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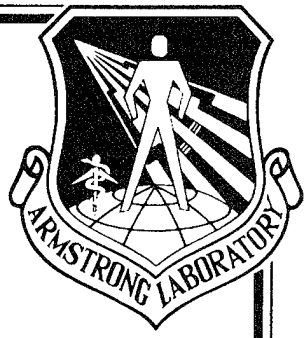
VOLUME III of IV



ARMSTRONG LABORATORY

GENETIC TOXICITY EVALUATION OF 1, 3, 3-TRINITROAZETIDINE

VOLUME III: RESULTS OF GENE MUTATION AT THE HGPRT LOCUS IN CULTURED CHINESE HAMSTER OVARY CELLS

I. J. Paika

TOXICON CORPORATION
225 WILDWOOD AVE
WOBURN, MA 01801

February 1994

FINAL REPORT FOR THE PERIOD JULY THROUGH DECEMBER 1992

AIR FORCE MATERIEL COMMAND
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TECHNICAL REVIEW AND APPROVAL

AL/OE-TR-1994-0069

VOLUME III

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

TERRY A. CHILDRESS, Lt Col, USAF, BSC
Director, Toxicology Division
Armstrong Laboratory

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Approved for public release; distribution is unlimited.

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13. ABSTRACT (Maximum 200 words)

The test substance, 1,3,3-Trinitroazetidine (TNAZ)both in the presence and absence of microsomal S-9 liver enzyme, failed to induce a significant increase in mutant colonies at the HGPRT locus in cultured Chinese hamster ovary cells. The effects of TNAZ at the dose levels selected based on the initial range finding assay were similar to those of the negative control (incomplete Ham's F12 medium) and solvent control (dimethylsulfoxide) substances. Both positive control substances (4-nitroquinoline-1-oxide and dimethylnitrosamine) in the presence and absence of an activation system, exhibited a significantly increased induction of mutant colonies. Confirmatory assays were performed utilizing the same test system conditions as the initial assays. The results were similar and consistent with the initial mutagenicity assay. The data indicates that TNAZ did not induce an increased number of mutants compared to the negative and solvent control substance values. Therefore, TNAZ is considered non-mutagenic under the conditions utilized in this study.

14. SUBJECT TERMS

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PREFACE

1,3,3-Trinitroazetidine (TNAZ) (CAS No. 97645-24-4) is a highly energetic castable explosive that is being considered by the Department of Defense for military and space applications. As a candidate replacement for select explosives, toxicity information is needed. A comprehensive literature search indicated that no information was available on the mutagenic potential of TNAZ. ManTech Environmental initiated a battery of three short-term assays that were utilized to assess the mutagenic and clastogenic potential of TNAZ. Protocols for these assays were in conformance with the Environmental Protection Agency's (Toxic Substances Control Act) Health Effects Testing Guidelines, 40 CFR, Part 798 (7-1-90 edition).

This document, Volume III of IV, serves as a final report detailing the results of gene mutation at the HGPRT locus in cultured Chinese hamster ovary cells in the genetic toxicity evaluation of TNAZ. Volumes I and II will describe, respectively, the results of the *salmonella typhimurium* reverse mutation assay (Ames assay) and the results of the mouse bone marrow micronucleus test. Volume IV will serve as a summary report presenting the pertinent findings of the three assays described in Volumes I through III.

The research described herein began in July 1992 and was completed in December 1993 by the Toxikon Corporation, Woburn, MA, under a subcontract to ManTech Environmental Technology Inc., Toxic Hazards Research Unit (THRU), and was coordinated by Darol E. Dodd, Ph.D., THRU Laboratory Director. This work was sponsored by the Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory, and was performed under Department of the Air Force Contract No. F33615-90-C-0532 (Study No. F19). Lt Col James N. McDougal and Lt Col Terry A. Childress, respectively, served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory, Toxicology Division.

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STUDY REPORT

Study Title

Chemical Induction of Gene Mutation at the HGPRT Locus
In Cultured Chinese Hamster Ovary (CHO) Cells

Company Name

ManTech Environmental Technology, Inc.
Toxic Hazards Research Unit
P. O. Box 31009
Dayton, OH 45437-0009

Product Identification

1, 3, 3 - Trinitroazetidine (TNAZ)

Data Requirement

TSCA, 40 CFR, Part 798

Author

Inder J. Paika, Ph.D.

Volume Number

_1_ of _1_

Final Report Completed On

December 09, 1993

Performing Laboratory

Toxikon Corporation
225 Wildwood Avenue
Woburn, MA 01801

Laboratory Project ID/Study Number

92G-1271

STUDY SUMMARY

The test substance, 1, 3, 3-Trinitroazetidine (TNAZ), both in the presence and absence of microsomal S-9 liver enzyme, failed to induce a significant increase in mutant colonies at the HGPRT locus in cultured Chinese Hamster Ovary (CHO) cells. The effects of this test substance, at the dose levels selected based on the initial Range Finding Assay test, were similar to those of the negative and solvent control substances. Both positive control substances, in the presence and absence of an activation system, exhibited a significantly increased induction of mutant colonies. Confirmatory Assays were performed utilizing the same test system conditions as the initial assays. The results were similar and consistent with the initial Mutagenicity Assay described above. The data indicates that the test substance did not induce an increased number of mutants compared to the negative and solvent control substance values. Therefore, the test substance is considered non-mutagenic under the conditions utilized in this study.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Company: ManTech Environmental Technology, Inc.
Toxic Hazards Research Unit
P.O. Box 31009
Dayton, OH 45437-0009

Performing Laboratory: Toxikon Corporation
225 Wildwood Avenue
Woburn, MA 01801

Test Substance:

Test Substance: 1, 3, 3 - Trinitroazetidine (TNAZ)

Lot/Batch #: Not Supplied

CAS/Code #: 97645-24-4

Project Officer: Darol E. Dodd 12/12/93
Darol Dodd, Ph.D. Date
ManTech Environmental
Technology, Inc.

This study was contracted to Toxikon to be conducted according to all applicable laws and regulations. Specific regulatory requirements included the current EPA (TSCA), 40 CFR, Part 792, Good Laboratory Practice Standards.

Study Director: Inder J. Paika 12/09/93
Inder J. Paika, Ph.D. Date:
Toxikon Corporation

SIGNATURE PAGE

The individuals below were involved in the generation and review of all data submitted for this project.

Inder J. Paika, Ph.D.
Study Director

Mark Turner, B.S.
Study Supervisor

QUALITY ASSURANCE STATEMENT

Company: ManTech Environmental Technology, Inc.
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P.O. Box 31009
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Performing Laboratory: Toxikon Corporation
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Woburn, MA 01801

Test Substance:

Test Substance: 1, 3, 3 - Trinitroazetidine (TNAZ)

Lot/Batch #: Not Supplied

CAS/Code #: 97645-24-4

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

INSPECTIONS	QUALITY ASSURANCE INSPECTIONS	REPORTS TO MANAGEMENT	REPORTS TO STUDY DIRECTOR
SCORING	11/12/92	11/12/92	11/12/92
RAW DATA	07/07/93	07/07/93	07/07/93
FINAL REPORT	12/09/93	12/09/93	12/09/93

Signature Of Authorized Personnel:

Naseem Kabir, M.S.
Toxikon Quality Assurance

12/09/93
Date

TABLE OF CONTENTS

1.0 PURPOSE

The CHO/HGPRT assay evaluated the mutagenic potential of the test substance 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. The gene mutations were induced at the HGPRT locus in cultured Chinese hamster ovary (CHO) cells.

2.0 REFERENCES

The assay was conducted based upon the following references:

Chu, E.H.Y. and Powell, S.S. *Adv. Human Genet.*7:189 (1976).

EPA (TSCA), 40 CFR, Part 798.5300.

Hsie, A.W., Brimer, P.A. Mitchell, T.J., and Gossless, D.G. *Somet. Cell Genet.*1:247 (1975).

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).

Mhyr, B.C. and DiPaolo, J.A. *Cancer Res.*38:2539 (1978).

Kastenbaum, M.A. and Bowman, K.O. *Mutation Res.*9:527 (1970).

3.0 COMPLIANCE

The study conformed to all applicable laws and regulations. Specific regulatory requirements included the current Good Laboratory Practice Standards, TSCA (EPA), 40 CFR, Part 792.

4.0 TEST AND CONTROL SUBSTANCES

The following information was supplied by the Sponsor wherever applicable. It did not apply to confidential information. The Sponsor was responsible for all test substance characterization data as specified in the GLP regulations.

4.1 Test Substance Name: 1,3,3-Trinitroazetidine (TNAZ)
CAS/Code #: 97645-24-4
Lot/Batch #: Not Supplied by the Sponsor (N/S)
Physical State: White granular solid
Color: White
Density: 1.84
pH: (N/S)
Stability: CAUTION: Class A Explosive (Refer to MSDS)
Solubility: Negligible in Water; DMSO
Source: Eglin AFB, FL 32542-5000

Storage Conditions: 0° to 120°F
Safety Precautions: Standard Procedures for Class A Explosives

4.2 Negative Control Article Name: Incomplete Ham's F12 medium
CLPR #: 9210005CC
Physical State: Liquid
Color: Pink
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Practices

4.3 Solvent Control Substance Name: Dimethylsulfoxide (DMSO)
CSC #: 90071CC
Physical State: Liquid
Color: clear
Storage Conditions: Room Temperature

4.4 Positive Control Substance Name: 4-Nitroquinoline-1-oxide (4-NQ)
CLPR #: 9209009CC
Physical State: Solid
Color: Pale-yellow
Storage Conditions: -20°C Freezer
Safety Precautions: Standard Laboratory Safety Practices

4.5 Positive Control Substance Name: Dimethylnitrosamine (DMN)
CSC #: 9109004CC
Physical State: liquid
Color: Clear
Storage Conditions: 4+1°C Refrigeration
Safety Precautions: Standard Laboratory Safety Practices

5.0 IDENTIFICATION AND JUSTIFICATION OF THE TEST SYSTEM

HGPRT is an enzyme that allows cells to salvage hypoxanthine and guanine from the culture medium for use in DNA synthesis. Since the purine analog 6-thioguanine (TG) was included in the growth medium, it was phosphorylated by this enzyme and incorporated into DNA, thereby causing cell death. The induction of a single-step forward mutation in wild-type cells ($HGPRT^+$) gave rise to mutant cells ($HGPRT^-$) which were incapable of utilizing hypoxanthine, guanine, or TG from the culture medium. Mutant cells retained their ability to grow as well as wild-type cells in normal medium because DNA synthesis was made possible by alternate or *de novo* purine synthetic pathways. Taken together, these findings indicated that the basis for selection of HGPRT mutants was the lack of any ability to utilize the toxic purine analog TG. Therefore, cells that grew to form colonies in the presence of TG were assumed to have undergone mutation either spontaneously or by the action of a test substance to the HGPRT phenotype.

The CHO-K1 cell line was originally derived from the ovary of a female Chinese hamster (*Cricetalus griseus*) obtained from

American Type Culture Collection, Rockville, MD.

The CHO/HGPRT Gene Mutation Assay has been used extensively and has been demonstrated to be effective in detecting the mutagenic activity of chemicals from a wide range of chemical classes.

6.0 ROUTE OF TEST SUBSTANCE ADMINISTRATION

The test substance was administered *in vitro* directly to the test system. This was the only route of administration available in this test system.

7.0 EXPERIMENTAL DESIGN

7.1 Indicator Cells:
The CHO-K1 cell line used in the study was selected for its high cloning ability (80%) and a doubling time of 12 to 14 hours. To reduce the negative control substance frequency of $HGPRT^-$ mutants to as low a level as possible, cell cultures were exposed to conditions that selected against the phenotype and were returned to normal growth medium for three or more days before use.

7.2 Culture Media:
The CHO cells were propagated in complete Ham's F-12 culture medium (Ham's F-12 nutrient medium supplemented with 10% heat inactivated fetal bovine serum, 2mM L-glutamine, 50 units/mL penicillin and 50 ug/mL streptomycin). Medium used for reducing the spontaneous frequency of $HGPRT^-$ mutants prior to the initiation of the study consisted of complete medium supplemented with 5.0×10^{-6}M thymidine, 1.0×10^{-5} hypoxanthine (Hx), 2.0×10^{-4}M glycine, and 3.2×10^{-6}M aminopterin. Recovery medium consisted of reducing medium without aminopterin. Selection medium was prepared from complete medium that lacked hypoxanthine, but contained 10 ug/mL TG and 5% fetal bovine serum.

7.3 Control Substances:

7.3.1 Negative Control Substance:
The negative control substance used was incomplete Ham's F-12 (serum free). The negative control consisted of assay procedures performed on untreated cells.

7.3.2 Solvent Control Substance:
The solvent control substance used was 0.5% DMSO. Since the test substance was insoluble in growth medium, DMSO (solvent) was used. The final concentration of solvent in the growth medium was assayed as the solvent control to determine any effects on survival or mutation caused by the solvent alone. For test substances assayed with activation, the negative and solvent control substances included the activation mixture.

7.3.3 Positive Control Substance (Without Activation):
The positive control substance, 4-Nitroquinoline-1-oxide (4-NQ),

is a stable chemical that has proven to be highly mutagenic to CHO-K1 cells. It was dosed at 0.03 ug/mL in the non-activated studies.

7.3.4 Positive Control Substance (With Activation):
Dimethylnitrosamine (DMN), which causes mutation at the HGPRT locus with metabolic activation, was used at 0.3 uL/mL in the activated systems.

7.4 Assay Procedure:

7.4.1 Preparation of Test Cultures:
The CHO stock cultures for the assay were grown in complete Hx-free medium. Cultures growing in T75 tissue culture flasks and showing approximately 50-90% confluency were harvested and used to prepare the test cultures. The medium from the T75 flasks was discarded, and the cells were washed with Ca^{++}-free and Mg^{++}-free Hank's Balanced Salt Solution (HBSS). The cells were dissociated by adding 2 ml of 0.05% trypsin to each flask. The cells were rinsed with trypsin, and the excess trypsin removed. The flasks were incubated at $37\pm1^{o}C$ until the cells dissociated. Complete Hx-free medium (5.0 mL) was added to each of the stock culture flasks, and the cell suspension was aspirated to obtain a single cell suspension. The cells from a number of stock culture flasks were pooled and centrifuged at 800 rpm for 5 minutes. The supernatant was removed, and the cells were resuspended in complete Hx-free culture medium. An aliquot of the cell suspension was diluted to the appropriate concentration and counted in a hemacytometer. Based on the cell counts, a separate cell suspension with 1×10^5 cells/mL was prepared to seed the test flasks. An appropriate number of T25 tissue culture flasks were seeded with 5.0 mL of cell suspension to obtain test cultures with a density of 5×10^5 cells/flask.

7.4.2 Preparation of Test Substance:
The test substance was dissolved in 0.5% DMSO solution in Ham's F-12 incomplete medium to prepare a stock solution concentration of 5.0 mg/mL.

7.4.3 Range Finding Assay:
A Range Finding Assay was performed with and without metabolic activation on 10 concentrations of test substance, negative and solvent control substances. Culture dishes (100 mm), seeded with 200 cells/dish, were exposed to the appropriate concentrations for 2 ± 0.5 hours. The dosing solutions were aspirated and the plates re-fed with complete Ham's F-12. The cells were allowed to grow for 7 days without any disturbance to minimize the formation of satellite colonies. The colonies were washed with phosphate buffered saline (PBS), fixed with methanol, stained with Giemsa stain and counted. A cluster of more than 50 cells growing within a confined area was considered a colony. The average number of colonies per dish was calculated and the Parallel Cloning Efficiency (PCE) (Colonies Observed each Plate/Cells Plated) was determined.

7.4.4 Preparation of Metabolic Activation System:
The S9 microsomal fraction, prepared from Sprague-Dawley rat livers induced with Aroclor 1254, was obtained commercially. The S9 rat liver homogenate was stored at -80^{o}C until use. Just prior to feeding the cells the S9 was thawed and the metabolic activation reaction mixture including the cofactors was prepared. The medium·cofactor mixture consisted of 10% 10X isocitrate·NADP cofactors and 90% incomplete Hx-free, serum-free medium. S9 fraction was added to the medium·cofactor mixture to give the following concentrations in the cell culture: 4.5 mg/mL isocitrate (trisodium salt), 2.4 mg/mL NADP (disodium salt), and 20 uL/mL S9 fraction.

7.4.5 Mutation Assay
Duplicate cultures seeded with 5 x 10^5 cells/flask were used at each dose level. The cells were treated with seven dose levels of test substance, one dose of positive control, one dose of the negative control, and one dose of the solvent control in the activated and non-activated systems. After the exposure time, the cells were washed with PBS, refed with complete Ham's F-12 medium, and allowed to grow for five days. The non-activated exposure period was 16 hours.

In the activated system, the medium was removed and 12.0 mL of the appropriate test or control solution and 600 uL of S-9 mixture was added to each of the culture flasks. After a five hour exposure period, the cells were washed with PBS and cultured for five days.

Post-expression, the cells were harvested and reseeded in selection medium at 2 x 10^5 cells per 100 mm dish. Five dishes were plated per dose level for a total of 1 x 10^6 cells. Concurrently, 200 cells per 100 mm dish were seeded in complete medium for the Parallel Cloning Efficiency Assay. The cultures were then incubated for six days without disturbing the dishes to minimize the formation of satellite colonies. The colonies were then washed with PBS, fixed, stained and counted for cloning efficiency, and mutant selection. The average number of clones from the triplicate dishes was calculated and expressed as the Parallel Cloning Efficiency. The number of TG-resistant mutants for 1 x 10^6 cells seeded at each dose level was calculated by totaling the number of mutants from the five replicate dishes. Based on the PCE, the number of TG-resistant mutants per 1 x 10^6 surviving cells was calculated for each dose level.

8.0 DOSAGE

8.1 Range Finding Assay:
Ten concentrations of the test substance (activated and non-activated system) were assayed in the Range Finding Assay. Concentrations assayed included 5.00, 2.00, 1.00, 0.500, 0.250, 0.125, 0.062, 0.031, 0.016 and 0.008 mg/mL.

8.2 Mutagenesis Assay: (Initial and Confirmatory Assays)
Based on the outcome of the Range Finding Assay, the following dose concentrations were selected for the mutagenicity assay: S-9 Activated Assay 0.500, 0.250, 0.125, 0.063, 0.031, 0.016, 0.008 mg/ml and Non-Activated Assay 0.250, 0.125, 0.063, 0.031, 0.016, 0.008 and 0.004 mg/ml.

9.0 EVALUATION CRITERIA

9.1 The Mutation Assay is considered valid if it meets the following conditions:

9.1.1 In the negative control substance, the average number of TG-resistant mutants per 1 x 10^6 surviving cells should not exceed 25.

9.1.2 The number of TG-resistant mutants in the positive control substances should exceed 100 mutants per 1 x 10^6 surviving cells.

9.1.3 At least one of the test substance doses should show more than 40% reduction in the PCE. This requirement should not be applied to test substances where no apparent toxicity could be achieved at the maximum dose analyzed.

9.1.4 The negative control should show a cloning efficiency of at least 65%.

9.1.5 The results should have at least three usable dose points.

9.2 Evaluation of Test Results:
The results of the CHO/HGPRT Locus Mutation Assay were evaluated on the basis of the number of TG-resistant mutants per 1x10^6 surviving cells. Mutant data from test substance doses which show more than 80% reduction in Parallel Cloning Efficiency was included in evaluating the results. The significance of the test results was determined by the following method:

9.2.1 The test results are considered significant if a dose shows more than a two-fold increase in the number of mutants per 1 x 10^6 surviving cells over that of the concurrent control (solvent control).

9.3 Positive Response:
The test substance is considered to have caused a positive response if:

9.3.1 The test substance shows a positive dose response and at least one test substance dose shows a statistically significant increase in the number of mutants per 1 x 10^6 surviving cells or a twofold increase in the number of mutants per 1 x 10^6 surviving cells. In the event that the test substance causes a significant increase in the number of mutants per 1 x 10^6 surviving cells due

to an unusually low number of mutants (less than 10 mutants per 1 x 10^6 surviving cells) in the concurrent solvent control substance, the data from the test substance-treated cultures will be compared to the historic negative control data.

9.3.2 In the absence of a positive dose-response trend, at least two consecutive test doses should show a significant increase in the number of mutants per 1 x 10^6 surviving cells.

9.4 Negative Response:
The test substance is considered to have caused a negative response if none of the test doses shows a significant increase in the number of mutants per 1 x 10^6 surviving cells.

10.0 RESULTS AND DISCUSSION

The data from the Range Finding Assay is presented in Tables 1-2. Of the doses tested, the higher dose concentrations at 5.00, 2.00, 1.00, and 0.500 mg/mL were cytotoxic in the activated system. Therefore, 0.500, 0.250, 0.125, 0.062, 0.031, 0.016 and 0.008 mg/mL doses were utilized. In the non-activated Mutagenicity Assay 0.250, 0.125, 0.062, 0.031, 0.016, 0.008 and 0.004 mg/mL doses were utilized.

The Mutagenicity Assay (Tables 3 and 4), both in the presence and absence of microsomal S-9 liver enzyme, demonstrated that the test substance failed to induce significantly large numbers of mutant colonies. The effect of the test substance on the expression of mutant colonies was similar to untreated (negative) and solvent controls, whereas both positive controls exhibited an increased induction of mutant colonies.

10.1 Confirmatory Assay:
The Mutagenicity Assay was repeated as a Confirmatory Assay (Tables 5 and 6). These results indicate that the test substance did not induce increased numbers of mutants and is comparable to that of the negative control substance under the conditions utilized in this test system.

11.0 CONCLUSION

Based on the methods described in the study report, the test substance, 1, 3, 3-Trinitroazetidine (TNAZ), is considered non-mutagenic.

12.0 RECORDS

Original Data: Toxikon Corporation Archives
Final Report: Toxikon Corporation Archives
Test Substance: All unused test substance will be returned to the Sponsor

13.0 CONFIDENTIALITY

This study and related information was authorized for confidential use by ManTech and appropriate regulatory agencies.

14.0 STORAGE LOCATION OF RECORDS

Original Data:	Toxikon Corporation Archives
Final Report:	Toxikon Corporation Archives
Test Substance:	All unused test substance will be returned to the Sponsor.

15.0 VERIFICATION OF DATA

Date of Signed Protocol:	07/27/92
Project Log Date:	08/21/92
Range Finding Assay:	04/02/93 - 04/09/93
Mutagenicity Assay:	04/13/93 - 04/28/93
Confirmatory Assay:	04/29/93 - 05/14/93
Final Report:	12/09/93

16.0 AUTHORIZED SIGNATURE

Inder J. Paika Ph.D.
Study Director

12/09/93
Date

TABLE 1

RANGE FINDING ASSAY (S-9 ACTIVATED)

TEST	AVE. COLONIES PER DISH	% PLATING EFFICIENCY
TEST SUBSTANCE (5.00 mg/mL)	0.0	0.0
TEST SUBSTANCE (2.00 mg/mL)	0.0	0.0
TEST SUBSTANCE (1.00 mg/mL)	0.0	0.0
TEST SUBSTANCE (0.500 mg/mL)	0.0	0.0
TEST SUBSTANCE (0.250 mg/mL)	149.0	74.5
TEST SUBSTANCE (0.125 mg/mL)	152.0	76.0
TEST SUBSTANCE (0.062 mg/mL)	157.0	78.5
TEST SUBSTANCE (0.031 mg/mL)	161.0	80.5
TEST SUBSTANCE (0.016 mg/mL)	158.0	79.0
TEST SUBSTANCE (0.008 mg/mL)	154.0	77.0
NEGATIVE CONTROL	165.0	82.0
SOLVENT CONTROL (0.5% DMSO)	162.0	81.0

TABLE 2

RANGE FINDING ASSAY (NON-ACTIVATED)

TEST	AVE. COLONIES PER DISH	% PLATING EFFICIENCY
TEST SUBSTANCE (5.00 mg/mL)	0.0	0.0
TEST SUBSTANCE (2.00 mg/mL)	0.0	0.0
TEST SUBSTANCE (1.00 mg/mL)	0.0	0.0
TEST SUBSTANCE (0.500 mg/mL)	0.0	0.0
TEST SUBSTANCE (0.250 mg/mL)	0.0	0.0
TEST SUBSTANCE (0.125 mg/mL)	0.0	0.0
TEST SUBSTANCE (0.062 mg/mL)	0.0	0.0
TEST SUBSTANCE (0.031 mg/mL)	131.0	65.5
TEST SUBSTANCE (0.016 mg/mL)	162.0	81.0
TEST SUBSTANCE (0.008 mg/mL)	159.0	79.5
NEGATIVE CONTROL	160.0	80.0
SOLVENT CONTROL (0.5% DMSO)	155.0	77.5

TABLE 3

MUTAGENICITY ASSAY
ACTIVATED ASSAY

	MUTANT SCORING		PARALLEL CLONING EFFICIENCY	
TEST	AVE. MUTANT COL/DISH	AVERAGE SURVIVING COLONIES	AVERAGE % PLATING EFFICIENCY	MEAN MUTANT FREQUENCY PER $1x10^6$ SURVIVORS
TEST SUBSTANCE (0.500 mg/mL)	0.0	29.0	14.5	0.00
TEST SUBSTANCE (0.250 mg/mL)	0.4	146.0	73.0	2.74
TEST SUBSTANCE (0.125 mg/mL)	0.6	156.0	78.0	3.87
TEST SUBSTANCE (0.062 mg/mL)	0.6	150.0	75.0	4.00
TEST SUBSTANCE (0.032 mg/mL)	1.0	159.0	79.5	6.29
TEST SUBSTANCE (0.016 mg/mL)	0.8	165.0	82.5	4.85
TEST SUBSTANCE (0.008 mg/mL)	0.6	135.0	67.5	4.44
NEGATIVE CONTROL	0.4	162.0	81.0	2.47
SOLVENT CONTROL (0.5% DMSO)	0.6	170.0	85.0	3.53
POSITIVE CONTROL DMN (0.3 ul/ml)	18.6	156.0	78.0	119.23

COL = Colonies

TABLE 4

MUTAGENICITY ASSAY
NON-ACTIVATED ASSAY

	MUTANT SCORING		PARALLEL CLONING EFFICIENCY	
TEST	AVE. MUTANT COL/DISH	AVERAGE SURVIVING COLONIES	AVERAGE % PLATING EFFICIENCY	MEAN MUTANT FREQUENCY PER $1x10^6$ SURVIVORS
TEST SUBSTANCE (0.250 mg/mL)	0.0	0.0	0.0	0.0
TEST SUBSTANCE (0.125 mg/mL)	0.0	0.0	0.0	0.0
TEST SUBSTANCE (0.062 mg/mL)	0.0	0.0	0.0	0.0
TEST SUBSTANCE (0.032 mg/mL)	0.6	124.0	62.0	4.84
TEST SUBSTANCE (0.016 mg/mL)	0.8	162.0	81.0	4.94
TEST SUBSTANCE (0.008 mg/mL)	0.4	158.0	79.0	2.53
TEST SUBSTANCE (0.004 mg/mL)	0.6	146.0	73.0	4.11
NEGATIVE CONTROL	0.4	163.0	81.5	2.45
SOLVENT CONTROl (0.25%)	0.4	156.0	78.0	2.56
POSITIVE CONTROL 4-NQ(0.03 ul/ml)	14.6	145.0	72.5	100.69

COL = Colonies,

TABLE 5

CONFIRMATORY ASSAY
(ACTIVATED)

	MUTANT SCORING	PARALLEL CLONING EFFICIENCY		
TEST	AVE. MUTANT COL/DISH	AVERAGE SURVIVING COLONIES	AVERAGE % PLATING EFFICIENCY	MEAN MUTANT FREQUENCY PER $1x10^6$ SURVIVORS
TEST SUBSTANCE (0.500 mg/mL)	0.0	25.0	12.5	0.0
TEST SUBSTANCE (0.250 mg/mL)	0.2	140.03	70.0	1.49
TEST SUBSTANCE (0.125 mg/mL)	0.6	152.0	76.0	3.95
TEST SUBSTANCE (0.062 mg/mL)	0.4	164.0	82.0	2.44
TEST SUBSTANCE (0.031 mg/mL)	0.8	170.0	85.0	4.71
TEST SUBSTANCE (0.016 mg/mL)	0.6	168.0	8.4	3.57
TEST SUBSTANCE (0.008 mg/mL)	0.4	164.0	82.0	2.44
NEGATIVE CONTROL	0.4	172.0	86.0	2.33
SOLVENT CONTROL (0.5% DMSO)	0.6	180.0	90.0	3.33
POSITIVE CONTROL DMN (0.3 ul/ml)	20.2	159.0	79.5	127.04

COL = Colonies

TABLE 6

CONFIRMATORY ASSAY
(NON-ACTIVATED)

	MUTANT SCORING		PARALLEL CLONING EFFICIENCY	
TEST	AVE. MUTANT COL/DISH	AVERAGE SURVIVING COLONIES	AVERAGE % PLATING EFFICIENCY	MEAN MUTANT FREQUENCY PER $1x10^6$ SURVIVORS
TEST SUBSTANCE (0.25 mg/mL)	0.0	0.0	0.0	0.0
TEST SUBSTANCE (0.125 mg/mL)	0.0	0.0	0.0	0.0
TEST SUBSTANCE (0.062 mg/mL)	0.0	0.0	0.0	0.0
TEST SUBSTANCE (0.031 mg/mL)	0.8	172.0	86.0	4.65
TEST SUBSTANCE (0.016 mg/mL)	0.4	160.0	80.0	2.50
TEST SUBSTANCE (0.008 mg/mL)	0.6	155.0	77.5	3.87
TEST SUBSTANCE (0.004 mg/mL)	0.4	158.0	79.0	2.53
NEGATIVE CONTROL	0.4	166.0	83.0	2.41
SOLVENT CONTROL (DMSO)	0.6	138.0	69.0	4.35
POSITIVE CONTROL 4-NQ(0.03 ug/ml)	17.0	146.0	73.0	116.44

COL = Colonies

PROTOCOL AMENDMENT 92G-1271

SPONSOR: ManTech Environmental Technology, Inc.
Toxic Hazards Research Unit
P.O. Box 31009
Dayton, OH 45437-0009

Performing Laboratory: Toxikon Corporation
225 Wildwood Avenue
Woburn, MA 01801

TEST SUBSTANCE:

Test Substance: 1, 3, 3 - Trinitroazetidine (TNAZ)

Lot/Batch #: Not Supplied

CAS/Code #: 97645-24-4

AMENDMENTS

Amendment 1:
Section 2.1 of the protocol states that the Sponsor's address would be:

ManTech Environmental Technology, Inc.
Toxic Hazards Research Unit
P.O. Box 31008
Dayton, OH 45431-0009

Per Sponsor's request, the address has been corrected to:

ManTech Environmental Technology, Inc.
Toxic Hazards Research Unit
P.O. Box 31009
Dayton, OH 45437-0009

Amendment 2:
The protocol does not include separate sections for Evaluation Criteria or the Range Finding Assay. These sections have been introduced in the report for ease of readability.

Amendment 3:
Section 8.2 of the protocol states the cells would be maintained in Eagle's MEM. During the assay the cells were maintained in Ham's F-12 medium. This is the media most recommended for culturing of this type of cells.

Amendment 4:
The protocol states that the Range Finding Assay would be performed without Activation, during the present study this assay was performed with and without (S9) activation in order to have a better understanding of the cytoxicity of the test substance.

Amendment 5:
Section 8.4 of the protocol states that 2 x 10^6 cells in 150 mm dishes would be exposed to the test substance. During the assay, 1 x 10^6 cells in 75 cm^2 flasks were used instead.

Amendment 6:
Section 8.4 of the protocol indicates that the exposure period of the cells to the test substance would be two hours. During the assay, the exposure period for the Range Finding study was five hours and 16 hours for the Activated Assay and Non-activated Assay, respectively.

Amendment 7:
Section 8.4 of the protocol states that three 60 mm dishes containing 200 cells each would be started concurrently. During the activated and non-activated Mutagenicity Assays, three 100 mm dishes containing 200 cells each were prepared per treatment (negative, solvent, positive and test substance articles). These dishes were utilized for the Parallel Cloning Efficiency Assay.

Amendment 8:
Section 9.0 of the protocol states that the concentrations tested should include a maximum dose of 5.0 mg/mL with two-fold dilutions. During the Range Finding Assay, the maximum concentration dose utilized was 5.0 mg/mL. The next concentration assayed was 2.0 mg/mL. All subsequent concentrations assayed (1.0, 0.5, 0.25, 0.125, 0.062, 0.031, 0.016 and 0.008 mg/mL) were two-fold dilutions thereof.

The Study Director assures that the above mentioned deviations do not alter the final outcome of the study.

SIGNATURES OF THE AUTHORIZED PERSONNEL:

Inder J. Paika, Ph.D.
Study Director

12/09/93
Date

Sponsor

12/12/93
Date

U. S. Government Printing Office 1996 750-071/00161