells prior to mutant selection, and is expressed as TG-resistant mutants per 10^6 clonable cells. For experimental conditions in which no mutant colonies were observed, mutant frequencies were expressed as less than the frequency obtained with one mutant colony. Mutant frequencies generated from doses giving ≤ 10% relative survival are presented in the data but were not considered as valid data points.

Because spontaneous mutant frequencies are very low for the CHO/HGPRT assay, calculation of mutagenic response in terms of fold increase in mutant frequency above the background rate does not provide a reliable indication of the significance of the observed response. The wide acceptable range in spontaneous mutant frequency also suggests the need to set a minimum mutant frequency for a response to be considered positive. Hsie *et al.* (1981) refer to a level of 50 mutants per 10^6 clonable cells. In this laboratory, a more conservative approach was used which set the minimum level at >40 mutants per 10^6 clonable cells.

The assay would be considered positive in the event of a dose-dependent increase in mutant frequencies with at least two consecutive doses showing mutant frequencies which were elevated above 40 mutants per 10^6 clonable cells. If a single point above 40 mutants per 10^6 clonable cells was observed at the highest dose, the assay would be considered suspect. If no culture exhibited a mutant frequency of >40 mutants per 10^6 clonable cells, the test article would be considered negative.

CRITERIA FOR A VALID TEST

The cloning efficiency of the solvent control must be greater than 50%. The spontaneous mutant frequency in the solvent control must fall within the range of 0-25 mutants per 10^6 clonable cells. The positive control must induce a mutant frequency at least three times that of the solvent control and must exceed 40 mutants per 10^6 clonable cells.

ARCHIVES

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

RESULTS AND CONCLUSIONS

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 concentration of 500 mg/ml, the maximum concentration tested.

PRELIMINARY TOXICITY ASSAY

The results of the preliminary toxicity assay are presented in Table 1. CHO cells were exposed to solvent alone and nine concentrations of test article ranging from 0.5 to 5000 μg/ml in the absence and presence of S9 reaction mixture. No test article precipitate was observed at any dose level in treatment medium. The osmolality of the solvent control and the highest soluble dose could not be determined due to equipment malfunction. This deviation from the protocol was determined by the Study Director to have had no adverse effect on the integrity or outcome of the study. Cloning efficiency relative to the solvent controls (RCE) was 89% at 5000 μg/ml without activation and 42% at 5000 μg/ml with S9 activation. Based on the results of the toxicity test, the doses chosen for the mutagenesis assay ranged from 1000 to 5000 μg/ml for the non-activated cultures and 500 to 5000 μg/ml for the S9-activated cultures.

MUTAGENESIS ASSAY

The cytotoxic effects of the test article (concurrent cytotoxicity) are presented in Table 2. Mutagenicity data are presented in Tables 3 and 4. In the non-activated system, cultures treated with concentrations of 1000, 2000, 3000, 4000 and 5000 μg/ml were cloned. In the S9-activated system, cultures treated with concentrations of 500, 2000, 3000, 4000 and 5000 μg/ml were cloned. No test article precipitate was observed at any dose level in treatment medium. Relative cloning efficiency was 75% and 44% at the highest dose tested in the non-activated and S9-activated systems, respectively. None of the treated cultures exhibited mutant frequencies of greater than 40 mutants per 10^6 clonable cells.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the CHO/HGPRT Mutation Assay indicate that, under the conditions of this study, Tertiary Amyl Methyl Ether (TAME) did not cause a positive response in the non-activated and S9-activated systems and was concluded to be negative.

REFERENCES

Hsie, A.W., D.A. Casciano, D.B. Couch, B.F. Krahn, J.P. O'Neill, and B.L. Whitfield. 1981. The Use of Chinese Hamster Ovary Cells to Quantify Specific Locus Mutation and to Determine Mutagenicity of Chemicals. A Report of the Gene-Tox Program. *Mutation Research.* 86:193-214.

O'Neill, J.P., P.A. Brimer, R. Machanoff, G.P. Hirsch, and A.W. Hsie. 1977. A Quantitative Assay of Mutation Induction at the Hypoxanthine-guanine Phosphoribosyl Transferase Locus in Chinese Hamster Ovary Cells (CHO/HGPRT System): Development and Definition of the System. *Mutation Research.* 45:91-101.

Table 1. Preliminary Toxicity Assay Using Tertiary Amyl Methyl Ether (TAME)

-S9						+S9					
Treatment[1] (μg/ml)	Colonies per Dish 1	2	3	Cloning Efficiency[2]	Relative Cloning Efficiency[3]	Treatment[1] (μg/ml)	Colonies per Dish 1	2	3	Cloning Efficiency[3]	Relative Cloning Efficiency[3] (%)
Ethanol	91	122	95	1.03	100	Ethanol	113	88	100	1.0	100
Tertiary Amyl Methyl Ether (TAME):											
0.5	109	118	119	1.15	112	0.5	73	76	70	0.73	73
1.5	123	126	119	1.23	119	1.5	78	76	83	0.79	79
5.0	102	102	109	1.04	102	5.0	a	69	76	0.73	72
15	105	92	99	0.99	96	15	93	104	84	0.94	93
50	116	123	104	1.14	111	50	92	100	95	0.96	95
150	105	81	108	0.98	95	150	97	100	109	1.02	102
500	111	119	106	1.12	109	500	101	94	95	0.97	96
1500	101	106	111	1.06	103	1500	71	63	69	0.68	67
5000	107	94	73	0.91	89	5000	46	38	42	0.42	42

[1] Cells were exposed to the test article for 5 hours at 37 ± 1°C

[2] Cloning efficiency = $\frac{\text{total colonies counted}}{\text{number of dishes x 100 cells/dish}}$

[3] Relative cloning efficiency = $\frac{\text{cloning efficiency of treatment group x 100}}{\text{cloning efficiency of solvent group}}$

Table 2. Concurrent Cytotoxicity Assay Using Tertiary Amyl Methyl Ether (TAME)

-S9						+S9					
Treatment[1] (μg/ml)	Colonies per Dish 1	2	3	Cloning Efficiency[2]	Relative Cloning Efficiency[3]	Treatment[1] (μg/ml)	Colonies per Dish 1	2	3	Cloning Efficiency[3]	Relative Cloning Efficiency[3] (%)
Ethanol	107 80	116 99	131 107	1.07	100	Ethanol	78 93	70 76	63 70	0.75	100
EMS 0.2μl/ml	48 a	67 131	72 112	0.86	81	B(a)P 4 μg/ml	44 29	39 35	29 45	0.37	49
Tertiary Amyl Methyl Ether (TAME):											
1000	84 98	76 108	63 104	0.89	83	500	65 61	67 57	50 60	0.60	80
2000	82 145	74 a	83 a	0.96	90	2000	47 46	67 61	48 51	0.53	71
3000	65 a	75 a	98 a	0.79	74	3000	33 42	36 38	42 a	0.38	51
4000	74 112	71 107	63 108	0.89	84	4000	39 32	46 35	40 30	0.37	49
5000	69 a	81 a	91 a	0.80	75	5000	32 28	30 35	38 33	0.33	44

[1] Cells were exposed to the test article for 5 hours at 37 ± 1°C

[2] Cloning efficiency = $\frac{\text{total colonies counted}}{\text{number of dishes x 100 cells/dish}}$

[3] Relative cloning efficiency = $\frac{\text{cloning efficiency of treatment group x 100}}{\text{cloning efficiency of solvent group}}$

EMS Ethyl Methanesulfonate

a Plate lost due to contamination

Table 3. Non-activated (-S9) Study Using Tertiary Amyl Methyl Ether (TAME)

Treatment[1] (μg/ml)	Colonies per Dish 1	2	3	Total Colonies	Cloning Efficiency[2]	Mutant Colonies/Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants/10^6 Clonable Cells[3]
Ethanol	86	101	78	556	0.93	0	0	0	0	0	0	<0.6[4]
	90	100	101			0	a	0	0	0		
EMS 0.2μl/ml	95	78	83	472	0.79	44	21	32	32	49	351	223.1
	58	86	72			38	34	49	18	34		
Tertiary Amyl Methyl Ether (TAME):												
1000	97	108	75	539	0.90	0	0	0	0	2	7	3.9
	90	87	82			2	2	0	1	0		
2000	99	123	121	584	0.97	0	0	0	0	0	0	<0.5[4]
	75	81	85			0	0	0	0	0		
3000	112	102	88	574	0.96	0	0	1	2	0	3	1.6
	89	98	85			0	0	0	0	0		
4000	102	91	90	517	0.86	0	1	0	0	1	11	6.4
	73	90	71			2	1	2	1	3		
5000	81	97	109	541	0.90	0	0	0	0	0	1	0.6
	76	81	97			0	0	0	0	1		

[1] Cells were exposed to the test article for 5 hours at 37±1°C

[2] Cloning efficiency = $\frac{\text{total colonies counted}}{\text{number of dishes x 100 cells/dish}}$

[3] Mutants/10^6 clonable cells = $\frac{\text{total mutant colonies x } 10^6}{\text{number selection dishes X cloning efficiency X 2 x } 10^3 \text{ cells}}$

[4] Calculated on the basis of <1 mutant colonies observed in the total number of dishes prepared

EMS Ethyl methanesulfonate

a Plate lost due to contamination

Table 4. Activated (+S9) Study Using Tertiary Amyl Methyl Ether (TAME)

Treatment[1] (μg/ml)	Colonies per Dish 1	2	3	Total Colonies	Cloning Efficiency[2]	Mutant Colonies/Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants/10^6 Clonable Cells[3]
Ethanol	69	86	104	531	0.89	1	0	3	1	0	14	7.9
	82	94	96			1	2	4	2	0		
B(a)P	69	85	80	423	0.71	15	20	14	20	21	203	144.0
4μl/ml	52	70	67			21	28	23	21	20		
Tertiary Amyl Methyl Ether (TAME):												
500	81	81	85	534	0.89	1	0	0	2	1	8	4.5
	98	103	86			0	0	0	0	4		
2000	82	97	89	504	0.84	2	2	1	2	1	9	5.4
	79	82	75			1	0	0	0	0		
3000	70	80	72	222	0.74	1	1	0	0	3	5	6.8
	a	a	a			a	a	a	a	a		
4000	68	76	84	405	0.68	2	0	0	0	0	6	4.4
	61	53	63			1	2	0	1	0		
5000	57	78	98	440	0.73	3	3	2	0	0	17	11.6
	68	60	79			0	3	1	3	2		

[1] Cells were exposed to the test article for 5 hours at 37±1°C

[2] Cloning efficiency = $\frac{\text{total colonies counted}}{\text{number of dishes x 100 cells/dish}}$

[3] Mutants/10^6 clonable cells = $\frac{\text{total mutant colonies} \quad \text{x } 10^6}{\text{number selection dishes X cloning efficiency X 2 x } 10^3 \text{ cells}}$

[4] Calculated on the basis of <1 mutant colonies observed in the total number of dishes prepared

B(a)P Benzo(a)pyrene

a Plate lost due to contamination

APPENDIX A

HISTORICAL CONTROL DATA

CHO/HGPRT Assay Historical Control Data

1993 - 1995

	Non-activated		S9-activated	
	Solvent Control	0.2µl/ml EMS	Solvent Control	4.0µg/ml B(a)P
Mean MF	6.5	234.9	8.0	140.0
SD	5.0	58.3	7.0	63.0
Maximum	19.9	453.9	24.2	327.5
Minimum	0.0	137.6	0.4	38.1

Solvent controls including: culture medium, distilled water, saline, DMSO, ethanol, acetone or vehicle supplied by Sponsor

EMS Ethyl methanesulfonate
B(a)P Benzo(a)pyrene
MF Mutant frequency per 10^6 clonable cells
SD Standard deviation

APPENDIX B

STUDY PROTOCOL

PROTOCOL AMENDMENT I

QAcc APPROVED 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:

Richard D	10/31/96
STUDY DIRECTOR	DATE
Robert A Kleinstein	11/8/96
SPONSOR REPRESENTATIVE	DATE

Toom 1-25-96 QA APPROVED

Received by RA/QA 01/23/96

MA Study Number: G95CA89.782

CHO/HGPRT MUTATION ASSAY

1.0 PURPOSE

The purpose of this study is to assess the mutagenic potential of a test article based on quantitation of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of 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:	Solvent:	Test article vehicle
		Positive:	Ethyl methanesulfonate (EMS)
			Benzo(a)pyrene (BaP)

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.
4.2	Address:	9900 Blackwell Road Rockville, MD 20850
4.3	Study Director:	Richard H. C. San, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 1/31/96

5.2 Proposed Experimental Completion Date: 3/25/96

5.3 Proposed Report Date: 3/29/96

6.0 TEST SYSTEM

The CHO-K1-BH4 cell line is a proline auxotroph with a modal chromosome number of 20, a population doubling time of 12-14 hours, and a cloning efficiency of usually greater than 80% (1). This subclone (D1) was derived by Dr. Abraham Hsie, Oak Ridge National Laboratories, Oak Ridge, TN. CHO cells were cleansed in medium supplemented with HAT (hypoxanthine, aminopterin and thymidine) then frozen. Cells used in the mutation assay will not exceed four subpassages from frozen stock. Each freeze lot of cells has been tested and found to be free of mycoplasma contamination.

The CHO/HGPRT assay was designed to select for mutant cells which have become resistant to such purine analogues as 6-thioguanine (TG) and 8-azaguanine as a result of mutation at the X-chromosome-linked HGPRT locus (1-4). This system has been demonstrated to be sensitive to the mutagenic action of a variety of chemicals (2).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be performed by exposing CHO cells for 5 hours to five concentrations of test article as well as positive and the solvent controls in the presence and absence of an exogenous source of metabolic activation. After a seven to nine day expression period, the treated cells will be cultured in the presence of 10 μM TG for selection of mutant colonies. The mutagenic potential of a test article will be determined by its ability to induce a dose-related increase in the number of TG-resistant mutant colonies when compared to the solvent control.

7.1 Selection of solvent

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

7.2 Dose Selection

The optimal dose levels for the mutation assay will be selected following a preliminary toxicity test based upon colony-forming efficiency. Approximately 5×10^5 CHO cells will be seeded into 25 cm^2 flasks and incubated at $37 \pm 1°C$ in a humidified atmosphere of $5 \pm 1\%$ CO_2 in air. Eighteen to 24 hours later, cells will be exposed to solvent alone and to no less than nine concentrations of test article, the highest concentration being the lowest insoluble dose in treatment medium not to exceed 5000 $\mu g/ml$. The pH of the treatment medium will be adjusted, if necessary, to maintain a neutral pH in the treatment medium. The osmolality of the highest soluble treatment condition will also be measured. Exposure will be for 5 hours at $37 \pm 1°C$ in a humidified atmosphere of $5 \pm 1\%$ CO_2 in air in the presence and absence of S-9 activation. Eighteen to 24 hours after removal of treatment medium, the treated cells will be trypsinized and reseeded at a density of 100 cells/60 mm dish. After 7-10 days incubation at $37 \pm 1°C$ in a humidified atmosphere of $5 \pm 1\%$ CO_2 in air, colonies will be fixed with 95% methanol, stained with 10% aqueous Giemsa, and counted. The cell survival of the test article-treated groups will be expressed relative to the solvent control (relative cloning efficiency).

Whenever possible, the high dose will be selected to give a cell survival of 10-30%. Four lower doses will be selected, at least one of which will be non-toxic. If the desired toxicity is not achieved in the preliminary toxicity test at the maximum concentration allowed by protocol, the Sponsor will be contacted prior to dose selection.

7.3 Frequency and Route of Administration

Cell cultures will be treated for 5 hours by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This technique of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S-9 will be used as the metabolic activation

system. The source of S-9 will be adult male Sprague-Dawley rats induced by a single injection of Aroclor 1254 at a dose level of 500 mg/kg body weight five days prior to sacrifice. The S-9 will be batch prepared and stored frozen approximately -70°C until used.

Immediately prior to use, the S-9 will be thawed and mixed with a cofactor pool to contain 100 μl S-9/ml reaction mixture of approximately 4 mM NADP, 5 mM glucose-6-phosphate, 10 mM $MgCl_2$, 30 mM KCl, 10 mM $CaCl_2$, and 50 mM sodium phosphate buffer, pH 8.0 (3). The S-9 reaction mixture will be stored on ice until used.

7.5 Controls

7.5.1 Solvent control

The solvent for the test article will be used as the solvent control.

7.5.2 Positive control

Ethyl methanesulfonate (EMS) will be used at one concentration within the range of 0.1 to 0.4 μl/ml as the positive control for the non-activated study. Benzo(a)pyrene (BaP) will be used at one or two concentrations within the range of 3 to 6 μg/ml as the positive control for the S-9 activated study.

7.6 Preparation of Target Cells

Exponentially growing CHO-K1-BH4 cells will be seeded in F12 medium, with or without hypoxanthine, supplemented with 5% dialyzed serum (F12FBS5 or F12FBS5-Hx) at a density of 5 x 10^5 cells/25 cm^2 surface area and will be incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 18-24 hours.

7.7 Identification of the Test System

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

7.8 Treatment of Target Cells

The time of initiation of chemical treatment will be designated as day 0. Cells will be exposed, in duplicate cultures, to five concentrations of test article for 5 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. For each 25 cm^2 of surface area treated, the treatment medium will consist of 5 ml F12FBS5 or F12FBS5-Hx and 50 μl of control or test article diluted to the appropriate concentration in solvent for the non-activated study, or 4 ml F12FBS5 or F12FBS5-Hx, 1 ml S-9 reaction mixture, and 50 μl of

control or test article diluted to the appropriate concentration in solvent for the activated study. After the treatment period, all media will be aspirated, the cells washed with Hank's Balanced Salt Solution (CMF-HBSS) and cultured in F12FBS5 or F12FBS5-Hx at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After 18-24 hours incubation, the cells will be subcultured to assess cytotoxicity and to continue the phenotypic expression period.

7.9 Estimation of Toxicity

For evaluation of cytotoxicity, the replicate cultures from each treatment condition will be subcultured independently in F12FBS5 or F12FBS5-Hx, in triplicate, at a density of 100 cells/60 mm dish. After 7-10 days incubation at 37±1°C in 5±1% CO_2 in air, colonies will be fixed with 95% methanol, stained with 10% aqueous Giemsa, and counted. Cytotoxicity will be expressed relative to the solvent-treated control cultures.

7.10 Expression of the Mutant Phenotype

For expression of the mutant phenotype, the replicates from each treatment condition will be subcultured independently in F12FBS5 or F12FBS5-Hx, at a density of no greater than 10^6 cells/100 mm dish. Subculture as above at 2-3 day intervals will be performed for the 7-9 day expression period. At this time, selection for the mutant phenotype will be performed.

7.11 Selection of the Mutant Phenotype

For selection of the TG-resistant phenotype, cells from each treatment condition will be plated into a maximum of five dishes at a density of 2 x 10^5 cells/100 mm dish in F12FBS5-Hx containing 10 μM TG. For cloning efficiency at the time of selection, 100 cells/60 mm dish will be plated in triplicate in medium free of TG. After 7-10 days of incubation, the colonies will be fixed, stained and counted for both cloning efficiency at selection and mutant selection.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The cloning efficiency of the solvent control must be greater than 50%. The spontaneous mutant frequency in the solvent control must fall within the range of 0-25 mutants per 10^6 clonable cells.

The positive control must induce a mutant frequency at least three times that of the solvent control and must exceed 40 mutants per 10^6 clonable cells.

9.0 EVALUATION OF TEST RESULTS

The cytotoxic effects of each treatment condition are expressed relative to the solvent-treated control (relative cloning efficiency). The mutant frequency (MF) for

each treatment condition is calculated by dividing the total number of mutant colonies by the number of cells selected, corrected for the cloning efficiency of cells prior to mutant selection, and is expressed as TG-resistant mutants per 10^6 clonable cells. For experimental conditions in which no mutant colonies are observed, mutant frequencies will be expressed as less than the frequency obtained with one mutant colony. Mutant frequencies generated from doses giving $\leq 10\%$ relative survival are not considered as valid data points and will not be included in the data analysis.

Spontaneous mutant frequencies in this assay range from 0 to 25 mutants per 10^6 clonable cells. As a result, calculation of mutagenic response in terms of fold increase in mutant frequency above the background rate does not provide a reliable indication of the significance of the observed response. The wide acceptable range in spontaneous mutant frequency also suggests the need to set a minimum mutant frequency for a response to be considered positive. Hsie *et al* (2) refer to a level of 50 mutants per 10^6 clonable cells. In this laboratory, a more conservative approach is used which sets the minimum significant level at >40 mutants per 10^6 clonable cells.

All conclusions will be based on sound scientific judgement; however, the following will be used as a guide to interpretation of the data. The test article will be considered to induce a positive response if there is a concentration-related increase in mutant frequencies with at least two consecutive doses showing mutant frequencies of > 40 mutants per 10^6 clonable cells. If a single point above 40 mutants per 10^6 clonable cells is observed at the highest dose, the assay will be considered suspect. If no culture exhibits a mutant frequency of > 40 mutants per 10^6 clonable cells, the test article will be considered 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 data.

Results presented will include, but not be limited to:

- cell type used, number of cultures, methods for maintenance of cell cultures
- rationale for selection of concentrations and number of cultures
- test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period, selective agent
- method used to enumerate numbers of viable and mutant cells
- dose-response relationship, if applicable
- positive and solvent control historical data

11.0 RECORDS AND ARCHIVES

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

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was written according to the OECD Guideline 476 (Genetic Toxicology: *In Vitro* Mammalian Cell Gene Mutation Tests), April, 1984; and the EPA Health Effects Testing Guidelines, Subpart 798.5300 (Detection of Gene Mutations in Somatic Cells in Culture) Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987. A confirmatory assay will be required for full OECD and EPA guideline compliance.

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, 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

1. Li, A. P., J. H. Carver, W. N. Choy, A. W. Hsie, R. S. Gupta, K. S. Loveday, J. P. O'Neill, J. C. Riddle, L. F. Stankowski, and L. L. Yang. 1987. A guide for the performance of Chinese hamster ovary cell/hypoxanthine-guanine phosphoribosyl transferase gene mutation assay. Mutation Res. 189: 135-141.

2. Hsie, A. W., D. A. Casciano, D. B. Couch, B. F. Krahn, J. P. O'Neill, and B. L. Whitfield. 1981. The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. A report of the Gen-Tox Program. Mutation Res. 86: 193-214.

3. Machanoff, R., J. P. O'Neill, and A. W. Hsie. 1981. Quantitative analysis of cytotoxicity and mutagenicity of benzo(a)pyrene in mammalian cells (CHO/HGPRT). Chem. Biol. Interactions. 34: 1-10.

4. O'Neill, J. P., P. A. Brimer, R. Machanoff, J. P. Hirsch, and A. W. Hsie. 1977. a quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Development and definition of the system. Mutation Res. 45: 91-101.

14.0 APPROVAL

SPONSOR REPRESENTATIVE	1/16/96 DATE
(Print or Type Name)	
MA STUDY DIRECTOR	1/23/96 DATE

Memo to the Cell History Record for CHO-K_1-BH_4 Cells

CHO-K_1-BH_4, subclone D1 cells were a gift to Microbiological Associates from Dr. Abraham W. Hsie, Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN in 1980. Upon receipt the cells were amplified in number by subculture, cells pooled and batch frozen on 9-9-80.

Since the GLPs did not go into effect until 1979, complete documentation procedures were not in place at the time the CHO cells were received from Dr. Hsie. Therefore this document will stand as the receipt and origination documentation for this cell line.

I was a member of the genetic toxicology management staff at the time the cells were received and can attest to these facts.

6-19-96

Donald L. Putman, Ph.D.
Director, Genetic Toxicology
June 19, 1996

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202-682-8000
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