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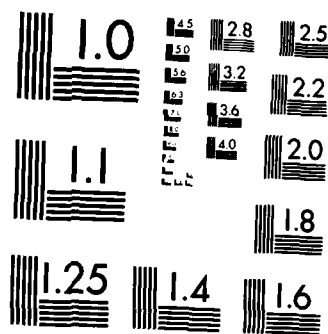
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INSTITUTE REPORT NO. 199

MUTAGENIC POTENTIAL OF GUANIDINE NITRATE

STEVEN K. SANO, BA, SP4
and
DON W. KORTE JR, PhD, MAJ MSC

TOXICOLOGY GROUP
DIVISION OF RESEARCH SUPPORT

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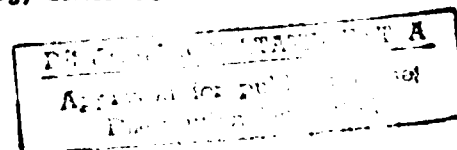
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MARCH 1985

Toxicology Series 79

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Mutagenic Potential of: Guanidine Nitrate--Sano and Korte
(Toxicology Series 75)

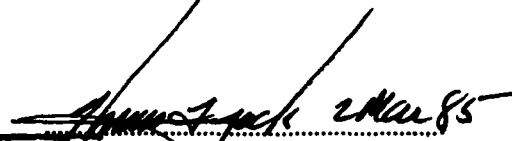
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ABSTRACT

The mutagenic potential of guanidine nitrate (TP030) was assessed by using the Ames Salmonella/Mammalian Microsome Mutagenicity Assay. Tester strains TA98, TA100, TA1535, TA1537, and TA1538 were exposed to doses ranging from 5 mg/plate to 0.0016 mg/plate. The test compound was not mutagenic under conditions of this assay.

Key Words: Mutagenicity, Toxicology, Ames Assay, Guanidine Nitrate

PREFACE

TYPE REPORT: Ames Assay 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 Medical Bioengineering Research and
Development Laboratory
Fort Detrick, Maryland 21701-5010
Project Officer: Jesse Barkley, MS

WORK UNIT: 3E162720A835 Nitrocellulose-Nitroguanidine
Projects; WU 180; APC: TL09

GLP STUDY NUMBER: 84020

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

PRINCIPAL INVESTIGATOR: SP4 Steven K. Sano, BA

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

TEST SUBSTANCE: Guanidine Nitrate

INCLUSIVE STUDY DATES: 12 - 31 March 1984

OBJECTIVE: The objective of this study was to determine the mutagenic
potential of guanidine nitrate (LAIR Code TP030).

ACKNOWLEDGMENTS

The authors wish to thank SP5 Thomas P. Kellner, BA; SP5 Lawrence Mullen, BS, and John Dacey for their assistance in performing the research. The authors would also like to thank Dr. Gunda Reddy, US Army Medical Research and Development Laboratory, for his technical and administrative assistance on this project.

SIGNATURES OF PRINCIPAL SCIENTISTS AND MANAGERS INVOLVED IN THE STUDY

We, the undersigned, declare that GLP Study 84020, 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. 18 DEC 84
DON W. KORTE, JR., Ph.D. / DATE
MAJ, MSC
Study Director

Steven K. Sano 18 Dec 1984
STEVEN K. SANO, B.A. / DATE
SP4, USA
Principal Investigator



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

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ATTENTION OF:

SGRD-ULZ-QA

10 Jul 84

MEMORANDUM FOR RECORD

SUBJECT: Report of GLP Compliance

I hereby certify that in relation to LAIR GLP study 84020 the following inspections were made:

16 Mar 84

28 Mar 84

The report and raw data for this study were audited on 10 Jul 84.

Routine inspections with no adverse findings are reported quarterly, thus these inspections are also included in the 11 Apr 84 report to Management and the Study Director.

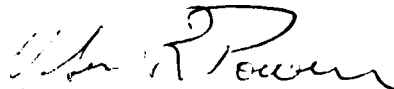

NELSON R. POWERS, Ph.D.
DAC
Chief, Quality Assurance Unit

TABLE OF CONTENTS

Abstract.....	1
Preface.....	iii
Acknowledgments.....	iv
Signatures of Principal Scientists.....	v
Report of Quality Assurance Unit.....	vi
Table of Contents.....	vii

BODY OF REPORT

INTRODUCTION

Objective of the Study.....	1
-----------------------------	---

METHODS

Test Compound.....	1
Test Solvent.....	2
Chemical Preparation.....	2
Test Strains.....	2
Test Format.....	2

RESULTS.....	4
--------------	---

DISCUSSION.....	11
-----------------	----

CONCLUSION.....	11
-----------------	----

RECOMMENDATION.....	11
---------------------	----

REFERENCES.....	12
-----------------	----

APPENDIX.....	13
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DISTRIBUTION LIST.....	16
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Mutagenic Potential of: Guanidine Nitrate--Sano and Korte

The Ames Salmonella/Mammalian Microsome Mutagenicity Assay is a short-term screening assay that utilizes histidine auxotrophic mutant strains of Salmonella typhimurium to detect those compounds which are potentially mutagenic in mammals. A mammalian microsomal enzyme system is incorporated in the assay to increase sensitivity by simulating in vivo metabolic activation of the test compound. The Ames assay is an inexpensive yet highly predictive and reliable assay for detecting mutagenic activity and thus carcinogenic potential (1).

Objective of the Study

The objective of this study was to determine the mutagenic potential of guanidine nitrate (LAIR Code TP030) by using the Ames Salmonella/Mammalian Microsome Mutagenic Assay.

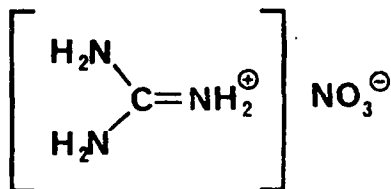
METHODS

Test Compound

Chemical name: Guanidine Nitrate

Chemical Abstract Service Registry No.: 506-93-4

Structural formula:



Empirical formula: $\text{CH}_5\text{N}_3\text{HNO}_3$

Storage: Five hundred grams of guanidine nitrate (Lot Number 123820) was received from Chemical Dynamics Corporation (South Plainfield, NJ) on 24 February 1984 and assigned the LAIR Code number TP030. The test compound was stored at room temperature (21°C) until use.

Chemical Properties/Analysis: Data characterizing the composition and stability of the test material are detailed in Appendix A.

Test Solvent

Positive control chemicals were dissolved in grade I dimethyl sulfoxide (lot 100F-0269) obtained from Sigma Chemical Co. (St Louis, MO).

Test compound was dissolved in sterile, deionized water obtained from a Polymetrics Model 200-3 Water Purifier (Sunnyvale, CA).

Chemical Preparation

Guanidine nitrate was stored at room temperature (21°C) until use. On the day before dosing, 300 mg of the test compound was measured into a sterile vial and again stored at room temperature. On the day of dosing, the 300 mg sample was dissolved in a 6 ml volume of autoclaved water from a polymetrics water filter system to achieve a 5% (w/v) solution. Aliquots of this solution were used to dose the test plates. The dosing procedure was completed within 20 minutes of dissolving the test compound.

Test Strains

Salmonella strains TA98, TA100, TA1535, TA1537, and TA1538, obtained directly from Dr. Bruce Ames, University of California, Berkeley, were used. These strains were maintained in our laboratory at -80°C. Quality controls were run concurrently with the test substance to establish the validity of their special features and to determine the spontaneous reversion rate. Descriptions of the mutants, their genetic markers, and the methods for strain validation is given in the LAIR SOP, OP-STX-1 (2).

Test Format

Guanidine nitrate was evaluated for mutagenic potential according to the methods of Ames et al (3). A detailed description of the methodology is given in LAIR SOP, OP-STX-1 (2).

Toxicity Tests

Toxicity tests were conducted to determine a sublethal concentration of the test substance. This toxicity level was found by using minimal glucose agar (MGA) plates, concentrations of guanidine nitrate ranging from 1.6×10^{-3} mg/plate to 5 mg/plate, and approximately 10^8 cells of TA100 per plate. Top agar containing trace amounts of histidine and biotin were placed on the plates. Strain verification was confirmed on the bacteria, along with a determination of the spontaneous reversion rate. After incubation, the growth on the plates was observed. Since none of the plates showed decreased macrocolony formation (below the level of the spontaneous reversion plates) or an observable reduction in the density of the background lawn, a maximum "limit" dose of 5 mg per plate was used in the mutagenicity assay.

Mutagenicity Assay

The test substance was evaluated over a 1000-fold range of concentrations, decreasing from the minimum toxic level (the maximum or limit dose) by a dilution factor of 5 both with and without 0.5 ml of the S-9 microsome fraction. The S-9 was purchased from Litton Bionetics (Kensington, MD). The optimal titer of this S-9, as determined by Litton Bionetics, was 0.75 mg protein/plate. After all the ingredients were added, the top agar was mixed, then overlaid on MGA plates. These plates contained 2% glucose and Vogel Bonner "E" Concentrate (4). The water used in this medium and in all reagents came from a polymetric system. Plates were incubated, upside down in the dark, at 37°C for 48 hours. Plates were prepared in triplicate and the average revertant counts were recorded. The average number of revertants at each dose level was compared to the average number of spontaneous revertants (negative control). No vehicle control was required as the test compound was diluted in water. The spontaneous reversion rate (with and without S-9) was monitored by averaging the counts from two determinations run simultaneously with the test compound. The spontaneous reversion rate was determined by inoculating a set of plates before and one set after the test compound assay, so that any change in spontaneous reversion rate during the dosing procedure would be detected. This spontaneous reversion rate was also compared with historical values for this laboratory and those cited in Ames et al (3). Concurrent sterility and strain verification controls were run. All reagents, test compounds, and media were checked for sterility by plating samples of each on MGA media and incubating them at 37°C with the test plates. The Salmonella strains were verified by a standard battery of tests.

The following tests were run to determine if:

- Lipopolysaccharide layer (LP) alteration causes growth inhibition in the presence of crystal violet.
- An ampicillin-resistant R factor has allowed growth in strains TA98 and TA100 in the presence of ampicillin impregnated disks.
- Absence of excision repair mechanism has inhibited growth in the presence of ultraviolet light.

Four known mutagens were tested as positive controls to confirm the responsiveness of the strains to the mutation process. These compounds, benzo [a] pyrene, 2-aminofluorene, aminoanthracene and N-methyl-n'-nitro-n-nitrosoguanidine, were obtained from Sigma Chemical Co (St Louis, MO). The test and mutagens compound were handled during this study in accordance with the standards published in NIH Guidelines for the Laboratory Use of Chemical Carcinogens (DHHS Publication No. (NIH) 81-2385, May 1981).

Data Interpretation

According to Ames et al (3), a compound is considered mutagenic if the following criteria are met:

- The test compound causes revertant colony counts greater than or equal to twice the spontaneous revertant rate.
- The test compound produces a correlated dose response relationship.

RESULTS

On 21 March 1984, the toxicity level determination was performed on guanidine nitrate (Table 1). For this experiment all sterility, strain verification, and negative controls were normal (Table 2). No toxicity was observed after exposure of the tester strain (TA100) to the highest dose used (5 mg/plate).

Normal results were obtained for all sterility, strain verification, and negative controls during the Ames Assay performed on 28-30 March 1984 (Tables 3-4). No revertant counts were obtained that exceeded double the spontaneous reversion rate following exposure of the bacterial strains to the test compound (Table 5).

TABLE 1

TOXICITY LEVEL DETERMINATION

Substance assayed: TP030 Guanidine Nitrate Substance dissolved in: H₂O
 Study Number: 84020 Date: 23 Mar 84 Performed by: Sano, Kellner

TA 100 REVERTANT PLATE COUNT

Test Compound Concentration	Plate #1	Plate #2	Plate #3	Average	Background Lawn (1)
5 mg/plate	87	93	100	93	NL
1 mg/plate	102	113	106	107	NL
0.2 mg/plate	86	111	124	107	NL
0.04 mg/plate	109	97	99	102	NL
0.008 mg/plate	129	110	120	120	NL
0.016 mg/plate	112	113	102	109	NL

(1) NG = No Growth ST = Slight Growth NL = Normal Lawn

TABLE 2

STRAIN VERIFICATION FOR TOXICITY LEVEL DETERMINATION

Strains	Histidine Requirement	Ampicillin Resistance	UV	Sensitivity to Crystal Violet	Sterility Control	Response (1)
100	NG	G	NG	NG (10nm)	NG	+
1537	NT	NT	NT	NT	NT	NT
WT	G	NT	G	NT	NA	+

STERILITY CONTROL FOR TOXICITY LEVEL DETERMINATION

His-Bio Mix Initial: NG End: NG MGA Plate: NG
 Top Agar Initial: NG End: NG
 Diluent: NG Nutrient Broth: NG
 Test Compound (a) TP030-NG (b) NA (c) NA (d) NA (e) NA

G = Growth NC = No Growth NT = Not Tested NA = Not Applicable WT = Wild Type

Spontaneous Revertants: TA 100 No S-9, (102,105,100)

(1) + = expected response - = unexpected response

Study Number: 84020 Date: 22 MAR 84 By: SANO, KELLNER

TABLE 3
STRAIN VERIFICATION CONTROL FOR ASSAY

Strains	Histidine Requirement	Ampicillin Resistance	UV	Sensitivity to Crystal Violet	Sterility Control		Response (1)
					start	end	
94	NG	G	NG	NG (10mm)	NG	NG	+
100	NG	G	NG	NG (13mm)	NG	NG	+
1535	NG	NT	NG	NG (15mm)	NG	NG	+
1537	NG	NG (24mm)	NC	NG (15mm)	NG	NG	+
1538	NG	NT	NG	NG (15mm)	NG	NG	+
WT	G	NT	G	NT	NT	NT	+

STERILITY CONTROL FOR ASSAY

His-Bio Mix Initial: NG End: NG Diluent: NG

Top Agar Initial: NG End: NG MCA Plate: NG

S-9 Mix Initial: NG End: NG Nutrient Broth: NG

Test Compound (a) TPD30-NG (b) (c) (d) (e) (f) (f)

G = Growth NG = No Growth NT = Not Tested NA = Not Applicable WT = Wild Type

Study Number: 84020 By: SANO, KELLNER (1) + = expected response

Date: 29 MAR 84 - = unexpected response

TABLE 4
POSITIVE AND NEGATIVE CONTROL TEST

Compd. *	Amount of Compd. Added	S-9 Added	(Revertants/Plate) Mean				Strain No. 1535	1537	1538
			98	100	100	1535			
AF	2 ug/plate	yes	(506,565,521) 531	(392,361,268) 340					(461,543,465) 490
BP	2 ug/plate	yes	(202,138, 95) 145	(695,759,618) 691			(59, 59, 61) 60		(114,155,246) 172
AA	2 ug/plate	yes	(970,945,967) 961	(1000,1022,1056) 1026			(239,222,237) 233		(994,976,869) 946
MNNG	2 ug/plate	no		(1550,1601,1503) 1551					
	20 ug/plate	no				(1602,1541,1551) 1565			

Spontaneous Reversion Rate/ Negative Control

before assay	yes	(17, 18, 16)	(115,107, 79)	(8, 8, 8)	(2, 2, 5)	(10, 19, 16)
after assay		(19, 9, 18)	(93, 87, 79)	(8, 8, 9)	(2, 2, 3)	(11, 7, 14)
		16	93	8	3	13
before assay	no	(30, 14, 20)	(115,113,119)	(24, 26, 17)	(3, 2, 2)	(14, 13, 8)
after assay		(10, 9, 7)	(77, 88, 76)	(15, 9, 18)	(1, 2, 2)	(6, 5, 13)
		15	98	18	2	10

Study Number: 84020

Date: 30 MAR 84 By: SANO, KELLNER

* Compounds: AF = 2-aminofluorene, BP = Benz(a)pyrene, AA = 2-aminoanthracene,
MNNG = N-methyl-N'-nitro-N-nitrosoguanidine

†, §§ Indicates colony counts exceeded 1000

TABLE 5

GUANIDINE NITRATE ASSAY
(Revertants/Plate)

Compd	Amount of Compd. Added	S-9 Added	Mean		Strain No.	
			98	100	1535	1537
TP030	5 mg/plate	yes	(20, 27, 20) 22	(101, 91, 105) 99	(11, 9, 8) 9	(5, 3, 5) 4
		no	(15, 17, 20) 17	(8, 82, 91) 85	(11, 8, 13) 11	(8, 3, 4) 5
TP030	1 mg/plate	yes	(20, 22, 21) 21	(85, 88, 110) 94	(11, 11, 12) 11	(4, 12, 7) 8
		no	(13, 15, 14) 14	(91, 105, 85) 94	(9, 7, 12) 9	(4, 8, 3) 5
TP030	0.2 mg/plate	yes	(28, 23, 28) 26	(102, 135, 103) 113	(10, 9, 9) 9	(1, 1, 7) 3
		no	(8, 8, 18) 11	(125, 134, 122) 127	(20, 13, 11) 15	(4, 4, 2) 3
TP030	0.04 mg/plate	yes	(21, 17, 26) 21	(102, 98, 120) 107	(12, 10, 6) 9	(7, 5, 6) 6
		no	(22, 10, 16) 16	(91, 98, 111) 100	(14, 18, 11) 14	(2, 6, 4) 4

Study No.: 84020

Date: 30 MAR 84

Performed by: SANO, KELLNER

TABLE 5 (concluded)
 GUANIDINE NITRATE ASSAY
 (Revertants/Plate)
 Mean

Compd	Amount of Compd. Added	S-9 Added	98	100	Strain No. 1535	1537	1538
TP030	0.008 mg/plate	yes	(10, 11, 19) 13	(91, 89, 83) 88	(10, 11, 14) 12	(1, 4, 3) 3	(15, 6, 6) 9
		no	(7, 13, 12) 11	(108, 104, 105) 106	(10, 16, 16) 14	(4, 2, 1) 2	(5, 10, 14) 10
TP050	0.0016 mg/plate	yes	(17, 20, 11) 16	(85, 90, 91) 89	(6, 9, 9) 8	(3, 1, 3) 2	(5, 8, 11) 8
			(8, 9, 12) 10	(88, 81, 80) 83	(20, 23, 9) 17	(6, 5, 4) 5	(12, 11, 12) 12

Study No.: 84020

Date: 30 MAR 84

Performed by: SANO, KELNER

DISCUSSION

Certain test criteria must be satisfied before an Ames assay can be considered a valid assessment of a compound's mutagenic potential. First, the special features of the Ames strains must be verified. These features include demonstration of ampicillin resistance, LP layer alterations, and DNA excision repair deficiencies. Second, the Salmonella strains must be shown to be responsive to the mutagenic process by exposing the strains to known mutagens. Third, the optimal concentration of the test compound must be determined by treating TA100 with a broad range of doses and observing the potential toxic effects on macrocolony and microcolony formation. If these tests are performed and expected data are obtained, then the resulting Ames test can be considered valid.

After validation of bacterial strains and selection of optimal sublethal doses, guanidine nitrate was evaluated in the Ames assay. In no instance did the test compound elicit a doubling of the spontaneous reversion rate or a correlated dose response relationship. Thus, the results of this study indicate guanidine nitrate is not mutagenic when evaluated in the Ames assay.

CONCLUSION

Guanidine nitrate is not mutagenic in the Ames assay at the dose levels tested.

RECOMMENDATION

Guanidine nitrate should undergo further toxicity testing in accordance with Toxic Substances Control Act regulations.

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Sano--13

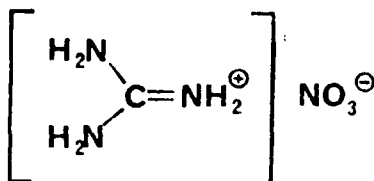
APPENDIX

Sano--14

CHEMICAL DATA

Chemical Name: Guanidine Nitrate

Structural formula:



Empirical formula: $\text{CH}_5\text{N}_3\cdot\text{HNO}_3$

Molecular weight: 122

Physical state: White crystalline powder

Stability: Extremely stable at room temperature

Melting point: 214°C

Source: Chemical Dynamics Corporation
Hadley Road PO Box 395
South Plainfield, NJ

Manufacturer's lot No.: 123820

Analytical data/purity: Infrared spectrophotometry was performed on 1 March 1984 and was identical with published standards (The Sadtler Research Laboratories. Sadtler standard spectra. Philadelphia: The Sadtler Research Laboratories, 1964: 14498). Major absorption peaks were observed at 3330 (broad), 1660, 1525, 1400, 1300, 1050, and 780 cm^{-1} .

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