

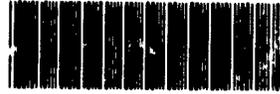
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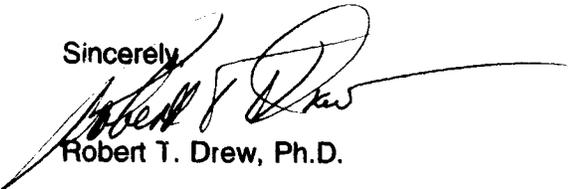
Dear FYI Coordinator:

In accordance with API's policy of providing the federal government with copies of research designed to determine whether any chemical substance or mixture manufactured, processed or distributed by API member companies may cause a risk of injury to health or the environment, we are enclosing copies of the following report:

(Identification no: FYI not assigned) Draft Report, CHO/HGPRT Mutation Assay.

Please note that this information is provided in accordance with the full disclosure policy of API and does not constitute a formal submission as required by a test rule. These documents do not contain confidential information. If you have any questions, please communicate with me.

Sincerely,


Robert T. Drew, Ph.D.

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DRAFT REPORT

Study Title

CHO/HGPRT MUTATION ASSAY

Test Article

Tertiary Amyl Methyl Ether (TAME)

Authors

Richard H. C. San, Ph.D.

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Study Completion Date

(Pending Final Report)

Performing Laboratory

Microbiological Associates, Inc.

9900 Blackwell Road

Rockville, MD 20850

Laboratory Study Number

G95CA89.782

Sponsor Project Number

HES1621-L-00860-MUTAGEN

Sponsor

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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 $\mu\text{g/ml}$ and in the presence of Aroclor-induced rat liver S9 at doses of 500 to 5000 $\mu\text{g/ml}$. 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 $\leq 50\%$ of the solvent control, was observed at doses of 4000 and 5000 $\mu\text{g/ml}$ 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.

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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

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

DBM
Diane B. Madser
QUALITY ASSURANCE

4-8-96
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.

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

4/8/96
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 10⁶ 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 is 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.

Ethyl methanesulfonate (EMS), CAS 62-50-0, was obtained from Eastman Laboratory Chemicals 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 and was used at a stock concentration of 400 µg/ml as the positive control for the S9-activated test system.

TEST SYSTEM

CHO-K₁-BH₄ 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. The freeze lot of cells was tested and found to be free of mycoplasma contamination. Cells used in the mutation assay were within four

subpassages from frozen stock in order to assure karyotypic stability.

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 $\leq -70^{\circ}\text{C}$ 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 $37 \pm 1^{\circ}\text{C}$ to the vehicle alone and nine concentrations of test article ranging from 0.5 to 5000 $\mu\text{g/ml}$ 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 $37\pm 1^\circ\text{C}$ to the vehicle alone, appropriate positive controls and 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-K₁-BH₄ cells were seeded in F12FBS5-Hx at a density of 5×10^5 cells/25 cm² flask and were incubated at $37\pm 1^\circ\text{C}$ in a humidified atmosphere of $5\pm 1\%$ CO₂ 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 treatment flasks with 5 ml F12FBS5-Hx/25 cm² flask for the non-activated study, 5 ml F12FBS5-Hx and 1 ml S9 reaction mixture/25 cm² flask for the S9-activated study, to which was added 50 µl dosing solution of test or control article in vehicle or vehicle alone. Duplicate flasks of cells were exposed to at least five concentrations of the test article for 5 hours at $37\pm 1^\circ\text{C}$. 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 $37\pm 1^\circ\text{C}$. 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 with methanol, stained with 10% aqueous Giemsa, counted and cloning efficiency 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 2×10^5 cells/100 mm dish in F12FBS5-Hx containing 10 μ M 6-thioguanine (TG, 2-amino-6-mercaptapurine). 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 2×10^6 cells: 10 plates at 2×10^5 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 $\leq 10\%$ 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 is used which sets the minimum level at >40 mutants per 10^6 clonable cells.

The assay will be considered positive in the event of a dose-dependent increase in mutant frequencies with at least two consecutive doses showing mutant frequencies which are elevated above 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.

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 47% 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 10⁶ 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
CHO/HGPRT MUTATION ASSAY
Preliminary Toxicity Assay Using Tertiary Amyl Methyl Ether (TAME)

Treatment ¹ (µg/ml)	Colonies per Dish			Cloning Efficiency ²	Relative Cloning Efficiency ³	Treatment ¹ (µg/ml)	Colonies per Dish			Cloning Efficiency ²	Relative Cloning Efficiency ³ (%)
	1	2	3				1	2	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	1.0	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 = $\frac{\text{total colonies counted}}{\text{number of dishes} \times 100 \text{ cells/dish}}$

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

^a Plate lost due to contamination

TABLE 2
CHO/HGPRT MUTATION ASSAY
Concurrent Cytotoxicity Assay Using Tertiary Amyl Methyl Ether (TAME)

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

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

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

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

a Plate lost due to contamination

TABLE 3

Ch HGPRT MUTATION ASSAY

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

Treatment ¹ (µg/ml)	Colonies per Dish			Total Colonies	Cloning Efficiency ²	Mutant Colonies/Selection Dish					Total Mutant Colonies	Mutants/10 ⁶ Clonable Cells ³
	1	2	3			1	2	3	4	5		
EtOH	86	101	78	556	0.93	0	0	0	0	0	0	<0.6 ⁴
	90	100	101			0	0	0	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	535	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 = $\frac{\text{total colonies counted}}{\text{number of dishes} \times 100 \text{ cells/dish}}$

³ Mutants/10⁶ clonable cells = $\frac{\text{total mutant colonies}}{\text{number selection dishes} \times \text{cloning efficiency} \times 2 \times 10^6 \text{ cells}}$ x 10⁶

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

EMS Ethyl methanesulphonate.

EtOH Ethyl alcohol.

µg µg/ml.

µl µl/ml.

TABLE 4
CH HGPRT MUTATION ASSAY

Treatment ¹ (µg/ml)	Colonies per Dish			Total Colonies	Cloning Efficiency ²	Mutant Colonies/Selection Dish					Total Mutant Colonies	Mutants/10 ⁶ Clonable Cells ³
	1	2	3			1	2	3	4	5		
EtOH	86	101	78	556	0.93	0	0	0	0	0	0	<0.6 ⁴
	90	100	101			0	0	0	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	535	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		

TABLE 4
CHO/HGPRT MUTATION ASSAY
Activated (+S9) Study Using Tertiary Amyl Methyl Ether (TAME)

Treatment ¹ (µg/ml)	Colonies per Dish			Total Colonies	Cloning Efficiency ²	Mutant Colonies/Selection Dish					Total Mutant Colonies	Mutants/10 ⁶ Clonable Cells ³
	1	2	3			1	2	3	4	5		
EtOH	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 4µl/ml	69	85	80	423	0.71	15	20	14	20	21	203	144.0
	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
	■	■	■			■	■	■	■	■		
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 = $\frac{\text{total colonies counted}}{\text{number of dishes} \times 100 \text{ cells/dish}}$

³ Mutants/10⁶ clonable cells = $\frac{\text{number of mutant colonies}}{\text{number selection dishes} \times \text{cloning efficiency} \times 2 \times 10^6 \text{ cells}} \times 10^6$

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

B(a)P Benzo(a)pyrene.

■ 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 10⁶ clonable cells

SD Standard deviation

APPENDIX B

Study Protocol

Dom
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: 3/20/96

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 (?).

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 μ M 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 cm² flasks and incubated at $37 \pm 1^\circ\text{C}$ in a humidified atmosphere of $5 \pm 1\%$ CO₂ 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 µg/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 $37 \pm 1^\circ\text{C}$ in a humidified atmosphere of $5 \pm 1\%$ CO₂ 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 $37 \pm 1^\circ\text{C}$ in a humidified atmosphere of $5 \pm 1\%$ CO₂ 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 MgCl₂, 30 mM KCl, 10 mM CaCl₂, 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 5×10^5 cells/25 cm² surface area and will be incubated at $37 \pm 1^\circ\text{C}$ in a humidified atmosphere of $5 \pm 1\%$ CO₂ 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 $37 \pm 1^\circ\text{C}$ in a humidified atmosphere of $5 \pm 1\%$ CO₂ in air. For each 25 cm² 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 $37 \pm 1^\circ\text{C}$ in a humidified atmosphere of $5 \pm 1\%$ 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 $37 \pm 1^\circ\text{C}$ in $5 \pm 1\%$ 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 2×10^6 cells/100 mm dish in F12FBS5-Hx containing $10 \mu\text{M}$ 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.

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 MICROBIOLOGICAL
ASSOCIATES, INC.

MA Study No. G95CA89.782

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14.0 APPROVAL

Richard L. ... 1/16/96
SPONSOR REPRESENTATIVE DATE

(Print or Type Name)
Richard L. ... 1/23/96
MA STUDY DIRECTOR DATE

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