National Cancer Institute
CARCINOGENESIS
Technical Report Series
NO. 165
1979

BIOASSAY OF 4-CHLORO-o-TOLUIDINE HYDROCHLORIDE FOR POSSIBLE CARCINOGENICITY

CAS No. 3165-93-3

NCI-CG-TR-165

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health

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Carcinogenesis Testing Program
Division of Cancer Cause and Prevention
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20014

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DHEW Publication No. (NIH) 79-1721

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FOREWORD: This report presents the results of the bioassay of 4-chloro-o-toluidine hydrochloride conducted for the Carcinogenesis Testing Program, Division of Cancer Cause and Prevention, National Cancer Institute (NCI), National Institutes of Health, Bethesda, Maryland. This is one of a series of experiments designed to determine whether selected chemicals have the capacity to produce cancer in animals. A negative result, in which the test animals do not have a greater incidence of cancer than control animals, does not necessarily mean that a test chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of circumstances. A positive result demonstrates that a test chemical is carcinogenic for animals under the conditions of the test and indicates that exposure to the chemical is a potential risk to man. The actual determination of the risk to man from chemicals found to be carcinogenic in animals requires a wider analysis.

CONTRIBUTORS: This bioassay of 4-chloro-o-toluidine hydrochloride was conducted at the NCI Frederick Cancer Research Center (FCRC) (1), Frederick, Maryland, operated for NCI (2) by Litton Bionetics, Inc.

The manager of the bioassay at FCRC was Dr. B. Ulland, the toxicologist was Dr. E. Gordon, and Drs. R. Cardy and D. Creasia compiled the data. Ms. S. Toms was responsible for management of data, Mr. D. Cameron for management of histopathology, Mr. L. Callahan for management of the computer branch, and Mr. R. Cypher for management of the facilities. Mr. A. Butler performed the computer services. Histopathologic evaluations for rats were performed by Dr. B. C. Zook and histopathologic evaluations for mice were performed by Dr. H. R. Seibold. The diagnoses included in this report represent their interpretations.

Animal pathology tables and survival tables were compiled at EG&G Mason Research Institute (3). Statistical analyses were performed by Dr. J. R. Joiner (4) and Ms. P. L. Yong (4), using methods selected for the bioassay program by Dr. J. J. Gart (5). The chemicals used in this bioassay were analyzed at FCRC by Dr. W. Zielinsky (1). The chemical analyses and narrative were reviewed and approved by Dr. W. Lijinsky (1).

This report was prepared at Tracor Jitco (4) under the direction of NCI. Those responsible for the report at Tracor Jitco were Dr. C. R. Angel, Acting Director of the Bioassay Program; Dr. S. S. Olin, Deputy Director for Science; Dr. J. F. Robens, toxicologist; Dr. R. L. Schueler, pathologist; Dr. G. L. Miller, Ms. L. A. Owen, Ms. M. S. King, and Mr. W. D. Reichardt, bioscience writers; and Dr. E. W. Gunberg, technical editor, assisted by Ms. Y. E. Presley.

The following scientists at NCI were responsible for evaluating the bioassay experiment, interpreting the results, and reporting the findings: Dr. Kenneth C. Chu, Dr. Cipriano Cueto, Jr., Dr. J. Fielding Douglas, Dr. Richard A. Griesemer, Dr. Thomas E. Hamm, Dr. William V. Hartwell, Dr. Morton H. Levitt, Dr. Harry A. Milman, Dr. Thomas W. Orme, Dr. A. R. Patel, Dr. Sherman F. Stinson, Dr. Jerrold M. Ward, and Dr. Carrie E. Whitmire.

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SUMMARY

A bioassay of 4-chloro-o-toluidine hydrochloride for possible carcinogenicity was conducted by administering the test chemical in feed to F344 rats and B6C3F1 mice.

Groups of 50 rats of each sex were administered 4-chloro-o-toluidine in the diet at one of two doses, either 1,250 or 5,000 ppm, for 107 weeks. Groups of 50 mice of each sex were administered the test chemical in the diet at one of two doses, either 3,750 or 15,000 ppm for the males and either 1,250 or 5,000 ppm for the females, for 99 weeks, except for the high-dose females (92 weeks). Matched controls consisted of 20 untreated rats and 20 untreated mice of each sex. All surviving animals were killed at the end of administration of the test chemical.

Mean body weights of the high-dose rats and the low- and high-dose mice of each sex were lower than those of corresponding controls, and those of the mice were dose related. Mortality was not significantly affected by administration of the test chemical to rats of either sex and survival was 75% or greater at the end of the study in dosed and control groups. Sufficient numbers of rats were at risk for the development of late-appearing tumors. In mice, mortality was dose related for each sex.

In rats no tumors occurred at incidences which could clearly be related to administration of the test chemical.

In both male and female mice, hemangiosarcomas occurred at incidences that were dose related (P less than or equal to 0.001), and in direct comparisons the incidences in the high-dose males and the low- and high-dose females were significantly higher (P less than 0.001) than those in the corresponding controls (males: controls 0/20, low-dose 3/50, high-dose 37/50; females: controls 0/18, low-dose 40/49, high-dose 39/50). The combined incidences of hemangiosarcomas and hemangiomas also were dose related and were significantly higher in the dosed groups of male and female mice than in corresponding controls. There was a high incidence of hemosiderin deposit in the renal tubular epithelium, particularly in mice with hemangiosarcomas.

It is concluded that under the conditions of this bioassay, 4-chloro-o-toluidine hydrochloride was not carcinogenic for F344 rats but was carcinogenic for B6C3F1 mice, inducing hemangiosarcomas and hemangiomas in both males and females.

TABLE OF CONTENTS

		Page
I.	Introduction	1
II.	Materials and Methods	3
	A. Chemical	3
	B. Dietary Preparation	3
	C. Animals	4
	D. Animal Maintenance	4
	E. Subchronic Studies	7
	F. Chronic Studies	10
	G. Clinical and Pathologic Examinations	10
	H. Data Recording and Statistical Analyses	13
III.	Results - Rats	19
	A. Body Weights and Clinical Signs (Rats)	19
	B. Survival (Rats)	19
	C. Pathology (Rats)	22
	D. Statistical Analyses of Results (Rats)	24
IV.	Results - Mice	27
	A. Body Weights and Clinical Signs (Mice)	27
	B. Survival (Mice)	27
	C. Pathology (Mice)	30
	D. Statistical Analyses of Results (Mice)	33
V.	Discussion	35
VI.	Bibliography	39

APPENDIXES

Appendix A	Summary of the Incidence of Neoplasms in Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet	41
Table A1	Summary of the Incidence of Neoplasms in Male Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet	43

Page

Table A2 Summary of the Incidence of Neoplasms in Female Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 47

Appendix B Summary of the Incidence of Neoplasms in Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 51

Table B1 Summary of the Incidence of Neoplasms in Male Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 53

Table B2 Summary of the Incidence of Neoplasms in Female Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 57

Appendix C Summary of the Incidence of Nonneoplastic Lesions in Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 61

Table C1 Summary of the Incidence of Nonneoplastic Lesions in Male Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 63

Table C2 Summary of the Incidence of Nonneoplastic Lesions in Female Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 67

Appendix D Summary of the Incidence of Nonneoplastic Lesions in Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 71

Table D1 Summary of the Incidence of Nonneoplastic Lesions in Male Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 73

Table D2 Summary of the Incidence of Nonneoplastic Lesions in Female Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 77

Appendix E Analyses of the Incidence of Primary Tumors in Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 81

Page

Table E1 Analyses of the Incidence of Primary Tumors in Male Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 83

Table E2 Analyses of the Incidence of Primary Tumors in Female Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 88

Appendix F Analyses of the Incidence of Primary Tumors in Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 91

Table F1 Analyses of the Incidence of Primary Tumors in Male Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 93

Table F2 Analyses of the Incidence of Primary Tumors in Female Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 98

TABLES

Table 1 4-Chloro-o-Toluidine Hydrochloride Subchronic Feeding Studies in Rats and Mice 8

Table 2 4-Chloro-o-Toluidine Hydrochloride Chronic Feeding Studies in Rats 11

Table 3 4-Chloro-o-Toluidine Hydrochloride Chronic Feeding Studies in Mice 12

FIGURES

Figure 1 Growth Curves for Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 20

Figure 2 Survival Curves for Rats Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 21

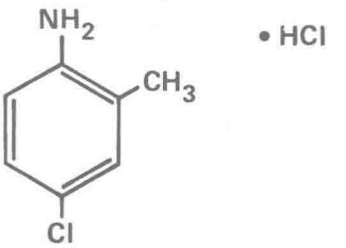
Page

Figure 3 Growth Curves for Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 28

Figure 4 Survival Curves for Mice Administered 4-Chloro-o-Toluidine Hydrochloride in the Diet 29

I. INTRODUCTION

4-Chloro-o-toluidine hydrochloride (CAS 3165-93-3: NCI C02368) is used commercially as an intermediate for dyestuffs intended for textiles, e.g., pigment yellow 49 and pigment red 7, as well as C.I. 12800, azoic coupling component 8, and azoic diazo component 11 (Society of Dyers and Colourists, 1971). These latter two components are used in the synthesis of some azoic dyes, which are made by a two-stage process involving diazotization of a primary amine component and coupling of the diazotized amine with a naphthol-derived coupling component (Society of Dyers and Colourists, 1971). The discovery of the greater number of these colorants derived from 4-chloro-o-toluidine was made in 1921 (Society of Dyers and Colourists, 1971). 4-Chloro-o-toluidine is currently produced in the United States by at least one manufacturer (USITC, 1977a); imports during 1976 amounted to 25,000 pounds (USITC, 1977b).



4-Chloro-o-toluidine hydrochloride

Studies were initiated by the NCI in the 1960's which were designed to assess the carcinogenic effects of monocyclic aromatic amines (Homburger, 1972; Weisburger et al., in press). During these studies, LD_{50}'s of 4-chloro-o-toluidine by intraperitoneal administration were determined to be as follows: male and female Charles River CD-1 mice, 720 and 680 mg/kg, respectively, and male and female Charles River CD rats, 560 and 700 mg/kg, respectively (Weisburger et al., in press). The chronic studies showed 4-chloro-o-toluidine to be carcinogenic in mice but not in rats. Because prior studies were conducted with relatively small numbers of animals, 4-chloro-o-toluidine was selected for study in the Carcinogenesis Testing Program, using an expanded bioassay protocol.

II. MATERIALS AND METHODS

A. Chemical

4-Chloro-o-toluidine hydrochloride (2-amino-4-chlorotoluene hydrochloride) was obtained from American Aniline Products as a fine, light-pink powder. The infrared spectrum was consistent with its chemical structure. The eluate from high-pressure liquid chromatography (reversed-pack packing; mobile phase of 50% methanol in 0.01M KH_2PO_4; with absorption determined at 280 nm) contained two components, one of which was greater than 99% of the total amount of material eluated. Thin-layer chromatography showed only one spot.

B. Dietary Preparation

Test diets containing 4-chloro-o-toluidine hydrochloride were prepared weekly in 6-kg batches at appropriate doses. A known weight of the chemical was first mixed with an equal weight of autoclaved Wayne® Sterilizable Lab Meal containing 4% fat (Allied Mills, Inc., Chicago, Ill.), using a mortar and pestle. The mixing was continued with second and third additions of feed,

and final mixing was performed with the remaining quantity of feed for a minimum of 15 minutes in a Patterson-Kelly twin-shell blender with an intensifier bar. The diets were stored at 7°C until used.

C. Animals

Male and female F344 (Fischer) rats and B6C3F1 mice were obtained as 4-week-old weanlings, all within 3 days of the same age, from the NCI Frederick Cancer Research Center (Frederick, Md.). The animals were housed within the test facility for 2 weeks and then were assigned four rats to a cage and five mice to a cage on a weight basis for each cage of animals of a given species and sex. The initial weight for male rats was 90 to 105 g, averaging at least 100 g; for female rats, 80 to 95 g, averaging at least 90 g; for male mice, 18 to 22 g, averaging at least 19.5 g; and for female mice, 17 to 21 g, averaging at least 18.5 g. Individual animals were identified by ear punch.

D. Animal Maintenance

The animals were housed in polycarbonate cages (Lab Products,

Inc., Garfield, N.J.), 19 x 10-1/2 x 8 inches for the rats and 11-1/2 x 7-1/2 x 5 inches for the mice. The cages were suspended from aluminum racks (Scientific Cages, Inc., Bryan, Tex.) and were covered by nonwoven, polyester-fiber, 12-mil-thick filter paper (Hoeltge, Inc., Cincinnati, Ohio). The bedding used was Absorb-dri® hardwood chips (Northeastern Products, Inc., Warrenburg, N.Y.). The feed supplied was presterilized Wayne® Sterilizable Lab Meal containing 4% fat, provided ad libitum in suspended stainless steel hoppers and replenished at least three times per week. Water, acidified to pH 2.5, was supplied ad libitum from glass bottles with sipper tubes (Lab Products, Inc.) suspended through the tops of the cages.

The contaminated bedding was disposed of through an enclosed vacuum line that led to a holding tank from which the bedding was fed periodically into an incinerator. The cages were sanitized twice per week and the feed hoppers twice per month at 82 to 88°C in a tunnel-type cagewasher (Industrial Washing Corp., Mataway, N. J.), using the detergents, Clout® (Pharmacal Research Laboratories, Greenwich, Conn.) or Oxford D'Chlor (Oxford Chemicals, Atlanta, Ga.). The glass bottles and sipper tubes were sanitized at 82 to 88°C in a tunnel-type bottle washer (Consolidated Equipment Supply Co., Mercersburg, Pa.) three times per week, using a Calgen Commercial Division

detergent (St. Louis, Mo.). The racks for the cages were sanitized at or above 82°C in a rack washer (Consolidated Equipment Supply Co.) once per month, using the Calgen Commercial Division detergent, and the filter paper was changed at the same time.

Animal rooms were maintained at 22 to 24°C and 45 to 55% relative humidity. Incoming air was passed through a filter of 65% efficiency and a bag filter of 95% efficiency at the intake and expelled without recirculation through a "Z"-type roughing filter of 30% efficiency and a bag system of 90 to 95% efficiency at the exhaust (American Air Filters, Louisville, Ky.; Mine Safety Appliances, Pittsburgh, Pa.). Room air was changed 15 times per hour. The air pressure was maintained negative to a clean hallway and positive to a return hallway. Fluorescent lighting was provided automatically on a 12-hour-per-day cycle.

Rats administered 4-chloro-o-toluidine hydrochloride and their corresponding controls were housed in the same room as rats on feeding studies of the following chemicals:

(CAS 156-62-7) calcium cyanamide
(CAS 86-30-6) N-nitrosodiphenylamine

Mice administered 4-chloro-o-toluidine hydrochloride and their

corresponding controls were housed in the same room as mice on feeding studies of the following chemicals:

(CAS 128-37-0) butylated hydroxytoluene (BHT)
(CAS 97-77-8) tetraethylthiuram disulfide
(CAS 148-18-5) sodium diethyldithiocarbamate
(CAS 95-53-4) o-toluidine hydrochloride

E. Subchronic Studies

Subchronic feeding studies were conducted to estimate the maximum tolerated doses (MTD's) of 4-chloro-o-toluidine hydrochloride, on the basis of which two concentrations (referred to in this report as "low" and "high" doses) were selected for administration in the chronic studies. Groups of five rats and mice of each sex were fed diets containing 4-chloro-o-toluidine hydrochloride at one of several doses for 7 weeks, followed by 1 week of additional observation, and groups of five control animals of each species and sex were administered basal diet only. In rats, two separate tests were conducted for males and three for females. Each animal was weighed twice per week. Table 1 shows the doses fed, the survival of animals in each dosed group at the end of the study, and the mean body weights of dosed animals at week 7 expressed as percentages of mean body weights of controls.

At the end of the subchronic studies, all animals were killed

Table 1. 4-Chloro-o-Toluidine Hydrochloride Subchronic Feeding Studies in Rats and Mice

	Male			Female		
	Dose (ppm)	Survival(a)	Mean Weight at Week 7 as % of Control	Dose (ppm)	Survival	Mean Weight at Week 7 as % of Control
RATS						
First Study						
	0	5/5	100			
	250	5/5	93			
	500	5/5	94			
	1,000	5/5	95			
	2,000	5/5	94			
	4,000	5/5	92			
Second Study				First Study		
	0	5/5	100	0	5/5	100
	6,000	5/5	91	6,000	5/5	92
	6,200	5/5	98	6,200	5/5	90
	6,500	5/5	99	6,500	5/5	93
	7,000	5/5	92	7,000	5/5	89
	8,000	5/5	89	8,000	5/5	91
	10,000	5/5	92	10,000	5/5	90
				Second Study		
				0	5/5	100
				1,000	5/5	103
				2,500	5/5	101
				3,000	5/5	98
				4,000	5/5	101
				Third Study		
				0	5/5	100
				6,200	5/5	81
				12,500	5/5	67
				25,000	5/5	55
				50,000	0/5	--
MICE						
	0	5/5	100			
	2,000	5/5	103			
	4,000	5/5	96			
	5,000	5/5	99			
	7,500	5/5	97			
	10,000	5/5	98			
	15,000	5/5	89	0	5/5	100
				15,000	5/5	90
				17,500	5/5	90
				20,000	5/5	78

(a) Number surviving/number in group.