TRADE SECRET

FINAL REPORT

Study Title

H-23005: *IN VITRO* MAMMALIAN CELL GENE MUTATION TEST WITH AN INDEPENDENT REPEAT ASSAY

LABORATORY PROJECT ID: HLO-1998-01211

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STUDY COMPLETED ON: March 19, 1998

PERFORMING LABORATORY: MA BioServices, Inc.
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for
E. I. du Pont de Nemours and Company
Haskell Laboratory for Toxicology and Industrial Medicine
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Newark, Delaware 19714-0050

PERFORMING LABORATORY STUDY NO.: G97CK07.782001

WORK REQUEST NO.: [redacted]

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with EPA FIFRA (40 CFR 160) and EPA TSCA (40 CFR 792) Good Laboratory Practice Standards, and OECD Principles of Good Laboratory Practice (C(81)30(Final), Annex 2) with the following exception:

Treatment solutions or suspensions were not analyzed for identity, composition, uniformity, or stability of the test and control articles. The procedures used by trained personnel to prepare the treatment solutions intended to ensure:

A. The accuracy of concentration because the test and control articles were weighed on an analytical balance accurate to 3 decimal places and the vehicle in which the test and control substances were dissolved was accurately measured with graduated pipettes or flasks;

B. Uniformity because all treatment solutions or suspensions were mixed prior to administration to the test system; and

C. Stability because treatment solutions or suspensions were prepared just prior to administration to the test system.

This deviation did not affect the validity or the integrity of the study.

Submitter/Submitter: E. I. du Pont de Nemours and Company

Study Director: _____ 3/19/98
Richard H.C. San, Ph.D. Date
Scientific Director

Study Monitor: _____ 3/20/98
Brian H. Mathison, Ph.D. Date

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CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Reported Approved and
Issued by Study Director: Richard 3/19/98

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

Date

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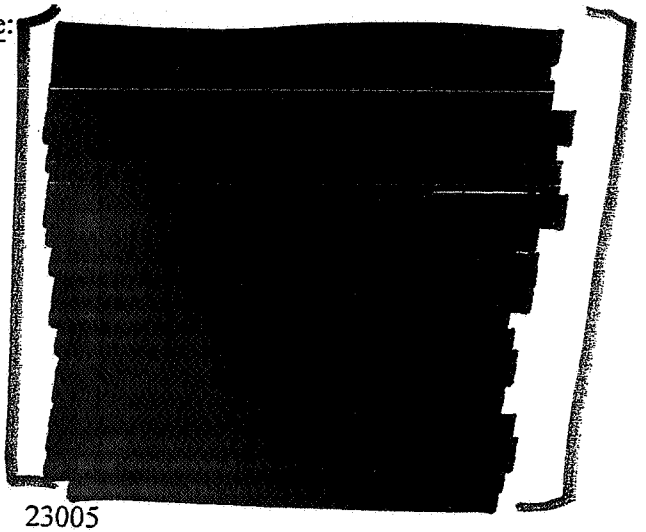
STUDY INFORMATION

Substance Tested: [redacted]

Synonyms/Codes: [redacted]

- H-23005

Structure: 

Haskell Number: 23005

Composition: [redacted]

Known Impurities: [redacted]

Physical Characteristics: Tan liquid

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Sponsor: E. I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

Study Initiated/Completed: December 11, 1997 / March 19, 1998

In-Life Initiated/Completed: December 16, 1997 / February 13, 1998

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QUALITY ASSURANCE STATEMENT

Study Title: *IN VITRO* MAMMALIAN CELL GENE MUTATION TEST WITH AN INDEPENDENT REPEAT ASSAY

Study Number: G97CK07.782001

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 11 DEC 97, TO STUDY DIR 11 DEC 97, TO MGMT 11 DEC 97
PHASE: Protocol Review

INSPECT ON 23 JAN 98-27 FEB 98, TO STUDY DIR 27 FEB 98, TO MGMT 27 FEB 98
PHASE: Scoring toxicity plates

INSPECT ON 23 FEB 98-24 FEB 98, TO STUDY DIR 24 FEB 98, TO MGMT 27 FEB 98
PHASE: Draft Report

INSPECT ON 19 MAR 98, TO STUDY DIR 19 MAR 98, TO MGMT 19 MAR 98
PHASE: Draft to Final Report

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

Becky D. Schreckengost 3/19/98

Becky D. Schreckengost, B.S.
QUALITY ASSURANCE

DATE

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SUMMARY

The test article, H-23005, was tested in the CHO/HGPRT Mutation Assay in the absence and presence of Aroclor-induced rat liver S9. The preliminary toxicity assay was used to establish the dose range for the initial mutagenesis assay. The initial and independent repeat mutagenesis assays were used to evaluate the mutagenic potential of the test article. The dosing solutions were adjusted to compensate for the purity of the test article.

Sterile distilled water was determined to be the solvent of choice based on solubility information from the Sponsor and compatibility with the target cells. The test article was soluble in sterile distilled water at a concentration of 50 mg/mL, the maximum concentration prepared in the preliminary toxicity assay.

In the preliminary toxicity assay, the maximum concentration of H-23005 tested was 5000 µg/mL. There was visible precipitate in the treatment medium at concentrations ≥1500 µg/mL at the end of treatment. Selection of dose levels for the mutagenesis assay was based on the cloning efficiency relative to the solvent control and the solubility limitations. Substantial toxicity, i.e., cloning efficiency ≤50% of the solvent control, was observed at ≥50 µg/mL without activation and at 5000 µg/mL with S9 activation. Based on these findings, the doses chosen for the initial mutagenesis assay ranged from 15 to 2500 µg/mL for both the non-activated and S9-activated cultures.

In the initial mutagenesis assay, no positive responses, i.e., treated cultures with mutant frequencies >40 mutants per 10^6 clonable cells, were observed. Visible precipitate was observed in treatment medium at concentrations ≥1500 µg/mL at the end of treatment. Concentrations of ≤500 µg/mL were soluble in treatment medium. Toxicity, i.e., cloning efficiency ≤50% of the solvent control, was observed at doses of ≥1500 µg/mL without S9 activation and at 2500 µg/mL with S9 activation. Based on these findings, the doses chosen for the independent repeat assay ranged from 50 to 1500 µg/mL for the non-activated cultures and from 50 to 2500 µg/mL for the S9-activated cultures.

In the independent repeat assay, no positive responses were observed. Visible precipitate was observed in treatment medium at concentrations ≥750 µg/mL at the end of treatment. Concentrations of ≤500 µg/mL were soluble in treatment medium. No toxicity was observed at any dose levels, with or without S9 activation.

Under the conditions of this study, test article H-23005 was concluded to be negative in the CHO/HGPRT Mutation Assay.

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PURPOSE

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.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, H-23005, was received by MA BioServices, Inc. (MA) on December 9, 1997 and was assigned the code number 97CK07. The test article was characterized by the Sponsor as a clear, tan liquid, which should be stored at room temperature. Its purity was given as [redacted] Upon receipt, the test article was described as pale yellow liquid with very tiny, dark particles and was stored at room temperature, protected from exposure to light.

The vehicle used to deliver H-23005 to the test system was sterile distilled water (CAS 7732-18-5) obtained from Life Technologies. The dosing solutions were adjusted to compensate for the purity of the test article.

Ethyl methanesulfonate (EMS), CAS 62-50-0, lot 04625BR, expiration 3/98, was obtained from Aldrich Chemical Company 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, lot 96H2600, expiration 9/98, 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.

MATERIALS AND METHODS

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. 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 ≤ -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

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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. The S9 reaction mixture for the preliminary toxicity assay contained only 2.5 mM glucose-6-phosphate due to a calculation error. Since the data from the preliminary toxicity assay provided a toxicity profile that was consistent with that in the initial mutation assay, this protocol deviation was not considered to have adversely impacted the integrity or conclusions of this study.

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 and nine concentrations of test article ranging from 0.5 to 5000 µg/mL in both the absence and presence of S9-activation.

Mutagenesis Assays

The initial and independent repeat mutagenesis assays were 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 and at least 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. The cells for the preliminary toxicity assay were seeded at $5.6x10^5$ cells/25 cm^2 flask due to a calculation error. Since the data from the preliminary toxicity assay provided a toxicity profile that was consistent with that in the initial mutation assay, this protocol deviation was not considered to have adversely impacted the integrity or conclusions of this study. 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 4.5 mL F12FBS5-Hx/25 cm^2 flask for the non-activated study and 3.5 mL F12FBS5-Hx and 1 mL S9 reaction mixture/25 cm^2 flask for the

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S9-activated study, to which was added 500 µl dosing solution of test article in vehicle or vehicle alone. The positive control cultures were treated with 50 µL of EMS or B(a)P in a total of 5 mL per 25 cm^2 flask. Duplicate flasks of cells were exposed to 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. In the preliminary toxicity assay, the cultures treated with 5000 and 1500 µg/mL were rinsed twice with CMF-HBSS instead of once. This is a deviation from the Standard Operating Procedures of the testing facility; however, the Study Director has determined that this deviation did not affect the integrity or conclusions of the study. 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 $2x10^5$ 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,

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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 is used which sets the minimum level at >40 mutants per 10^6 clonable cells.

All conclusions were based on sound scientific judgement; however, the following criteria are presented as a guide to interpretation of the data:

- The test article was considered to induce a positive response if there was 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 was observed at the highest dose, the assay was considered suspect.
- If no culture exhibited a mutant frequency of >40 mutants per 10^6 clonable cells, the test article was 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. There must be at least four analyzable test article concentrations with mutant frequency data.

RESULTS AND DISCUSSION

Solubility

Sterile distilled water was determined to be the solvent of choice based on solubility information from the Sponsor and compatibility with the target cells. The test article was soluble in sterile distilled water at a concentration of 50 mg/mL, the maximum concentration prepared in the preliminary toxicity assay.

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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. There was visible precipitate in the treatment medium at concentrations ≥1500 µg/mL at the end of treatment. The osmolality of the solvent control was 319 mmol/kg and the osmolality of the top dose, 5000 µg/mL (which was non-precipitating at the beginning of treatment), was 307 mmol/kg. Cloning efficiency relative to the solvent controls (RCE) was 0% at 5000 µg/mL without activation and 1% at 5000 µg/mL with S9 activation. Based on the results of the toxicity test, the doses chosen for the initial mutagenesis assay ranged from 15 to 2500 µg/mL for both the non-activated and S9-activated cultures.

Mutagenesis Assays

The cytotoxic effects of the test article (concurrent cytotoxicity) from the initial mutagenesis assay are presented in Table 2. Mutagenicity data are presented in Tables 3 and 4. In both the non-activated and S9-activated systems, cultures treated with concentrations of 15, 50, 150, 500, 1500 and 2500 µg/mL were cloned. Visible precipitate was observed in treatment medium at concentrations ≥1500 µg/mL at the end of treatment. Concentrations of ≤500 µg/mL were soluble in treatment medium. Relative cloning efficiency was 0% and 24% at the highest dose tested in the non-activated and S9-activated systems, respectively. The non-activated cultures treated with 2500 µg/mL were not seeded for mutant selection due to toxicity. None of the treated cultures that were cloned for mutant selection exhibited mutant frequencies of greater than 40 mutants per 10^6 clonable cells.

The cytotoxic effects of the test article from the independent repeat assay are presented in Table 5. Mutagenicity data are presented in Tables 6 and 7. In the non-activated system, cultures treated with concentrations of 50, 150, 500, 750, 1000 and 1500 µg/mL were cloned. In the S9-activated system, cultures treated with concentrations of 50, 150, 500, 1000, 2000 and 2500 µg/mL were cloned. Visible precipitate was observed in treatment medium at concentrations ≥750 µg/mL at the end of treatment. Concentrations of ≤500 µg/mL were soluble in treatment medium. Relative cloning efficiency was 68% and 73% 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, H-23005 did not cause a positive response in the non-activated and S9-activated systems and was concluded to be negative.

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RECORDS AND SAMPLE STORAGE

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

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.

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TABLE 1

CHO/HGPRT MUTATION ASSAY

Preliminary Toxicity Assay Using H-23005

-S9						+S9					
Treatment[1]	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[2]	Relative Cloning Efficiency[3] (%)
Water (100 µL/mL):											
	82	77	87	0.82	100		83	82	73	0.79	100
H-23005 (µg/mL):											
0.5	80	89	60	0.76	93	0.5	60	59	70	0.63	79
1.5	57	62	77	0.65	80	1.5	59	72	64	0.65	82
5.0	74	67	64	0.68	83	5.0	78	79	52	0.70	88
15	58	76	56	0.63	77	15	78	52	52	0.61	76
50	32	28	52	0.37	46	50	61	85	76	0.74	93
150	33	45	38	0.39	47	150	49	77	59	0.62	78
500	18	23	25	0.22	27	500	48	56	35	0.46	58
1500*	27	22	11	0.20	24	1500*	46	57	44	0.49	62
5000*	0	0	0	0.00	0	5000*	0	1	1	0.01	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] Relative cloning efficiency = $\frac{\text{cloning efficiency of treatment group}}{\text{cloning efficiency of solvent group}} \times 100$

* visible precipitation, as observed at the end of the treatment period

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TABLE 2

CHO/HGPRT MUTATION ASSAY

Concurrent Cytotoxicity Test Using H-23005
Initial Assay

Treatment[1]	-S9 Colonies per dish 1	2	3	Cloning Efficiency[2]	Relative Cloning Efficiency[3] (%)	Treatment[1]	+S9 Colonies per dish 1	2	3	Cloning Efficiency[2]	Relative Cloning Efficiency[3] (%)
Water (100 µL/mL):											
A	47	75	63			A	80	86	81		
B	56	65	36	0.57	100	B	81	92	82	0.84	100
EMS (0.2 µL/mL):						B(a)P (4.0 µg/mL):					
A	23	31	38			A	10	17	28		
B	27	34	25	0.30	52	B	14	12	19	0.17	20
H-23005 (µg/mL):											
15 A	65	88	45			15 A	87	93	77		
B	50	50	49	0.58	101	B	89	96	73	0.86	103
50 A	78	62	54			50 A	91	78	76		
B	46	43	42	0.54	95	B	80	86	76	0.81	97
150 A	57	62	46			150 A	110	113	109		
B	36	39	40	0.47	82	B	76	85	99	0.99	118
500 A	52	61	46			500 A	91	73	98		
B	46	42	28	0.46	80	B	80	77	82	0.84	100
1500* A	9	12	12			1500* A	60	70	58		
B	1	5	2	0.07	12	B	66	77	65	0.66	79
2500* A	0	0	0			2500* A	11	13	16		
B	0	0	0	0.00	0	B	27	30	23	0.20	24

A and B are duplicate cultures

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

* visible precipitation, as observed at the end of the treatment period

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TABLE 3

CHO/HGPRT MUTATION ASSAY

Non-activated (-S9) Study Using H-23005

Treatment[1]		Cloning Efficiency at Selection: 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]
Water (100 µL/mL):													
	A	43	40	59			4	2	a	a	2		
	B	56	57	63	318	0.53	0	1	3	2	1	15	17.7
EMS (0.2 µL/mL):													
	A	65	71	57			32	44	57	56	37		
	B	75	68	77	413	0.69	41	37	43	41	35	423	307.3
H-23005 (µg/mL):													
15	A	64	72	78			0	1	0	0	0		
	B	57	63	73	407	0.68	0	2	2	1	0	6	4.4
50	A	47	61	63			0	0	0	0	0		
	B	47	49	46	313	0.52	3	4	3	6	1	17	16.3
150	A	43	33	43			0	0	0	0	2		
	B	58	68	62	307	0.51	0	0	0	1	0	3	2.9
500	A	70	72	71			1	1	a	0	0		
	B	66	79	58	416	0.69	0	0	0	1	1	4	3.2
1500*	A	56	58	47			4	2	4	4	2		
	B	71	81	68	381	0.64	0	0	1	0	0	17	13.4

A and B are duplicate cultures

[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 = $\frac{\text{total mutant colonies}}{\text{number selection dishes X cloning efficiency X 2 x } 10^5 \text{ cells}} \times 10^6$

[a] plate lost to contamination

* visible precipitation, as observed at the end of the treatment period

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TABLE 4

CHO/HGPRT MUTATION ASSAY

Activated (+S9) Study Using H-23005

Treatment[1]		Cloning Efficiency at Selection: 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]
Water (100 µL/mL):													
	A	78	84	70			1	2	0	3	1		
	B	63	67	75	437	0.73	0	0	0	0	0	7	4.8
B(a)P (4.0 µg/mL):													
	A	51	71	53			24	26	22	30	23		
	B	54	78	60	367	0.61	30	22	36	27	23	263	215.0
H-23005 (µg/mL):													
15	A	78	84	69			0	0	0	0	0		
	B	67	55	47	400	0.67	0	1	0	1	0	2	1.5
50	A	86	74	72			1	1	1	4	1		
	B	62	51	59	404	0.67	2	4	2	3	1	20	14.9
150	A	71	76	67			0	0	0	0	0		
	B	72	76	72	434	0.72	0	0	1	1	2	4	2.8
500	A	63	66	65			0	1	0	0	0		
	B	54	75	64	387	0.65	0	1	3	2	3	10	7.8
1500*	A	60	63	69			1	0	0	0	0		
	B	98	88	53	431	0.72	0	0	0	0	0	1	0.7
2500*	A	67	63	67			0	0	0	0	0		
	B	60	63	49	369	0.62	0	0	0	0	0	0	<0.8[4]

A and B are duplicate cultures

[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 = $\frac{\text{total mutant colonies}}{\text{number selection dishes X cloning efficiency X 2 x } 10^5 \text{ cells}} \times 10^6$

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

* visible precipitation, as observed at the end of the treatment period

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TABLE 5

CHO/HGPRT MUTATION ASSAY

Concurrent Cytotoxicity Test Using H-23005
Independent Repeat Assay

	-S9						+S9					
Treatment[1]	Colonies per dish			Cloning Efficiency[2]	Relative Cloning Efficiency[3] (%)		Colonies per dish			Cloning Efficiency[2]	Relative Cloning Efficiency[3] (%)	
	1	2	3				1	2	3			
Water (100 µL/mL):												
A	64	54	60			A	65	57	74			
B	52	72	56	0.60	100	B	61	69	71	0.66	100	
EMS (0.2 µL/mL):						B(a)P (4.0 µg/mL):						
A	33	34	46			A	28	35	29			
B	24	42	37	0.36	60	B	18	19	12	0.24	36	
H-23005 (µg/mL):												
50 A	63	75	46			50 A	55	59	82			
B	80	80	61	0.68	113	B	62	65	58	0.64	96	
150 A	36	48	29			150 A	80	81	65			
B	39	43	63	0.43	72	B	49	67	46	0.65	98	
500 A	56	54	62			500 A	76	75	79			
B	59	48	55	0.56	93	B	56	66	65	0.70	105	
750* A	57	73	52			1000* A	52	68	60			
B	58	35	45	0.53	89	B	46	60	51	0.56	85	
1000* A	39	44	53			2000* A	61	73	46			
B	50	42	64	0.49	82	B	60	48	58	0.58	87	
1500* A	38	36	51			2500* A	55	65	59			
B	38	39	42	0.41	68	B	28	42	39	0.48	73	

A and B are duplicate cultures

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

* visible precipitation, as observed at the end of the treatment period

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TABLE 6

CHO/HGPRT MUTATION ASSAY

Non-activated (-S9) Study Using H-23005
Independent Repeat Assay

Treatment[1]	Cloning Efficiency at Selection: Colonies per Dish 1	2	3	Total Colonies	Cloning Efficiency[2]	Mutant Colonies/Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants/10^5 Clonable Cells[3]
Water (100 µL/mL):												
A	63	60	58			2	0	0	1	0		
B	36	66	65	348	0.58	0	0	0	0	0	3	2.6
EMS (0.2 µL/mL):												
A	36	46	51			84	75	79	76	85		
B	31	36	32	232	0.39	56	54	49	61	54	67	870.3
H-23005 (µg/mL):												
50 A	52	76	56			1	0	0	0	0		
B	56	50	74	364	0.61	1	0	1	1	0	4	3.3
150 A	60	96	69			1	1	0	0	1		
B	60	56	59	400	0.67	0	1	0	0	0	4	3.0
500 A	48	50	46			0	0	0	0	0		
B	55	49	73	321	0.54	0	0	0	0	0	0	<0.9[4]
750* A	57	69	73			1	0	0	1	2		
B	54	61	67	381	0.64	0	0	0	0	0	4	3.1
1000* A	63	44	63			0	0	0	0	0		
B	84	61	73	388	0.65	0	0	0	0	0	0	<0.8[4]
1500* A	68	63	82			0	0	0	1	0		
B	49	60	53	375	0.63	0	0	0	0	0	1	0.8

[1] A and B are duplicate cultures
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 = $\frac{\text{total mutant colonies}}{\text{number selection dishes X cloning efficiency X 2 x } 10^3 \text{ cells}} \times 10^5$

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

* visible precipitation, as observed at the end of the treatment period

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TABLE 7

CHO/HGPRT MUTATION ASSAY

Activated (+S9) Study Using H-23005
Independent Repeat Assay

Treatment[1]		Cloning Efficiency at Selection: 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]
Water (100 µL/mL):													
	A	108	127	118			0	0	0	0	0		
	B	75	82	64	574	0.96	0	0	1	0	1	2	1.0
B(a)P (4.0 µg/mL):													
	A	121	81	103			33	26	19	24	30		
	B	72	96	82	555	0.93	15	17	21	21	19	225	121.6
H-23005 (µg/mL):													
50	A	84	115	109			2	1	4	4	1		
	B	76	92	82	558	0.93	0	0	0	1	1	14	7.5
150	A	97	110	115			0	2	1	2	0		
	B	75	99	100	596	0.99	0	0	0	0	0	5	2.5
500	A	87	79	115			3	3	2	2	2		
	B	76	89	96	542	0.90	0	0	0	0	0	12	6.6
1000*	A	76	80	98			0	0	0	0	0		
	B	79	81	65	479	0.80	1	0	1	1	1	4	2.5
2000*	A	86	91	95			0	0	0	0	0		
	B	86	71	79	508	0.85	0	3	0	0	0	3	1.8
2500*	A	55	77	66			0	0	0	0	0		
	B	104	91	111	504	0.84	0	0	0	0	0	0	<0.6[4]

A and B are duplicate cultures

[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 = $\frac{\text{total mutant colonies}}{\text{number selection dishes X cloning efficiency X 2 x } 10^5 \text{ cells}} \times 10^6$

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

* visible precipitation, as observed at the end of the treatment period

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APPENDIX I

Historical Control Data

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CHO/HGPRT Assay Historical Control Data

1995 - 1997

	Non-activated		S9-activated	
	Solvent Control	0.2µL/mL EMS	Solvent Control	4.0µg/mL B(a)P
Mean MF	7.0	312.3	6.1	180.3
SD	6.2	97.3	6.0	63.0
Maximum	22.9	725.7	23.1	349.0
Minimum	0.0	144.0	0.5	38.1

Solvent control (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

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APPENDIX II

Study Protocol

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Received by RA/QA 12-4-97

MA Study Number: G97CK07.782001

QA UC 12-11-97
APPROVED

In Vitro Mammalian Cell Gene Mutation Test with an Independent Repeat 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: **E. I. du Pont de Nemours and Company**

2.2 Address: **Haskell Laboratory for Toxicology and Industrial Medicine**
P.O. Box 50, Elkton Road
Newark, DE 19714

2.3 Representative: **Brian H. Mathison, Ph.D.**

2.4 Sponsor Project #: [redacted]

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **H-23005**

3.2 Controls:
Negative: Test article solvent (or 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: Toxicology Testing Facility
MA BioServices, Inc.

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4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

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

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 12/16/97

5.2 Proposed Experimental Completion Date: 2/18/98

5.3 Proposed Report Date: 2/27/98

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% (Li *et al.*, 1987). 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 (O'Neill *et al.*, 1977; Hsie *et al.*, 1981; Machanoff *et al.*, 1981; Li *et al.*, 1987). This system has been demonstrated to be sensitive to the mutagenic action of a variety of chemicals (Hsie *et al.*, 1981).

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,

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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 x 10^5 CHO cells will be seeded into 25 cm^2 flasks and incubated at 37±1°C in a humidified atmosphere of 5±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 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±1°C in a humidified atmosphere of 5±1% CO_2 in air in the presence and absence of S9 activation. After the treatment period, all media will be aspirated, the cells washed, and cultures given fresh culture medium. Eighteen to 24 hours later, the treated cells will be trypsinized and reseeded at a density of 100 cells/60 mm dish. After 7-10 days incubation at 37±1°C in a humidified atmosphere of 5±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 10-20%. Four lower doses will be selected, at least one of which will be non-toxic. For freely soluble, non-toxic test articles, the highest concentration will be 5000 µg/ml. For relatively insoluble, non-toxic test articles, the highest concentration will be the lowest insoluble dose in treatment medium but not to exceed 5000 µg/ml. If dose-related cytotoxicity or mutagenicity is noted, irrespective of solubility, then the top concentration will be based on toxicity as described above. In all cases, precipitation will be evaluated at the beginning and at the end of the treatment period using the naked eye (ICH, 1995).

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 S9 will be used as the metabolic activation system. The source of S9 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 S9 will be batch prepared and stored frozen approximately -70°C until used.

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Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 100 µl S9/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 (Machanoff *et al.*, 1981). The S9 reaction mixture will be stored on ice until used.

7.5 Controls

7.5.1 Negative Control

The solvent (or vehicle) for the test article will be used as the negative control.

7.5.2 Positive Control

Ethyl methanesulfonate (EMS) will be used at a concentration of 0.2 µl/ml as the positive control for the non-activated study. Benzo(a)pyrene (BaP) will be used at a concentration of 4 µg/ml as the positive control for the S9 activated study.

7.6 Preparation of Target Cells

Exponentially growing CHO-K1-BH4 cells will be seeded in F12 medium without hypoxanthine, supplemented with 5% dialyzed serum, 2mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) (F12FBS5-Hx), at a density of 5×10^5 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-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-Hx, 1 ml S9 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-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.

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7.9 Estimation of Toxicity

For evaluation of cytotoxicity, the replicate cultures from each treatment condition will be subcultured independently in 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-Hx, in duplicate, 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 2×10^5 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.

7.12 Independent Repeat Assay

An independent repeat mutation assay, with and without a metabolic activation system, will be conducted.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

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

8.2 Positive Controls

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.

8.3 Test Article-Treated Cultures

A minimum of four analyzable concentrations with mutant frequency data will be required.

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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 at the time of 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 ≤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.* (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 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:

- test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.
- solvent/vehicle: justification for choice of vehicle; solubility and stability of test article in solvent/vehicle, if known.
- cell type used, number of cultures, methods for maintenance of cell cultures
- rationale for selection of concentrations and number of cultures

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- 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 Quality Assurance Unit of MA BioServices, Inc., Rockville, MD in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with EPA Health Effects Testing Guidelines, Draft Document OPPTS 870.5300 (Detection of Gene Mutations in Somatic Cells in Culture), Fed. Register, vol. 61, June 1996, with OECD Guideline for the Testing of Chemicals, Guideline 476 (*In Vitro* Mammalian Cell Gene Mutation Test), Proposal for Updating Guideline 476, February 1997, and with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, Step 4 Final Draft, July 18, 1995.

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? YES

If so, to which agency or agencies? EPA (TSCA), EU, OECD

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

EPA Health Effects Testing Guidelines, Draft Document OPPTS 870.5300 (Detection of Gene Mutations in Somatic Cells in Culture), Fed. Register, vol. 61, June 1996.

Hsie, A.W., Casciano, D.A., Couch, D.B., Krahn, B.F., O'Neill, J.P. and Whitfield, B.L. (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 Research 86: 193-214.

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International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, Step 4 Final Draft. July 18, 1995.

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

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

O'Neill, J.P., Brimer, P.A., Machanoff, R., Hirsch, J.P. and Hsie, A.W. (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.

OECD Guideline for the Testing of Chemicals, Guideline 476 (*In Vitro* Mammalian Cell Gene Mutation Test), Proposal for Updating Guideline 476, February 1997.

14.0 APPROVAL

SPONSOR REPRESENTATIVE	DATE 12/8/97
Brian H. Matthison (Print or Type Name)	
MA STUDY DIRECTOR	DATE 12/11/97

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