

TOXIKON FINAL REPORT: 01-7019-G1

**CHO/HGPRT FORWARD MUTATION ASSAY - ISO
(T-6889.7)**

Author
Devaki Sadhu, Ph.D.

Final Report Date:
March 28, 2002

MANAGEMENT OF THE STUDY

Performing Laboratory
Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730

Sponsor:
3M
3M Center 220-2E-02
Saint Paul, MN 55144

RECEIVED
DPPT NCIC
2002 JUL 29 AM 9:25

CONTAIN NO CBI

000003

TABLE OF CONTENTS

Title Page

Table of Contents

Study Summary

Quality Assurance Statement

Study Director Signature and Verification Dates

- 1.0 Purpose
- 2.0 References
- 3.0 Compliance
- 4.0 Identification of the Test and Control Articles
- 5.0 Identification of the Test System
- 6.0 Justification of Test System and Route of Administration
- 7.0 Experimental Design
- 8.0 Dosage
- 9.0 Evaluation Criteria
- 10.0 Results
- 11.0 Conclusion
- 12.0 Records
- 13.0 Confidentiality Agreement

- Table I: Parallel Cytotoxicity Assay
- Table II: Parallel Cloning Efficiency (Non-Activated)
- Table III: Parallel Cloning Efficiency (Activated)
- Table IV: HGPRT Mutagenesis Assay (Non-Activated)
- Table V: HGPRT Mutagenesis Assay (Activated)
- Table VI: Summary of Mutant Colony Frequency in HGPRT Assay (Non-Activated)
- Table VII: Summary of Mutant Colony Frequency in HGPRT Assay (Activated)

STUDY SUMMARY

The test article, Ammonium Perfluorooctanoate (FL-143), did not induce mutations at the HGPRT locus as evidenced by the absence of a statistically significant increase in the number of mutant colonies at the highest analyzable test concentration as compared to the negative controls in the presence or absence of an exogenous mammalian metabolic activation system. The statistically significant increase in the number of mutant colonies in positive controls in both the activated and non-activated assays verified the proper functioning of the test system. Therefore, based on the evaluation criteria of the protocol, the test article is considered non-mutagenic, under the experimental conditions employed.

000005

QUALITY ASSURANCE STATEMENT

This study was conducted in compliance with U.S. Food and Drug Administration regulations set forth in 21 CFR, Part 58.

The sections of the regulations not performed by or under the direction of Toxikon Corporation, exempt from this Good Laboratory Practice Statement, included characterization and stability of the test article and its mixture with carriers, 21 CFR, Parts 58.105 and 58.113.

The Quality Assurance Unit conducted inspections on the following dates. The findings were reported to the Study Director and to Toxikon's Management.

INSPECTIONS	DATE OF INSPECTION	DATE REPORTED STUDY DIRECTOR	DATE REPORTED MANAGEMENT
DOSING	01/02/02	01/02/02	01/02/02
CELL COUNTING	02/07/02	02/07/02	02/07/02
RAW DATA	03/28/02	03/28/02	03/28/02
FINAL REPORT	03/28/02	03/28/02	03/28/02


Mansi Desai, B.Sc.
Quality Assurance

03/28/02
Date

000006

STUDY DIRECTOR SIGNATURE AND VERIFICATION DATES

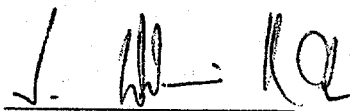
This study met with the technical requirements of the protocol. The study also met with the requirements of the Good Laboratory Practice Regulations, 21 CFR, Part 58, with the exemptions noted in the QA Statement.

Protocol Number: 3MC/VITRO/003-01/000

Study Director: Devaki Sadhu, Ph.D.

Company: Toxikon Corporation

Signature:



Date:

03/28/02

Study Supervisor: Leigh Waugh-Cohen, B.A.

VERIFICATION DATES:

The study dates were as follows:

Protocol Effective Date:	12/12/01
Test Article Receipt:	12/12/01
Project Log Date:	12/12/01
Technical Initiation:	02/05/02
Technical Completion:	02/20/02
Final Report Date:	03/28/02

000007

1.0 PURPOSE

The CHO/HGPRT Forward Mutation Assay evaluated the mutagenic potential of a test article via its ability to induce forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus. The CHO assay system utilized toxic purine analogs to select for resistant cells that are presumed deficient in the purine salvage enzyme HGPRT.

2.0 REFERENCES

The test was conducted based upon the following references:

- 2.1 Biological Evaluation of Medical Devices – Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity, ANSI/AAMI/ISO 10993-3: 1993.
- 2.2 OECD Guidelines for the testing of Chemicals, "Genetic Toxicology: *In vitro* Mammalian Cell Gene Mutation Test," Test Guideline # 476, current version.
- 2.3 Hsie, A.W., Casciano, D.A., Couch, D.B., Krahn, D.F., O'Neill, J.P., Whitefield, B.L. "EPA's Gene Tox Program," Mutation Research, 86: 193-214 (1981).

3.0 COMPLIANCE

The present study conformed to all applicable laws and regulations. Specific regulatory requirements included the current FDA, 21 CFR, Part 58 - Good Laboratory Practice for Nonclinical Laboratory Studies.

4.0 IDENTIFICATION OF THE TEST AND CONTROL ARTICLES

The following information was supplied by the Sponsor wherever applicable. Confidential information does not apply. The Sponsor was responsible for all test article characterization data as specified in the GLP regulations.

4.1 Test Article: Ammonium Perfluorooctanoate (FL-143)
CAS/Code #: 3825-26-1 / (T-6889.7)
Lot/Batch#: Lot 332
Physical State: Solid
Color: White / Crystal
Density: Not Supplied by Sponsor (N/S)
pH: 4 - 7
Stability: Stable
Solubility: >1g/mL
Storage Conditions: Room Temperature, -20°C or 4°C
Safety Precautions: Standard Toxikon Laboratory Safety Precautions

000008

4.2 Control Articles - (Toxikon Supplied)

4.2.1 Negative Control Article Name: Ham's F-12 Complete Medium

QC Inventory #: LPR-01-11-001-CC

Physical State: Liquid

Color: Colorless

Storage Conditions: Room Temperature

Safety Precautions: Standard Laboratory Safety Precautions

4.2.2 Positive Control Article Name (Non-Activated Assay): Ethylmethanesulfoxide (EMS)

QC Inventory #: CSC-01-03-004-CC

Physical State: Liquid

Color: Clear

Storage Conditions: 4±2°C

Safety Precautions: Standard Laboratory Safety Precautions

4.2.3 Positive Control Article Name (Activated Assay): Dimethylbenzanthracene (DMBA)

QC Inventory #: CSC-97-09-013-CC

Physical State: Liquid

Color: Clear

Storage Conditions: 4±2°C

Safety Precautions: Standard Laboratory Safety Precautions

5.0 IDENTIFICATION OF THE TEST SYSTEM

Chinese Hamster Ovary (CHO) cells utilized in this assay were obtained from the American Type Culture Collection, Manassas, Virginia. The cells were derived from an ovarian biopsy of a chinese hamster.

6.0 JUSTIFICATION OF TEST SYSTEM AND ROUTE OF ADMINISTRATION

6.1 This assay evaluated the mutagenic potential of a test article via its ability to induce mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus. This locus is responsible for production of HGPRT, an enzyme that is present in normal cells and allows cells to salvage hypoxanthine and guanine from the culture medium in order to synthesize DNA. In normal cells, when a toxic substance called 6-thioguanine is included in the growth medium, it is salvaged by the HGPRT enzyme along with hypoxanthine and guanine and incorporated into DNA, thereby causing cell death. Exponentially growing cells, sensitive to the toxic effects of 6-thioguanine, were exposed to a various concentrations of the test material. If the test material is potentially mutagenic, normal cells (which can utilize hypoxanthine, guanine and 6-thioguanine) mutate to become incapable of utilizing hypoxanthine, guanine, or 6-thioguanine from the culture medium. However, mutant cells retain their ability to grow as well as normal cells in culture medium because DNA synthesis is made possible by alternate synthetic pathways. Taken together, these findings indicate that the basis for selection of HGPRT mutants is the lack of any ability to utilize the toxic 6-thioguanine. Therefore, cells that grow to form colonies in the presence of 6-thioguanine are assumed to have undergone mutation either spontaneously or by exposure to the test material.

000009

The CHO/HGPRT Assay has been used extensively in the detection of mutagenic activity of a wide range of chemical classes.

6.2 The test article was administered *in vitro*, through a solvent compatible with the test system, per Sponsor's specification. This was the optimal route of administration available in this test system.

7.0 EXPERIMENTAL DESIGN

7.1 Cell Line

The CHO-K1 cell line was selected for its high cloning ability and rapid doubling time. It was not necessary to select the cell cultures to reduce the background frequency of HGPRT⁻ mutants.

7.2 Maintenance of CHO Cells

The CHO cells were grown in complete Ham's F-12 medium, buffered with 10 mM HEPES. Complete medium consisted of Ham's F-12 medium containing 10% fetal bovine serum (FBS), 1-2 mM L-glutamine, 100-units/mL penicillin, and 100-μg/mL streptomycin. Incomplete medium was serum-free complete medium. The cells were incubated at 37±1°C, 5±1% CO₂, and saturated humidity.

7.3 Forward Mutation Assay

7.3.1 Metabolic Activation System (S9):

The S9 microsomal fraction was prepared from Sprague Dawley rat livers induced with Aroclor^R 1254. The S9 rat liver homogenate was purchased from Molecular Toxicology Inc (157 Industrial Park drive, Boone, NC 28607) and stored at -80±5°C until use. A combination of S9 fraction, isocitric cofactors and incomplete medium was prepared just before exposure and used as the metabolic activation system. The cofactor mixture consisted of isocitrate (trisodium salt) and NADP (disodium salt) at a final concentration of 4.5 mg/mL and 2.4 mg/mL, respectively. The S9 fraction was added to each flask at a concentration of 20 μL/mL.

7.3.2 Parallel Cytotoxicity Assay

A parallel cytotoxicity assay was performed along with the Mutation assay. The cultures were treated exactly as the mutation plates, incubated for 9 days following the removal of the test article, fixed, stained, and colonies counted.

7.3.3 Preparation of Test Cultures:

Approximately 24 hours prior to exposure, duplicate 100 mm plates were seeded at a density of 1 x 10⁶ cells/dish and triplicate parallel cytotoxicity plates were seeded at 200 cells/60 mm dish.

7.3.4 Exposure Periods:

The cells were exposed to the test article for 17 hours in the non-activated assay and 5 hours in the activated assay. After exposure, cells were washed twice with PBS and supplemented with complete medium.

000010

7.3.5 Phenotype Expression:

Approximately 48 hours following termination of non-activated exposure period and approximately 64 ½ hours following termination of activated exposure period, cells were trypsinized, counted, and plated at 1×10^6 cells per 100 mm dish. The cells were passed every 48-72 hours to maintain exponential growth during phenotypic expression for 6 days.

7.3.6 Selective Growth:

Following the phenotypic expression period, cells were grown in selective medium to select for mutant cells. The selective medium included hypoxanthine-free Ham's F-12 with 10% fetal bovine serum, penicillin-streptomycin (P/S) and 10 μ M 6-thioguanine. Ten dishes (five from each duplicate) were used for each test condition. Cell density was 2×10^5 /100 mm petri dish. The cultures were incubated for 9 days to allow colonies to develop.

7.3.7 Parallel Cloning Efficiency (PCE):

Concurrently, the cloning efficiency was determined by plating cells in selective medium without 6-thioguanine. Six dishes were seeded for each concentration (three from each duplicate) at 200-cells/60 mm dish. The cultures were then incubated for 9 days to allow colony formation.

7.3.8 Termination:

At the end of the incubation period, plates were rinsed with PBS, fixed in methanol and stained with Giemsa. Only colonies with 50 or more cells were counted.

7.4 Control Articles

7.4.1 Positive Control Article:

The positive control for the non-activated system was Ethylmethanesulfoxide (EMS). For the activated system, Dimethylbenzanthracene (DMBA) was used as the positive control.

7.4.2 Negative Control Article:

Ham's F-12 Complete Medium served as the negative control article for the non-activated and activated assays.

8.0 DOSAGE

8.1 The test article was dissolved in the culture medium at a concentration of 5 mg/mL as per OECD guidelines. 1:128, 1:256 and 1:512 dilutions of 5 mg/mL concentration of the test article were used for dosing.

8.2 Dose Selection

8.2.1 A cytotoxicity preliminary dose-range finding was performed using several dilutions of the 5 mg/mL test article and 1:128, 1:256 and 1:512 dilutions were selected for testing. The positive control for the non-activated assay, EMS, was used at a concentration of 244 μ g/mL. The positive control for the non-activated assay, DMBA, was used at a final concentration of 9 μ g/mL.

000011

9.0 EVALUATION CRITERIA

9.1 Evaluation of Test Results

The results of the CHO/HGPRT Locus Mutation Assay were evaluated based on the number of TG-resistant mutants per 1×10^6 surviving cells. The significance of the test results were determined by using the statistical program, Tallarida, R.S. and R.B. Murray's Pharmacological Calculations Procedure, ANOVA (analysis of variance) and Newman-Keuls Test for confirmation. This statistical method determined if there was a significant ($p \leq 0.05$) increase in the mutation frequency of the test article compared to the negative control article.

The test article was considered to have caused a positive response in the assay if the test article had exhibited a reproducible and statistically significant increase in the number of mutants per 1×10^6 cells over its concurrent negative control article.

10.0 RESULTS

A preliminary Range Finding Assay was conducted to determine the dilutions of the test article to be used in the Forward Mutation Assay. Exposure to dilutions of the test article resulted in cytotoxicity ranging from 50% cell survival at 1:128 and 80% greater at 1:256 and 1:512. Based on these findings, three dilutions ranging from 1:128 to 1:512 were utilized in the Forward Mutation Assay.

The parallel cytotoxicity assay indicated that the positive control article exhibited a cell survival percentage of 10% and 45% in the non-activated and activated assays respectively (Table I). The dilutions of the test article extract, 1:128, 1:256 and 1:512 exhibited cell survival values of 105, 99 and 102 respectively in the non-activated assay and 94, 104 and 118% respectively in the activated assay. The negative control article exhibited values of 79 and 110% in the non-activated and activated assays respectively.

The test article was tested at the three dilutions (1:128, 1:256, 1:512) established in the Range Finding Assay in non-activated assay. None of the three dilutions tested showed a statistically significant increase in the number of mutants per 1×10^6 surviving cells as compared to the corresponding negative controls in both the non-activated and activated assays (Tables II-VII). The positive controls did show a statistically significant increase in the number of mutants per 1×10^6 surviving cells as compared to the corresponding negative controls.

11.0 CONCLUSION

The test article, Ammonium Perfluorooctanoate (FL-143), did not induce mutations at the HGPRT locus as evidenced by the absence of a statistically significant increase in the number of mutant colonies at the highest analyzable test concentration as compared to the negative controls in the presence or absence of an exogenous mammalian metabolic activation system. The statistically significant increase in the number of mutant colonies in positive controls in both the activated and non-activated assays verified the proper functioning of the test system. Therefore, based on the

evaluation criteria of the protocol, the test article is considered non-mutagenic, under the experimental conditions employed.

12.0 RECORDS

- 12.1 Original raw data is archived at Toxikon Corporation.
- 12.2 A copy of the final report and any amendments is archived at Toxikon Corporation.
- 12.3 The original final report and a copy of any protocol amendments or deviations is forwarded to the Sponsor.
- 12.4 All unused test articles shall be returned to the Sponsor by Toxikon, per sponsor's specification.
- 12.5 Final reports will not be reproduced except in full, without the written authorization/approval from Toxikon.

13.0 CONFIDENTIALITY AGREEMENT

Statements of confidentiality were as agreed upon prior to study initiation.

**Table I: Parallel Cytotoxicity Assay
 (Mutagen Assay)**

Dilution of Extract	Metabolic Activation	Total # of Foci			Average # Foci per Plate	Percentage Survival*
Negative	Non-Activated	147	155	170	157	79
Positive (EMS)	Non-Activated	10	12	35	19	10
1:128	Non-Activated	232	194	201	209	105
1:256	Non-Activated	187	202	202	197	99
1:512	Non-Activated	200	213	199	204	102
Negative	Activated	222	208	230	220	110
Positive (DMBA)	Activated	95	90	87	90	45
1:128	Activated	182	184	200	188	94
1:256	Activated	194	232	198	208	104
1:512	Activated	232	240	237	236	118

* Percentage Survival= Average colonies per plate / number of cells plated (200 cells) x 100 %

000014

TABLE II
PARALLEL CLONING EFFICIENCY
NON-ACTIVATED

	# COLONIES/PLATE						SURVIVING FRACTION*	AVERAGE COLONIES
	1	2	3	4	5	6		
NEGATIVE CONTROL (MEDIUM)	70	80	106	103	117	110	0.488	98
POSITIVE CONTROL (EMS)	20	19	8	8	5	7	0.056	11
TEST ARTICLE EXTRACT (1:128)	79	98	91	89	87	75	0.433	87
TEST ARTICLE EXTRACT (1:256)	94	99	112	91	116	102	0.512	102
TEST ARTICLE EXTRACT (1:512)	109	84	93	116	117	72	0.493	99

* SURVIVING FRACTION= AVERAGE COLONIES PER PLATE/NUMBER OF CELLS PLATED (200 CELLS/PLATE)

TABLE III
PARALLEL CLONING EFFICIENCY
ACTIVATED

	# COLONIES/PLATE						SURVIVING FRACTION*	AVERAGE COLONIES
	1	2	3	4	5	6		
NEGATIVE CONTROL (MEDIUM)	116	113	91	87	101	106	0.512	102
POSITIVE CONTROL (EMS)	6	10	15	12	2	13	0.048	10
TEST ARTICLE EXTRACT (1:128)	76	72	71	72	96	93	0.400	80
TEST ARTICLE EXTRACT (1:256)	101	95	108	79	77	106	0.472	94
TEST ARTICLE EXTRACT (1:512)	108	75	112	76	103	113	0.489	98

* SURVIVING FRACTION= AVERAGE COLONIES PER PLATE/NUMBER OF CELLS PLATED (200 CELLS/PLATE)

TABLE IV
HGPRT MUTAGENESIS ASSAY
(NON-ACTIVATED)

TREATMENT	# MUTANT COLONIES/PLATE									
	1	2	3	4	5	6	7	8	9	10
NEGATIVE (ACTUAL)	1	1	0	0	0	0	0	1	1	0
NEGATIVE (NORMALIZED)+	2.05	2.05	0.00	0.00	0.00	0.00	0.00	2.05	2.05	0.00
POSITIVE (ACTUAL)	40	39	33	54	47	12	12	19	14	11
POSITIVE (NORMALIZED)+	716.42	698.51	591.04	967.16	841.79	214.93	214.93	340.30	250.75	197.01
TEST ARTICLE EXTRACT (1:128 ACTUAL)	0	0	0	0	0	2	1	1	0	1
TEST ARTICLE EXTRACT (NORMALIZED)+	0.00	0.00	0.00	0.00	0.00	4.62	2.31	2.31	0.00	2.31
TEST ARTICLE EXTRACT (1:256 ACTUAL)	1	0	0	0	1	1	0	2	0	0
TEST ARTICLE EXTRACT (NORMALIZED)+	1.95	0.00	0.00	0.00	1.95	1.95	0.00	3.91	0.00	0.00
TEST ARTICLE EXTRACT (1:512 ACTUAL)	0	0	0	2	0	1	1	0	0	0
TEST ARTICLE EXTRACT (NORMALIZED)+	0.00	0.00	0.00	4.06	0.00	2.03	2.03	0.00	0.00	0.00

+ NORMALIZED MUTATION FREQUENCY= NORMALIZED TO 1×10^6 CELLS PER PLATE BASED UPON CORRESPONDING SURVIVAL FRACTION (SEE TABLE II)

000017

TABLE V
HGPRT MUTAGENESIS ASSAY
(ACTIVATED)

TREATMENT	# MUTANT COLONIES/PLATE									
	1	2	3	4	5	6	7	8	9	10
NEGATIVE (ACTUAL)	0	0	1	1	1	1	0	0	0	0
NEGATIVE (NORMALIZED)+	0.00	0.00	1.95	1.95	1.95	1.95	0.00	0.00	0.00	0.00
POSITIVE (ACTUAL)	45	32	26	22	20	18	15	36	31	29
POSITIVE (NORMALIZED)+	931.03	662.07	537.93	455.17	413.79	372.41	310.34	744.83	641.38	600.00
TEST ARTICLE EXTRACT (1:128 ACTUAL)	2	0	0	2	1	0	0	3	0	0
TEST ARTICLE EXTRACT (NORMALIZED)+	5.00	0.00	0.00	5.00	2.50	0.00	0.00	7.50	0.00	0.00
TEST ARTICLE EXTRACT (1:256 ACTUAL)	0	0	1	0	2	0	0	0	0	2
TEST ARTICLE EXTRACT (NORMALIZED)+	0.00	0.00	2.12	0.00	4.24	0.00	0.00	0.00	0.00	4.24
TEST ARTICLE EXTRACT (1:512 ACTUAL)	1	1	0	0	0	1	0	0	1	0
TEST ARTICLE EXTRACT (NORMALIZED)+	2.04	2.04	0.00	0.00	0.00	2.04	0.00	0.00	2.04	0.00

+ NORMALIZED MUTATION FREQUENCY= NORMALIZED TO 1×10^6 CELLS PER PLATE BASED UPON CORRESPONDING SURVIVAL FRACTION (SEE TABLE III)

000018

TABLE VI
SUMMARY OF MUTANT COLONY FREQUENCY IN HGPRT ASSAY
(NON-ACTIVATED ASSAY)

TREATMENT	TOTAL # PLATES	TOTAL # FOCI	AVERAGE FOCI PER PLATE	AVERAGE FOCI* PER PLATE (NORMALIZED)
NEGATIVE CONTROL	10	4	0.4	0.82
POSITIVE CONTROL	10	281	28.1	503.28
TEST ARTICLE (1:8)	10	5	0.5	1.16
TEST ARTICLE (1:16)	10	5	0.5	0.98
TEST ARTICLE (1:32)	10	4	0.4	0.81

* NORMALIZED TO 1×10^6 CELLS PER PLATE BASED UPON THE CORRESPONDING SURVIVAL FRACTION (SEE TABLE II)

TABLE VII
SUMMARY OF MUTANT COLONY FREQUENCY IN HGPRT ASSAY
(ACTIVATED ASSAY)

TREATMENT	TOTAL # PLATES	TOTAL # FOCI	AVERAGE FOCI PER PLATE	AVERAGE FOCI* PER PLATE (NORMALIZED)
NEGATIVE CONTROL	10	4	0.4	0.78
POSITIVE CONTROL	10	274	27.4	566.90
TEST ARTICLE (1:8)	10	8	0.8	2.00
TEST ARTICLE (1:16)	10	5	0.5	1.06
TEST ARTICLE (1:32)	10	4	0.4	0.82

* NORMALIZED TO 1×10^6 CELLS PER PLATE BASED UPON THE CORRESPONDING SURVIVAL FRACTION (SEE TABLE III)

**TOXIKON TEST PROTOCOL
FDA GLP GUIDELINES**

**CHO/HGPRT FORWARD MUTATION ASSAY - ISO
(T-6889.7)**

TOXIKON PROTOCOL NUMBER: 3MC/VITRO/003-01/000

PROTOCOL DATE: 12/03/01

EFFECTIVE DATE: 12/12/01

COMPLIANCE

21 CFR, Part 58

Good Laboratory Practice for Nonclinical Laboratory Studies

MANAGEMENT OF THE STUDY

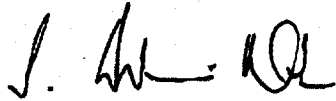
**Performing Laboratory:
Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730**

**Sponsor:
3M Company
3M Center
Corporate Toxicology
Building 0220-02-E-02
St. Paul, MN 55144**

000021

Toxikon Corporation
CHO/HGPRT Forward Mutation Assay - ISO (T-6889.7)
Protocol Number: 3MC/VITRO/002-01/000
Protocol Date: 12/03/01
Effective Date: 12/12/01

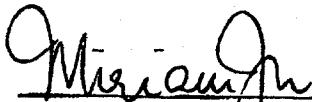
PROTOCOL ACCEPTANCE



Devaki Sadhu, Ph.D.
Study Director
Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730

12/12/01

Date



Quality Assurance
Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730

12/12/01

Date



Sponsor's Representative
3M Company
3M Center
Corporate Toxicology
Building 0220-02-E-02
St. Paul, MN 55144

12/13/01

Date

Toxikon Corporation
CHO/HGPRT Forward Mutation Assay - ISO (T-6899.7)
Protocol Number: 3MC/VITRO/002-01/000
Protocol Date: 12/03/01
Effective Date: 12/12/01

TABLE OF CONTENTS

Title Page

Protocol Acceptance

Table of Contents

- 1.0 Purpose
- 2.0 References
- 3.0 Compliance
- 4.0 Identification of Test and Control Articles
- 5.0 Identification of Test System
- 6.0 Justification of the Test System and Route of Administration
- 7.0 Experimental Design
- 8.0 Dosage
- 9.0 Evaluation Criteria
- 10.0 Records
- 11.0 Confidentiality Agreement
- 12.0 Protocol Amendments/Deviations

Toxikon Corporation
CHO/HGPRT Forward Mutation Assay - ISO (T-6889.7)
Protocol Number: 3MC/VITRO/002-01/000
Protocol Date: 12/03/01
Effective Date: 12/12/01

1.0 PURPOSE

The CHO/HGPRT Forward Mutation Assay (with Confirmation) evaluates the mutagenic potential of a test article via its ability to induce forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus. The CHO assay system utilizes toxic purine analogs to select for resistant cells that are presumed deficient in the purine salvage enzyme HGPRT. The gene mutations are induced at the HGPRT locus in cultured Chinese hamster ovary (CHO) cells.

2.0 REFERENCES

The test will be conducted based upon the following references:

- 2.1 Biological Evaluation of Medical Devices-Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity, ANSI/AAMI/ISO 10993-3: 1993.
- 2.2 OECD Guidelines for the testing of Chemicals, "Genetic Toxicology: *In vitro* Mammalian Cell Gene Mutation Test," Test Guideline # 476, current version.
- 2.3 Hsie, A.W., Casciano, D.A., Couch, D.B., Krahn, D.F., O'Neill, J.P., Whitefield, B.L. "EPA's Gene Tox Program," Mutation Research, 86: 193-214 (1981).
- 2.4 Extraction procedures, if applicable, will be based upon the standard titled Biological Evaluation of Medical Devices-Part 12: Sample Preparation and Reference Materials, EN/ISO 10993-12 (1997).

3.0 COMPLIANCE

The study will conform to all applicable laws and regulations. Specific regulatory requirements include the current Good Laboratory Practice for Nonclinical Laboratory Studies, FDA, 21 CFR, Part 58.

4.0 IDENTIFICATION OF THE TEST AND CONTROL ARTICLES

The following information will be supplied by the Sponsor on a test requisition form or other correspondence wherever applicable; it does not apply to confidential information. The Sponsor will be responsible for all test article characterization data as specified in the GLP regulations. Test and control articles (exclusive of extracts) that are mixed with carriers require verification of concentration, homogeneity and stability. Samples of test and control article mixtures will be returned to the Sponsor for characterization and verification, wherever applicable.

Toxikon Corporation
CHO/HGPRT Forward Mutation Assay - ISO (T-6889.7)
Protocol Number: 3MC/VITRO/002-01/000
Protocol Date: 12/03/01
Effective Date: 12/12/01

4.1 Test Article: To Be Determined (TBD)

Lot/Batch #:
CAS/Code #:
Physical State:
Color:
Density:
pH:
Stability:
Solubility:
Expiration Date:
Storage Conditions:
Safety Precautions:

4.2 Control Articles (Toxikon Supplied):**4.2.1 Negative Control Article Name: TBD**

Toxikon QC #: TBD
Physical State: Liquid
Color: Colorless
Storage: Room Temperature
Safety Precautions: Standard Laboratory Safety Precautions

4.2.2 Positive Control Articles:**4.2.2.1 Positive Control Article Name: TBD**

Toxikon QC #: TBD
Physical State:
Color:
Storage:
Safety Precautions: Known Mutagen. Appropriate Laboratory Safety
Precautions

4.2.2.2 Positive Control Article Name: TBD

Toxikon QC #: TBD
Physical State:
Color:
Storage:
Safety Precautions: Known Mutagen. Appropriate Laboratory Safety
Precautions

Toxikon Corporation
CHO/HGPRT Forward Mutation Assay - ISO (T-6889.7)
Protocol Number: 3MC/VITRO/002-01/000
Protocol Date: 12/03/01
Effective Date: 12/12/01

5.0 IDENTIFICATION OF THE TEST SYSTEM

Chinese Hamster Ovary (CHO) cells utilized in this assay are obtained from the American Type Culture Collection, Rockville, Maryland. The cells are derived from an ovarian biopsy of a chinese hamster.

6.0 JUSTIFICATION OF TEST SYSTEM AND ROUTE OF ADMINISTRATION

6.1 This assay evaluates the mutagenic potential of a test article via its ability to induce mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus. This locus is responsible for production of HGPRT, an enzyme that is present in normal cells and allows cells to salvage hypoxanthine and guanine from the culture medium in order to synthesize DNA. In normal cells, when a toxic substance called 6-thioguanine is included in the growth medium, it is salvaged by the HGPRT enzyme along with hypoxanthine and guanine and incorporated into DNA, thereby causing cell death. Exponentially growing cells, sensitive to the toxic effects of 6-thioguanine, are exposed to several concentrations of the test material. If the test material is potentially mutagenic, normal cells (which can utilize hypoxanthine, guanine and 6-thioguanine) mutate to become incapable of utilizing hypoxanthine, guanine, or 6-thioguanine from the culture medium. However, mutant cells retain their ability to grow as well as normal cells in culture medium because DNA synthesis is made possible by alternate synthetic pathways. Taken together, these findings indicate that the basis for selection of HGPRT mutants is the lack of any ability to utilize the toxic 6-thioguanine. Therefore, cells that grow to form colonies in the presence of 6-thioguanine are assumed to have undergone mutation either spontaneously or by exposure to the test material. The CHO/HGPRT Assay has been used extensively in the detection of mutagenic activity of a wide range of chemical classes.

6.2 The test article will be administered *in vitro*, directly or through a solvent compatible with the test system. These are the only routes of administration available in this test system.

7.0 EXPERIMENTAL DESIGN

7.1 Cell Line

The CHO-K1 cell line is selected for its high cloning ability and rapid doubling time. To reduce the negative control article frequency of HGPRT⁻ mutants to as low as possible, cell cultures can be selected against this phenotype and returned to normal growth medium for three or more days before use.

7.2 Maintenance of CHO Cells

The CHO cells will be grown in complete Ham's F-12 medium, buffered with 10 mM HEPES. Complete medium will consist of 10% fetal bovine serum (FBS), 1-2 mM L-glutamine, 100 units/ml penicillin, and 100 ug/ml streptomycin. Incomplete medium is serum-free complete medium. The cells will be incubated at 37±1°C, 5±1% CO₂, and saturated humidity.

Toxikon Corporation**CHO/HGPRT Forward Mutation Assay - ISO (T-6889.7)****Protocol Number: 3MC/VITRO/002-01/000****Protocol Date: 12/03/01****Effective Date: 12/12/01**

7.3 Cytotoxicity Assay

The Cytotoxicity Assay (if necessary) is conducted without metabolic activation, prior to the Mutation Assay. The highest concentration is based on maximum solubility, 10 mg/ml, or Sponsor specifications. Liquid test articles will be tested at 100 μ l/ml-0.01 μ l/ml. Each test substance concentration, negative control and solvent control (if applicable) is tested in triplicate.

Cultures are initiated in 60 mm dishes at 200 cells/plate approximately 24 hours prior to dosing. The cultures are exposed to the test article and controls for 15-17 hours. At the end of the exposure period, dishes are rinsed with medium or Phosphate Buffered Saline (PBS) and incubated for an additional 6-9 days to allow colonies to develop. At the end of the incubation period, the dishes are fixed in methanol, stained with Giemsa and colonies counted. Relative survival is obtained by comparing the number of surviving colonies for each dose to that of the negative or solvent control.

7.4 Forward Mutation Assay**7.4.1 Metabolic Activation System (S9)**

The S9 microsomal fraction is prepared from Sprague Dawley rat livers induced with Aroclor® 1254. The S9 rat liver homogenate is stored at $-80 \pm 5^\circ\text{C}$ until use. A combination of S9 fraction, isocitric cofactors and incomplete medium is prepared just prior to exposure and used as the metabolic activation system. The cofactor mixture consists of isocitrate (trisodium salt) and NADP (disodium salt) at a final concentration of 4.5 mg/ml and 2.4 mg/ml, respectively. The S9 fraction is added to each flask or directly to the cofactor mixture at a concentration of 20 μ l/ml.

7.4.2 Parallel Cytotoxicity Assay

A parallel cytotoxicity assay is performed along with the Mutation assay. The cultures are treated exactly as the mutation plates, incubated for 6-9 days following the removal of the test article, fixed, stained, and colonies counted.

7.4.3 Preparation of Test Cultures

Approximately 24 hours prior to exposure, duplicate 100 mm mutation plates are seeded at a density of 5×10^5 cells/dish and triplicate parallel cytotoxicity plates are seeded at 200 cells/60 mm dish.

7.4.4 Exposure Periods

The cells will be exposed to the test article for 15-17 hours (4-6 hours for highly reactive chemicals) in the non-activated assay and 4-6 hours in the activated assay. After exposure, cells are washed at least once with medium or PBS and supplemented with complete medium.

Toxikon Corporation
CHO/HGPRT Forward Mutation Assay - ISO (T-6889.7)
Protocol Number: 3MC/VITRO/002-01/000
Protocol Date: 12/03/01
Effective Date: 12/12/01

7.4.5 Phenotype Expression

Approximately 24-48 hours following termination of exposure period, cells are trypsinized, counted, and plated at 1×10^6 cells per 100 mm dish. The cells are passed every 48-72 hours to maintain exponential growth during phenotypic expression for approximately 7-9 days.

7.4.6 Selective Growth

Following the phenotypic expression period, cells are grown in selective medium to select for mutant cells. The selective medium includes hypoxanthine-free HAM'S F-12 with 10% dialyzed fetal bovine serum, penicillin-streptomycin (P/S) and 10μ 6-thioguanine. A total of ten dishes (five from each duplicate) are used for each test condition. Cell density is 2×10^5 / 100 mm petri dish. The cultures are incubated for 6-9 days to allow colonies to develop.

7.4.7 Parallel Cloning Efficiency (PCE)

Concurrently, the cloning efficiency is determined by plating cells in selective medium without 6-thioguanine. Six dishes are seeded for each concentration (three from each duplicate) at 200 cells/60 mm dish. The cultures are then incubated for 6-9 days to allow colony formation.

7.4.8 Termination

At the end of the incubation period, plates are rinsed with PBS, fixed in methanol and stained with Giemsa. Only colonies with 50 or more cells are counted.

7.5 Control Articles

7.5.1 Positive Control Article

The positive control for the non-activated system is Ethylmethanesulfoxide (EMS) or 4-Nitroquinoline-1-oxide (4NQ). For the activated system, 9,10,- Dimethyl-1,2-benzanthracene (DMBA) or Dimethylnitrosamine (DMN) can be used as the positive control.

7.5.2 Negative Control Article

The appropriate solvent, extractant or cell culture medium will serve as the negative control article.

7.6 Confirmatory Assay

The results of the Forward Mutation Assay will be confirmed through an independent Confirmatory Assay. The Confirmatory Assay will be performed as described in Sections 7.0 and 8.0.

Toxikon Corporation
CHO/HGPRT Forward Mutation Assay - ISO (T-6889.7)
Protocol Number: 3MC/VITRO/002-01/000
Protocol Date: 12/03/01
Effective Date: 12/12/01

8.0 DOSAGE

8.1 Preparation of Test Article

8.1.1 Medical Devices:

The test article will be extracted at ratios specified by EN/ISO 10993-12. Extraction vehicles may be one of the following media: serum-free medium, complete medium, or 0.9% USP Sodium Chloride for Injection, USP (NaCl).

Extraction conditions will be as specified by Sponsor: (please check desired condition)

- $121 \pm 2^{\circ}\text{C}$ for one hour
- $70 \pm 2^{\circ}\text{C}$ for 24 hours
- $50 \pm 2^{\circ}\text{C}$ for 72 hours
- $37 \pm 1^{\circ}\text{C}$ for 72 hours
- $37 \pm 1^{\circ}\text{C}$ for 24 hours
- per Sponsor's directions (_____ $^{\circ}\text{C}$ for _____ hours).

Extracts prepared with medium will be tested at 100% (neat) concentration. Extracts prepared with Sodium Chloride will be diluted with 2X medium and tested at 50% extract concentration (considered neat). Modifications to test article preparation will be as specified by the Sponsor.

8.1.2 Solid/Liquid Test Articles

Solid test articles will be dissolved or suspended in a vehicle appropriate for the test system or as specified by the Sponsor. Liquid test articles will be administered as received at predetermined concentrations or as specified by the Sponsor.

8.2 Dose Selection

8.2.1 The concentrations for the Mutation Assay are selected based on toxicity information, maximum solubility of the test article or Sponsor specifications. If necessary, a Cytotoxicity Assay will be performed to determine toxicity. If toxicity is evident, the doses should include the highest concentration which causes a low level of survival (approximately 10%) and four lower doses, including one dose with no apparent cytotoxicity. If no apparent cytotoxicity is observed, the maximum concentration will be based on the limit of solubility or 10 mg/ml, whichever is lower (or as specified by the Sponsor). Subsequent doses will decrease in approximate half-log increments. In the absence of toxicity, liquid test articles will be tested at 100 ul/ml-0.01 ul/ml. Dose selection modifications will be as specified by the Sponsor.

8.2.2 Medical device extracts will be analyzed at one concentration only (neat extract). Dilutions of medical device extracts are not analyzed unless otherwise required, in which case the justification shall be indicated in the final report. An initial Cytotoxicity Assay is not performed for device extracts.

Toxikon Corporation
CHO/HGPRT Forward Mutation Assay - ISO (T-6889.7)
Protocol Number: 3MC/VITRO/002-01/000
Protocol Date: 12/03/01
Effective Date: 12/12/01

9.0 EVALUATION CRITERIA

The results of the CHO/HGPRT Locus Mutation Assay will be evaluated on the basis of the number of TG-resistant mutants per 1×10^6 surviving cells. The significance of the test results will be determined by either one of the following methods:

9.1 The test results are analyzed using a statistical program such as Tallarida, R.S. and R.B. Murray's Pharmacological Calculations Procedure, ANOVA (analysis of variance) and Newman-Keuls Test for confirmation of pairwise comparisons. This statistical method determines if there is a significant ($p < 0.05$) increase in the mutation frequency of the test article compared to the negative control article. The results obtained for the mutation frequency at the various dose levels will be analyzed by the method of Linear Regression using a program such as "Linear Regression I" by R.J. Tallarida and R.B. Murray, (Manual of Pharmacologic Calculations with Computer Programs, Springer-Verlag, New York, 1986, pp 10-13). This will determine if there is a positive dose response.

9.2 The test article is considered to have caused a positive response if the test article shows a statistically significant positive dose response or at least one test article dose shows a reproducible statistically significant increase in the number of mutants per 1×10^6 surviving cells as compared to the corresponding negative control article.

9.3 Confirmation Assay

The confirmation assay will validate the reproducibility of the mutagenesis assay.

10.0 RECORDS

- 10.1 Original raw data will be archived at Toxikon Corporation.
- 10.2 A copy of the final report and any amendments will be archived at Toxikon Corporation.
- 10.3 The original final report and a copy of any protocol amendments or deviations will be forwarded to the Sponsor.
- 10.4 All unused test article will be handled as specified in the Test Requisition Form. Otherwise, all remaining test article will be discarded.

11.0 CONFIDENTIALITY AGREEMENT

Statements of confidentiality may be agreed upon prior to study initiation.

Toxikon Corporation
CHO/HGPRT Forward Mutation Assay - ISO (T-6889.7)
Protocol Number: 3MC/VITRO/002-01/000
Protocol Date: 12/03/01
Effective Date: 12/12/01

12.0 PROTOCOL AMENDMENTS/DEVIATIONS

All changes to the approved protocol and the reason for the changes will be documented in writing, signed by the Study Director, dated, and maintained with the protocol. No protocol amendments will be made without written approval in the form of a Sponsor Communication Log between the Sponsor and the Study Director which will be generated as closely as possible to the time of the change.