

NTP REPORT
ON THE
TOXICOLOGY STUDY
OF DIISOPROPYLCARBODIIMIDE
(CAS NO. 693-13-0)

IN GENETICALLY MODIFIED
(FVB Tg.AC HEMIZYGOUS) MICE
AND CARCINOGENICITY STUDY
OF DIISOPROPYLCARBODIIMIDE
IN GENETICALLY MODIFIED
[B6.129-*Trp53*^{tm1Brd} (N5) HAPLOINSUFFICIENT] MICE
(DERMAL STUDIES)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

March 2007

NTP GMM 10

NIH Publication No. 07-4427

National Institutes of Health
Public Health Service
U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

FOREWORD

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA). Established in 1978, the NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

The Genetically Modified Model (GMM) Report series began in 2005 with studies conducted by the NTP. The studies described in the GMM Report series are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected agents in laboratory animals that have been genetically modified. These genetic modifications may involve inactivation of selected tumor suppressor functions or activation of oncogenes that are commonly observed in human cancers. This may result in a rapid onset of cancer in the genetically modified animal when exposure is to agents that act directly or indirectly on the affected pathway. An absence of a carcinogenic response may reflect either an absence of carcinogenic potential of the agent or that the selected model does not harbor the appropriate genetic modification to reduce tumor latency and allow detection of carcinogenic activity under the conditions of these subchronic studies. Substances selected for NTP toxicity and carcinogenicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. The interpretive conclusions presented in NTP GMM Reports are based only on the results of these NTP studies. Extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection *per se* is not an indicator of a substance's carcinogenic potential.

The NTP conducts its studies in compliance with its laboratory health and safety guidelines and FDA Good Laboratory Practice Regulations and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. Studies are subjected to retrospective quality assurance audits before being presented for public review.

NTP GMM Reports are indexed in the NIH/NLM PubMed database and are available free of charge electronically on the NTP website (<http://ntp.niehs.nih.gov>) or in hardcopy upon request from the NTP Central Data Management group at cdm@niehs.nih.gov or (919) 541-3419.

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CONTENTS

ABSTRACT	5
EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY	8
TECHNICAL REPORTS REVIEW SUBCOMMITTEE	9
SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS	10
INTRODUCTION	11
MATERIALS AND METHODS	17
RESULTS	23
DISCUSSION AND CONCLUSIONS	27
REFERENCES	29
APPENDIX A Summary of Lesions in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Diisopropylcarbodiimide	33
APPENDIX B Summary of Lesions in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Diisopropylcarbodiimide	39
APPENDIX C Organ Weights and Organ-Weight-to-Body-Weight Ratios	43
APPENDIX D Chemical Characterization and Dose Formulation Studies	45

SUMMARY

Background

Diisopropylcarbodiimide is used as a chemical reagent. We tested if diisopropylcarbodiimide could cause cancer in two different strains of genetically modified mice.

Methods

We applied solutions containing diisopropylcarbodiimide in ethanol to the backs of female Tg.AC mice five times per week for 20 weeks and to female p53 haploinsufficient mice for 27 weeks. The daily doses were 4.38, 8.75, 17.5, 35, or 70 milligrams of diisopropylcarbodiimide per kilogram body weight. Animals given the ethanol solution alone served as the control groups. Tissues from over 20 sites were examined for every animal.

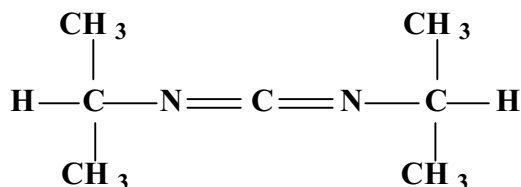
Results

Exposure to diisopropylcarbodiimide had no effect on the Tg.AC mice. In p53 haploinsufficient mice, the only response seen was an increase in minimal epidermal hyperplasia in the skin at the site of dermal application in mice receiving 70 milligrams of diisopropylcarbodiimide per kilogram of body weight.

Conclusions

We conclude that diisopropylcarbodiimide did not cause cancer in the genetically modified mice used in these studies.

ABSTRACT



Diisopropylcarbodiimide

CAS No. 693-13-0

Chemical Formula: $\text{C}_7\text{H}_{14}\text{N}_2$ Molecular Weight: 126.20

Synonyms: 1,3-Diisopropylcarbodiimide; *N,N'*-diisopropylcarbodiimide; *N,N'*-methanetetraylbis (2-propanamine)

Diisopropylcarbodiimide is used as a reagent for a variety of reactions including peptide syntheses. The National Cancer Institute nominated diisopropylcarbodiimide for study as a representative chemical in the alkylcarbodiimide class because of its acute toxicity, widespread low-level human exposure, and the absence of data on health effects. Female Tg.AC hemizygous or p53 haploinsufficient mice were administered diisopropylcarbodiimide (greater than 99% pure) dermally for 20 or 27 weeks, respectively.

20-WEEK STUDY IN Tg.AC HEMIZYGOUS MICE

Groups of 10 female Tg.AC hemizygous mice received dermal applications of 0, 4.38, 8.75, 17.5, 35, or 70 mg diisopropylcarbodiimide/kg body weight in ethanol, 5 days a week for 20 weeks. Twelve animals died or were sacrificed moribund prior to the end of the study; two each from vehicle controls, 4.38, 8.75, and 17.5 mg/kg groups, and four from the 35 mg/kg group. Premature deaths were not associated with chemical-related lesions. Odontoma, a common spontaneous finding in Tg.AC hemizygous mice, resulting in jaw malformation, malocclusion, and loss of body condition,

occurred in the majority of control, 4.38, 8.75, and 17.5 mg/kg animals that died prematurely. Of the surviving animals, mean body weights were similar to those of vehicle controls. There were no significant changes in organ weights and no treatment-related clinical findings. No neoplasms or nonneoplastic lesions were attributed to administration of diisopropylcarbodiimide.

27-WEEK STUDY IN p53 HAPLOINSUFFICIENT MICE

Groups of 15 female p53 haploinsufficient mice received dermal applications of 0, 4.38, 8.75, 17.5, 35, or 70 mg/kg diisopropylcarbodiimide in ethanol, 5 days a week for 27 weeks. All animals survived to the end of the study. Mean body weights were similar to those of vehicle controls, and there were no treatment-related clinical findings. At necropsy there were no treatment-related gross lesions. Microscopically, there was a higher incidence of treatment-related, predominantly minimal epidermal hyperplasia at the site of application in 70 mg/kg mice than in vehicle controls. No neoplasms were attributed to the administration of diisopropylcarbodiimide.

CONCLUSIONS

Under the conditions of this 27-week study, there was *no evidence of carcinogenic activity** of diisopropylcarbodiimide in female p53 haploinsufficient mice administered 4.38, 8.75, 17.5, 35, or 70 mg/kg in ethanol.

There were no treatment-related neoplasms or nonneoplastic lesions in female Tg.AC hemizygous mice administered 4.38, 8.75, 17.5, 35, or 70 mg/kg in ethanol for 20 weeks.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 8. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Report appears on page 10.

Summary of the 20-Week Toxicology Study of Diisopropylcarbodiimide in Female Tg.AC Hemizygous Mice

Doses in ethanol by dermal application	Vehicle control, 4.38, 8.75, 17.5, 35, and 70 mg/kg
Body weights	Exposed groups similar to the vehicle control group
Survival rates	8/10, 8/10, 8/10, 8/10, 6/10, 10/10
Nonneoplastic effects	None
Neoplastic effects	None

Summary of the 27-Week Carcinogenesis Study of Diisopropylcarbodiimide in Female p53 Haploinsufficient Mice

Doses in ethanol by dermal application	Vehicle control, 4.38, 8.75, 17.5, 35, and 70 mg/kg
Body weights	Exposed groups similar to the vehicle control group
Survival rates	15/15, 15/15, 15/15, 15/15, 15/15, 15/15
Nonneoplastic effects	<u>Skin</u> : epidermal hyperplasia (0/15, 0/15; 0/15, 0/15, 0/15, 8/15)
Neoplastic effects	None
Level of evidence of carcinogenic activity	No evidence

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in this Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (**clear evidence and some evidence**); one category for uncertain findings (**equivocal evidence**); one category for no observable effects (**no evidence**); and one category for experiments that cannot be evaluated because of major flaws (**inadequate study**). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

For studies showing multiple chemical-related neoplastic effects that if considered individually would be assigned to different levels of evidence categories, the following convention has been adopted to convey completely the study results. In a study with clear evidence of carcinogenic activity at some tissue sites, other responses that alone might be deemed some evidence are indicated as “were also related” to chemical exposure. In studies with clear or some evidence of carcinogenic activity, other responses that alone might be termed equivocal evidence are indicated as “may have been” related to chemical exposure.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

**NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE**

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Report on diisopropylcarbodiimide on September 28, 2005, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On September 28, 2005, the draft Report on the toxicology and carcinogenesis studies of diisopropylcarbodiimide in genetically modified mouse models received public review by the National Toxicology Program's Board of Scientific Counselors' Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. R.S. Chhabra, NIEHS, introduced the toxicology and carcinogenicity studies of diisopropylcarbodiimide in genetically modified mice by explaining the NTP's exploration of the use of transgenic mice as a possible alternative test model by comparing results with the standard 2-year bioassay. He described the design and results of studies of diisopropylcarbodiimide in two transgenic mouse strains. No carcinogenic responses were noted in the genetically modified mouse studies or the 2-year rat and mouse studies. The proposed conclusions were *no evidence of carcinogenic activity* of diisopropylcarbodiimide in female p53 haploinsufficient mice administered 4.38, 8.75, 17.5, 35, or 70 mg/kg in ethanol. There were no treatment-related neoplasms or nonneoplastic lesions in female Tg.AC hemizygous mice administered 4.38, 8.75, 17.5, 35, or 70 mg/kg in ethanol for 20 weeks.

Dr. Vore, the first principal reviewer, thought the study was well conducted and written and had no scientific or editorial criticisms.

Dr. Sikka, the second principal reviewer, noted that the p53 protein was protective, rather than enhancing malignancy, as stated in the background information. He inquired why the study durations were shorter than in some other GMM studies reported.

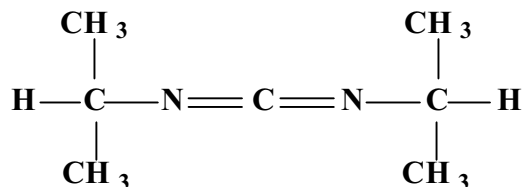
Dr. Crump, the third principal reviewer, also agreed with the proposed conclusions. He inquired why one of the transgenic mouse studies was called a carcinogenicity study and the other a toxicology study.

Dr. Chhabra replied that the ideal study duration was being developed at the time the studies started; often papillomas were seen as early as 9 weeks for positive controls. Dr. J.R. Bucher, NIEHS, added that subsequent information has indicated that 9 months was closer to the optimum duration for maximizing study sensitivity.

Dr. Chhabra explained that a previous Technical Reports Review Subcommittee had felt the Tg.AC model, as a promotion reporter, was not a sufficient indicator of carcinogenicity while the p53 model might be; therefore, studies with the former were called toxicology studies and the latter carcinogenicity studies.

Dr. Vore moved and Dr. Elwell seconded that the conclusions be accepted as written. The motion was accepted unanimously with five votes.

INTRODUCTION



Diisopropylcarbodiimide

CAS No. 693-13-0

Chemical Formula: $\text{C}_7\text{H}_{14}\text{N}_2$ Molecular Weight: 126.20

Synonyms: 1,3-Diisopropylcarbodiimide; *N,N'*-diisopropylcarbodiimide; *N,N'*-methanetetraylbis (2-propanamine)

CHEMICAL AND PHYSICAL PROPERTIES

Diisopropylcarbodiimide is a colorless liquid, with a boiling point of 145° to 148° C, a flash point of 33° C (closed cup), a refractive index of 1.433, and a density of 0.806 g/mL (*Aldrich*, 1988). It is flammable; soluble in chloroform, methylene chloride, acetonitrile, dioxane, dimethylformamide, and tetrahydrofuran; and reacts with water to form 1,3-diisopropylurea. Diisopropylcarbodiimide is a member of the carbodiimide class of chemicals.

Diisopropylcarbodiimide is available in purities ranging from 97% to 99% in quantities up to 1,000 kg. The main impurities are unreacted isocyanates and polymerized carbodiimides (*Janssen Chimica*, 1990; *Kuney*, 1990).

PRODUCTION, USE, AND HUMAN EXPOSURE

Diisopropylcarbodiimide is manufactured primarily by four processes. In the first process, diisopropylcarbodiimide is produced by extended or excessive heating of isopropyl isocyanate from 100° to 250° C under anhy-

drous conditions to condense the carbodiimide with elimination of carbon dioxide. A number of catalysts are effective in accelerating this reaction to the extent of making it a practical synthesis for this symmetrical carbodiimide. The phospholine oxides are particularly effective catalysts, although simple trialkylphosphine oxides or triethyl phosphate may be used (*Chadwick and Cleveland*, 1979). Other organometallic catalysts, including tetraisopropyltitanate and tetraisopropylzirconate, are also used to produce diisopropylcarbodiimide (*Budnick*, 1968; *Smeltz*, 1969).

In a second process, *N,N'*-diisopropylthiourea is reacted with cyanuric chloride in dichloromethane to yield an oily product, which, when hydrolyzed with sodium hydroxide and heated, yields diisopropylcarbodiimide and trithiocyanuric acid (*Furumoto*, 1971a). Thirdly, diisopropylcarbodiimide can be obtained by treating *N,N'*-diisopropylthiourea in dichloromethane with dichlorodicyanobenzoquinone; the resultant mixture is evaporated and heated in sodium hydroxide to yield diisopropylcarbodiimide (*Furumoto*, 1971b). In a fourth process patented by Celanese Corporation in 1967, a reaction mass consisting of diisopropylthiourea, lead

oxide, and water is heated to refluxing temperature, the mixture is distilled, and diisopropylcarbodiimide is separated by decantation (White and Mullin, 1967).

Although carbodiimides were discovered in 1873, it was not until the early 1950s that they were used in industry. Reactivity of these compounds with free carboxyl groups made them valuable as stabilizing agents in elastomers, natural rubber, and many types of polyolefins, polyesters, resins, fibers, cellulose esters, and foam materials to protect against deterioration. In 1953, it was discovered that carbodiimides are potent condensing agents for mono- and diesters of phosphoric acid and for the corresponding di- and tetraesters of pyrophosphoric acid. Since then, these chemicals have been widely used in the synthesis of *ortho*- and pyrophosphate esters, nucleotides, cyclic phosphates, oligoribonucleotides, polynucleotides, nucleoside-5'-phosphoramidates, and mixed anhydrides (Azzi *et al.*, 1984).

Diisopropylcarbodiimide is a useful reagent for peptide syntheses, especially solid-phase peptide synthesis. For example, diisopropylcarbodiimide is used as a peptide coupling reagent in the synthesis of protected peptide proteins of scorpion neurotoxin II, the *N*-hydroxysuccinamide active ester of diethylenetriaminepentaacetic acid (DTPA) (which is subsequently used in a process for conjugating DTPA to proteins), *N*-acyl ureas, and 2-alkoxyoxazolones from alkoxycarbonylamino acids and as a condensing reagent in dipeptide synthesis (Bates *et al.*, 1981; Orlowska *et al.*, 1983; Izdebski and Pelka, 1984; Kricheldorf *et al.*, 1985; Paxton *et al.*, 1985; Sabatier *et al.*, 1987). In addition, diisopropylcarbodiimide is used as a chemical intermediate in the synthesis of *N*-silylformamides (Ojima *et al.*, 1974) and in the preparation of polyimide precursor coatings for electrophoretic image display fabrication (Minnema and Van der Zande, 1988).

There are numerous other proposed uses for diisopropylcarbodiimide. It has been reported that insoluble resin-bound diisopropylcarbodiimide, in the presence of 1-hydroxybenzotriazole, catalyzed the synthesis of the cyclic peptide gramicidin S (Nutt, 1978). Also, alpha, beta-dehydroamino acid derivatives can be made from serine, threonine, or cysteine using diisopropylcarbodiimide (Miller, 1980). Diisopropylcarbodiimide has been used as a stabilization reagent for a solution of *S*-(diisopropylaminoethyl)-*O*-ethyl methylphosphonothioate (Buckles and Lewis, 1977). In organic synthesis, diisopropylcarbodiimide has been used in cycloaddition reactions to form a number of heterocyclic compounds

(Aldrich, 1988). Like most carbodiimides, diisopropylcarbodiimide has also been used in dehydration reactions for conjugated alkadienoic acid and anhydride preparations. In the presence of (dimethylamino)pyridinium toluenesulfonate as a catalyst, diisopropylcarbodiimide has been used to prepare polyester from hydroxyphenyl-terminated carboxylic acids (Moore and Stupp, 1990). Diisopropylcarbodiimide is used as a stabilizer for the military nerve agent Sarin (Nasr *et al.*, 1988).

Human exposure to diisopropylcarbodiimide could occur during the extensive handling of the compound that occurs during the synthesis of peptides and other compounds in the chemical, pharmaceutical, and recombinant DNA industries.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

NTP conducted absorption and distribution studies via the dermal route in mice and rats (NTP, 2006). Data indicated that only approximately 1% to 2% of the radiolabeled dose was absorbed due to the high volatility of diisopropylcarbodiimide. The majority of the dose was recovered in the charcoal-impregnated appliances used to cover the site of application. Approximately 90.7% of the dose was unabsorbed and 90.5% was contained in the appliance and skin wash. Less than 0.2% of the administered dose was recovered from any tissue site. The majority of the dose rapidly volatilized from the dose site and was not available for absorption.

TOXICITY

Experimental Animals

The oral LD₅₀ for diisopropylcarbodiimide in mice is 36 mg/kg (RTECS, 1991a). While the chemistry of diisopropylcarbodiimide and dicyclohexylcarbodiimide are virtually identical, interactions with biomolecules differ. Two cases in point involve ATPase. The Ca²⁺-ATPase of sarcoplasmic reticulum vesicles is readily inactivated by diisopropylcarbodiimide (Murphy, 1981). Although the related chemical, dicyclohexylcarbodiimide, readily inactivates *Escherichia coli* BF1-ATPase at 0.05 mM, diisopropylcarbodiimide shows almost no inactivation at this concentration (Satre *et al.*, 1979).

The National Toxicology Program (NTP, 2006) conducted 2-week and 3-month dermal studies of diisopropylcarbodiimide in male and female F344/N rats and B6C3F₁ mice. In the 2-week studies, rats and mice were

administered 0, 3, 9, 27, 81, or 242 mg or 0, 1, 3, 9, 27, or 81 mg diisopropylcarbodiimide per animal, respectively. All rats administered 27 mg or greater and all mice administered 9 mg or greater died before the end of the studies. Nonneoplastic lesions were observed at the site of application in dosed animals. In the 3-month studies, rats were administered 0, 10, 20, 40, 80, or 160 mg/kg, and mice were administered 0, 17.5, 35, 70, 140, or 280 mg/kg. All rats administered 160 or 80 mg/kg, all mice administered 280 mg/kg, and most mice administered 140 mg/kg died early. Nonneoplastic lesions were observed at the site of application and in the brain, lung, and liver of rats and at the site of application and in the thymus of mice. No chemical-related changes in hematology or clinical chemistry parameters were reported in rats or mice.

Humans

Delayed, temporary blindness was reported in a worker following an acute occupational exposure to diisopropylcarbodiimide vapor (Moyer, 1990). The worker had cleaned up a 1 L spill of diisopropylcarbodiimide while wearing a respirator, laboratory coat, and impervious gloves. Approximately 12 to 18 hours later, the worker experienced hazy vision followed by mild pain that maximized 34 hours after the exposure. Damage to the outer layer of the cornea resulted in blindness that subsided over a 2-week period. Ellis (1991) noted that this injury resembled mild to moderate mustard gas injury, for which the postulated mechanism of action is alkylation of nucleophilic functional groups of intracellular components, occurring within minutes of exposure and leading to cellular dysfunction and even cell death. This author noted that it is reasonable to assume that all alkylcarbodiimides are capable of functioning as alkylating agents and are therefore potential vesicants and carcinogens.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Experimental Animals

No chemical-related differences in reproductive parameters were observed in F344/N rats administered 0, 10, 20, 40, 80, or 160 mg/kg or B6C3F₁ mice administered 0, 17.5, 35, 70, 140, or 280 mg/kg in 3-month dermal studies (NTP, 2006). No other information on the reproductive or developmental toxicity of diisopropylcarbodiimide in animals was found in the literature.

A single oral reproductive study in rats of the related chemical carbodiimide has been reported (RTECS, 1991b). In this study, a dose of 2,450 mg carbodiimide/kg caused preimplantation mortality, 1,750 mg/kg caused paternal effects (testes, epididymis, sperm duct, prostate, seminal vesicle, Cowper's gland, and accessory glands), 208 mg/kg affected postimplantation mortality and the live birth index. In the same study, 2,600 mg/kg affected live birth index and growth statistics in newborn rats.

Humans

No information on the reproductive or developmental toxicity of diisopropylcarbodiimide in humans was found in the literature.

CARCINOGENICITY

Experimental Animals

In 2-year dermal studies of diisopropylcarbodiimide conducted by the NTP (2006), male and female F344/N rats and B6C3F₁ mice were administered 0, 10, 20, or 40 mg/kg. No neoplastic effects occurred in either rats or mice. Because of severe neurological signs exhibited by 40 mg/kg male rats, a neuropathological review of these animals was performed, which revealed neuronal necrosis, cerebral hemorrhage, and/or fibrinoid vascular necrosis of the brain, and spinal axonopathy was also present. Other chemical-related nonneoplastic lesions in rats included hemorrhage in the lung of males and chronic lung inflammation and alveolar epithelium hyperplasia in females; at the site of application, epidermal hyperplasia and chronic inflammation occurred in males and females. In male mice, epidermal hyperplasia and focal dermal inflammation occurred at the site of application.

Humans

No epidemiology studies or case reports associating diisopropylcarbodiimide exposure with cancer risk in humans were found in the literature.

GENETIC TOXICITY

Diisopropylcarbodiimide was not mutagenic in *Salmonella typhimurium* strains TA97, TA98, TA100, or TA1535 with or without induced rat or hamster liver S9 activation enzymes when tested at 3 to 1,000 µg/plate (NTP, 2006). Witt *et al.* (1999) described the results of

a series of *in vivo* mutagenicity tests with diisopropylcarbodiimide. The chemical was shown to induce micronuclei in erythrocytes of male and female mice treated by skin painting for a period of 3 months. An additional subchronic micronucleus investigation was performed with diisopropylcarbodiimide in male mice, in which the chemical was administered by skin painting for 4 months and weekly or biweekly counts of micronucleated erythrocytes, as well as percent polychromatic erythrocytes (% PCEs), were obtained. Results of this study confirmed the activity of diisopropylcarbodiimide that was observed in the 3-month skin painting study. However, short-term tests using a three-injection protocol with diisopropylcarbodiimide in rats and in mice showed no evidence of micronucleus induction in bone marrow PCEs. Results of a single-injection mouse micronucleus test with diisopropylcarbodiimide gave results that were concluded to be equivocal; frequencies of micronucleated PCEs were significantly increased in peripheral blood samples obtained 48 hours after injection, but in bone marrow, the frequency of micronucleated PCEs, although elevated at 24 and 48 hours in two of three trials, did not differ significantly from the control levels. The authors suggested that one interpretation of these results might be that diisopropylcarbodiimide induces chromosomal damage in erythrocytes soon after treatment, and that, due to the kinetics of erythrocyte maturation, this damage is detectable as micronucleated cells in blood but not bone marrow at the standard time points assayed for micronucleus induction. Alternatively, the spleen might be a target organ for diisopropylcarbodiimide, and damage to splenic cells might result in increased frequencies of micronucleated erythrocytes in the circulating erythrocyte population compared to the population of erythrocytes residing in the bone marrow.

BACKGROUND ON GENETICALLY ALTERED MICE

Mutation and/or deletions of tumor suppressor genes or activation of protooncogenes can disrupt cell function and predispose an animal to cancer. In the current studies, two genetically altered mouse models with either a loss of heterozygosity in a critical cancer gene (*Trp53*) or a gain of oncogene function (*Ha-ras*) were used to determine how these animals would respond to diisopropylcarbodiimide exposure. The Tg.AC hemizygous and p53 haploinsufficient mice have been shown to be susceptible to the rapid development of cancer and

are being evaluated by the National Institute of Environmental Health Sciences (NIEHS) and the NTP as models for identifying chemical toxicity and/or chemical carcinogenic processes (Tennant *et al.*, 1996; Pritchard *et al.*, 2003).

FVB/N-TgN(v-Ha-ras)Led (Tg.AC) Hemizygous Mouse Model

The Tg.AC hemizygous mouse (on an FVB/N background) was developed by Leder *et al.* (1990) by introduction via pronuclear injection of a tripartite transgene composed of the promoter of the mouse embryonic zeta-globin gene, through the *v-Ha-ras* coding sequence, with point mutations in codons 12 and 59, and an SV40 polyadenylation sequence.

The Tg.AC hemizygous transgenic mouse model has been evaluated as a reporter phenotype (skin papillomas) in response to either genotoxic or nongenotoxic carcinogens, including tumor promoters (Spalding *et al.*, 1993, 1999; Tennant *et al.*, 1999). The Tg.AC strain of mice is hemizygous for a mutant *v-Ha-ras* transgene. The model was developed by Leder *et al.* (1990) with an inducible zeta-globin promoter driving the expression of a mutated *v-Ha-ras* oncogene and is regarded as a genetically initiated model. With the exception of bone marrow, constitutive expression of the transgene cannot be detected in adult tissues. The transgene is usually transcriptionally silent until activated by certain treatments including full-thickness wounding, ultraviolet irradiation, or exposure to some chemicals (Cannon *et al.*, 1997; Trempus *et al.*, 1998). Point mutations in the *Ha-ras* gene are believed to be early events in the induction of skin papillomas and malignancies. Topical application of carcinogens to the shaved dorsal surface of Tg.AC hemizygous mice induces epidermal squamous cell papillomas or carcinomas, a reporter phenotype that defines the activity of the chemical. The oral route of administration can also generate tumor responses in the skin of Tg.AC hemizygous mice and lead to squamous cell papillomas and/or carcinomas of the forestomach (Wyde *et al.*, 2004). To date, the appearance of either spontaneous or induced tumors has been shown to involve transgene expression. However, the mechanism of response by the Tg.AC hemizygous model to chemical carcinogens is not yet understood.

In NIEHS studies, mice are exposed beginning at 2 months of age for a total of 6 to 9 months. Cutaneous papillomas at various sites have been reported at 10% and 7% incidence in 33-week-old control male and

female Tg.AC hemizygous mice, respectively (Mahler *et al.*, 1998). Cutaneous papillomas occurring at sites such as the lip, pinnae, prepuce, and vulva suggest a possible relationship to grooming and chronic irritation. Up to 32% of Tg.AC homozygous and heterozygous male or female mice can develop odontogenic tumors as early as 33 weeks (Wright *et al.*, 1995; Mahler *et al.*, 1998). A number of different tumor types occur in untreated Tg.AC hemizygous mice at an incidence of greater than 3% including odontogenic tumors, forestomach papillomas, cutaneous papillomas, alveolar-bronchiolar adenomas, salivary gland duct carcinomas, and erythro-leukemia (Mahler *et al.*, 1998). In the FVB mouse (the background strain for the Tg.AC hemizygous mouse), alveolar/bronchiolar neoplasms occur at 14 months of age (Mahler *et al.*, 1996).

The Tg.AC hemizygous mouse model was used in the current Report for studies of diisopropylcarbodiimide because this model has been reported to detect both nongenotoxic and genotoxic carcinogens (Spalding *et al.*, 1993; Tennant *et al.*, 1995, 1996; Pritchard *et al.*, 2003).

B6.129-Trp53^{tm1Brd} (N5)

Haploinsufficient Mouse Model

The heterozygous B6.129-Trp53 (N12)^{tm1Brd(+/-)} mouse (on a B6.129S7 background) was developed by Donehower *et al.* (1992). A null mutation was introduced into one p53 allele by homologous recombination in murine embryonic stem cells. Insertion of a neo cassette resulted in deletion of a 450-base pair gene fragment containing 106 nucleotides of exon 5 and approximately 350 nucleotides of intron 4.

Trp53, a nuclear protein, plays an essential role in the regulation of the cell cycle, specifically in the transition from G₀ to G₁, as well as G₂ to M, and the spindle apparatus. The p53 protein has a short half-life and exists at a very low concentration under normal cell physiological conditions. However, in DNA damaged cells that are able to replicate, p53 is expressed in high amounts with a significant increase in half-life due to posttranslational modification (phosphorylation or acetylation). Mutations in p53 may also increase the protein half-life and alter functions that may contribute to transformation and development of the malignant phenotype. The p53 protein is a DNA-binding protein containing DNA-binding, oligomerization, and transcription activation domains. Many amino acid residues in different p53 domains may

be phosphorylated or acetylated, which may determine specific p53 functions. It is postulated to bind as a tetramer to a p53-binding site and activate expression of downstream genes that inhibit growth and/or invasion or promote apoptosis, functioning as a tumor suppressor. This protein is critical to tumor suppression in humans and rodents. Mutants of p53 that fail to bind the consensus DNA binding site, and hence are unable to function as tumor suppressors, frequently occur in human cancers. Alterations of the *Trp53* gene occur not only as somatic mutations in human malignancies, but also as germline mutations in some cancer-prone families with Li-Fraumeni syndrome.

The mouse heterozygous for a p53 null allele (+/-) has only a single functional wild-type p53 allele, which provides a target for mutagens. The p53 protein has a short half-life and exists at a very low concentration under normal cell physiological conditions. However, in DNA damaged cells that are able to replicate, p53 is expressed in high amounts with a significant increase in half-life due to posttranslational modification (phosphorylation or acetylation). Mutations in p53 may also increase the protein half-life and alter functions that may contribute to transformation and development of the malignant phenotype. The p53 tumor suppressor gene is one of the most common sites for mutations and gene alterations in human cancer (Harris, 1996a,b,c). Many amino acid residues in different p53 domains may be phosphorylated or acetylated, which may determine specific p53 functions.

Heterozygous p53^(+/-) mice develop normally, and like humans and other mammals, develop cancer (primarily lymphomas or sarcomas) with age, but often with decreased latency.

STUDY RATIONALE

Diisopropylcarbodiimide and dicyclohexylcarbodiimide were nominated by the National Cancer Institute for toxicity and carcinogenicity studies as representatives of the carbodiimide chemical class. The results of the dicyclohexylcarbodiimide studies will be presented in separate reports.

In the studies presented in this Report, the dermal route of exposure was chosen to mimic the primary route of human exposure. The studies in Tg.AC hemizygous and p53 haploinsufficient genetically modified mouse

models were performed as part of the NTP effort to evaluate alternative test models that require shorter exposure times, are less expensive to study, and use fewer animals when compared to the traditional 2-year study design.

The NTP has completed 2-year carcinogenicity studies of diisopropylcarbodiimide in rats and mice. The results of those studies have been reported in a separate Technical Report (NTP, 2006).

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION

Diisopropylcarbodiimide

Diisopropylcarbodiimide was obtained from Aldrich Chemical Company (Milwaukee, WI) in two lots. Lot 01207BG was used during the 20-week study; lot 13016JS was used during the 27-week study. Identity and purity analyses were conducted by the analytical chemistry laboratory at Midwest Research Institute (Kansas City, MO) and by the study laboratories at Microbiological Associates, Inc. (Bethesda, MD; 20-week study), and BioReliance Corporation (Rockville, MD; 27-week study); physical properties, moisture content, and stability of the bulk diisopropylcarbodiimide were determined by the analytical chemistry laboratory. Reports on analyses performed in support of the diisopropylcarbodiimide studies are on file at the National Institute of Environmental Health Sciences.

Lot 01207BG, a colorless liquid, was identified as diisopropylcarbodiimide by the study laboratory using infrared (IR) spectroscopy. Lot 13016JS was identified as diisopropylcarbodiimide by the study laboratory using IR spectroscopy and by the analytical chemistry laboratory using IR, proton nuclear magnetic resonance (NMR), and ultraviolet/visible spectroscopy and gas chromatography (GC)/mass spectrometry. All spectra were consistent with the structure of diisopropylcarbodiimide and with literature references.

The purity of lot 01207BG was determined by the study laboratory using GC. The purity of lot 13016JS was determined by the study laboratory using GC and by the analytical chemistry laboratory using thin layer chromatography (TLC) and GC. The moisture content of lot 13016JS was determined by the analytical chemistry laboratory using Karl Fischer titration; the boiling point and relative density of this lot were also measured by the analytical chemistry laboratory.

For lot 01207BG, GC indicated a major peak and five impurity peaks with areas ranging from 0.05% to 0.27%

of the total peak area. Fourteen minor impurities were present in the sample chromatograms. The overall purity of lot 01207BG was determined to be 99.35%.

For lot 13016JS, the boiling point and relative density were consistent with the literature value for diisopropylcarbodiimide. Karl Fischer titration indicated 0.06% water in the bulk chemical. TLC detected a major, a minor, and two trace spots. GC at the study laboratory indicated a relative purity of 100.4% when compared to a frozen reference sample. GC indicated a major peak and five impurity peaks with a combined area of approximately 0.5% of the total peak area; the purity of the test article was determined to be approximately 99.5%. The overall purity of lot 13016JS was determined to be greater than 99%.

The analytical chemistry laboratory conducted accelerated stability studies of lot 13016JS with samples stored for 2 weeks in amber vials with Teflon[®]-lined septa at approximately 5°, 25°, and 60° C compared to frozen samples from the same lot stored at -20° C. Analysis using GC indicated that the test article was stable when protected from light at temperatures up to approximately 60° C for 2 weeks. To ensure stability, the bulk chemical was stored at room temperature under nitrogen, protected from light as recommended by the manufacturer. Periodic purity analyses of the bulk chemical were performed during both studies using GC. No degradation of the bulk chemical was detected.

Anhydrous Ethanol

Anhydrous ethanol was obtained from Pharmco (Brookfield, CT) in one lot (number unknown) used for the 20-week study and two lots (9901074 and 9801193) that were used for the 27-week study. Identity and purity analyses of all lots were conducted by the study laboratories. The chemical, a clear liquid, was identified as ethanol using IR spectroscopy; the sample spectra were a good match for the reference spectrum of ethanol. The purity of each lot was determined using GC. No impurities were detected that exceeded a relative concentration of 0.1% in any lot.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared by mixing diisopropylcarbodiimide and anhydrous ethanol to give the required concentrations; formulations were prepared once not more than 7 days prior to the study start and every 3 weeks thereafter for the 20-week study or monthly for the 27-week study. The dose formulations were stored at room temperature for the 20-week study and the 27-week study until April 6, 1999, or later, when formulations were stored at -20°C for up to 35 days.

Because the dose formulations were true solutions of the test article in ethanol, homogeneity studies were not performed. Prior to the 20-week study, a stability study of 2.19 mg/mL dose formulations of lot 01207BG was conducted by the study laboratory using GC; stability was confirmed for up to 35 days for the dose formulation stored at ambient temperature in sealed containers under a nitrogen headspace and for up to 3 hours when exposed to light and air at ambient temperature.

Periodic analyses of the dose formulations of diisopropylcarbodiimide were conducted by the study laboratories using GC. During the 20-week study, the dose formulations were analyzed four times; animal room samples of these dose formulations were also analyzed. All 20 dose formulations analyzed were within 10% of the target concentrations; all 20 of the animal room samples analyzed were within 10% of the target concentrations. Dose formulations were analyzed four times during the 27-week study; animal room samples of these dose formulations were also analyzed. Of the 20 dose formulations analyzed, all were within 10% of the target concentrations; 9 of 20 animal room samples were within 10% of the target concentrations. Unusual degradation was observed in the animal room samples from the formulations prepared on February 17, 1999, and June 7, 1999. Attempts to discover the cause of the degradation in the formulations were not conclusive, but the most likely cause, water in the anhydrous ethanol, was eliminated. To prevent degradation of the test chemical, formulations prepared on or after April 6, 1999, were stored at -20°C .

20-WEEK STUDY

Female FVB/N-TgN(v-Ha-ras)Led (Tg.AC) hemizygous mice were obtained from Taconic Farms, Inc. (Germantown, NY). On receipt, the mice were 4 weeks

old. Animals were quarantined for 13 days and were 6 weeks old on the first day of the study. Before the study began, five mice were randomly selected for parasite evaluation and gross observation for evidence of disease. Blood was collected from five randomly selected vehicle control mice at study termination. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b); all results were negative.

The dose levels selected for this study were based on results from a 3-month toxicity study in B6C3F₁ mice (NTP, 2006). Groups of 10 male and 10 female mice were dermally administered 0, 17.5, 35, 70, 140, or 280 mg/kg diisopropylcarbodiimide 5 days per week for 3 months. All 280 mg/kg males and females and nine 140 mg/kg males and females died before the end of the study. The final body weight gain of 70 mg/kg males was significantly less than that of vehicle controls. At the site of application, there were significant increases in the incidences of epidermal hyperplasia in males and females administered 70 mg/kg or greater, chronic inflammation in 140 and 280 mg/kg males and 70 mg/kg or greater females, and sebaceous gland hyperplasia in 140 mg/kg males. Based on these results, 70 mg/kg was selected as the high dose for the current studies with four lower doses of 35, 17.5, 8.75, and 4.38 mg/kg.

Groups of 10 female mice received dermal applications of 0, 4.38, 8.75, 17.5, 35, or 70 mg/kg in ethanol, 5 days per week for 20 weeks. A dosing volume of 2 mL ethanol per kg body weight was applied to the center of a shaved dorsal area extending from the posterior of the scapulae to the base of the tail, with the application site no greater than 10% of the animal's body surface. A minimum of two consecutive days of dosing were performed prior to terminal sacrifice, with the last dose within 24 hours of sacrifice. Animals were housed individually, with feed and water available *ad libitum*. Body weights were recorded initially, weekly, and at the end of the study, and clinical findings were recorded weekly. Details of the study design and animal maintenance are summarized in Table 1.

Necropsies were performed on all animals. The heart, right kidney, liver, lung, and thymus were weighed. Microscopic examinations were performed on all vehicle control and 70 mg/kg animals and all animals that died early. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned

to a thickness of 4 to 6 μm , and stained with hematoxylin and eosin. Table 1 lists the tissues and organs routinely examined.

27-WEEK STUDY

Female B6.129-*Trp53*^{tm1Brd} (N5) haploinsufficient mice were obtained from Taconic Farms, Inc. (Germantown, NY). On receipt, the animals were approximately 5 weeks old. Animals were quarantined for 13 days and were 7 weeks old on the first day of the study. Before initiation of the study, five mice were randomly selected for parasite evaluation and gross observation for evidence of disease. Blood was collected at study termination from five randomly selected vehicle control mice. The sera were analyzed for antibody titers to rodent viruses; all results were negative.

The dose levels selected for this study were based on results from a 3-month toxicity study in B6C3F₁ mice (NTP, 2006) as described for the 20-week study. Groups of 15 female mice received dermal applications of 0, 4.38, 8.75, 17.5, 35, or 70 mg/kg in ethanol 5 days per week for 27 weeks. The dosing volume of 2 mL/kg was applied to the center of a shaved dorsal area posterior of the scapulae to the base of the tail, with the application site no greater than 10% of the animal's body surface. A minimum of two consecutive days of dosing were performed prior to terminal sacrifice, with the last dose given within 24 hours of sacrifice. Mice were housed individually, with feed and water available *ad libitum*. Body weights were recorded initially, weekly, and at the

end of the study, and clinical findings were recorded weekly. Details of the study design and animal maintenance are summarized in Table 1.

Necropsies were performed on all animals. Histopathologic examinations were performed on all mice. Skin samples from the site of application were cut in an anterior-posterior direction, trimmed or marked with indelible ink to indicate the anterior end, and placed on an index card to keep them flattened during fixation. Control skin samples from an untreated area were also taken. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μm , and stained with hematoxylin and eosin. Table 1 lists the tissues and organs routinely examined.

Upon completion of the laboratory pathologist's histopathologic evaluation, the slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were sent to an independent pathology laboratory where quality assessment was performed. Results were reviewed and evaluated by the NTP Pathology Working Group (PWG); the final diagnoses represent a consensus of contractor pathologists and the PWG. Details of these review procedures have been described by Maronpot and Boorman (1982) and Boorman *et al.* (1985).

TABLE 1
Experimental Design and Materials and Methods in the Dermal Studies of Diisopropylcarbodiimide

20-Week Study	27-Week Study
Study Laboratory Microbiological Associates, Inc. (Bethesda, MD)	BioReliance Corporation, (Rockville, MD)
Strain and Species FVB/N-TgN(v-Ha-ras)Led (Tg.AC) hemizygous mice	B6.129-Trp53 ^{tm1Brd} (N5) haploinsufficient mice
Animal Source Taconic Farms, Inc. (Germantown, NY)	Taconic Farms, Inc. (Germantown, NY)
Time Held Before Studies 13 days	13 days
Average Age When Studies Began 6 weeks	7 weeks
Date of First Dose June 14, 1995	January 4, 1999
Duration of Dosing 20 weeks	27 weeks
Date of Last Dose October 31, 1995	July 6-7, 1999
Necropsy Dates November 1, 1995	July 7-8, 1999
Average Age at Necropsy 26 weeks	34 weeks
Size of Study Groups 10 females	15 females
Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 20-week studies
Animals per Cage 1	1
Method of Animal Identification Tail tattoo	Tail tattoo
Diet NIH-07 open formula meal/pelleted diet (Zeigler Brothers, Inc., Gardners, PA), available <i>ad libitum</i> , changed weekly	Irradiated NTP-2000 open formula meal/pelleted diet (Ziegler Brothers, Inc., Gardners, PA), available <i>ad libitum</i> , changed weekly
Water Tap water (Washington Suburban Sanitary Commission Potomac Plant) via automatic watering system(Edstrom Industries, Inc., Waterford, WI), available <i>ad libitum</i>	Tap water (Washington Suburban Sanitary Commission Potomac Plant) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available <i>ad libitum</i>
Cages Polycarbonate (Lab Products, Inc., Seaford, DE), rotated every 2 weeks	Polycarbonate (Lab Products, Inc., Seaford, DE), rotated every 2 weeks

TABLE 1
Experimental Design and Materials and Methods in the Dermal Studies of Diisopropylcarbodiimide

20-Week Study	27-Week Study
Bedding	
Sani-chips [®] (P.J. Murphy Forest Products Corp., Montville, NJ), changed weekly	Same as 20-week study
Cage Filters	
Remay 2016 (Snow Filtration, West Chester, OH), changed weekly	Same as 20-week study
Racks	
Stainless (Lab Products, Inc., Seaford, DE), changed every 2 weeks	Same as 20-week study
Animal Room Environment	
Temperature: 72° ± 3° F	Temperature: 72° ± 3° F
Relative humidity: 50% ± 15%	Relative humidity: 50% ± 15%
Room fluorescent light: 12 hours/day	Room fluorescent light: 12 hours/day
Room air changes: 10/hour	Room air changes: 10/hour
Doses	
0, 4.38, 8.75, 17.5, 35, or 70 mg/kg administered in ethanol (2 mL/kg body weight) 5 days per week	0, 4.38, 8.75, 17.5, 35, or 70 mg/kg administered in ethanol (2 mL/kg body weight) 5 days per week
Type and Frequency of Observation	
Observed twice daily; animals were weighed initially, weekly, and at the end of the studies; clinical findings were recorded weekly.	Observed twice daily; animals were weighed initially, weekly, and at the end of the studies; clinical findings were recorded weekly.
Method of Sacrifice	
Carbon dioxide asphyxiation	Same as 20-week study
Necropsy	
Necropsies were performed on all animals. Organs weighed were heart, right kidney, liver, lung, and thymus.	Necropsies were performed on all animals.
Histopathology	
Complete histopathologic examinations were performed on all vehicle control and 70 mg/kg animals and all animals that died early. In addition to gross lesions and tissue masses the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, gallbladder, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung with mainstream bronchi, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, salivary gland, skin (site of application), spleen, stomach (forestomach and glandular), thymus, thyroid gland, trachea, urinary bladder, and uterus. Chemical-related lesions (target organs) were identified and these organs plus gross lesions and tissue masses were examined in the remaining dose groups.	Histopathologic examinations were performed on all mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, kidney, liver, lymph nodes (mandibular, mediastinal, and mesenteric), lung and bronchi, mammary gland, ovary, parathyroid gland, pituitary gland, skin (site of application and control site), spleen, stomach (forestomach and glandular), thymus, thyroid gland, trachea, and uterus. Tissues with chemical-related lesions (target organs) were identified in the high dose group and successively lower dose groups were examined until a group with no lesions was reached.

STATISTICAL METHODS

Calculation and Analysis

of Lesion Incidences

The incidences of lesions are presented in Tables A1, A2, B1, and B2 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. The Fisher exact test (Gart *et al.*, 1979), a procedure based on the overall proportion of affected animals, was used to determine significance.

Analysis of Continuous Variables

Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Average

severity values were analyzed for significance with the Mann-Whitney U test (Hollander and Wolfe, 1973).

QUALITY ASSURANCE METHODS

The 20- and 27-week studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from these studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assurance contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Report.

RESULTS

MICE

20-WEEK STUDY

There were no deaths considered related to diisopropylcarbodiimide administration. Twelve animals died or were sacrificed moribund prior to the end of the study; two each from the vehicle control, 4.38, 8.75, and 17.5 mg/kg groups, and four from the 35 mg/kg group (Table 2). There were no treatment-related clinical findings that contributed to the morbidity/mortality of these animals. The most consistent incidental finding was odontoma (odontogenic tumor), which occurred without a dose relationship in animals from vehicle control and several dose groups. Odontogenic tumors are spontaneous neoplasms in Tg.AC mice with a reported incidence of 17.3% in control female Tg.AC hemizygous mice at 33 weeks of age (Mahler *et al.*, 1998). They arise in the mandible or maxilla from primitive tooth structures resulting in progressive malformation of the jaw, malocclusion and difficulty in eating with loss of

body condition. All other animals survived to the end of the study. Of the surviving animals, mean body weights were similar to those of vehicle controls (Table 2 and Figure 1). There were no significant differences in organ weights between dosed groups and the vehicle control group (Table C1).

No neoplasms or nonneoplastic lesions were observed in female Tg.AC hemizygous mice that were considered related to administration of diisopropylcarbodiimide, including in the skin at the site of application. In the forestomach, single squamous cell papillomas occurred in two of 10 animals macroscopically examined from each of the 8.75, 17.5, and 70 mg/kg groups. Although this is slightly higher than the reported background incidence of 10.3% in control female Tg.AC hemizygous mice (Mahler *et al.*, 1998), there was no dose-related increase in the number or multiplicity of tumors, and they were considered unrelated to administration of diisopropylcarbodiimide.

TABLE 2
Survival and Body Weights of Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Diisopropylcarbodiimide

Dose (mg/kg)	Survival ^a	Mean Body Weight ^b (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
0	8/10 ^c	20.9 ± 0.5	27.8 ± 0.5	7.1 ± 0.6	
4.38	8/10 ^d	20.7 ± 0.4	29.1 ± 1.6	7.8 ± 1.5	105
8.75	8/10 ^e	21.1 ± 0.4	30.0 ± 2.7	8.9 ± 2.4	108
17.5	8/10 ^f	21.5 ± 0.6	26.9 ± 1.0	5.3 ± 0.8	97
35	6/10 ^g	20.3 ± 0.4	26.0 ± 1.4	5.7 ± 1.4	94
70	10/10	20.6 ± 0.4	25.6 ± 0.5	5.0 ± 0.5	92

^a Number of animals surviving at 20 weeks/number initially in group

^b Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study. Differences from the vehicle control group are not significant by Dunnett's test.

^c Week of death: 14, 16

^d Week of death: 14, 20

^e Week of death: 15, 19

^f Week of death: 12, 16

^g Week of death: 7, 7, 8, 10

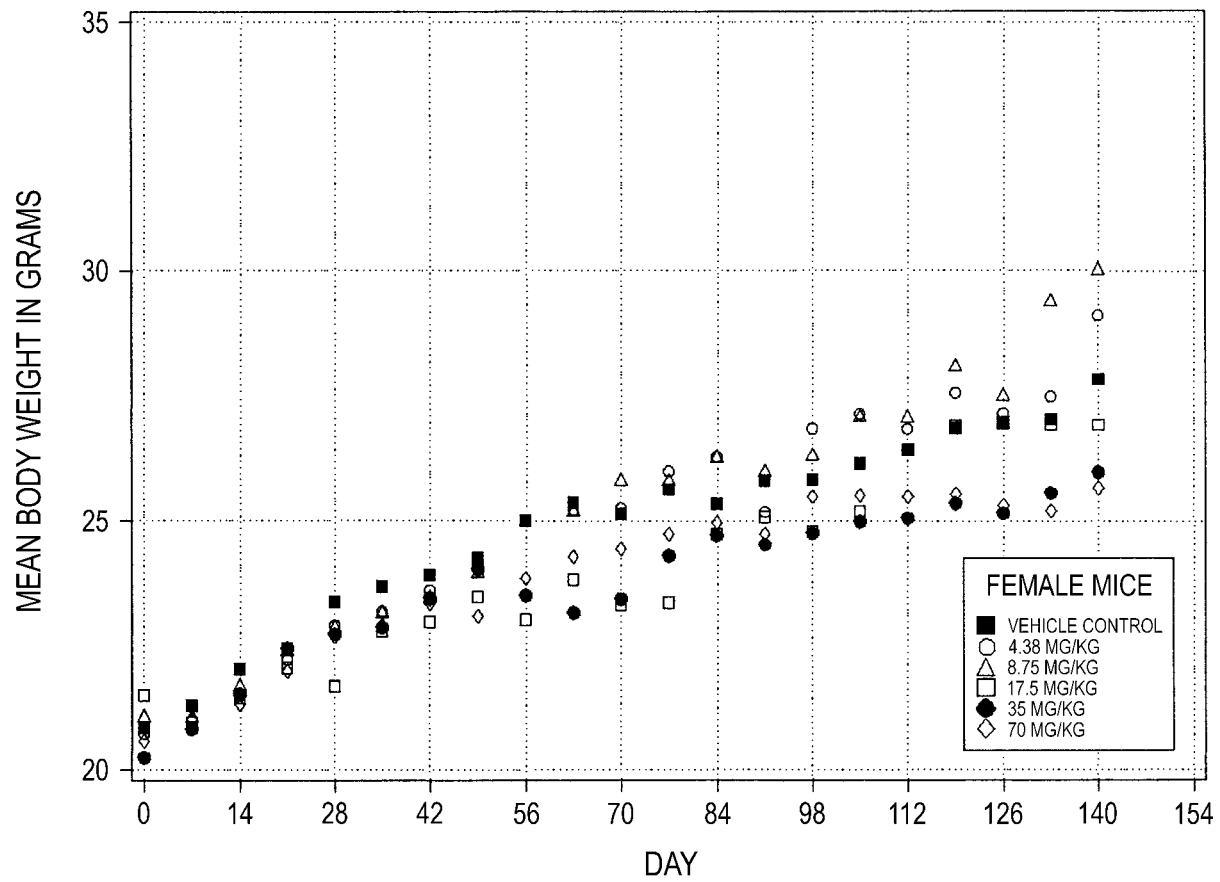


FIGURE 1
Growth Curves for Female Tg.AG Hemizygous Mice
Dermally Administered Diisopropylcarbodiimide for 20 Weeks

27-WEEK STUDY

All animals survived to the end of the study (Table 3). Mean body weights of the dosed groups were similar to that of the vehicle control group (Table 3 and Figure 2). There were no clinical findings related to diisopropylcarbodiimide administration. There were no treatment-related gross lesions.

Epidermal hyperplasia of the skin occurred at the site of application in 8 of 15 mice receiving 70 mg/kg while none were seen in any other dose groups or controls

(Table B2). Epidermal hyperplasia was of minimal severity and focal to multifocal in six animals; one had mild hyperplasia, and one had moderate hyperplasia. The moderate hyperplasia was a more extensive/diffuse change. Epidermal hyperplasia was characterized as an increase in epidermal thickness from the normal one or two cells up to three cells thick (minimal hyperplasia), four cells thick (mild hyperplasia), or five cells thick (moderate hyperplasia). No neoplasms were observed in female p53 haploinsufficient mice that were considered related to administration of diisopropylcarbodiimide.

TABLE 3
Survival and Body Weights of Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Diisopropylcarbodiimide

Dose (mg/kg)	Survival ^a	Mean Body Weight ^b (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
0	15/15	18.1 ± 0.4	32.1 ± 1.7	14.0 ± 1.5	
4.38	15/15	17.9 ± 0.4	30.9 ± 1.1	13.0 ± 0.9	96
8.75	15/15	18.1 ± 0.3	32.6 ± 1.1	14.5 ± 1.0	101
17.5	15/15	17.7 ± 0.4	30.2 ± 1.5	12.5 ± 1.3	94
35	15/15	17.9 ± 0.3	32.5 ± 1.4	14.6 ± 1.2	101
70	15/15	17.8 ± 0.3	30.6 ± 1.7	12.8 ± 1.6	95

^a Number of animals surviving at 27 weeks/number initially in group

^b Weights and weight changes are given as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test.

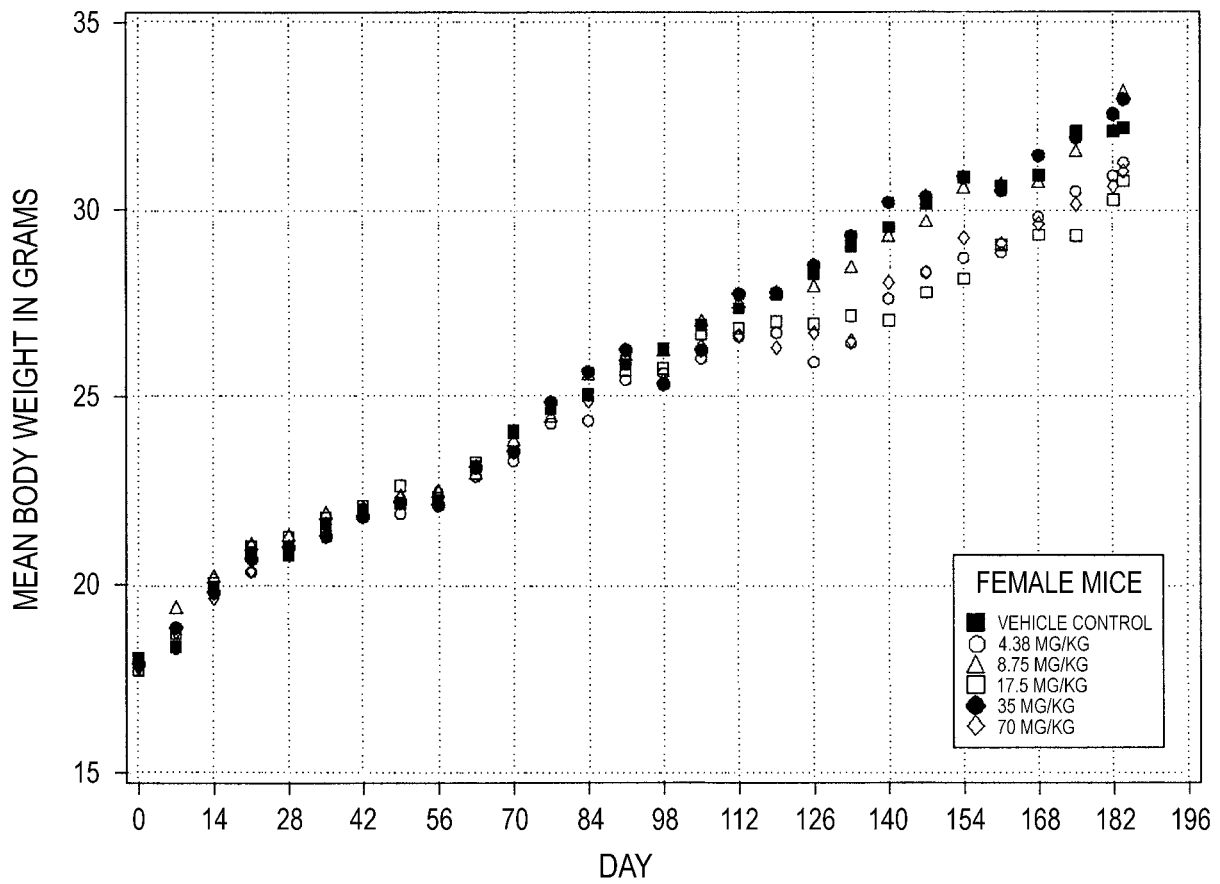


FIGURE 2
Growth Curves for Female p53 Haploinsufficient Mice
Dermally Administered Diisopropylcarbodiimide for 27 Weeks

DISCUSSION AND CONCLUSIONS

Genetically altered mouse models carry activated oncogenes or inactivated tumor suppressor genes known to be involved in neoplastic processes in both humans and rodents. For some chemicals, this trait allows these mice to respond or show the effects of carcinogens in less than the usual 2 years of conventional rodent carcinogenicity tests and within a time frame in which few, if any, spontaneous tumors would arise. Target or reporter genes also allow direct molecular and cellular analysis of a chemical's effects and can provide additional mechanistic information about its mode of action. The National Toxicology Program (NTP) has been using a number of transgenic rodent models for studies of carcinogenesis [p16^{Ink4a}, p53^{+/-}, Tg.AC (v-Ha-ras), and Tg.NK (MMTV/c-neu)] and mutagenesis [C57BL/6J-TgN (phiX174am3, cs70) 54Hvm] for the last few years. The p53 haploinsufficient model has demonstrated preferential identification of genotoxic/mutagenic carcinogens, and the Tg.AC hemizygous model has responded to both genotoxic and nongenotoxic carcinogens. The goal of the NTP is to select transgenic animal models that best mimic human tissue processes, providing a firmer foundation for applying hazard data from animals to humans (Chhabra *et al.*, 2003).

Studies on diisopropylcarbodiimide and dicyclohexylcarbodiimide are part of the database the NTP is establishing to help in developing more rapid and economical assays to reduce dependence on the 2-year bioassay. Dicyclohexylcarbodiimide and diisopropylcarbodiimide are representatives of the carbodiimide chemical class, are widely used reagents in the chemical and pharmaceutical industries, and are used increasingly in the field of bioenergetics. Dicyclohexylcarbodiimide and diisopropylcarbodiimide are widely used as coupling and condensation agents, especially in peptide synthesis, and as stabilizing agents. Of the two chemicals, dicyclohexylcarbodiimide currently has the greater usage. However, this use pattern might change because the reaction side products of diisopropylcarbodiimide are easier to remove than those of dicyclohexylcarbodiimide, and the use of diisopropylcarbodiimide is increasing. Dicyclohexylcarbodiimide and diisopropylcarbodi-

imide are listed in the Toxic Substances Control Act (TSCA) Inventory and on the European Inventory of existing commercial chemical substances, but information on specific production volumes is not available. Research scientists in the bioenergetics field are particularly at risk. Occupational exposure to these chemicals occurs primarily by dermal contact or inhalation.

The NTP has completed traditional dermal toxicity and carcinogenicity studies on diisopropylcarbodiimide and reported the results in a Technical Report (NTP, 2006). There were no diisopropylcarbodiimide treatment-related increases in the incidences of neoplastic lesions in those studies. In the current studies, two genetically altered mouse models with either a loss of heterozygosity in a critical cancer suppressor gene (p53) or a gain of oncogene function (Tg.AC) were used to determine how these animals would respond to diisopropylcarbodiimide treatment. The dose concentrations used in the studies were derived from the 3-month toxicity study performed in the B6C3F₁ mice that showed skin as the primary target site for diisopropylcarbodiimide toxicity (NTP, 2006). The dose concentrations selected for the 2-year bioassay were 0, 10, 20, and 40 mg/kg. Because of different strain backgrounds in the transgenic mouse models and shorter durations of treatment, the doses selected for these studies were 0, 4.38, 8.75, 17.5, 35, and 70 mg/kg. This broader dosing regimen consisting of five dose groups was expected to achieve maximum tolerated doses at the upper end of the dosing range.

The findings from the Tg.AC hemizygous mouse study show that with diisopropylcarbodiimide treatment there were no neoplastic or nonneoplastic lesions. Similarly, there were no diisopropylcarbodiimide treatment-related neoplastic or nonneoplastic lesions in the p53 haploinsufficient mouse model with the exception of epidermal hyperplasia in the 70 mg/kg group at the site of application. These findings agree with the lack of diisopropylcarbodiimide carcinogenic activity in the traditional 2-year rodent bioassay (NTP, 2006). However, the diisopropylcarbodiimide studies performed in the Tg.AC hemizygous model were some of the earliest NTP

studies exploring the value of the model as an alternate to the traditional bioassay. The protocol used does not meet the current study design standards with respect to the number of animals per group. Also, the animals could have tolerated higher doses than 70 mg/kg since no chemical-related effects were seen at this concentration.

Pritchard *et al.* (2003), in a review of the role of transgenic mouse models in carcinogen identification, reported that if a testing strategy of evaluating chemicals in both the p53 haploinsufficient and Tg.AC hemizygous mouse models were adopted, predictions of the carcinogenic potential of chemicals in humans could be made with approximately 83% accuracy. If the 2-year bioassay were added to this combination, approximately the same percentage of predictability could be expected. In comparison, the 2-year bioassay alone yielded a correct determination for 69% of chemicals tested. This lower predictability could be due to a lack of sufficient information in humans for classification of the chemicals that have been identified as carcinogens in the 2-year bioassay. Although transgenic models had a high percentage of correct determinations, they did fail to respond to a number of known or probable human carcinogens, whereas the traditional bioassay identified all of these chemicals. The studies reported by Pritchard *et al.* (2003) are based on a limited database of approximately 100 chemicals from human and animal studies with varying study designs and approaches. Therefore, acceptance of transgenic mouse models individually or in combination as a substitute for the 2-year bioassay remain controversial. Additional studies such as those reported here, as well as basic research in the suitability of these models for identification of chemical carcinogens, is needed. In the meantime, studies from genetically modified models may contribute to the weight of evidence in the risk assessment of chemicals causing adverse health effects in humans.

Diisopropylcarbodiimide is not mutagenic in the *Salmonella* assay (NTP, 2006) and therefore, the chemical might be expected to be negative for carcinogenic activity in the transgenic mouse strains used in this study. However, results of *in vivo* mutagenicity tests showed clear evidence of increased micronucleus frequencies in blood of male and female mice following three or more months of exposure via skin painting (Witt *et al.*, 1999). The results of the peripheral blood micronucleus tests are somewhat surprising because a strong correlation has been reported between positive results in subchronic peripheral blood micronucleus tests and rodent carcinogenicity (Witt *et al.*, 2000). The number of positive studies from which this correlation derives is small, although additional support for the relationship between positive rodent micronucleus test data and carcinogenicity was provided by Morita *et al.* (1997), who reported a 90.5% correlation between carcinogenic activity in humans and positive results in the rodent micronucleus test when data were corrected for known structure-activity considerations with regard to micronucleus assay sensitivity. In addition, Zeiger (1998) reported a 70% correlation between rodent carcinogenicity and positive results in the mouse bone marrow micronucleus test in an unadjusted dataset of 83 NTP chemicals. Thus, the pattern of activity shown by diisopropylcarbodiimide is unusual.

CONCLUSIONS

Under the conditions of this 27-week study, there was *no evidence of carcinogenic activity** of diisopropylcarbodiimide in female p53 haploinsufficient mice administered 4.38, 8.75, 17.5, 35, or 70 mg/kg in ethanol.

There were no treatment-related neoplasms or nonneoplastic lesions in female Tg.AC hemizygous mice administered 4.38, 8.75, 17.5, 35, or 70 mg/kg in ethanol for 20 weeks.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 8. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Report appears on page 10.

REFERENCES

- The Aldrich Library of ^{13}C and ^1H FT-NMR Spectra* (1993). 1st ed., p. 1412(C). Aldrich Chemical Co., Inc., Milwaukee, WI.
- The Aldrich Library of FT-IR Spectra* (1985). 1st ed. (C.J. Pouchert, Ed.), Vol. 1, p. 876 (A). Aldrich Chemical Co., Inc., Milwaukee, WI.
- The Aldrich Library of Infrared Spectra* (1981). 3rd ed. (C.J. Pouchert, Ed.), Vol. 1, spectrum 524C (1). Aldrich Chemical Co., Inc., Milwaukee, WI.
- Aldrich Technical Information Bulletin No. AL-168: 1,3-Diisopropylcarbodiimide (DIC)* (1988). Aldrich Chemical Co., Inc., Milwaukee, WI
- Anonymous (1977). *Spectrosc. Lett.* **10**, 959-970.
- Azzi, A., Casey, R.P., and Nalecz, M.J. (1984). The effect of N,N'-dicyclohexylcarbodiimide on enzymes of bioenergetic relevance. *Biochim. Biophys. Acta* **768**, 209-226.
- Bates, H.S., Jones, J.H., Ramage, W.I., and Witty, M. (1981). Some observations on the activation of alkoxy-carbonylamino acids by diisopropylcarbodiimide. In *Peptides, Proceedings of the 16th European Peptide Symposium* (K. Brunfeldt, Ed.), pp. 185-190.
- Boorman, G.A., Montgomery, C.A., Jr., Eustis, S.L., Wolfe, M.J., McConnell, E.E., and Hardisty, J.F. (1985). Quality assurance in pathology for rodent carcinogenicity studies. In *Handbook of Carcinogen Testing* (H.A. Milman and E.K. Weisburger, Eds.), pp. 345-357. Noyes Publications, Park Ridge, NJ.
- Boorman, G.A., Hickman, R.L., Davis, G.W., Rhodes, L.S., White, N.W., Griffin, T.A., Mayo, J., and Hamm, T.E., Jr. (1986). Serological titers to murine viruses in 90-day and 2-year studies. In *Complications of Viral and Mycoplasmal Infections in Rodents to Toxicology Research and Testing* (T.E. Hamm, Jr., Ed.), pp. 11-23. Hemisphere Publishing Corporation, Washington, DC.
- Buckles, L.C., and Lewis, S.M. (1977). S-(2-Diisopropylamino-ethyl)-O-ethyl methylphosphonothioate stabilized with soluble carbodiimides. U.S. Patent No. 4012464.
- Budnick, E.G. (1968). Triarylsines as catalysts for converting isocyanates to carbodiimides. U.S. Patent No. 3406198.
- Cannon, R.E., Spalding, J.W., Trempus, C.S., Szczesniak, C.J., Virgil, K.M., Humble, M.C., and Tennant, R.W. (1997). Kinetics of wound-induced v-Ha-ras transgene expression and papilloma development in transgenic Tg.AC mice. *Mol. Carcinog.* **20**, 108-114.
- Chadwick, D.H., and Cleveland, T.H. (1979). Isocyanates, Organic. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 3rd ed. (M. Grayson, D. Eckroth, H.F. Mark, D.F. Othmer, C.G. Overberger, and G.T. Seaborg, Eds.), Vol. 13, pp. 797-799. John Wiley and Sons, New York.
- Chhabra, R.S., Bucher, J.R., Wolfe, M., and Portier, C. (2003). Toxicity characterization of environmental chemicals by the U.S. National Toxicology Program: An overview. *Int. J. Hyg. Environ. Health* **206**, 437-445.
- Code of Federal Regulations (CFR) **21**, Part 58.
- Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**, 215-221.
- Dunnnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* **50**, 1096-1121.
- Ellis, H.V. (1991). Treatment for eye irritants. *Chem. Eng. News* **69**, 2.

- Furumoto, S. (1971a). Novel method for the synthesis of carbodiimide. *Nippon Kagaku Zasshi* **92**, 357-360.
- Furumoto, S. (1971b). Novel method for the synthesis of carbodiimide. *Nippon Kagaku Zasshi* **92**, 1005-1007.
- Gart, J.J., Chu, K.C., and Tarone, R.E. (1979). Statistical issues in interpretation of chronic bioassay tests for carcinogenicity. *JNCI* **62**, 957-974.
- Harris, C.C. (1996a). Structure and function of the p53 tumor suppressor gene: Clues for rational cancer therapeutic strategies. *J. Natl. Cancer Inst.* **88**, 1442-1455.
- Harris, C.C. (1996b). p53 tumor suppressor gene: From the basic research laboratory to the clinic—an abridged historical perspective. *Carcinogenesis* **17**, 1187-1198.
- Harris, C.C. (1996c). The 1995 Walter Hubert Lecture – molecular epidemiology of human cancer: Insights from the mutational analysis of the p53 tumour-suppressor gene. *Brit. J. Cancer* **73**, 261-269.
- Hollander, M., and Wolfe, D.A. (1973). *Nonparametric Statistical Methods*, pp. 120-123. John Wiley and Sons, New York.
- Izdebski, J., and Pelka, J. (1984). Peptide synthesis using disubstituted carbodiimides which form dichloromethane-soluble urea derivatives. In *Peptides, Proceedings of the 18th European Peptide Symposium*, (U. Ragnarsson, Ed.) pp. 113-116.
- The Janssen Chimica Catalog Handbook of Fine Chemicals for Research and Industry 1991-1992* (1990). p. 457, Janssen Chimica or Spectrum Chemical Manufacturing Corp., Research Triangle Park, NC.
- Kricheldorf, H.R., Au, M., and Mang, T. (1985). Models of molecular evolution. 2. Stereospecificity of dipeptide syntheses by means of cyanamides and carbodiimides. *Int. J. Pept. Protein Res.* **2**, 149-157.
- Kuney, J.H., Ed. (1990). *Chemyclopedia 91: The Manual of Commercially Available Chemicals*, p. 409. American Chemical Society, Washington, DC.
- Leder, A., Kuo, A., Cardiff, R.D., Sinn, E., and Leder, P. (1990). v-Ha-ras transgene abrogates the initiation step in mouse skin tumorigenesis: Effects of phorbol esters and retinoic acid. *Proc. Natl. Acad. Sci.* **87**, 9178-9182.
- Mahler, J.F., Stokes, W., Mann, P.C., Takaoka, M., and Maronpot, R.R. (1996). Spontaneous lesions in aging FVB/N mice. *Toxicol. Pathol.* **24**, 710-716.
- Mahler, J.F., Flagler, N.D., Malarkey, D.E., Mann, P.C., Haseman, J.K., and Eastin, W. (1998). Spontaneous and chemically induced proliferative lesions in Tg.AC transgenic and p53-heterozygous mice. *Toxicol. Pathol.* **26**, 501-511.
- Maronpot, R.R., and Boorman, G.A. (1982). Interpretation of rodent hepatocellular proliferative alterations and hepatocellular tumors in chemical safety assessment. *Toxicol. Pathol.* **10**, 71-80.
- Miller, M.J. (1980). Isoarea-mediated preparation of dehydro amino acids. *J. Org. Chem.* **45**, 3131-3132.
- Minnema, L., and Van der Zande, J.M. (1988). Pattern generation in polyimide coatings and its application in an electrophoretic image display. *Polym. Eng. Sci.* **28**, 815-822.
- Moore, J.S., and Stupp, S.I. (1990). Room temperature polyesterification. *Macromolecules* **23**, 65-70.
- Morita, T., Asano, N., Awogi, T., Sasaki, Y.F., Sato, S., Shimada, H., Sutou, S., Suzuki, T., Wakata, A., Sofuni, T., and Hayashi, M. (1997). Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (groups 1, 2A and 2B) the summary report of the 6th collaborative study by CSGMT/JEMS MMS. Collaborative Study of the Micronucleus Group Test. Mammalian Mutagenicity Study Group. *Mutat. Res.* **389**, 3-122 (Erratum, 1997, **391**, 259-267).
- Moyer, R.C. (1990). A carbodiimide eye irritant. *Chem. Eng. News* **68**, 2.
- Murphy, A.J. (1981). Kinetics of the inactivation of the ATPase of sarcoplasmic reticulum by dicyclohexylcarbodiimide. *J. Biol. Chem.* **256**, 12,046-12,050.

- Nasr, M.L., Goldman, M., Klein, A.K., and Dacre, J.C. (1988). SCE induction in Chinese hamster ovary cells (CHO) exposed to G agents. *Mutat. Res.* **204**, 649-654.
- National Institute of Standards Technology (NIST) (1995). Finnigan MAT™ NIST™ Library for GCQ/ICIS.
- National Toxicology Program (NTP) (2007). Toxicology and Carcinogenesis Studies of Diisopropylcarbodiimide (CAS No. 693-13-0) in F344/N Rats and B6C3F₁ Mice (Dermal Studies). Technical Report No. 523, NIH Publication No. 07-4473. National Institutes of Health, Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC.
- Nutt, R.F. (1978). Cyclization of peptides; reaction of an oligopeptide with isopropylcarbodiimide bound to polystyrene. U.S. Patent No. 4102877.
- Ojima, I., Inaba, S., and Nagai, Y. (1974). Syntheses of N-silylformamidines by the hydrosilylation of carbodiimides. *Organometal. Chem.* **72**, C11-C13.
- Orlowska, A., Holodowicz, E., and Drabarek, S. (1983). Comparison of dicyclohexylcarbodiimide and diisopropylcarbodiimide as coupling reagents in solid phase peptide syntheses. *Pol. J. Chem.* **56**, 1067-1070.
- Paxton, R.J., Jakowatz, J.G., Beatty, J.D., Beatty, B.G., Vlahos, W.G., Williams, L.E., Clark, B.R., and Shively, J.E. (1985). High-specific-activity ¹¹¹In-labeled anticarcinoembryonic antigen monoclonal antibody: Improved method for the synthesis of diethylenetriaminepentaacetic acid conjugates. *Cancer Res.* **45**, 5694-5699.
- Pritchard, J.B., French, J.E., Davis, B.J., and Haseman, J.K. (2003). The role of transgenic mouse models in carcinogen identification. *Environ. Health Perspect.* **111**, 444-454.
- Rao, G.N., Haseman, J.K., and Edmondson, J. (1989a). Influence of viral infections on body weight, survival, and tumor prevalence in Fischer 344/NCr rats on two-year studies. *Lab. Anim. Sci.* **39**, 389-393.
- Rao, G.N., Piegorsch, W.W., Crawford, D.D., Edmondson, J., and Haseman, J.K. (1989b). Influence of viral infections on body weight, survival, and tumor prevalence of B6C3F₁ (C57BL/6N × C3H/HeN) mice in carcinogenicity studies. *Fundam. Appl. Toxicol.* **13**, 156-164.
- Registry of Toxic Effects of Chemical Substances (RTECS)* [database online] (1991a). Cyanamide. Bethesda, MD: National Institute for Occupational Safety and Health; 1971 to present. Updated quarterly. Available from the National Library of Medicine, Bethesda, MD.
- Registry of Toxic Effects of Chemical Substances (RTECS)* [database online] (1991b). Carbodiimide, diisopropyl. Bethesda, MD: National Institute for Occupational Safety and Health; 1971 to present. Updated quarterly. Available from the National Library of Medicine, Bethesda, MD.
- Sabatier, J.M., Tessier-Rochat, M., Granier, C., Van Rietschoten, J., Pedroso, E., Grandas, A., Albericio, F., and Giralt, E. (1987). Convergent solid phase peptide synthesis. VI. Synthesis by the Fmoc procedure with a modified protocol of two protected segments, sequence 5-17 and 18-31 of the neurotoxin II of the scorpion *Androctonus australis Hector*. *Tetrahedron* **43**, 5973-5980.
- Satre, M., Lunardi, J., Pougeois, R., and Vignais, P.V. (1979). Inactivation of *Escherichia coli* BF1-ATPase by dicyclohexylcarbodiimide. Chemical modification of the beta-subunit. *Biochemistry* **18**, 3134-3140.
- Smeltz, K.C. (1969). Catalysts for preparing carbodiimides. U.S. Patent No. 3426025.
- Spalding, J.W., Momma, J., Elwell, M.R., and Tennant, R.W. (1993). Chemically induced skin carcinogenesis in a transgenic mouse line (TG.AC) carrying a v-HA-ras gene. *Carcinogenesis* **14**, 1335-1341.
- Spalding, J.W., French, J.E., Tice, R.R., Furedi-Machacek, M., Haseman, J.K., and Tennant, R.W. (1999). Development of a transgenic mouse model for carcinogenesis bioassays: Evaluation of chemically induced skin tumors in Tg.AC mice. *Toxicol. Sci.* **49**, 241-254.
- Tennant, R.W., French, J.E., and Spalding, J.W. (1995). Identifying chemical carcinogens and assessing potential risk in short-term bioassays using transgenic mouse models. *Environ. Health Perspect.* **103**, 942-950.
- Tennant, R.W., Spalding, J., and French, J.E. (1996). Evaluation of transgenic mouse bioassays for identifying carcinogens and noncarcinogens. *Mutat. Res.* **365**, 119-127.

- Tennant, R.W., Stasiewicz, S., Mennear, J., French, J.E., and Spalding, J.W. (1999). Genetically altered mouse models for identifying carcinogens. *IARC Sci. Publ.* **146**, 123-150.
- Trempeus, C.S., Mahler, J.F., Ananthaswamy, H.N., Loughlin, S.M., French, J.E., and Tennant, R.W. (1998). Photocarcinogenesis and susceptibility to UV radiation in the v-Ha-ras transgenic Tg.AC mouse. *J. Invest. Dermatol.* **111**, 445-451.
- White, G.T., and Mullin, K.B. (1967). Diisopropylcarbodiimide. U.S. Patent No. 3352908.
- Williams, D.A. (1971). A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics* **27**, 103-117.
- Williams, D.A. (1972). The comparison of several dose levels with a zero dose control. *Biometrics* **28**, 519-531.
- Witt, K.L., Tice, R.R., Shelby, M.D., Chhabra, R.S., and Zeiger, E. (1999). Induction of micronucleated erythrocytes in rodents by diisopropylcarbodiimide and dicyclohexylcarbodiimide: Dependence on exposure protocol. *Environ. Mol. Mutagen.* **33**, 65-74.
- Witt, K.L., Knapton, A., Wehr, C.M., Hook, G.J., Mirsalis, J., Shelby, M.D., and MacGregor, J.T. (2000). Micronucleated erythrocyte frequency in peripheral blood of B6C3F₁ mice from short-term, prechronic, and chronic studies of the NTP Carcinogenesis Bioassay Program. *Environ. Mol. Mutagen.* **36**, 163-194.
- Wright, J.T., Hansen, L., Mahler, J., Szczesniak, C., and Spalding, J.W. (1995). Odontogenic tumours in the v-HA-ras (TG.AC) transgenic mouse. *Arch. Oral Biol.* **40**, 631-638.
- Wyde, M.E., Braen, A.P.J.M., Hejtmancik, M., Johnson, J.D., Toft, J.D., Blake, J.C., Cooper, S.D., Mahler, J., Vallant, M., Bucher, J.R., and Walker, N.J. (2004). Oral and dermal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces cutaneous papillomas and squamous cell carcinomas in female hemizygous Tg.AC transgenic mice. *Toxicol. Sci.* **82**, 34-45.
- Zeiger, E. (1998). Identification of rodent carcinogens and noncarcinogens using genetic toxicity tests: Premises, promises, and performance. *Regul. Toxicol. Pharmacol.* **2**, 85-95.

APPENDIX A
SUMMARY OF LESIONS
IN FEMALE Tg.AC HEMIZYGOUS MICE
IN THE 20-WEEK DERMAL STUDY
OF DIISOPROPYLCARBODIIMIDE

TABLE A1	Summary of the Incidence of Neoplasms in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Diisopropylcarbodiimide	34
TABLE A2	Summary of the Incidence of Nonneoplastic Lesions in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Diisopropylcarbodiimide	36

TABLE A1
Summary of the Incidence of Neoplasms in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Diisopropylcarbodiimide^a

	Vehicle Control	4.38 mg/kg	8.75 mg/kg	17.5 mg/kg	35 mg/kg	70 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Moribund	1	2	2	1	2	
Natural deaths	1			1	2	
Survivors						
Terminal sacrifice	8	8	8	8	6	10
Animals examined microscopically	10	8	10	9	9	10
Alimentary System						
Intestine small, jejunum	(9)	(2)	(2)	(1)	(2)	(10)
Carcinoma	1 (11%)					
Liver	(10)	(6)	(7)	(7)	(6)	(10)
Leukemia erythrocytic	1 (10%)					
Salivary glands	(10)	(2)	(10)	(10)	(10)	(10)
Duct, squamous cell carcinoma					1 (25%)	
Stomach, forestomach	(10)	(2)	(3)	(3)	(2)	(10)
Squamous cell papilloma			2 (67%)	2 (67%)		2 (20%)
Tooth	(3)	(4)	(4)	(6)	(3)	(7)
Odontoma	3 (100%)	2 (50%)	4 (100%)	6 (100%)	1 (33%)	6 (86%)
Genital System						
Ovary	(10)	(2)	(2)	(2)	(4)	(10)
Teratoma benign					2 (50%)	
Yolk sac carcinoma			1 (50%)			
Hematopoietic System						
Spleen	(9)	(5)	(3)	(3)	(6)	(10)
Leukemia erythrocytic	1 (11%)					
Integumentary System						
Skin	(10)	(3)	(4)	(4)	(4)	(10)
Squamous cell papilloma		1 (33%)		1 (25%)		
Squamous cell papilloma, multiple	1 (10%)		1 (25%)			
Dermis, skin, site of application, fibrosarcoma			1 (25%)			
Skin, site of application, squamous cell papilloma						1 (10%)
Skin, site of application, squamous cell papilloma, multiple	1 (10%)		1 (25%)	1 (25%)		
Respiratory System						
Nose	(10)	(5)	(1)	(2)	(4)	(10)
Respiratory epithelium, adenoma	1 (10%)					
Systemic Lesions						
Multiple organs ^b	(10)	(8)	(10)	(9)	(9)	(10)
Leukemia erythrocytic	1 (10%)					

TABLE A1
Summary of the Incidence of Neoplasms in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Diisopropylcarbodiimide

	Vehicle Control	4.38 mg/kg	8.75 mg/kg	17.5 mg/kg	35 mg/kg	70 mg/kg
<i>Systems Examined with no Neoplasms Observed</i>						
Cardiovascular System						
Endocrine System						
General Body System						
Musculoskeletal System						
Nervous System						
Special Senses System						
Urinary System						
Neoplasm Summary						
Total animals with primary neoplasms ^c	6	3	6	7	3	8
Total primary neoplasms	8	3	10	10	4	9
Total animals with benign neoplasms	5	3	6	7	3	8
Total benign neoplasms	6	3	8	10	3	9
Total animals with malignant neoplasms	2		2		1	
Total malignant neoplasms	2		2		1	

^a Number of animals examined microscopically at site and number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Diisopropylcarbodiimide^a

	Vehicle Control	4.38 mg/kg	8.75 mg/kg	17.5 mg/kg	35 mg/kg	70 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Moribund	1	2	2	1	2	
Natural deaths	1			1	2	
Survivors						
Terminal sacrifice	8	8	8	8	6	10
Animals examined microscopically	10	8	10	9	9	10
Alimentary System						
Intestine small, jejunum	(9)	(2)	(2)	(1)	(2)	(10)
Ulcer, chronic active	1 (11%)					
Liver	(10)	(6)	(7)	(7)	(6)	(10)
Hematopoietic cell proliferation	1 (10%)		1 (14%)			1 (10%)
Inflammation			1 (14%)			
Inflammation, focal	7 (70%)	4 (67%)	3 (43%)	7 (100%)	2 (33%)	8 (80%)
Necrosis	1 (10%)		1 (14%)			
Pancreas	(9)	(2)	(2)	(1)	(4)	(10)
Inflammation, focal	1 (11%)					
Stomach, glandular	(10)	(2)	(2)	(2)	(2)	(10)
Inflammation						1 (10%)
Tongue					(1)	
Epithelium, hyperkeratosis					1 (100%)	
Tooth	(3)	(4)	(4)	(6)	(3)	(7)
Hyperplasia, odontogenic					1 (33%)	1 (14%)
Cardiovascular System						
Blood vessel	(10)	(2)	(2)	(2)	(4)	(10)
Aorta, adventitia, inflammation, focal	1 (10%)					
Genital System						
Clitoral gland	(7)	(2)	(1)	(1)	(2)	(8)
Atrophy		1 (50%)		1 (100%)		
Uterus	(10)	(2)	(2)	(2)	(4)	(10)
Hydrometra	1 (10%)					2 (20%)
Bilateral, hydrometra	2 (20%)				1 (25%)	1 (10%)

^a Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Diisopropylcarbodiimide

	Vehicle Control	4.38 mg/kg	8.75 mg/kg	17.5 mg/kg	35 mg/kg	70 mg/kg
Hematopoietic System						
Bone marrow	(10)	(2)	(2)	(2)	(4)	(10)
Myeloid cell, hyperplasia	1 (10%)		1 (50%)			
Lymph node			(1)	(1)		
Infiltration cellular, plasma cell			1 (100%)			
Infiltration cellular, histiocyte			1 (100%)			
Mediastinal, infiltration cellular, histiocyte				1 (100%)		
Lymph node, mandibular	(10)	(2)	(2)	(1)	(4)	(10)
Infiltration cellular, plasma cell	1 (10%)		1 (50%)	1 (100%)		
Infiltration cellular, polymorphonuclear	1 (10%)					1 (10%)
Lymph node, mesenteric	(9)	(2)	(2)	(1)	(4)	(10)
Infiltration cellular, polymorphonuclear	1 (11%)					
Infiltration cellular, histiocyte	1 (11%)		1 (50%)			
Inflammation						1 (10%)
Inflammation, focal			1 (50%)		1 (25%)	
Pigmentation		1 (50%)				
Spleen	(9)	(5)	(3)	(3)	(6)	(10)
Fibrosis				1 (33%)		
Hematopoietic cell proliferation	7 (78%)	5 (100%)	2 (67%)	1 (33%)	5 (83%)	9 (90%)
Lymphoid follicle, necrosis					1 (17%)	
Thymus	(9)	(2)	(2)		(3)	(10)
Atrophy	1 (11%)	2 (100%)	1 (50%)			
Thymocyte, necrosis	1 (11%)		1 (50%)		1 (33%)	
Integumentary System						
Skin	(10)	(3)	(4)	(4)	(4)	(10)
Dermis, atrophy			1 (25%)			
Dermis, skin, site of application, inflammation			1 (25%)		1 (25%)	1 (10%)
Epidermis, hyperplasia			1 (25%)			
Epidermis, skin, site of application, hyperplasia			1 (25%)		1 (25%)	
Epidermis, skin, site of application, inflammation			2 (50%)			
Epidermis, skin, site of application, inflammation, chronic active						1 (10%)
Skin, site of application, ulcer, chronic active			1 (25%)			
Respiratory System						
Lung	(10)	(2)	(2)	(3)	(4)	(10)
Inflammation, chronic, focal	1 (10%)					
Inflammation, chronic active, focal				1 (33%)		1 (10%)
Inflammation, focal	2 (20%)		1 (50%)			
Nose	(10)	(5)	(1)	(2)	(4)	(10)
Inflammation	2 (20%)	3 (60%)				2 (20%)
Systems Examined with no Lesions Observed						
Endocrine System						
General Body System						
Musculoskeletal System						
Nervous System						
Special Senses System						
Urinary System						

APPENDIX B
SUMMARY OF LESIONS
IN FEMALE p53 HAPLOINSUFFICIENT MICE
IN THE 27-WEEK DERMAL STUDY
OF DIISOPROPYLCARBODIIMIDE

TABLE B1	Summary of the Incidence of Neoplasms in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Diisopropylcarbodiimide	40
TABLE B2	Summary of the Incidence of Nonneoplastic Lesions in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Diisopropylcarbodiimide	41

TABLE B1
Summary of the Incidence of Neoplasms in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Diisopropylcarbodiimide^a

	Vehicle Control	4.38 mg/kg	8.75 mg/kg	17.5 mg/kg	35 mg/kg	70 mg/kg
Disposition Summary						
Animals initially in study	15	15	15	15	15	15
Survivors						
Terminal sacrifice	15	15	15	15	15	15
Animals examined microscopically	15	15	15	15	15	15
Hematopoietic System						
Lymph node, mandibular Hemangioma	(15)	(15)	(15)	(14)	(15) 1 (7%)	(15)
Systems Examined with no Neoplasms Observed						
Alimentary System						
Cardiovascular System						
Endocrine System						
General Body System						
Genital System						
Integumentary System						
Musculoskeletal System						
Nervous System						
Respiratory System						
Special Senses System						
Urinary System						
Neoplasm Summary^b						
Total animals with primary neoplasms					1	
Total primary neoplasms					1	
Total animals with benign neoplasms					1	
Total benign neoplasms					1	

^a Number of animals examined microscopically at site and number of animals with neoplasm

^b Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE B2
Summary of the Incidence of Nonneoplastic Lesions in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Diisopropylcarbodiimide^a

	Vehicle Control	4.38 mg/kg	8.75 mg/kg	17.5 mg/kg	35 mg/kg	70 mg/kg
Disposition Summary						
Animals initially in study	15	15	15	15	15	15
Survivors						
Terminal sacrifice	15	15	15	15	15	15
Animals examined microscopically	15	15	15	15	15	15
Alimentary System						
Liver	(15)	(15)	(15)	(15)	(15)	(15)
Infiltration cellular, focal, lymphocyte	4 (27%)	1 (7%)	1 (7%)		2 (13%)	5 (33%)
Infiltration cellular, lymphocyte			1 (7%)			
Inflammation, chronic active, focal	1 (7%)					
Hepatocyte, necrosis, focal	1 (7%)	1 (7%)	3 (20%)	8 (53%)	4 (27%)	3 (20%)
Endocrine System						
Adrenal cortex	(15)	(15)	(15)	(14)	(14)	(15)
Subcapsular, hyperplasia, focal	12 (80%)	7 (47%)	10 (67%)	12 (86%)	12 (86%)	15 (100%)
Zona glomerulosa, hyperplasia, focal						1 (7%)
Parathyroid gland	(10)	(5)	(4)	(4)	(9)	(5)
Cyst						1 (20%)
Thyroid gland	(15)	(14)	(14)	(15)	(15)	(15)
Ectopic thymus	2 (13%)		1 (7%)	2 (13%)	1 (7%)	1 (7%)
Follicular cell, hyperplasia, focal	1 (7%)					
Genital System						
Uterus	(15)	(15)	(15)	(15)	(15)	(15)
Hydrometra						1 (7%)
Endometrium, hyperplasia, cystic	14 (93%)	14 (93%)	14 (93%)	14 (93%)	14 (93%)	13 (87%)
Hematopoietic System						
Bone marrow					(1)	(1)
Myeloid cell, hyperplasia						1 (100%)
Lymph node				(1)		
Inguinal, hyperplasia, lymphoid				1 (100%)		
Lymph node, mandibular	(15)	(15)	(15)	(14)	(15)	(15)
Hyperplasia, lymphoid	2 (13%)		2 (13%)			1 (7%)
Lymph node, mediastinal	(15)	(14)	(13)	(15)	(12)	(15)
Hyperplasia, lymphoid	2 (13%)					
Spleen	(15)	(15)	(15)	(15)	(15)	(15)
Hematopoietic cell proliferation	14 (93%)	15 (100%)	15 (100%)	15 (100%)	14 (93%)	15 (100%)
Pigmentation	1 (7%)					

^a Number of animals examined microscopically at the site and the number of animals with lesion

TABLE B2
Summary of the Incidence of Nonneoplastic Lesions in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Diisopropylcarbodiimide

	Vehicle Control	4.38 mg/kg	8.75 mg/kg	17.5 mg/kg	35 mg/kg	70 mg/kg
Integumentary System						
Skin	(15)	(15)	(15)	(15)	(15)	(15)
Dermis, fibrosis						1 (7%)
Dermis, infiltration cellular, mast cell						2 (13%)
Epidermis, control, hyperplasia, focal	1 (7%)					
Epidermis, skin, site of application, hyperplasia						8 (53%)
Epidermis, skin, site of application, hyperplasia, diffuse						1 (7%)
Epidermis, skin, site of application, hyperplasia, focal						1 (7%)
Sebaceous gland, hypertrophy						1 (7%)
Subcutaneous tissue, metaplasia, focal, osseous						1 (7%)
Vulva, degeneration						1 (7%)
Vulva, ulcer, chronic, focal						1 (7%)
Respiratory System						
Lung	(15)	(15)	(15)	(15)	(15)	(15)
Alveolus, hemorrhage, focal	1 (7%)	1 (7%)				
Alveolus, inflammation, chronic active, focal				1 (7%)	1 (7%)	
Perivascular, infiltration cellular, focal, lymphocyte					1 (7%)	
Urinary System						
Kidney	(15)	(15)	(15)	(15)	(15)	(15)
Infiltration cellular, focal, lymphocyte	1 (7%)	2 (13%)				
Pelvis, infiltration cellular, focal, lymphocyte					1 (7%)	
Renal tubule, degeneration, focal	1 (7%)					
Renal tubule, dilatation, focal	4 (27%)	1 (7%)	1 (7%)			3 (20%)
Renal tubule, hyperplasia						1 (7%)
Systems Examined with no Lesions Observed						
Cardiovascular System						
General Body System						
Musculoskeletal System						
Nervous System						
Special Senses System						

APPENDIX C ORGAN WEIGHTS AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE C1	Organ Weights and Organ-Weight-to-Body-Weight Ratios for Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Diisopropylcarbodiimide	44
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TABLE C1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Female Tg.AC Hemizygous Mice
in the 20-Week Dermal Study of Diisopropylcarbodiimide^a

	Vehicle Control	4.38 mg/kg	8.75 mg/kg	17.5 mg/kg	35 mg/kg	70 mg/kg
n	8	8	8	8	6	10
Necropsy body wt	27.8 ± 0.5	29.1 ± 1.6	30.0 ± 2.7	26.9 ± 1.0	26.0 ± 1.4	25.6 ± 0.5
Heart						
Absolute	0.142 ± 0.005	0.144 ± 0.004	0.149 ± 0.007	0.142 ± 0.003	0.132 ± 0.008	0.135 ± 0.003
Relative	5.113 ± 0.144	5.031 ± 0.205	5.087 ± 0.305	5.296 ± 0.137	5.083 ± 0.136	5.274 ± 0.131
R. Kidney						
Absolute	0.235 ± 0.011	0.233 ± 0.007	0.243 ± 0.015	0.235 ± 0.008	0.218 ± 0.011	0.220 ± 0.006
Relative	8.453 ± 0.328	8.108 ± 0.249	8.260 ± 0.472	8.790 ± 0.395	8.427 ± 0.252	8.597 ± 0.216
Liver						
Absolute	1.536 ± 0.080 ^b	1.637 ± 0.081	1.769 ± 0.135	1.593 ± 0.057	1.555 ± 0.088	1.428 ± 0.032
Relative	55.278 ± 2.147 ^b	56.534 ± 1.411	59.502 ± 2.645	59.658 ± 2.758	59.950 ± 1.781	55.828 ± 1.106
Lung						
Absolute	0.198 ± 0.008	0.196 ± 0.006	0.229 ± 0.018	0.212 ± 0.018	0.195 ± 0.009	0.175 ± 0.008
Relative	7.122 ± 0.289	6.852 ± 0.400	7.811 ± 0.607	8.021 ± 0.879	7.591 ± 0.403	6.858 ± 0.342
Thymus						
Absolute	0.036 ± 0.003	0.044 ± 0.002	0.038 ± 0.004	0.036 ± 0.003	0.032 ± 0.002	0.035 ± 0.002
Relative	1.277 ± 0.097	1.524 ± 0.044	1.251 ± 0.075	1.338 ± 0.122	1.249 ± 0.072	1.364 ± 0.086

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error). Differences from the vehicle control group are not significant by Dunnett's test.

^b n=7

APPENDIX D

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION	46
PREPARATION AND ANALYSIS OF DOSE FORMULATIONS	47
TABLE D1 Gas Chromatography Systems Used in the Dermal Studies of Diisopropylcarbodiimide	48
FIGURE D1 Infrared Absorption Spectrum of Diisopropylcarbodiimide	49
FIGURE D2 Proton Nuclear Magnetic Resonance Spectrum of Diisopropylcarbodiimide	50
TABLE D2 Preparation and Storage of Dose Formulations in the Dermal Studies of Diisopropylcarbodiimide	51
TABLE D3 Results of Analyses of Dose Formulations Administered to Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Diisopropylcarbodiimide	52
TABLE D4 Results of Analyses of Dose Formulations Administered to Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Diisopropylcarbodiimide	53

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION

Diisopropylcarbodiimide

Diisopropylcarbodiimide was obtained from Aldrich Chemical Company (Milwaukee, WI) in two lots. Lot 01207BG was used during the 20-week studies; lot 13016JS was used during the 27-week studies. Identity and purity analyses were conducted by the analytical chemistry laboratory at Midwest Research Institute (Kansas City, MO) and by the study laboratories at Microbiological Associates, Inc. (Bethesda, MD; 20-week study), and BioReliance Corporation (Rockville, MD; 27-week study); physical properties, moisture content, and stability of the bulk diisopropylcarbodiimide were determined by the analytical chemistry laboratory. Reports on analyses performed in support of the diisopropylcarbodiimide studies are on file at the National Institute of Environmental Health Sciences.

Lot 01207BG, a colorless liquid, was identified as diisopropylcarbodiimide by the study laboratory using infrared (IR) spectroscopy. Lot 13016JS was identified as diisopropylcarbodiimide by the study laboratory using IR spectroscopy and by the analytical chemistry laboratory using IR, proton nuclear magnetic resonance (NMR), and ultraviolet/visible spectroscopy and gas chromatography (GC)/mass spectrometry by system A (Table D1). All spectra were consistent with the structure of diisopropylcarbodiimide and with literature references (Anonymous, 1977; *Aldrich* 1981, 1985, 1993; NIST, 1995). Representative IR and proton NMR spectra are presented in Figures D1 and D2.

The purity of lot 01207BG was determined by the study laboratory using GC system B. The purity of lot 13016JS was determined by the study laboratory using GC system C and by the analytical chemistry laboratory using thin layer chromatography (TLC) and GC system D. The moisture content of lot 13016JS was determined by the analytical chemistry laboratory using Karl Fischer titration; the boiling point and relative density of this lot were also measured by the analytical chemistry laboratory.

For lot 01207BG, GC by system B indicated a major peak and five impurity peaks with areas ranging from 0.05% to 0.27% of the total peak area. Fourteen minor impurities were present in the sample chromatograms. The overall purity of lot 01207BG was determined to be 99.35%.

For lot 13016JS, the boiling point and relative density were consistent with the literature value for diisopropylcarbodiimide (*Aldrich*, 1988). Karl Fischer titration indicated 0.06% water in the bulk chemical. TLC detected a major, a minor, and two trace spots. GC by system C indicated a relative purity of 100.4% when compared to a frozen reference sample from the analytical chemistry laboratory. GC by system D indicated a major peak and five impurity peaks with a combined area of approximately 0.5% of the total peak area; the purity of the test article was determined to be approximately 99.5%. The overall purity of lot 13016JS was determined to be greater than 99%.

The analytical chemistry laboratory conducted accelerated stability studies of lot 13016JS with samples stored for 2 weeks in amber vials with Teflon[®]-lined septa at approximately 5°, 25°, and 60° C compared to frozen samples from the same lot stored at -20° C. Analysis using GC system E indicated that the test article was stable when protected from light at temperatures up to approximately 60° C for 2 weeks. To ensure stability, the bulk chemical was stored at room temperature under nitrogen, protected from light as recommended by the manufacturer. Periodic purity analyses of the bulk chemical were performed during both studies using GC by system B. No degradation of the bulk chemical was detected.

Anhydrous Ethanol

Anhydrous ethanol was obtained from Pharmco (Brookfield, CT) in one lot (number unknown) used for the 20-week study and two lots (9901074 and 9801193) that were used for the 27-week study. Identity and purity analyses of all lots were conducted by the study laboratories. The chemical, a clear liquid, was identified as ethanol using IR spectroscopy; the sample spectra were a good match for the reference spectrum of ethanol (*Aldrich*, 1985). The purity of each lot was determined using GC by system F. No impurities were detected that exceeded a relative concentration of 0.1% in any lot.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared by mixing diisopropylcarbodiimide and anhydrous ethanol to give the required concentrations; formulations were prepared once not more than 7 days prior to the study start and every 3 weeks thereafter for the 20-week study or monthly for the 27-week study (Table D2). The dose formulations were stored at room temperature for the 20-week study and for the 27-week study until April 6, 1999, or later, when formulations were stored at -20°C for up to 35 days.

Because the dose formulations were true solutions of the test article in ethanol, homogeneity studies were not performed. Prior to the 20-week study, a stability study of 2.19 mg/mL dose formulations of lot 01207BG was conducted by the study laboratory using GC by system G; stability was confirmed for up to 35 days for the dose formulation stored at ambient temperature in sealed containers under a nitrogen headspace and for up to 3 hours when exposed to light and air at ambient temperature.

Periodic analyses of the dose formulations of diisopropylcarbodiimide were conducted by the study laboratories using GC by system G. During the 20-week study, the dose formulations were analyzed four times; animal room samples of these dose formulations were also analyzed. All 20 dose formulations analyzed were within 10% of the target concentrations (Table D3); all 20 animal room samples analyzed were within 10% of the target concentrations. Dose formulations were analyzed four times during the 27-week study; animal room samples of these dose formulations were also analyzed (Table D4). Of the 20 dose formulations analyzed, all were within 10% of the target concentrations; 9 of 20 animal room samples were within 10% of the target concentrations. Unusual degradation was observed in the animal room samples from the formulations prepared on February 17, 1999, and June 7, 1999. Attempts to discover the cause of the degradation in the formulations were not conclusive, but the most likely cause, water in the anhydrous ethanol, was eliminated. To prevent degradation of the test chemical, formulations prepared on or after April 6, 1999, were stored at -20°C .

TABLE D1
Gas Chromatography Systems Used in the Dermal Studies of Diisopropylcarbodiimide^a

Detection System	Column	Carrier Gas	Oven Temperature Program
System A Mass spectrometer	DB-5, 30 m × 0.25 mm, 0.25 μm (J&W Scientific, Folsom, CA)	Helium 40 cm ³ /second	50° C, held for 5 minutes, then 10° C/minute to 250° C, held for 3 minutes
System B Flame ionization	HP-1, 5 m × 0.53 mm, (Hewlett-Packard, Palo Alto, CA)	Nitrogen at 7 mL/minute	35° C, then 10° C/minute to 185° C, held for 5 minutes
System C Flame ionization	SPB-5, 30 m × 0.53 mm, 1 μm (Supelco, Bellefonte, PA)	Nitrogen at 7 mL/minute	50° C, held for 5 minutes, then 10° C/minute to 250° C
System D Flame ionization	Rtx-5, 30 m × 0.53 mm, 1.0 μm (Restek, Bellefonte, PA)	Helium at 10 mL/minute	50° C, held for 5 minutes, then 10° C/minute to 250° C, held for 3 minutes
System E Flame ionization	Rtx-5, 30 m × 0.53 mm, 1.0 μm (Restek)	Helium at 10 mL/minute	70° C isothermal
System F Flame ionization	Carbowax, 30 m × 0.53 mm, 1.2 μm (Alltech; Deerfield, Ill)	Nitrogen at 6 mL/minute	40° C, held for 4 minutes, then 10° C/minute to 220° C
System G Flame ionization	J&W DB-1, 30 m × 0.53 mm, 3 μm (J&W Scientific)	Nitrogen at 17.5 mL/minute	115° C isothermal

^a Gas chromatographs manufactured by Thermo Electron Corp. (Finnigan), San Jose, CA (system A), Hewlett-Packard, Palo Alto, CA (systems B, C, F, G), Varian, Palo Alto, CA (systems D, E); mass spectrometer was manufactured by Finnigan.

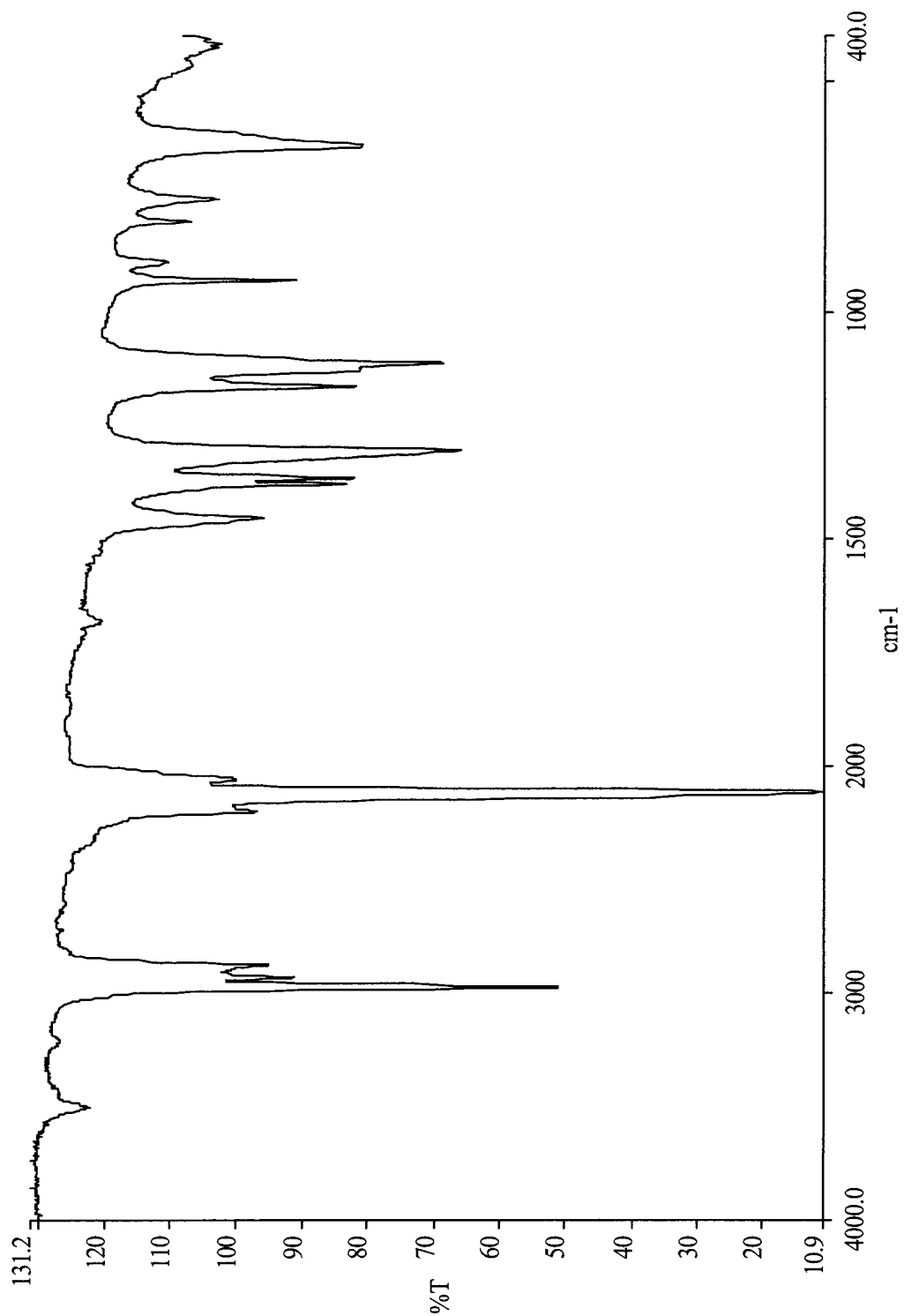


FIGURE D1
Infrared Absorption Spectrum of Diisopropylcarbodiimide

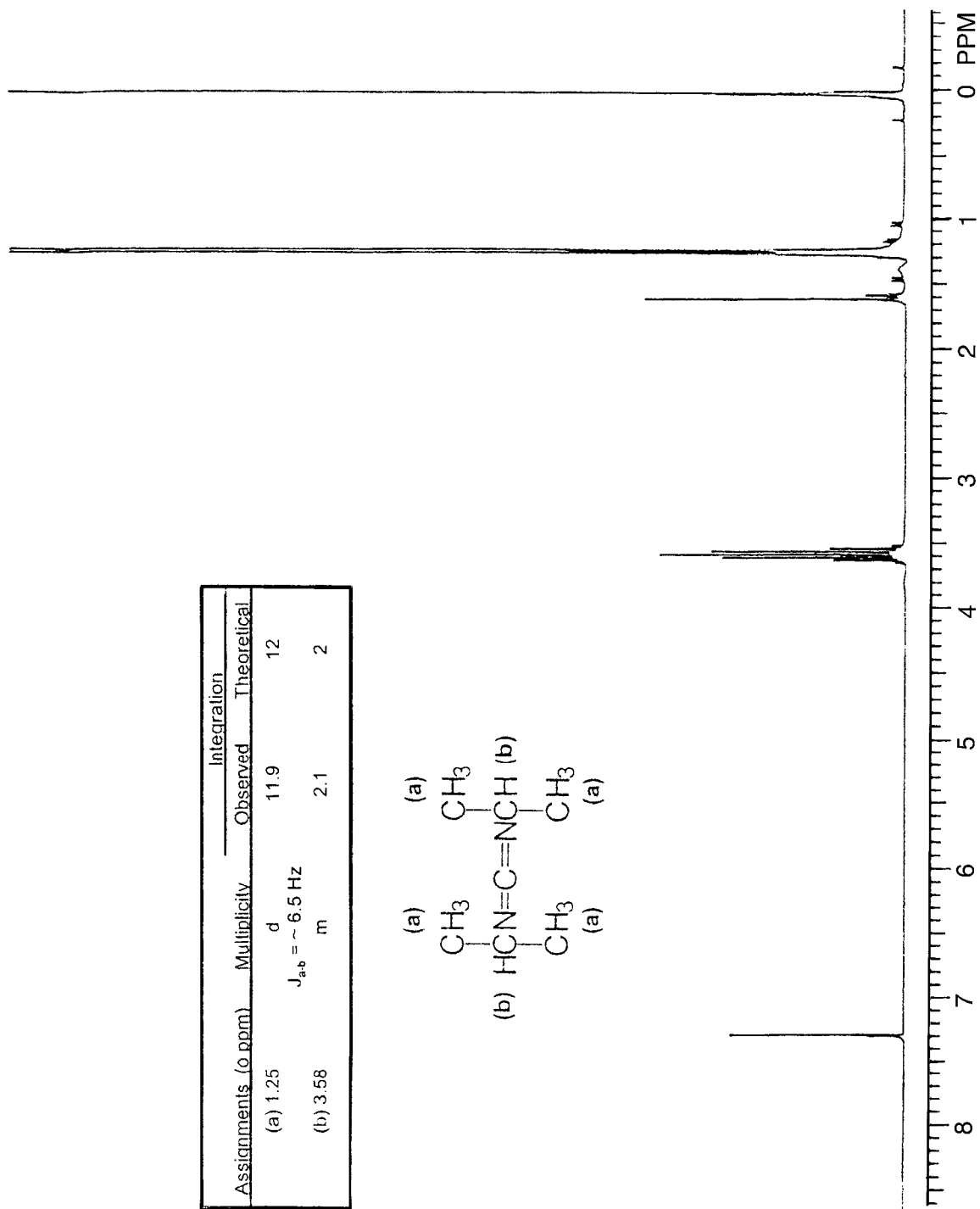


FIGURE D2
Proton Nuclear Magnetic Resonance Spectrum of Diisopropylcarbodiimide

TABLE D2
Preparation and Storage of Dose Formulations in the Dermal Studies of Diisopropylcarbodiimide

Tg.AC Hemizygous Mice 20-Week Study	p53 Haploinsufficient Mice 27-Week Study
<p>Preparation The first dose formulations were prepared in anhydrous ethanol no more than 7 days prior to study start, then every 3 weeks during the study. The highest dose formulation was prepared by weighing a specified amount of diisopropylcarbodiimide and dissolving in anhydrous ethanol to produce the appropriate concentration. Aliquots of this formulation were diluted with anhydrous ethanol to give the appropriate concentrations for the lower dose formulations.</p>	<p>Dose formulations were prepared every 4 weeks by weighing a specified amount of diisopropylcarbodiimide and adding anhydrous ethanol to produce the appropriate concentration for each dose formulation.</p>
<p>Chemical Lot Number 01207BG</p>	<p>13016JS</p>
<p>Maximum Storage Time 35 days</p>	<p>35 days</p>
<p>Storage Conditions Aliquots of dose formulations were transferred to serum vials, crimp-sealed under a headspace of nitrogen gas, and stored at room temperature.</p>	<p>Aliquots of each dose level were transferred to serum vials, sealed under a headspace of inert gas, and stored at room temperature; formulations prepared on or after April 6, 1999, were stored at -20° C.</p>
<p>Study Laboratory Microbiological Associates, Inc., Bethesda, MD</p>	<p>BioReliance Corporation, Rockville, MD</p>

TABLE D3
Results of Analyses of Dose Formulations Administered to Female Tg.AC Hemizygous Mice
in the 20-Week Dermal Study of Diisopropylcarbodiimide

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration ^a (mg/mL)	Difference from Target (%)
June 8, 1995	June 8, 1995	2.19	2.23	+2
		4.38	4.35	-1
		8.75	8.65	-1
		17.5	16.9	-3
		35.0	34.0	-3
	July 7, 1995 ^b	2.19	2.10	-4
		4.38	4.27	-2
		8.75	8.31	-5
		17.5	16.6	-5
		35.0	34.3	-2
July 20, 1995	July 20, 1995	2.19	2.29	+5
		4.38	4.59	+5
		8.75	8.63	-1
		17.5	17.0	-3
		35.0	34.9	0
	August 17, 1995 ^b	2.19	2.27	+4
		4.38	4.52	+3
		8.75	8.59	-2
		17.5	16.9	-3
		35.0	34.6	-1
August 31, 1995	August 31, 1995	2.19	2.20	0
		4.38	4.43	+1
		8.75	8.61	-2
		17.5	17.6	+1
		35.0	35.0	0
	September 28, 1995 ^b	2.19	2.17	-1
		4.38	4.40	0
		8.75	8.61	-2
		17.5	17.4	-1
		35.0	34.5	-1
October 12, 1995	October 12, 1995	2.19	2.18	0
		4.38	4.34	-1
		8.75	8.55	-2
		17.5	16.9	-3
		35.0	34.2	-2
	October 31, 1995 ^b	2.19	1.87	-15
		4.38	4.36	0
		8.75	8.50	-3
		17.5	17.2	-2
		35.0	34.1	-3
November 6, 1995 ^c	2.19	2.29	+5	

^a Results of duplicate analyses. Dosing volume=2.0 mL/kg; 2.19 mg/mL=4.38 mg/kg, 4.38 mg/mL=8.76 mg/kg, 8.75 mg/mL=17.5 mg/kg, 17.5 mg/mL=35 mg/kg, 35 mg/mL=70 mg/kg.

^b Animal room samples

^c Repeat analysis of 2.19 mg/kg animal room sample (October 31, 1995)

TABLE D4
Results of Analyses of Dose Formulations Administered to Female p53 Haploinsufficient Mice
in the 27-Week Dermal Study of Diisopropylcarbodiimide

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration ^a (mg/mL)	Difference from Target (%)
December 23, 1998	December 23, 1998	2.19	2.22	+1
		4.38	4.28	-2
		8.75	8.54	-2
		17.5	17.3	-1
		35.0	33.5	-4
	January 28, 1999 ^b	2.19	2.00	-9
		4.38	3.82	-13
		8.75	7.83	-10
		17.5	16.0	-9
		35.0	32.8	-6
February 17, 1999	February 17, 1999	2.19	2.24	+2
		4.38	4.39	0
		8.75	9.19	+5
		17.5	17.9	+2
		35.0	35.3	+1
	March 25, 1999 ^b	2.19	0.47	-78
		4.38	0.28	-94
		8.75	0.27	-97
		17.5	4.0	-77
		35.0	16.0	-54
April 6, 1999	April 6, 1999	2.19	2.11	-4
		4.38	4.32	-1
		8.75	8.65	-1
		17.5	17.1	-2
		35.0	34.4	-2
	May 6, 1999 ^b	2.19	1.97	-10
		4.38	4.11	-5
		8.75	8.05	-7
		17.5	17.7	+1
		35.0	34.1	-3
June 7, 1999	June 7, 1999	2.19	2.27	+4
		4.38	4.34	-1
		8.75	8.60	-2
		17.5	17.2	-2
		35.0	34.2	-2
	July 13, 1999 ^b	2.19	1.72	-22
		4.38	3.44	-23
		8.75	7.22	-17
		17.5	13.7	-23
		35.0	28.3	-23

^a Results of duplicate analyses. Dosing volume=2.0 mL/kg; 2.19 mg/mL=4.38 mg/kg, 4.38 mg/mL=8.76 mg/kg, 8.75 mg/mL=17.5 mg/kg, 17.5 mg/mL=35 mg/kg, 35 mg/mL=70 mg/kg.

^b Animal room samples

