Institute Report No. 252

Mutagenic Potential of Nitroguanidine in the Mouse Lymphoma Forward Mutation Assay

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and
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Genetic and Cellular Toxicology Branch
Division of Toxicology

December 1987

Toxicology Series: 159

LETTERMAN ARMY INSTITUTE OF RESEARCH
PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

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SECURITY CLASSIFICATION OF THIS PAGE

AD-A190926

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30, 1

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) Institute Report No. 252	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Division of Toxicology 6b. OFFICE SYMBOL (If applicable) SGRD-UL-TO	7a. NAME OF MONITORING ORGANIZATION USA Biomedical Research and Development Laboratory
6c. ADDRESS (City, State, and ZIP Code) Letterman Army Institute of Research San Francisco, Ca 94129-6800	7b. ADDRESS (City, State, and ZIP Code) Frederick, MD 21701-5010
8a. NAME OF FUNDING/SPONSORING ORGANIZATION USA Medical Research and Development Cmd. 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 3E16270A835 / PROJECT NO. 835 / TASK NO. AB / WORK UNIT ACCESSION NO. DA30391

11. TITLE (Include Security Classification)
Mutagenic Potential of Nitroguanidine (TP036) in the Mouse Lymphoma Forward Mutation Assay

12. PERSONAL AUTHOR(S)
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13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 11Mar85 TO 160ct85		

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Mutagenicity, Genetic Toxicology, Mouse Lymphoma Assay, Mutagenesis, In vitro, Nitroguanidine; teratology; toxic tolerances

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

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Keywords:
milligram/milliliter

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Edwin S. Beatrice	22b. TELEPHONE (Include Area Code) 415-561-3600 / 22c. OFFICE SYMBOL SGRD-UL-Z

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

ABSTRACT

The mutagenic potential of nitroguanidine (TP036) was assessed in the mouse lymphoma thymidine kinase forward mutation assay both with and without metabolic activation by rat liver S-9. In the initial range-finding assay, cells were exposed to test compound concentrations ranging from 4 mg/ml to 0.01 mg/ml. The confirmatory assay employed doses from 4 mg/ml to 1 mg/ml. Nitroguanidine did not induce a statistically significant increase in the mutant frequency in either of the two assays. These results indicate that nitroguanidine was not mutagenic under the conditions of this study.

Key Words: Mutagenicity, Genetic Toxicology, Mouse Lymphoma Assay, Nitroguanidine

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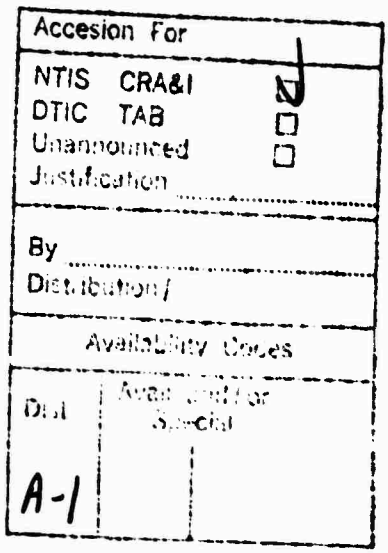

Title on cover is correct

PREFACE

TYPE REPORT: Mouse Lymphoma GLP Study Report

TESTING FACILITY: US Army Medical Research and Development Command
Letterman Army Institute of Research
Presidio of San Francisco, CA 94129-6800

SPONSOR: US Army Medical Research and Development Command
US Army Biomedical Research and Development Laboratory
Frederick, MD 21701-5010
Project Officer: Gunda Reddy, PhD

PROJECT/WORK UNIT/APC: #3E16270A835/180/TLB0

GLP STUDY NUMBER: 85035

STUDY DIRECTOR: MAJ Don W. Korte, Jr., PhD, MS

PRINCIPAL INVESTIGATOR: MAJ John W. Harbell, PhD, MS

REPORT AND DATA MANAGEMENT: A copy of the final report, retired SOPs, study protocol, retired stability and purity data on the test compound, and an aliquot of the test compound will be retained in the LAIR Archives.

TEST SUBSTANCE: Nitroguanidine CAS # 556-88-7

OBJECTIVE: The objective of this study was to determine the mutagenic potential of Nitroguanidine (TP036) by using the Mouse Lymphoma Forward Mutation Assay

ACKNOWLEDGMENTS

SGT Steven K. Sano and John Dacey provided research assistance during this study.

SIGNATURES OF PRINCIPAL SCIENTISTS AND MANAGERS INVOLVED IN THE STUDY

We, the undersigned, declare that GLP study number 85035 was performed under our supervision, according to the procedures described herein, and that this report is an accurate record of the results obtained.

Don W. Korte, Jr. 2DEC87

DON W. KORTE, JR. PhD / Date
MAJ, MS
Study Director

6 Aug 87

JOHN W. HARBELL, PhD / Date
MAJ, MS
Principal Investigator

4 Nov 87

CONRAD R. WHEELER, PhD / Date
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REPLY TO
ATTENTION OF:

SGRD-ULZ-QA 19 Nov 87
MEMORANDUM FOR RECORD

SUBJECT: Report of GLP Compliance for Study 85Ø35

1. I hereby certify that the protocol for GLP Study 85Ø35 was reviewed on 11 March 1985 and the study inspected 2 April 1985. The phase inspected was exposure of cells to compound.

2. The report entitled "Mutagenic Potential of Nitroguanidine (TPO36) in the Mouse Lymphoma Forward Mutation Assay," Toxicology Series 159, and the raw data were audited on 7 July 1987 and 5 November 1987.

Carolyn M. Lewis

CAROLYN M. LEWIS
C, Quality Assurance

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Mutagenic Potential of Nitroguanidine (TP036) in the Mouse Lymphoma Forward Mutation Assay -- Harbell and Korte

Nitroguanidine, a primary component of US Army triple-base propellants, is now produced in a Government-owned contractor-operated ammunition plant. The US Army Biomedical Research and Development Laboratory (USABRDL), as part of its mission to evaluate the environmental and health hazards of military-unique propellants generated by US Army munitions manufacturing facilities, conducted a review of the nitroguanidine database and identified significant gaps in the toxicity data (1). The Division of Toxicology, LAIR, was tasked by USABRDL to develop a genetic and mammalian toxicity profile for nitroguanidine, related intermediates/by-products of its manufacture, and its environmental degradation products.

Objective of the Study

The objective of this study was to determine the mutagenic potential of nitroguanidine (TP036) by using the Mouse Lymphoma Forward Mutation Assay.

MATERIALS AND METHODS

Nitroguanidine was evaluated for cytotoxicity and mutagenicity according to LAIR SOP, OP-STX-71 (2).

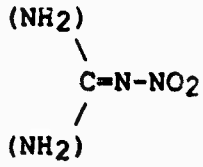
Test Compound

Chemical name: Nitroguanidine

LAIR Code name: TP036

Chemical Abstracts Service Registry No.: 556-88-7

Structural formula:



Empirical formula: $CH_4N_4O_2$

Storage: Nitroguanidine was obtained from Hercules Aerospace Division, Sunflower Army Ammunition Plant, DeSoto, Kansas (lot no. SOW84k101 A001 for the initial and SOW83H001-004 for the confirmatory assay) and was assigned the LAIR

Code number TP036. The test compound was stored at room temperature in a bunker on the Presidio of San Francisco. Small amounts of test compound were stored at room temperature in the laboratory just before use.

Chemical Properties/Analysis: Data characterizing the chemical composition and purity of the test material were prepared by the Division of Toxicology, LAIR, Presidio of San Francisco, CA (Appendix A).

Chemical Preparation

Nitroguanidine was dissolved directly in Fisher's Medium without serum with a final concentration of 4 mg/ml. This concentration is close to saturation for nitroguanidine in an aqueous medium (1).

Positive Controls

Ethyl methanesulfonate (EMS) (Sigma lot no. 83F-0279), added directly to the culture medium so as to provide a final concentration of 0.32 mg/ml, was used as the positive control for the assays conducted without metabolic activation. A stock solution of 2-acetamide fluorene (2AAF) (Sigma lot no. 113F-3679) in DMSO (Sigma lot no. 113F-0450) of 50 mg/ml (first assay) or 5 mg/ml (confirmatory assay) was prepared. One hundred microliters of this stock were used (0.5 or 0.05 mg/ml final concentration, respectively, in the two assays) as the positive control for assays conducted with metabolic activation. The final DMSO concentration of the 2AAF-treated cultures did not exceed 1%. Both positive controls were prepared fresh on the day of assay.

Cells

Mouse lymphoma cells L5178Y 3.7.2C $TK^{+/-}$ were provided by Dr. Donald Clive, PhD, Burroughs Wellcome Co, Research Triangle Park, NC 27709. These cells were maintained in antibiotic-free Fisher's Medium for Leukemic Cells of Mice (Fisher's Medium) supplemented with 10% horse serum. Six days before each assay began, the cell population was cleared of spontaneous thymidine kinase negative mutants by methotrexate treatment (2) and screened for mycoplasma and other contaminants by using the 3T6 co-culture technique (3). No nonnuclear DNA was detected after four days of co-culture, and thus the cell line was presumed to be uncontaminated.

Medium

Powdered Fisher's Medium (basic) was purchased from Sigma Chemical Co (lot no. 113F-4710-1) and prepared in 10 mM HEPES buffered glass distilled water (pH 7.3). The medium

was immediately filter sterilized. The sterile medium was supplemented with l-glutamine (2 mM) and sodium pyruvate (1 mM). Sterile horse serum (lot no. 310437) was obtained from Sterile Systems Inc, Logan, Utah, and was heat inactivated (56°C for 30 minutes) before use. Fisher's Medium was supplemented with horse serum at 5%, 10%, or 20% (volume/volume) final concentration. These were designated F_{5P}, F_{10P}, and F_{20P}, respectively, after the standard notation of Clive (4).

Metabolic Activation System

The metabolic activation system was composed of Aroclor-induced rat liver 9000 g supernatant fraction (S-9) and an NADPH-regenerating system provided by the cofactor mixture. Cofactor mixture, consisting of 2 mg/ml of NADP (Sigma lot no. 123F-7095 and 100F-7225) and 11.25 mg/ml of sodium isocitrate (Sigma lot no 64F-3825), was prepared in Fisher's Medium without serum. In order to achieve the desired final nitroguanidine concentrations, cofactor mixture containing nitroguanidine was also prepared. This solution was prepared separately and mixed with normal cofactor mixture to obtain the desired concentration. These solutions were prepared immediately before use. When metabolic activation was used, 3 ml of cofactor solution were combined with 6 ml of cell suspension containing the treatment compound. Then 1 ml of S-9 was added to each group. Litton Aroclor-induced rat liver S-9 lot no. (RDK120) was used for each assay. Vials were thawed immediately before use.

Assay Format

Dosing: Stock cultures of L5178Y 3.7.2C cells were prepared for use by clearing spontaneous mutants and checking for contamination (see "Cells" above). Only cleared and noncontaminated cell populations were used for these assays. L5178Y cells were counted with a Coulter Counter model ZM (Coulter Electronic Inc, Hialeah, Florida) and resuspended in Fisher's Medium with 5% horse serum (F_{5P}) at a concentration of 10^6 cells/ml. After one hour, 6 ml of the cell suspension were pipetted into each culture tube. The cells were then centrifuged down and the supernatant medium drawn off. The cells were then resuspended in F_{0P} containing the desired concentration of nitroguanidine. For groups treated without metabolic activation, 9.7 ml were added, while those with metabolic activation received 5.7 ml. Three hundred microliters of horse serum were added to bring the serum concentration to 3%. Positive controls were added (see Tables 1 and 3 for concentrations). Negative controls were prepared for both the metabolic activation series and the nonactivation series. The groups of the metabolic activation series received 3 ml of cofactor mixture and 1 ml of freshly thawed S-9 suspension. The cofactor mixture also contained

nitroguanidine in some cases to achieve the desired final compound concentration. The low serum (3%) concentration was intended to reduce the possible interaction (and inactivation) of test compounds with the serum proteins (4).

These cultures were maintained at 37°C on a roller drum for 4 hours, washed twice with Fisher's Medium containing 10% horse serum (F_{10P}), resuspended in 20 ml of F_{10P}, and returned to the roller drum. Ten percent serum in the medium provided for rapid growth in suspension culture.

Culturing: Approximately 24 hours after the cultures were first exposed, a sample of each culture was trypsin-treated for 10 minutes to produce a single cell suspension for counting. This suspension was then diluted to the appropriate concentration range and counted (average of three counts). The remaining cells from each culture were then diluted to 3×10^5 cells/ml in 20 ml of F_{10P} and returned to the roller drum. After approximately 48 hours, an aliquot from each culture was again counted. All cultures to be cloned at this point were diluted to 3×10^5 cells/ml in Fisher's Medium with 20% horse serum (F_{20P}). Twenty percent serum was used during cloning to enhance the absolute cloning efficiency.

Cloning

Nonselective: Soft agar cloning was used to determine the percentage of viable cells (viable count) and thymidine kinase negative mutants (mutant count) in each control and treated culture. To determine the percentage of viable cells, a portion of each freshly diluted culture (3×10^5 cells/ml) was further diluted to 600 cells/ml in F_{20P}. One milliliter of this suspension was diluted in 105 ml of F_{20P} containing 0.4% agar (Sigma lot no. 123F-0293) at 37°C. After vigorous mixing, this suspension of 5.7 cells/ml was dispensed into three 100 mm petri dishes (33 ml/dish). The extra 5 ml were provided to compensate for medium that foamed or adhered to the sides and thus could not readily be dispensed into the petri plates. The agar was allowed to harden at room temperature in the laminar flow hood (about 10 minutes).

Selective: To determine the percentage of thymidine kinase negative mutants, a similar but selective cloning procedure was performed. Ten milliliters of the 3×10^5 cells/ml suspension were diluted with 95 ml of F_{20P} with 0.4% agar (final concentration) which contained 1 µg/ml of trifluorothymidine (TFT) (Sigma lot no. 94F-0351). TFT was used to arrest the growth of all cells that contained thymidine kinase. After mixing, 33 ml of this 2.86×10^4

cells/ml suspension were placed into each of three 100-mm petri dishes.

After hardening, both the mutant and viable count dishes were incubated for 11 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The number of colonies on each plate was then determined by using a Biotran II Automated Colony Counter (New Brunswick Scientific Co, Edison, New Jersey) with the size setting on zero.

Assay Acceptance Criteria

The following criteria are required, according to Brusick (5), for a valid assay.

Cloning Efficiency: The minimum negative control viable count cloning efficiency (mean count/mean number of cells plated) should be 70% or greater for the negative control cultures not treated with the activation mixture. A 100% cloning efficiency may be exceeded due to the delay between cell counts and dilutions during which the cells continue to divide. However, since the dilutions for the selective and nonselective cloning suspensions are made at the same time, the ratio between the two should not change even with the delay.

Cell Replication: The cells in the negative control cultures (without S-9 activation) should undergo at least a 15-fold increase in cell number over the two days of suspension culture. Negative control cultures treated with the metabolic activation mixture characteristically show slightly less growth and therefore may not undergo the 15-fold increase.

Positive Control Responses: A statistically significant mutagenic response (see below) must be induced by the positive controls. Failure to induce a mutagenic response by the positive activation control (2AAF) would invalidate only the activation series provided that the EMS (nonactivation control) induced an appropriate response.

Treatment Concentration: In the absence of strong mutagenic activity (e.g. possible nonmutagen), cells should be exposed to the test compound concentration to the limits of solubility (usually up to 5 mg/ml) or to the point where suspension growth is reduced by cytotoxicity to 10% of controls. In these assays, the solubility of nitroguanidine limited the upper dose concentration.

DATA EVALUATION

Cell Replication and Survival

The combined activity of cell replication and survival for each control and treatment group is the product of suspension growth during the two days after exposure and the viable count cloning efficiency (2). Absolute suspension growth (ASG) is measured as a fold increase (usually 15- to 20-fold) in the control cultures over the 48-hour period. For example, the EMS treated culture in the initial assay (Table 1) grew from 3×10^5 cells/ml to 1.059×10^6 cells/ml, a 3.53-fold increase, during the first 24 hours. The culture was then diluted to 3×10^5 cells/ml and allowed to continue growing for another 24 hours. At that point, the cell concentration was 1.320×10^6 cells/ml, a 4.4-fold increase. Thus the total growth was $3.53 \times 4.4 = 15.4$-fold increase over two days. Relative suspension growth (RSG) compares the treated groups against the appropriate negative controls. Absolute cloning efficiency (ACE) is the observed number of viable count clones compared to the expected number of 189 per plate (5.7 cells/ml x 33 ml = 189 cells). Relative cloning efficiency (RCE) compares the treated groups with their respective negative controls. Thus, absolute cell survival (ACS) is the product of the suspension growth and absolute cloning efficiency while the relative cell survival (RCS) is the treated ACS compared to the control ACS.

Mutant Frequency

The mutant frequency (MF) is the mean selective plate count divided by the mean nonselective plate count multiplied by the dilution factor (2×10^{-4}). The dilution factor is derived from the ratio of the number of cells plated per ml in the nonselective plates divided by the number of cells plated per ml in the selective plates ($5.7/[2.86 \times 10^4] = 2 \times 10^{-4}$). The induced mutant frequency (IMF) in the treated groups is the observed mutant frequency less the spontaneous mutant frequency of the negative controls. Again, nonactivation and activation series are compared separately. The variance and standard error (SE) of the mutant frequency are calculated by using the mean selective and nonselective plate counts and with an assumed dilution variance of 10% (2,4).

Criteria for a Positive or Negative Response

An individual treatment concentration is considered positive if the assay is valid, the cell survival is at least 10% of controls, and the induced mutant frequency is at least three times ($p < 0.01$) the standard error of that mutant frequency. A test compound is considered mutagenic if it

yields a correlated positive dose response through several (usually three) treatment concentrations (4-6).

A compound is considered nonmutagenic in this system if a valid assay does not yield a positive response and the limits of compound solubility (up to 5 mg/ml) or 90% reduction in cell survival has been reached. Normally, a determination of mutagenic potential is not made on the basis of only one assay. Both positive and negative assays are confirmed.

Deviations from the Protocol/SOP

In the initial assay, the concentration of 2AAF was 0.5 mg/ml instead of 0.05 mg/ml, which accounts for the extreme toxicity observed. This toxicity and the mutant frequency does, however, demonstrate that the activation system was functioning.

RESULTS

Nitroguanidine was assayed two times, one initial assay and one confirmatory assay. The compound exposure concentration and resulting data are presented in Tables 1 through 4, while the raw data are contained in Appendix B.

The initial assay (Tables 1 and 2) covered a dose range of 4 to 0.01 mg/ml. Absolute suspension growth and absolute cloning efficiency for the negative controls were within prescribed limits. The apparent greater-than-100% absolute cloning efficiency was the result of the time delay between cell counting and dilution of these samples. Both positive controls produced a statistically significant mutagenic response as required for a valid assay. The test compound, nitroguanidine, did not produce significant mutagenic activity at any concentration tested.

The confirmatory assay (Tables 3 and 4) covered a dose range of 4 to 1 mg/ml. This assay was also valid in terms of cell growth, cloning efficiency, and positive control-induced mutagenic activity. Again no statistically significant mutagenic activity was induced by nitroguanidine.

Table 1

Cell Survival Data from the Initial Assay

Treatment			S-9	Cell Count Day 1 (x 10^3/ml)	Cell Count Day 2 (x 10^3/ml)	ASG[a]	RSG[b]	VC[c]	ACE[d]	RCE[e]	RCS[f]
Control[g]			-	1032	1347	15.30	100	250	132	100	100
EMS	0.32	mg/ml	-	1059	1320	15.40	101	217	115	87	88
TP036	4.0	mg/ml	-	891	1254	12.60	82	276	146	110	90
TP036	2.0	mg/ml	-	848	1484	13.72	90	228	120	91	81
TP036	1.0	mg/ml	-	949	1337	14.40	94	245	129	98	92
TP036	0.5	mg/ml	-	994	1333	14.52	95	246	130	98	92
TP036	0.1	mg/ml	-	1029	1359	15.30	100	259	137	104	104
TP036	0.05	mg/ml	-	1155	1271	16.38	107	270	143	108	116
TP036	0.01	mg/ml	-	1112	1257	15.54	102	246	130	98	100
Control			+	880	1394	13.34	100	235	124	100	100
2AAF	0.5	mg/ml	+	77	136	0.45	3	28	15	12	0
TP036	3.66	mg/ml	+	756	1360	11.25	84	257	136	109	92
TP036	2.0	mg/ml	+	819	1299	11.61	87	268	142	114	100
TP036	1.0	mg/ml	+	867	1221	11.89	88	275	146	117	104
TP036	0.5	mg/ml	+	808	1394	12.42	93	250	132	106	99
TP036	0.1	mg/ml	+	906	1326	13.20	98	249	132	106	104
TP036	0.05	mg/ml	+	1002	1147	12.54	94	232	123	99	93
TP036	0.01	mg/ml	+	929	1127	11.78	86	307	162	131	113

a Absolute suspension growth = Total fold increase in suspension culture
b Relative suspension growth = treated/control x 100
c Viable Clone Count = mean of 3 plates
d Absolute Cloning Efficiency = viable clone count/189 x 100
e Relative Cloning Efficiency = treated/control x 100
f Relative Cell Survival = absolute cell survival treated/absolute cell survival control x 100
g Mean values from both negative controls

Table 2

Mutagenesis Data from the Initial Assay

Treatment			S-9	RCS[a]	MF[b] ($x10^{-6}$)	IMF[c] ($x10^{-6}$)	SE[d] ($x10^{-6}$)	IMF/SE[e]
Control[f]			-	100	23.9			
EMS	0.32	mg/ml	-	88	50.1	26.2	6.66	3.93
TP036	4.0	mg/ml	-	90	15.9	-7.9		
TP036	2.0	mg/ml	-	81	14.3	-9.5		
TP036	1.0	mg/ml	-	92	17.7	-6.2		
TP036	0.5	mg/ml	-	92	14.1	-9.8		
TP036	0.1	mg/ml	-	104	11.6	-12.3		
TP036	0.05	mg/ml	-	116	20.5	-3.4		
TP036	0.01	mg/ml	-	100	24.7	0.8	3.69	0.21
Control			+	100	46.0	0.0		
2AAF	0.5	mg/ml	+	0	178.6	132.6	33.52	3.96
TP036	3.66	mg/ml	+	92	34.0	-12.0		
TP036	2.0	mg/ml	+	100	30.8	-15.2		
TP036	1.0	mg/ml	+	104	30.0	-15.9		
TP036	0.5	mg/ml	+	99	38.5	-7.6		
TP036	0.1	mg/ml	+	104	24.9	-21.1		
TP036	0.05	mg/ml	+	93	33.3	-12.7		
TP036	0.01	mg/ml	+	113	41.1	-5.0		

a Relative Cell Survival = absolute cell survival treatment/absolute cell survival controls x 100
b Mutant Frequency = mutant clone count/viable clone count x dilution factor
c Induced Mutant Frequency = treated mutant frequency - control mutant frequency
d Standard Error is calculated only when the IMF >0.
e Ratio of the IMF to SE > 3 indicates a positive treatment response
f Mean values from both negative controls

Table 3

Cell Survival Data from the Confirmatory Assay

Treatment			S-9	Cell Count Day 1 ($\times 10^3$/ml)	Cell Count Day 2 ($\times 10^3$/ml)	ASG[a]	RSG[b]	VC[c]	ACE[d]	RCE[e]	RCS[f]
Control[g]			-	1178	1662	21.65	100	169	89	100	100
EMS	0.32	mg/ml	-	705	1610	12.96	60	160	84	95	57
TP036	4.0	mg/ml	-	841	1576	14.84	69	177	94	105	72
TP036	2.0	mg/ml	-	1028	1657	18.70	86	163	86	97	83
TP036	1.0	mg/ml	-	1074	1649	19.80	91	172	91	102	93
Control[g]			+	793	1590	14.05	100	173	92	100	100
2AAF	0.05	mg/ml	+	589	1492	10.00	71	169	89	98	70
TP036	3.84	mg/ml	+	630	1552	10.92	78	157	83	91	71
TP036	2.0	mg/ml	+	689	1602	12.19	87	130	69	75	65
TP036	1.0	mg/ml	+	654	1632	11.88	85	157	83	91	77

[a] Absolute suspension growth = Total fold increase in suspension culture

[b] Relative suspension growth = treated/control x 100

[c] Viable Clone Count = mean of 3 plates

[d] Absolute Cloning Efficiency = viable clone count/189 x 100

[e] Relative Cloning Efficiency = treated/control x 100

[f] Relative Cell Survival = absolute cell survival treated/absolute cell survival control x 100

[g] Mean values from both negative controls

Table 4

Mutagenesis Data from the Confirmatory Assay

Treatment			S-9	RCS[a]	MF[b] ($x10^{-6}$)	IMF[c] ($10x^{-6}$)	SE[d] ($x10^{-6}$)	IMF/SE[e]
Control[f]			-	100	60.8			
EMS	0.32	mg/ml	-	57	395.4	334.6	45.33	7.38
TP036	4.0	mg/ml	-	72	42.1	-18.7		
TP036	2.0	mg/ml	-	83	44.3	-16.5		
TP036	1.0	mg/ml	-	93	67.3	6.5	8.95	0.73
Control[f]			+	100	59.4			
2AAF	0.05	mg/ml	+	70	118.2	58.8	14.63	4.02
TP036	3.84	mg/ml	+	71	58.7	-0.7		
TP036	2.0	mg/ml	+	65	63.1	3.7	9.07	0.41
TP036	1.0	mg/ml	+	77	65.8	6.4	8.97	0.71

[a] Relative Cell Survival = absolute cell survival treatment/absolute cell survival controls x 100

[b] Mutant Frequency = mutant clone count/viable clone count x dilution factor

[c] Induced Mutant Frequency = treated mutant frequency - control mutant frequency

[d] Standard Error of the mutant frequency calculated only when the IMF >0.

[e] Ratio of the IMF to SE > 3 indicates a positive treatment response

[f] Mean values from both negative controls

DISCUSSION

The mutagenicity of nitroguanidine was evaluated in a mouse lymphoma forward mutation study consisting of an initial and a confirmatory assay. The results of this study indicated that nitroguanidine was not mutagenic in the mouse lymphoma test system. Both assays met the acceptance criteria for cloning efficiency, cell replication, positive control responses, and maximum test compound concentrations. The cloning efficiency of the negative controls without metabolic activation was greater than 70%. The total cell replication in suspension culture of these same controls was greater than 15 fold for the two days of culture. Both EMS and 2AAF induced statistically significant mutagenic responses. Spontaneous mutation rates were well within published values of 25-115 x 10^{-6} without activation and 25-135 x 10^{-6} with activation (3). The highest concentration of nitroguanidine tested was 4 mg/ml, which is nearly the limit of solubility for this compound.

Metabolic activation with rat liver S-9 did not significantly alter either the cytotoxicity or mutagenicity of nitroguanidine. Even at the highest doses, cytotoxicity was mild, with relative cell survivals of 70% or greater in all cases.

Nitroguanidine has been reported to cause significant chromosome damage in Chinese hamster fibroblasts (7). For this study, Ishidata and Odashima used a dose of 4 mg/ml for 24 hours and found 26% of the metaphases to have chromosomal aberrations, principally gaps, breaks and translocations. Our data from the CHO Sister Chromatid Exchange Assay (SCE) showed nitroguanidine to be very toxic at that dose over a 24-hour exposure (8). Most of the metaphases detected were from first division cells, and even those that showed some differential staining had not gone through two complete replication cycles in the BrdU-containing medium. Examination of the metaphases from this dose group did not reveal the chromosomal aberrations reported previously. Furthermore, lower concentrations that were less cytotoxic did not induce an increase in SCEs. Thus our data are at odds with those of Ishidata and Odashima (7) though the test systems were by no means identical (e.g. different cell lines, presence of BrdU, and different chromosome preparation).

Nitroguanidine was also negative in the Ames Salmonella/ Mammalian Microsome Assay (8).

CONCLUSION

The mutagenic potential of nitroguanidine was evaluated in the mouse lymphoma thymidine kinase forward mutation assay. Nitroguanidine was not mutagenic under conditions of this study.

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3. Detection of mycoplasma in cell cultures. LAIR Standard Operating Procedure OP-STX-92. Presidio of San Francisco, California: Letterman Army Institute of Research, 1 March 1985.

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Glossary

Absolute Cell Survival: The product of the population's total fold increase in growth in suspension culture multiplied by the absolute cloning efficiency under nonselective conditions.

Absolute Cloning Efficiency: The number of colonies counted on the nonselective plates divided by the number of cells originally plated x 100.

Fold Increase in Suspension Growth: The quotient of the cell concentration at the end of the growth period divided by the starting cell concentration.

Induced Mutant Frequency: The mutant frequency of the treated population less the mutant frequency of the control population.

Mutant Frequency: The ratio of the number of colonies on the selective plates divided by the number of colonies on the nonselective plates multiplied by the dilution factor. The dilution factor is the ratio of the number of cells plated on the nonselective plates divided by the number plated on the selective plates.

Relative Cell Survival: The absolute cell survival of the treated population divided by the absolute cell survival of the negative control population x 100.

Relative Cloning Efficiency: The absolute cloning efficiency of the treated population divided by the absolute cloning efficiency of the control population x 100.

Relative Suspension Growth: Total fold increase in cell number during suspension growth of the treated population divided by the total fold increase in cell number during suspension growth of the negative control population x 100.

Total Fold Increase in Suspension Growth: The product of the fold increase for the first day times the fold increase for the second day.

APPENDICES

CHEMICAL DATA

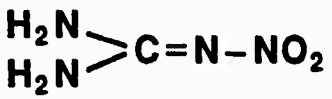
Chemical name: Nitroguanidine (NGu)

Other listed names: Guanidine, Nitro; alpha-Nitroguanidine; beta-Nitroguanidine

Chemical name: Nitroguanidine (NGu)

LAIR Code: TP036A

Structural formula:



Molecular formula: $CH_4N_4O_2$

Molecular weight: 104.1

pH range of dosing suspensions: 6.7 - 7.4[1]

Physical state: White Powder

Melting point: 232°[2]

Source: Hercules Aerospace Division
Sunflower Ammunition Plant
DeSoto, Kansas

Lot No. SOW84K010-A-001

1. Wheeler CR. Nitrocellulose-Nitroguanidine Projects Laboratory Notebook #85-12-022, p 26. Letterman Army Institute of Research, Presidio of San Francisco, CA.

2. Fedoroff BT, Sheffield OE. Encyclopedia of explosives and related items. Vol V. Dover, New Jersey: Picatinny Arsenal 1975: G154.

Nitroguanidine Chemical Data (continued)

Analytical data/purity: The major peaks in the infrared spectrum of the compound were observed at 3450, 3396, 3342, 3278, 3201, 1666, 1634, 1525, 1404, 1314, 1151, 1045, 782 cm^{-1} (3). The spectrum obtained for the test compound in our laboratory was identical to the Sadtler standard spectrum for nitroguanidine (4). HPLC showed only one peak (retention time 4.9 min) (5). The conditions employed were as follows: column, Brownlee RP-18 (4.6 x 250 mm); solvent 10% methanol/90% water, flow rate 0.7 ml/min; oven temperature, 50°C; monitoring wavelength, 265 nm.

3. Wheeler CR. Nitrocellulose-Nitroguanidine Projects. Laboratory Notebook #85-12-022, p. 22-23. Letterman Army Institute of Research, Presidio of San Francisco, CA.

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Raw Data from the Initial Assay without Activation
2 April 1985

Treatment			S-9	Cell Counts Day 1 (x 10^3/ml)	Cell Counts Day 2 (x 10^3/ml)	Viable Clone Counts	Selective Clone Counts
Control			-	961	1207	261	24
				955	1196	253	23
				1010	1152	252	20
EMS	0.32	mg/ml	-	1078	1356	223	55
				1081	1308	211	41
				1017	1297	217	67
TP036	4.0	mg/ml	-	925	1321	291	23
				859	1232	263	20
				889	1209	274	23
TP036	2.0	mg/ml	-	845	1509	236	14
				852	1472	200	16
				847	1472	247	19
TP036	1.0	mg/ml	-	942	1341	252	13
				945	1305	252	27
				961	1364	230	25
TP036	0.5	mg/ml	-	953	1364	240	22
				1015	1345	260	15
				1013	1290	238	15
TP036	0.1	mg/ml		1015	1373	250	16
				1040	1394	278	11
				1032	1310	248	18
TP036	0.05	mg/ml	-	1149	1286	300	22
				1158	1277	248	30
				1159	1250	263	31
TP036	0.01	mg/ml	-	1130	1277	243	26
				1086	1232	234	31
				1120	1263	261	34
Neg			-	1077	1526	242	37
				1119	1493	252	36
				1022	1509	239	38

Raw Data from the Initial Assay with Activation
2 April 1985

Treatment			S-9	Cell Counts Day 1 (x 10^3/ml)	Cell Counts Day 2 (x 10^3/ml)	Viable Clone Counts	Selective Clone Counts
Control			+	898	1418	227	53
				886	1403	245	60
				857	1361	233	49
2AAF	0.5	mg/ml	+	65	158	37	23
				91	125	24	21
				74	125	23	31
TP036	3.66	mg/ml	+	755	1360	260	43
				700	1355	260	44
				813	1365	250	44
TP036	2.0	mg/ml	+	856	1357	285	40
				799	1297	254	44
				803	1244	266	40
TP036	1.0	mg/ml	+	869	1200	279	32
				875	1212	263	44
				858	1251	284	48
TP036	0.5	mg/ml	+	781	1348	236	51
				827	1418	258	47
				815	1417	255	46
TP036	0.1	mg/ml	+	901	1322	238	23
				916	1362	266	35
				901	1295	243	35
TP036	0.05	mg/ml	+	997	1168	197	38
				1000	1152	252	39
				1010	1120	247	39
TP036	0.01	mg/ml	+	951	1140	320	73
				940	1110	307	58
				896	1132	293	58

Raw Data from the Confirmatory Assay without Activation
3 October 1985

Treatment			S-9	Cell Counts Day 1 (x 10^3/ml)	Cell Counts Day 2 (x 10^3/ml)	Viable Clone Counts	Selective Clone Counts
Control			-	1135	1744	177	35
				1166	1745	159	44
				1146	1709	163	41
EMS	0.32	mg/ml	-	706	1597	144	294
				715	1594	149	320
				693	1640	186	333
TP036	4.0	mg/ml	-	837	1576	167	41
				865	1565	171	39
				820	1586	194	32
TP036	2.0	mg/ml	-	977	1674	157	25
				1075	1667	156	36
				1031	1629	179	48
TP036	1.0	mg/ml	-	1109	1695	162	60
				1024	1662	167	56
				1088	1590	188	58
Control			-	1226	1557	174	64
				1192	1591	172	58
				1202	1621	168	66

Raw Data from the Confirmatory Assay with Activation
3 October 1985

Treatment			S-9	Cell Counts Day 1 (x 10^3/ml)	Cell Counts Day 2 (x 10^3/ml)	Viable Clone Counts	Selective Clone Counts
Control			+	777	1566	186	57
				736	1546	159	52
				736	1455	166	48
EMS	0.05	mg/ml	+	605	1483	162	90
				576	1524	169	104
				585	1470	175	105
TP036	3.84	mg/ml	+	641	1524	159	54
				612	1582	165	38
				637	1549	146	46
TP036	2.0	mg/ml	+	719	1560	133	40
				650	1637	137	38
				698	1609	120	45
TP036	1.0	mg/ml	+	657	1675	164	58
				642	1612	160	45
				664	1610	147	52
Control			+	853	1714	162	43
				831	1668	175	56
				823	1593	189	57

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