Institute Report No. 357

Dermal Sensitization Potential of Nitrosoguanidine in Guinea Pigs

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MAMMALIAN TOXICOLOGY BRANCH
DIVISION OF TOXICOLOGY

September 1989

Toxicology Series: 173

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

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This research was conducted in compliance with the "Guide for the Care and Use of Laboratory Animals," NIH Publication No. 85-23, as prepared by the Institute of Laboratory Animal Resources, National Research Council.

This material has been reviewed by Letterman Army Institute of Research and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. *(AR 360-5)*

William C Corby 15 Sept 89

for Donald G. Corby (date)
COL, MC
Commanding

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ABSTRACT

Nitrosoguanidine was evaluated for its potential to produce dermal sensitization in male guinea pigs. The Buehler test, which utilizes repeated closed patch inductions with the test compound, was used for this evaluation. No evidence of nitrosoguanidine-induced sensitization was obtained in the study.

Key Words: Dermal Sensitization, Mammalian Toxicology, Nitrosoguanidine, Buehler Test, Guinea Pigs, Nitroguanidine

PREFACE

TYPE REPORT: Dermal Sensitization GLP Study Report

TESTING FACILITY:

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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
Fort Detrick, MD 21701-5010
Project Officer: Gunda Reddy, PhD

PROJECT/WORK UNIT/APC: 3E162720A835/180/TLB0

GLP STUDY NO.: 85013

STUDY DIRECTOR: Don W. Korte, Jr., PhD, LTC, MSC
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PRINCIPAL INVESTIGATOR: Earl W. Morgan, DVM, MAJ, VC, Diplomate, American College of Veterinary Preventive Medicine, American Board of Toxicology.

CO-PRINCIPAL INVESTIGATOR: James D. Justus, MPA, SSG

CO-AUTHOR: Denzil F. Frost, MS, DVM, CPT, VC, Diplomate, American College of Veterinary Preventive Medicine

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: Nitrosoguanidine

INCLUSIVE STUDY DATES: 30 January - 14 March 1986

OBJECTIVE:

The objective of the study was to evaluate the dermal sensitization potential of nitrosoguanidine in guinea pigs.

ACKNOWLEDGMENTS

SP4 Paul B. Simboli, BS, and SGT John R. G. Ryabik, BS, for chemical analyses; SP4 James J. Fischer for technical assistance; SP4 Scott Schwebe, SP4 Theresa L. Polk, Richard A. Spieler, Charolette L. Speckman, and Obie Goodrich for animal care and facilities management; and Marie Rogers for secretarial assistance.

SIGNATURES OF PRINCIPAL SCIENTISTS INVOLVED IN THE STUDY

We, the undersigned, declare that GLP Study 85013 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. 7 Sep 89
DON W. KORTE, JR., PhD / DATE
LTC, MSC
Study Director

Denzil F. Frost 17 Aug 89
DENZIL F. FROST, MS, DVM / DATE
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CONRAD R. WHEELER, PhD / DATE
DAC
Analytical Chemist

DEPARTMENT OF THE ARMY
LETTERMAN ARMY INSTITUTE OF RESEARCH
PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129-6800

REPLY TO
ATTENTION OF:

SGRD-ULZ-QA 15 September 1989

MEMORANDUM FOR RECORD

SUBJECT: GLP Compliance for GLP Study 85Ø13

1. This is to certify that in relation to LAIR GLP Study 85Ø13 the following inspections were made:

1Ø May 1985	-	Protocol Review
11 March 1986	-	Dosing

2. The institute report entitled "Dermal Sensitization Potential of Nitrosoguanidine in Guinea Pigs," Toxicology Series 173, was audited on 14 October 1987.

Walter G. Bell
WALTER G. BELL
SFC, USA
Quality Assurance Officer

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INTRODUCTION

Nitrosoguanidine is a potential anaerobic degradation product of nitroguanidine (1), a primary component of US Army triple-base propellants, which 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 data base and identified significant gaps in the toxicity data (2). 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 Study

The objective of this study was to determine the dermal sensitization potential of nitrosoguanidine in guinea pigs.

MATERIALS

Test Substance

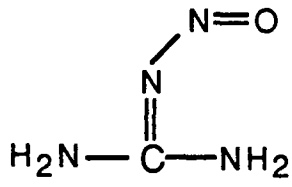
Chemical Name: Nitrosoguanidine

Chemical Abstracts Service Registry No.: 674-81-7

LAIR Code Number: TP48

Physical State: Yellow powder

Chemical Structure:



Molecular Formula: CH_4N_4O

Source: Alan Rosencrance
US Army Biomedical Research and Development Laboratory
Ft. Detrick, Frederick, MD 21701-5010

Other test substance information is presented in Appendix A.

Vehicle for Test Substance

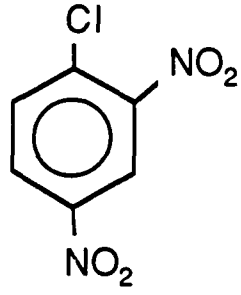
The vehicle for nitrosoguanidine was sterile isotonic saline (Abbott Laboratories, North Chicago, IL 60064). The expiration date for this lot (65-914-DM-03) was 1 June 1986.

Positive Control

Chemical Name: Dinitrochlorobenzene (DNCB)

Chemical Abstracts Service Registry No.: 97-00-7

Chemical Structure:



Molecular Formula: $C_6H_3N_2O_4Cl$

Other positive control substance information is presented in Appendix A.

Vehicle for Positive Control

The vehicle for DNCB was a propylene glycol (3%) and isotonic saline (97%) mixture. Propylene glycol (lot number 36485, exp. date 1991) was obtained from Certified Laboratories, Inc. (Philadelphia, PA). Sterile, isotonic saline (lot number 65-914-DM-03, exp. date 1 June 1986) was obtained from Abott Laboratories (North Chicago, IL).

Animal Data

Male albino guinea pigs, Hartley strain (Simonsen Laboratories, Inc., Gilory, CA), were used for this study. They were identified individually with ear tags. Two animals (86E0038, 86E0059) were selected for quality control necropsy evaluation on receipt. Animal weights on the day of receipt ranged from 184 to 250 g. Additional animal data appear in Appendix B.

Husbandry

Guinea pigs assigned to this study were caged individually in stainless steel, wire mesh cages in racks equipped with automatically flushing dump tanks. The diet, fed *ad libitum*, consisted of Certified Purina Guinea Pig Chow® Diet 5026 (Ralston Purina Company, Checkerboard Square, St. Louis, MO); water was provided by continuous drip from a central line. Temperature within the animal room was maintained in the range from 22.2 to 23.9°C. Relative humidity was maintained in the range of 25% to 54% with occassional spikes as high as 75% (room washing). The photoperiod was 12 hours of light per day.

METHODS

This study was conducted in accordance with LAIR SOP-OP-STX-82 "Buehler Dermal Sensitization Test" (3) and EPA guidelines (4).

Group Assignment/Acclimation

The guinea pigs were quarantined for 12 days before administration of the first induction dose. During the quarantine period, they were checked daily for signs of illness and weighed once a week. Animals were assigned to four groups by a stratified randomization technique based on their body weights.

Dose Levels

Dermal sensitization potential was evaluated in a test group receiving three weekly induction doses of 100% nitrosoguanidine in saline and, after a two-week delay, a challenge dose at the same concentration. Pilot studies indicated that this concentration was not irritating under conditions of the sensitization test. Three control groups were used in the study. Dinitrochlorobenzene, a known potent sensitizing agent (5), was applied to one control group, at a 0.1% concentration, as a positive control. Isotonic saline was applied to another group as a vehicle control. A negative control group received 100% nitrosoguanidine only on the day of challenge dosing.

Compound Preparation

The test compound was prepared by mixing 0.5 g nitrosoguanidine with 0.5 ml of isotonic saline to make a paste. The dinitrochlorobenzene (DNCB) dosing solution was prepared by first adding 30 mg DNCB to 1.0 ml of propylene glycol and heating until it dissolved (approximately 40°C). To this, 29 ml of 0.9% sodium chloride solution were added, to give a final concentration of 0.1% (w/v). This solution was heated to 65°C and vortexed before application to keep the DNCB in solution. Dosing solutions were prepared fresh for each application day.

Test Procedures

The closed patch dermal sensitization test procedures utilized in this study were developed by Buehler and Griffith (6-8) to mimic the repeated-insult patch test for humans. Test compounds were applied for six hours under a closed patch once a week for three weeks during the induction phase.

The same application site was used for each induction dose. To distinguish between reactions from repeated insult and sensitization, duplicate patches of the challenge dose were applied, one on the old site and one on a new site. To distinguish between reactions from primary irritation and sensitization, a negative control group was added which received only the challenge dose.

During the induction phase, the test and positive control groups were dosed with 0.5 ml of the appropriate compound/vehicle applied topically under a 2.5-cm^2 gauze patch. This procedure was performed for three consecutive weeks (11, 18, and 25 Feb 86). Twenty-four hours before each dosing, a 7.6-cm^2 area on the left flank of the animal was clipped with electric clippers (Oster® Model A5, size 40 blade, Sunbeam Corp., Milwaukee, WI) and then shaved with an electric razor (Norelco® Speed Razor Model HP1134/S, North American Phillips Corp., Stamford, CT). The patch was taped with Blenderm® hypoallergenic surgical tape (3M Corp., St. Paul, MN) to the same site each time, and the animal was wrapped several times with Vetrap® (3M Corp., St. Paul, MN). The patch was left in place for six hours. When the wrap and patch were removed, the area under the patch was gently wiped of any excess compound using a saline-moistened gauze and the site was marked for scoring.

Animals were challenged two weeks (11 Mar 86) following the third induction dose. Test group and positive control group animals received two 0.5-ml doses each of nitrosoguandine or DNCB, respectively, one applied to the old site on the left flank and the other to a new site on the right flank. Negative control animals received only a single 0.5-ml dose of nitrosoguanidine, applied to the left flank. Procedures for clipping, shaving, and wrapping and the exposure period remained the same.

In Buehler's procedure, skin reactions are scored 24 and 48 hours after the challenge dose only. In the present study, skin reactions were scored 24, 48, and 72 hours after each induction dose as well as 24, 48, and 72 hours after the challenge dose. Skin reactions were assigned scores according to Buehler's grading system: 0 (no reaction), 1 (slight erythema), 2 (moderate erythema), and 3 (marked erythema). Results are expressed in

terms of both incidence (the number of animals showing responses of 1 or greater at either 24, 48, or 72 hours) and severity (the sum of the test scores divided by the number of animals tested). Results from the left flank were compared with right flank and with the negative control group.

Some modifications of Buehler's procedures were made. Instead of placing animals in restraint during the 6-hour exposure period, the animals were wrapped several times with an elasticized tape to hold the patch in place. Consequently, the animals were able to move about freely in their cages during the exposure period. Buehler and Griffith (8) also recommended depilating the day before the challenge dose. For consistency with induction procedures, this step was replaced by clipping and shaving the fur of the animals.

The animals were observed daily for clinical signs and weight gain was monitored during the study. At the conclusion of the study, a necropsy was performed on each animal. A historical listing of study events appears in Appendix C.

Changes/Deviations

The DNCB solution was maintained at approximately 65°C before dosing the guinea pigs in order to keep it in solution for accurate dosing. There was little chance of thermal insult to the animal because the aliquot cooled quickly during pipetting before application of the patch. Two animals died during the quarantine period. Consequently, the number of animals in the vehicle control group was reduced by 2 to 13. It is believed that these deviations from the protocol did not adversely affect the study results.

Storage of Raw Data and Final Report

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.

RESULTS

Experimental

Table 1 summarizes the incidence of reactions 24, 48, and 72 hours after each dose. Two animals showed slight irritation to the third induction dose of nitrosoguanidine at 24 hours. However, this irritation had cleared by 48 hours. No reaction was observed in response to nitrosoguanidine after any other induction dose or the challenge dose. This lack of response is reflected in Table 2 which depicts the severity of skin reactions. Response severity for each group is calculated by summing the scores of responding animals and dividing by the total number of animals within that group. For nitrosoguanidine no response to the challenge dose was obtained; therefore, severity scores were zero.

Positive Control

Dinitrochlorobenzene produced a marked response at all time points after the second induction dose (Table 1). Between 80% and 100% of the DNCB-treated animals exhibited a response 24 hours following the second or third induction and challenge doses. These reactions persisted, yielding scorable effects in 27-93% of the animals at 48 hours after dosing and 13-73% of the animals at 72 hours after dosing. Severity scores for these responses to DNCB ranged from 0.9 to 1.7 at the 24-hour scoring period (Table 2). The highest score, 1.7, was observed in response to the challenge dose on the left flank. By 48 hours the reactions had subsided slightly; consequently, the severity range decreased to between 0.3 and 1.7. At 72 hours the reactions diminished further to a range of 0.1 to 1.3.

Negative and Vehicle Controls

No response was observed in the negative control (challenge dose of nitrosoguanidine) or vehicle control groups. Individual 24-hour, 48-hour, and 72-hour dermal scores for all animals appear, by group, in Appendix D.

TABLE 1: Incidences of Skin Reactions

Test Group	Induction			Challenge	
	First	Second	Third	Left	Right
		24 Hours			
Nitrosoguanidine	0/15	0/15	2/15	0/15	0/15
DNCB	0/15	12/15	15/15	15/15	13/15
Negative Control*	—	—	—	0/15	—
Vehicle Control	0/13	0/13	0/13	0/13	—
		48 Hours			
Nitrosoguanidine	0/15	0/15	0/15	0/15	0/15
DNCB	0/15	4/15	13/15	14/15	12/15
Negative Control*	—	—	—	0/15	—
Vehicle Control	0/13	0/13	0/13	0/13	—
		72 Hours			
Nitrosoguanidine	0/15	0/15	0/15	0/15	0/15
DNCB	0/15	2/15	6/15	11/15	7/15
Negative Control*	—	—	—	0/15	—
Vehicle Control	0/13	0/13	0/13	0/13	—

*The Negative Control Group received only a challenge dose of the test compound.

TABLE 2: Severity of Skin Reactions

Test Group	Induction			Challenge	
	First	Second	Third	Left	Right
		24 Hours			
Nitrosoguanidine	0.0	0.0	0.1	0.0	0.0
DNCB	0.0	0.9	1.3	1.7	1.4
Negative Control*	—	—	—	0.0	—
Vehicle Control	0.0	0.0	0.0	0.0	—
		48 Hours			
Nitrosoguanidine	0.0	0.0	0.0	0.0	0.0
DNCB	0.0	0.3	1.1	1.7	1.1
Negative Control*	—	—	—	0.0	—
Vehicle Control	0.0	0.0	0.0	0.0	—
		72 Hours			
Nitrosoguanidine	0.0	0.0	0.0	0.0	0.0
DNCB	0.0	0.1	0.5	1.3	0.7
Negative Control*	—	—	—	0.0	—
Vehicle Control	0.0	0.0	0.0	0.0	—

*The Negative Control Group received only a challenge dose of the test compound.

Clinical Signs

All animals were healthy and gained weight during the study. Individual body weight data are presented in Appendix E.

Pathology Findings

A necropsy was performed on all study animals. No lesions were found at necropsy that could be attributed to the test compound. The complete pathology report is presented in Appendix F.

DISCUSSION

Dermal Irritation and Sensitization

Most skin reactions occurring from contact with chemicals can be classified as either irritation or sensitization. Both reactions present as inflammation of the skin; the difference between irritation and sensitization is the mechanism responsible for this inflammation. Primary irritation is direct inflammation in response to injury to the skin produced by the eliciting chemical. Irritation is a locally mediated response ranging from mild reversible inflammation to severe ulceration progressing to necrosis. Sensitization is manifested as indirect inflammation mediated by components of the immune system in response to activation by the eliciting chemical (9). Dermal sensitization is usually a delayed hypersensitivity or cellular immunologic reaction. Although both types of reactions can appear grossly similar in experimental animals and may even be produced by the same agent, it is possible to distinguish between them. Irritation is an immediate response and can be produced upon first contact with the chemical, whereas sensitization requires at least one innocuous "conditioning" exposure before a reaction can be elicited.

Irritative responses usually require a relatively high concentration or dose of the offending chemical, whereas sensitization reactions may occur in response to minute quantities. Essentially all individuals in a population will express an irritative response to a reactive chemical, provided the dose is high

enough, whereas only a fraction of the population normally becomes sensitized to the same chemical. A fully developed response can be produced by first contact with an irritant, but initial contact with a sensitizer produces no reaction (a conditioning exposure is necessary). Unless there is accumulation of damage, subsequent exposures to an irritant produce inflammation of essentially similar intensity/severity, whereas the reaction to a sensitizer often increases over 2 to 4 exposures after the initial contact. An irritant produces inflammation of rapid onset with short duration, whereas a sensitization reaction is somewhat delayed and prolonged. The inflammatory response to an irritant may spread beyond the area of contact, whereas sensitization reactions are usually circumscribed.

The features of irritation and sensitization have been used to establish guidelines for differentiation between the two (6-9). In evaluating a dermal sensitization study it is recommended that the results from a challenge dose in the experimental group (sensitization) be compared with those for the negative control group (irritation) in accordance with the following criteria:

Irritative Responses:

- occur in a large proportion of test animals.
- develop in response to the first or second exposure.
- usually fade within 24 to 48 hours, unless damage is severe.
- may be stronger at challenge to a previously unexposed area of skin (contralateral flank).

Sensitization Reactions:

- occur in only a few animals, unless the compound is a potent sensitizer.
- are absent after the initial (conditioning) exposure, but appear in response to subsequent exposures.
- develop slowly, the intensity/severity of inflammation often is greater at 72 to 96 than at 24 to 48 hours.
- increase in intensity/severity from one exposure to the next (at sites previously exposed or unexposed).

Dermal irritancy potential is evaluated by the method of Draize *et al.* (10) in which the chemical is applied once, at high concentration, and the resulting acute inflammatory reaction is graded. Evaluation of sensitizing

potential is accomplished by repeated application, at lower nonirritating concentrations, over a few weeks. There is then a latent period, usually two weeks, to allow the immune system to elaborate and increase its specific response to the chemical. A challenge dose is then given, and the resulting inflammatory response is graded. Analysis of the incidence, severity, and timing of the response to the challenge dose estimates the sensitizing potential of the study compound.

Nitrosoguanidine

Nitrosoguanidine was evaluated for its ability to elicit a sensitization reaction via contact with the skin. A skin reaction (slight) to nitrosoguanidine was observed in two test animals at the third 24-hour induction period, but had dissipated by 48 hours. However, the results of the challenge dose (no response) indicated that nitrosoguanidine was negative in the Buehler test. If nitrosoguanidine had appreciable sensitizing potential it would have been detected by this test as control groups exhibited expected responses. The DNCB response was characteristic of that observed previously within the Institute (11). Although DNCB is capable of producing primary irritation, the characteristics of the responses observed in this study are indicative of a reaction due to sensitization since the concentration of DNCB used for induction and challenge was too low to produce primary irritation. Also, the response to DNCB was observed after two or more exposures. Since the Buehler test predicts predominately moderate to severe sensitizers, these results do not guarantee that nitrosoguanidine will not sensitize humans. However, it does indicate that nitrosoguanidine is unlikely to sensitize humans and its potential is low enough to permit its evaluation in man.

CONCLUSION

Nitrosoguanidine possesses minimal sensitizing potential, as it did not induce a dermal sensitization reaction under conditions of this study.

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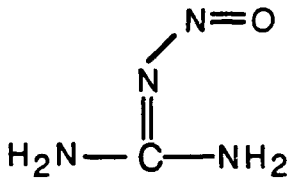
Appendix A: CHEMICAL DATA

Chemical Name: Nitrosoguanidine
Chemical Abstracts Service Registry No.: 674-81-7
Lot Number: WCC-2-002
LAIR Code: TP48
Chemical Structure:



Molecular Formula: CH_4N_4O
Molecular Weight: 88
Physical State: Yellow powder
Analytical Data:

HPLC: Nitrosoguanidine was analyzed using conditions similar to those employed by Burrows *et al.*[1] Conditions were as follows: column, Brownlee RP-18 (4.6 mm x 25 cm); mobile phase, water; flowrate, 0.8 ml/min. The effluent was monitored at 255 nm. The retention times for nitrosoguanidine and nitroguanidine were 4.4 and 6 min, respectively. The HPLC data demonstrated that the nitrosoguanidine contained approximately 2.5% nitroguanidine.[2]

IR (KBr): 3378, 3096, 1690, 1649, 1508, 1341, 1266,1134, 1088, 1035, 690, 668 cm^{-1}.[3]

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[1] Burrows EP, Brueggeman EE, Hoke SH. Chromatographic trace analysis of guanidine, substituted guanidines and striazines in water. Chromatog 1984;16:494-8.

[2] Wheeler, CR. Nitrocellulose-Nitroguanidine Projects. Laboratory Notebook #84-05-010.3, p 37. Letterman Army Institute of Research, Presidio of San Francisco, CA.

[3] *Ibid.* p 30.

Appendix A (cont.): CHEMICAL DATA

Solubility:

A saturated solution of nitrosoguanidine in water was prepared at room temperature. A 1:500 dilution of this solution produced an absorbance of 0.533 units. Using an extinction coefficient of 13,305 L/moles·cm, the concentration of nitrosoguanidine in the original saturated solution was calculated to be 1.76 mg/ml.[4]

Stability:

Stable for at least 4 hours in water at room temperature.[5]

Source: Alan Rosencrance
US Army Biomedical Research and Development Laboratory
Fort Detrick, Maryland

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[4] Wheeler CR. Nitrocellulose-Nitroguanidine Projects. Laboratory Notebook #85-01-006, p 66. Letterman Army Institute of Research, Presidio of San Francisco, CA.

[5] Wheeler, CR. Nitrocellulose-Nitroguanidine Projects. Laboratory Notebook #84-05-010.3, p 32-36. Letterman Army Institute of Research, Presidio of San Francisco, CA.

Appendix A (cont.): CHEMICAL DATA

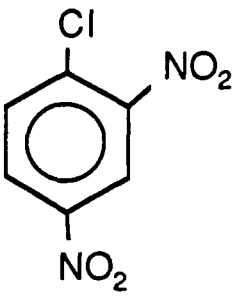
POSITIVE CONTROL

Chemical Name: 1-Chloro-2,4-dinitrobenzene

Alternate Chemical Name: 2,4-Dinitrochlorobenzene

Chemical Abstracts Service Registry Number: 97-00-7

Chemical Structure:



Molecular Formula: $C_6H_3N_2O_4Cl$

Molecular Weight: 202.6

Physical State: Yellow crystals

Melting Point: 52–54° C[1]

Purity: The compound was designated as 95% pure by source.

Analytical Data: Chemical analysis was performed as follows: Infrared spectra were obtained with a Perkin-Elmer 983 spectrometer.[2] Proton magnetic resonance (NMR) spectra were recorded on a Varian XL300 instrument with tetramethylsilane as the internal standard and chemical shifts expressed as parts per million (d).[3] Low resolution GC-MS analysis was performed with a Kratos MS-25RFA (30 m DB-1 capillary column).[4]

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1 Windholz M, ed. The Merck Index. 10th ed. Rahway, NJ: Merck and Co., Inc., 1983:300.

2 Wheeler CR. Toxicity Studies of Water Disinfectant. Laboratory Notebook #85-12-021, p. 9-10. Letterman Army Institute of Research, Presidio of San Francisco, CA.

3 *Ibid.* p. 11-12.

4 *Ibid.* p. 13-16.

Appendix A (cont.): CHEMICAL DATA

The following data were obtained: IR (KBr): 3443, 3104, 2877, 1963, 1829, 1801, 1756, 1705, 1604, 1591, 1542, 1349, 1246, 1156, 1046, 917, 902, 850, 835, 749, 732 cm^{-1}. The IR spectrum was very close to the Sadtler reference spectrum.[5] Differences were due to the much finer spectral resolution obtained on the P-E 983 instrument. NMR ($CDCl_3$): d 7.78 (1 H, d, J = 8.7 Hz), 8.38 (1 H, q, J_{ortho} = 8.7 Hz, J_{meta} = 3.6 Hz), 8.74 (1 H, d, J_{meta} = 2.4 Hz). The spectrum of DNCB was identical to the Aldrich reference spectrum.[6] GC-MS Analysis: A plot of the total ion current versus scan number showed one major peak for DNCB with only traces of other compounds (not identified). Molecular ion masses (m/z) of 202 and 204 confirmed the identity of the major peak as DNCB.[7]

Lot Number: 11F-0543

Source: Sigma Chemical Co.
St. Louis, MO

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[5] Sadtler Research Laboratory, Inc., Sadtler standard spectra. Philadelphia: The Sadtler Research Laboratory, Inc., 1962: Infrared spectrogram #964.

[6] Pouchert CJ. The Aldrich Library of NMR Spectra. Vol. 1, 2nd ed. Milwaukee: Aldrich Chemical Co., 1981:1173, spectrum D.

[7] Wheeler CR. Toxicity Studies of Water Disinfectant. Laboratory Notebook #85-12-021, p. 13-15. Letterman Army Institute of Research, Presidio of San Francisco, CA.

Appendix B: ANIMAL DATA

Species: *Cavia porcellus*

Strain: Hartley, albino

Source: Simonsen Laboratories, Inc.
Gilory, CA

Sex: Male

Date of Birth: 6 January 1986

Method of randomization: Weight bias, stratified animal allocation

Animals in each group: 15 male animals

Condition of animals at start of study: Normal

Identification procedures: Ear tag.

Pretest conditioning: Quarantine/acclimation 30 January - 10 February 1986

Justification: The laboratory guinea pig has proven to be a sensitive and reliable model for detection of delayed hypersensitivity from dermal contact.

Appendix C: HISTORICAL LISTING OF STUDY EVENTS

Date	Event
30 Jan 86	Animals arrived at LAIR. Animals were examined, weighed, placed in cages, and fed. Animals were assigned ear tags. Two animals were submitted for necropsy quality control.
31 Jan - 14 Mar 86	Animals were checked daily.
3, 10, 17, 24 Feb, 10, 14 Mar 86	Animals were weighed.
10 Feb 86	Animals were randomized into four groups (vehicle control, experimental, positive control, negative control).
10, 17, 24 Feb 86	Study animals, except negative control group, were clipped and shaved.
11, 18, 25 Feb 86	Study animals, except negative control group, were given induction dose.
12, 19, 26 Feb 86	Study animals, except negative control group , were scored for 24-hr skin reaction.
13, 20, 27 Feb 86	Study animals, except negative control group , were scored for 48-hr reaction.
14, 21, 28 Feb 86	Study animals, except negative control group , were scored for 72-hr reaction.
10 Mar 86	Study animals were clipped and shaved.
11 Mar 86	Study animals were given challenge dose.
12 Mar 86	Study animals were scored for 24-hr reaction.
13 Mar 86	Study animals were scored for 48-hr reaction.
14 Mar 86	Study animals were scored for 72-hr reaction. All animals were delivered to Necropsy Suite.

Appendix D: INDIVIDUAL ANIMAL SCORES

GROUP: ONE

COMPOUND: Nitrosoguanidine

ANIMAL NUMBER	FIRST INDUCTION			SECOND INDUCTION			THIRD INDUCTION			CHALLENGE DOSE					
										LEFT FLANK			RIGHT FLANK		
	24 H	48 H	72 H	24 H	48 H	72H	24 H	48 H	72H	24 H	48 H	72 H	24 H	48 H	72 H
86E0001	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
86E0002	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0009	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
86E0010	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0011	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0019	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0032	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0035	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0042	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0043	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0047	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0055	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0057	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0060	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86E0061	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix D (cont.): INDIVIDUAL ANIMAL SCORES

GROUP: TWO

COMPOUND: DNCB Positive Control

ANIMAL NUMBER	FIRST INDUCTION			SECOND INDUCTION			THIRD INDUCTION			CHALLENGE DOSE					
										LEFT FLANK			RIGHT FLANK		
	24 H	48 H	72 H	24 H	48 H	72H	24 H	48 H	72H	24 H	48 H	72 H	24 H	48 H	72 H
86E0003	0	0	0	1	1	0	1	1	0	1	1	0	1	1	1
86E0006	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
86E0007	0	0	0	1	0	0	1	1	0	2	1	1	2	0	0
86E0012	0	0	0	1	0	0	1	1	0	1	1	0	1	0	0
86E0013	0	0	0	1	0	0	2	1	0	1	2	2	2	1	0
86E0015	0	0	0	1	0	0	1	1	0	1	1	0	1	1	0
86E0022	0	0	0	1	1	1	1	2	2	2	1	1	2	1	1
86E0025	0	0	0	1	1	1	2	2	2	3	3	3	3	3	3
86E0028	0	0	0	0	0	0	1	0	0	1	2	2	0	1	1
86E0034	0	0	0	0	0	0	1	1	0	2	2	2	1	1	0
86E0039	0	0	0	1	0	0	1	1	0	3	3	2	3	2	1
86E0040	0	0	0	1	0	0	1	1	1	1	2	1	1	1	0
86E0050	0	0	0	1	0	0	2	1	1	3	3	2	1	1	1
86E0051	0	0	0	2	1	0	2	1	1	2	2	2	2	2	2
86E0056	0	0	0	1	0	0	2	2	1	2	1	1	1	1	0

Appendix D (cont.): INDIVIDUAL ANIMAL SCORES

GROUP: THREE

COMPOUND: NEGATIVE CONTROL

ANIMAL NUMBER	FIRST INDUCTION			SECOND INDUCTION			THIRD INDUCTION			CHALLENGE DOSE					
										LEFT FLANK			RIGHT FLANK		
	24 H	48 H	72 H	24 H	48 H	72H	24 H	48 H	72H	24 H	48 H	72 H	24 H	48 H	72 H
86E0005	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0008	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0018	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0023	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0024	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0026	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0031	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0037	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0044	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0046	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0048	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0049	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0053	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A
86E0058	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	N/A	N/A	N/A

Appendix D: INDIVIDUAL ANIMAL SCORES

GROUP: Four

COMPOUND: Vehicle Control

ANIMAL NUMBER	FIRST INDUCTION			SECOND INDUCTION			THIRD INDUCTION			CHALLENGE DOSE					
										LEFT FLANK			RIGHT FLANK		
	24 H	48 H	72 H	24 H	48 H	72H	24 H	48 H	72H	24 H	48 H	72 H	24 H	48 H	72 H
86E0004	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0016	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0017	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0020	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0021	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0027	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0029	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0033	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0036	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0041	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0045	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0052	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A
86E0054	0	0	0	0	0	0	0	0	0	0	0	0	N/A	N/A	N/A

Appendix E: INDIVIDUAL BODY WEIGHTS (grams)

Nitrosoguanidine

Animal Number	DAY OF STUDY						
	0*0	04	011	7	14	28	32
86E0001	217	248	310	363	379	493	500
86E0002	192	228	299	352	417	547	532
86E0009	226	267	341	404	467	574	587
86E0010	218	252	315	357	410	508	528
86E0011	187	227	285	326	383	478	479
86E0019	238	246	326	381	430	514	530
86E0032	250	289	347	368	416	480	487
86E0035	184	207	263	295	350	433	449
86E0042	229	162	294	342	401	514	532
86E0043	246	271	336	395	446	537	538
86E0047	219	238	290	325	372	448	446
86E0055	215	240	306	351	383	439	455
86E0057	227	252	325	370	434	530	546
86E0060	206	243	303	319	426	490	508
86E0061	190	221	274	–†	355	485	454
MEAN	216.3	239.4	307.6	353.4	404.6	498.0	504.7
Standard Deviation	21.0	29.7	24.5	30.3	33.8	40.1	42.1
Standard Error	5.4	7.7	6.3	8.1	8.7	10.4	10.9

* Q represents quarantine period.
† Weight not recorded.

Appendix E (cont.): INDIVIDUAL BODY WEIGHTS (grams)

DNCB

Animal Number	DAY OF STUDY						
	0*0	06	013	7	14	28	32
86E0003	240	309	322	389	453	587	601
86E0006	222	252	310	371	427	530	534
86E0007	230	272	346	407	469	552	573
86E0012	191	233	288	326	356	451	456
86E0013	210	223	294	331	387	537	543
86E0015	236	265	330	377	427	532	544
86E0022	222	243	288	319	352	405	414
86E0025	220	256	333	375	406	269	293
86E0028	238	283	298	361	416	420	512
86E0034	228	248	316	356	412	520	526
86E0039	186	210	274	327	373	477	486
86E0040	250	280	341	381	425	507	514
86E0050	236	261	302	330	382	455	469
86E0051	223	172	283	332	380	447	473
86E0056	233	237	308	370	423	525	536
MEAN	224.3	249.6	308.9	356.8	405.9	480.9	480.3
Standard Deviation	17.5	33.0	22.1	27.4	33.8	78.3	135.1
Standard Error	4.5	8.5	5.7	7.1	8.7	20.2	34.9

* Q represents quarantine period.

Appendix E (cont.): INDIVIDUAL BODY WEIGHTS (grams)

Negative Control

Animal Number	DAY OF STUDY						
	Q*0	Q4	Q11	7	14	28	32
86E0005	230	257	338	389	451	560	561
86E0008	234	266	321	363	424	519	529
86E0014	234	272	327	376	431	499	495
86E0018	186	215	281	331	387	496	489
86E0023	201	226	306	326	387	473	507
86E0024	208	244	235	332	390	495	508
86E0026	240	275	331	372	410	500	494
86E0031	216	243	288	325	376	456	456
86E0037	204	219	284	345	408	490	495
86E0044	216	243	301	360	392	517	528
86E0046	227	256	319	373	439	556	560
86E0048	212	233	302	341	391	470	469
86E0049	201	274	362	396	475	570	579
86E0053	224	250	308	366	404	500	499
86E0058	210	237	293	341	389	487	498
MEAN	216.2	247.3	306.4	355.7	410.3	505.9	511.1
Standard Deviation	15.2	19.4	29.6	23.0	28.2	33.4	34.4
Standard Error	3.9	5.0	7.7	5.9	7.3	8.6	8.9

* Q represents quarantine period.

Appendix E (cont.): INDIVIDUAL BODY WEIGHTS (grams)

Vehicle Control

Animal Number	DAY OF STUDY						
	Q*0	04	011	7	14	28	32
86E0004	225	246	297	327	360	443	454
86E0016	222	252	304	345	390	480	491
86E0019	238	246	326	381	430	514	530
86E0020	218	256	324	385	444	485	512
86E0021	188	165	250	292	350	453	464
86E0027	203	225	279	316	370	451	458
86E0029	238	279	338	370	417	513	456
86E0033	188	219	286	338	404	491	516
86E0036	206	237	292	332	360	427	442
86E0041	202	240	293	342	399	489	504
86E0045	210	241	304	345	387	488	493
86E0052	218	258	325	378	427	508	512
86E0054	222	247	310	353	397	464	476
MEAN	213.7	239.3	302.2	346.5	395.0	477.4	485.2
Standard Deviation	16.1	26.9	23.5	27.2	29.5	27.7	28.6
Standard Error	4.5	7.5	6.5	7.5	8.2	7.7	7.9

* Q represents quarantine period.

Appendix F: PATHOLOGY REPORT

GLP Study 85013

Study: Buehler Dermal Sensitization
APC#: LLB0
Substance: Nitrosoguanidine (CAS #674-81-7)
Animal: Guinea Pig/Hartley/Male.
Reference: SOP-OP-STX-78.

Euthanasia: Sodium pentobarbital.
Fixative: 10% buffered formalin.
Histopathology: Routine (39315 only)
Clinical Lab: None.

Gross findings:

GROUP 1 - VEHICLE CONTROL
(All live animals)

LAIR ACC#	ANIMAL ID#	OBSERVATION
39296	86E00004	Liver - multiple foci of necrosis.
39308	86E00016	Not remarkable (NR)
39309	86E00017	NR
39312	86E00020	NR
39313	86E00021	Liver - multiple foci of necrosis.
39319	86E00027	Liver - multiple foci of necrosis. Abdominal cavity - contained 3ml of clear yellow liquid.
39321	86E00029	Liver - multiple foci of necrosis. NR - No ID tag.
39324	86E00033	Liver - single linear area of necrosis.
39327	86E00036	NR
39331	86E00041	Liver - multiple foci of necrosis.
39335	86E00045	NR
39342	86E00052	Liver - multiple foci of necrosis. No ID tag.
39344	86E00054	Liver - single area of necrosis.

Appendix F (cont.): PATHOLOGY REPORT

Pathology Report
GLP Study 85013

GROUP 2 - POSITIVE TEST COMPOUND
(All live animals)

LAIR ACC#	ANIMAL ID#	OBSERVATION
39293	86E00001	Liver - pale multiple irregular areas of necrosis.
39294	86E00002	Liver - Pale multiple irregular areas of necrosis.
39301	86E00009	Liver - multiple foci of necrosis. Abdominal cavity - filled with 3-5m clear, yellow fluid.
39302	86E00010	NR
39303	86E00011	Liver - multiple foci of necrosis.
39311	86E00019	Liver - multiple foci of necrosis.
39323	86E00032	Liver - area of necrosis\
39326	86E00035	NR - No ID tag.
39332	86E00042	Liver - multiple foci of necrosis.
39333	86E00043	Liver - multiple foci of necrosis.
39337	86E00047	Liver - multiple foci of necrosis. No ID tag.
39345	86E00055	NR
39347	86E00057	NR
39349	86E00060	Liver - multiple foci of necrosis.
39350	86E00061	Liver - multiple foci of necrosis.

GROUP 3 - POSITIVE (DNCB) CONTROL
(All live animals)

LAIR ACC#	ANIMAL ID#	OBSERVATION
39295	86E00003	Liver - multiple foci of necrosis.
39298	86E00006	NR
39299	86E00007	Liver - multiple foci of necrosis.
39304	86E00012	NR
39305	86E00013	Liver - multiple foci of necrosis.
39307	86E00015	Liver - multiple foci of necrosis.
39314	86E00022	Liver - multiple foci of necrosis.
39317	86E00025	Liver - multiple foci of necrosis. Emaciated carcass.
39320	86E00028	Liver - focal area of necrosis.
39325	86E00034	NR
39329	86E00039	Liver - multiple foci of necrosis.
39330	86E00040	Liver - multiple foci of necrosis.
39340	86E00050	Liver - multiple foci of necrosis.
39341	86E00051	Liver - multiple foci of necrosis.
39346	86E00056	Liver - multiple foci of necrosis.

Appendix F (cont.): PATHOLOGY REPORT

Pathology Report
GLP Study 85013

GROUP 4 - NEGATIVE TEST COMPOUND
(All live animals)

LAIR ACC#	ANIMAL ID#	OBSERVATION
39297	86E00005	Liver - few, small multiple foci of necrosis.
39300	86E00008	Liver - multiple foci of necrosis.
39306	86E00014	Liver - multiple areas of necrosis. No ID tag.
39310	86E00018	Liver - multiple foci of necrosis.
39315	86E00023	Liver - two, firm, white, expansile masses. (Micro exam performed.)
39316	86E00024	Liver - multiple foci of necrosis.
39318	86E00026	Liver - multiple foci of necrosis.
39322	86E00031	Liver - multiple foci of necrosis. No ID tag.
39328	86E00037	Liver - multiple foci of necrosis.
39334	86E00044	Liver - multiple foci of necrosis.
39336	86E00046	Liver - multiple foci of necrosis.
39338	86E00048	Liver - multiple foci of necrosis.
39339	86E00049	Liver - multiple foci of necrosis.
39343	86E00053	Liver - multiple foci of necrosis.
39348	86E00058	Liver - multiple foci of necrosis.

Microscopic findings:

86E00023: Liver - multiple granulomas, minimal, etiology unknown.

Comments: The areas of liver necrosis are a frequently observed incidental finding in guinea pigs. The cause has not been determined. No post mortem findings are present that would confound or complicate the results of this project.

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C, Comparative Pathology Branch

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5 May 1986

Distribution List

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Cameron Station
Alexandria, VA 22304-6145

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