NTP

National Toxicology Program

U.S. Department of Health and Human Services

NTP GENETICALLY MODIFIED MODEL REPORT ON THE

TOXICOLOGY STUDIES OF DICYCLOHEXYLCARBODIIMIDE (CASRN 538-75-0) IN F344/N RATS, B6C3F1 MICE, AND GENETICALLY MODIFIED (FVB TG.AC HEMIZYGOUS) MICE AND CARCINOGENICITY STUDY OF DICYCLOHEXYLCARBODIIMIDE IN GENETICALLY MODIFIED [B6.129-TRP53TM1BRD (N5) HAPLOINSUFFICIENT] MICE (DERMAL STUDIES)

NTP GMM 09

SEPTEMBER 2007

NTP REPORT

ON THE

TOXICOLOGY STUDIES
OF DICYCLOHEXYLCARBODIIMIDE
(CAS NO. 538-75-0)

IN F344/N RATS, B6C3F$_1$ MICE,
AND GENETICALLY MODIFIED
(FVB Tg.AC HEMIZYGOUS) MICE

AND CARCINOGENICITY STUDY
OF DICYCLOHEXYLCARBODIIMIDE

IN GENETICALLY MODIFIED
[B6.129-*Trp53*^{tm1Brd} (N5) HAPLOINSUFFICIENT] MICE
(DERMAL STUDIES)

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

September 2007

NTP GMM 9

NIH Publication No. 07-4426

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.

CONTRIBUTORS

National Toxicology Program
Evaluated and interpreted results and reported findings

R.S. Chhabra, Ph.D., Study Scientist
S.A. Elmore, D.V.M., M.S., Study Pathologist
D.W. Bristol, Ph.D.
J.R. Bucher, Ph.D.
L.T. Burka, Ph.D.
J.E. French, Ph.D.
A.P. King-Herbert, D.V.M.
G.E. Kissling, Ph.D.
D.E. Malarkey, D.V.M., Ph.D.
R.R. Maronpot, D.V.M.
J.C. Peckham D.V.M., M.S., Ph.D.
S.D. Peddada, Ph.D.
C.S. Smith, Ph.D.
G.S. Travlos, D.V.M.
M.K. Vallant, B.S., M.T.
K.L. Witt, M.S.

Microbiological Associates, Inc.
Conducted 20-week study and evaluated pathology findings

M.L. Wenk, Ph.D., Principal Investigator
J.M. Pletcher, D.V.M., M.P.H.

BioReliance Corporation
Conducted 27-week study and evaluated pathology findings

M.L. Wenk, Ph.D., Principal Investigator
L.L. Lanning, D.V.M.

Experimental Pathology Laboratories, Inc.
Provided pathology review

M.H. Hamlin, II, D.V.M., Principal Investigator
E.T. Gaillard, D.V.M., M.S.

Dynamac Corporation
Prepared quality assurance audits

S. Brecher, Ph.D., Principal Investigator

NTP Pathology Working Group
Evaluated slides and prepared 13- and 20-week pathology reports
(February 11, 1997)

P.K. Hildebrandt, D.V.M., Chairperson
  PATHCO, Inc.
E.T. Gaillard, D.V.M., M.S.
  Experimental Pathology Laboratories, Inc.
J.E. Leininger, D.V.M., Ph.D.
  National Toxicology Program
J. Mahler, D.V.M.
  National Toxicology Program
D.E. Malarkey, D.V.M., Ph.D.
  National Toxicology Program
A. Radovsky, D.V.M., Ph.D.
  National Toxicology Program
D. Wolfe, D.V.M.
  Chemical Industry Institute of Toxicology

Evaluated slides and prepared 27-week pathology report
(September 7, 2000)

C.A. Picut, V.M.D., J.D., Chairperson
  ILS, Inc.
J. Mahler, D.V.M.
  National Toxicology Program

Constella Group, Inc.
Provided statistical analyses

P.W. Crockett, Ph.D., Principal Investigator
L.J. Betz, M.S.
K.P. McGowan, M.B.A.

Biotechnical Services, Inc.
Prepared Report

S.R. Gunnels, M.A., Principal Investigator
B.F. Hall, M.S.
L.M. Harper, B.S.
D.C. Serbus, Ph.D.

CONTENTS

SUMMARY

Background
Dicyclohexylcarbodiimide is used as a stabilizing agent in elastics and fibers and for the chemical synthesis of peptides. We tested if dicyclohexylcarbodiimide could cause cancer in two different strains of genetically modified mice.

Methods
We applied solutions containing dicyclohexylcarbodiimide dissolved in ethanol to the backs of female Tg.AC hemizygous mice for 20 weeks and to female p53 haploinsufficient mice for 27 weeks.   The daily doses were 0.75, 1.5, 3, 6, or 12 milligrams of dicyclohexylcarbodiimide per kilogram of body weight. Besides the skin where the solutions were applied, tissues from over 15 organs were examined for every animal.

Results
Exposure to dicyclohexylcarbodiimide caused inflammation and hyperplasia of the skin at the site of application in both strains of genetically modified mice. Female Tg.AC hemizygous mice given dicyclohexylcarbodiimide developed squamous cell papillomas of the skin at the site the chemical was applied. Female p53 haploinsufficient mice receiving dicyclohexylcarbodiimide did not have any increase in tumors related to the chemical.

Conclusions
We conclude that dicyclohexylcarbodiimide caused cancer of the skin in female Tg.AC hemizygous mice but not in female p53 haploinsufficient mice.

ABSTRACT
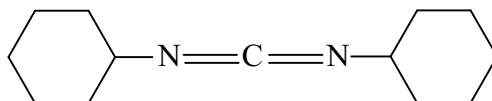
DICYCLOHEXYLCARBODIIMIDE

CAS No. 538-75-0

Chemical Formula: $C_{13}H_{22}N_2$ Molecular Weight: 206.32

Synonyms: Carbodicyclohexylimide; DCC; DCCI; *N,N′*-methanetetraylbis(cyclohexanamine)

Dicyclohexylcarbodiimide is used in industry as a stabilizing agent, coupling agent, and condensing agent. Its widespread use during protein synthesis in the recombinant DNA industry and in the synthesis of polypeptides in the chemical and pharmaceutical industries provides an increasing potential for low-level human exposure. Dicyclohexylcarbodiimide was nominated for study by The National Cancer Institute as a key representative of the carbodiimide chemical class because of its acute toxicity and the absence of data on potential health effects. Male and female F344/N rats and B6C3F$_1$ mice were administered dicyclohexylcarbodiimide (greater than 98% pure) dermally for 3 or 13 weeks. Female Tg.AC hemizygous and p53 haploinsufficient mice were administered dicyclohexylcarbodiimide dermally for 20 or 27 weeks, respectively. Genetic toxicology studies were conducted in *Salmonella typhimurium,* male F344/N rat bone marrow cells, and B6C3F$_1$ mouse peripheral blood erythrocytes.

3-WEEK STUDY IN F344/N RATS
Groups of five male and five female rats were dermally administered 0.3 mL ethanol containing 0, 0.6, 1.8, 5.1, 15, or 45 mg dicyclohexylcarbodiimide, 5 days per week for 3 weeks. All males and females in the 15 and 45 mg groups, four 5.1 mg males, and all 5.1 mg females died

before the end of the study. Of the surviving groups, final mean body weights were similar to those of the vehicle controls, although the one surviving 5.1 mg male rat lost weight during the study. Histopathologic examination of rats dosed with 5.1 mg dicyclohexylcarbodiimide or less revealed treatment-related lesions of the skin at the site of application including epidermal hyperplasia, epidermal necrosis, or chronic active inflammation in the dermis.

3-WEEK STUDY IN B6C3F$_1$ MICE
Groups of five male and five female mice were dermally administered 0.1 mL of ethanol containing 0, 0.2, 0.6, 1.7, 5, or 15 mg dicyclohexylcarbodiimide, 5 days per week for 3 weeks. One 0.6 mg female mouse and all mice in the 1.7, 5, and 15 mg groups died before the end of the study. Final mean body weights of the 0.6 mg groups were significantly less than those of the vehicle controls, and animals in these groups generally lost weight during the study. Histopathologic examination of mice dosed with 1.7 mg dicyclohexylcarbodiimide or less revealed treatment-related lesions of the skin at the site of application including epidermal hyperplasia, epidermal necrosis, and acute or chronic active dermal inflammation.

13-Week Study in F344/N Rats

Groups of 10 male and 10 female core study rats were dermally administered 0, 0.75, 1.5, 3, 6, or 12 mg dicyclohexylcarbodiimide/kg body weight in ethanol, 5 days per week for 13 weeks; groups of 10 male and 10 female clinical pathology study rats were administered the same doses for 22 days. All 12 mg/kg male and female core study rats died or were found moribund and sacrificed prior to day 45. Final mean body weight and body weight gain of 6 mg/kg males were significantly less than those of the vehicle controls. The predominant clinical pathology changes suggest a secondary, treatment-related inflammatory leukogram and minimal decreased erythron of chronic inflammation that would be consistent with necrosis and chronic active inflammation of the skin. Significantly increased incidences of skin lesions at the site of application included epidermal hyperplasia in 3 mg/kg or greater males and 1.5 mg/kg or greater females, chronic active inflammation in 6 and 12 mg/kg males and 1.5 mg/kg or greater females, and epidermal necrosis in 12 mg/kg males. The incidences and severities of epidermal hyperplasia increased in a dose-related manner in both sexes of rats.

13-Week Study in B6C3F$_1$ Mice

Groups of 10 male and 10 female mice were dermally administered 0, 1.5, 3, 6, 12, or 24 mg dicyclohexylcarbodiimide/kg body weight in ethanol, 5 days per week for 13 weeks. All 24 mg/kg male and female mice died or were found moribund and sacrificed prior to day 16. Final mean body weights of 6 and 12 mg/kg males and mean body weight gains of 6 and 12 mg/kg males and females were significantly less than those of the vehicle controls. The predominant clinical pathology changes suggest a secondary, treatment-related inflammatory leukogram and minimal decreased erythron of chronic inflammation that would be consistent with necrosis and chronic active inflammation of the skin. Dermal administration of dicyclohexylcarbodiimide significantly decreased the weight of the epididymis in 6 and 12 mg/kg males and significantly decreased epididymal spermatozoal motility in 6 mg/kg males. Significantly increased incidences of skin lesions at the site of application included epidermal hyperplasia in all dosed groups except those administered 24 mg/kg, chronic active inflammation in all dosed groups except 1.5 mg/kg females, and epidermal necrosis in 24 mg/kg males and females.

20-Week Study in Female Tg.AC Hemizygous Mice

Groups of 10 female Tg.AC hemizygous mice were dermally administered 0, 0.75, 1.5, 3, 6, or 12 mg dicyclohexylcarbodiimide/kg body weight in ethanol, 5 days per week for up to 20 weeks. Due to the severity of skin lesions observed in 12 mg/kg animals, the application of dicyclohexylcarbodiimide was discontinued after eight dermal applications in this group. There were no deaths considered related to dicyclohexylcarbodiimide administration, although 13 animals died or were sacrificed moribund prior to the end of the study: three each from the vehicle control and 0.75 mg/kg groups, four from the 3 mg/kg group, two from the 6 mg/kg group, and one from the 12 mg/kg group. Overall, the survival was within the range known for the Tg.AC hemizygous mouse. Mean body weights of dosed groups of mice were similar to those of the vehicle controls. At the site of application, the incidences of squamous cell papilloma were increased in a dose-related manner. The incidences of chronic active inflammation of the dermis and epidermal hyperplasia were significantly increased in mice administered 3 or 6 mg/kg.

27-Week Study in Female p53 Haploinsufficient Mice

Groups of 15 female mice were dermally administered 0, 0.75, 1.5, 3, 6, or 12 mg dicyclohexylcarbodiimide/kg body weight in ethanol, 5 days per week for up to 27 weeks. Dosing of the 6 and 12 mg/kg groups was discontinued after 11 and 8 days, respectively, because of the severity of skin lesions at the site of application. Twelve animals died or were sacrificed moribund prior to the end of the study: three from the 3 mg/kg group, one from the 6 mg/kg group, and eight from the 12 mg/kg group. Mean body weights of dosed groups of mice were similar to those of the vehicle controls. No neoplasms were attributed to administration of dicyclohexylcarbodiimide. At the site of application, the incidences of focal epidermal hyperplasia were significantly increased in 1.5, 3, and 12 mg/kg mice, the incidences of focal chronic active inflammation of the dermis were increased in groups administered 3 or 12 mg/kg, and the incidences of focal ulcer and focal chronic active inflammation of the subcutaneous tissue were increased in the 12 mg/kg group.

GENETIC TOXICOLOGY

Dicyclohexylcarbodiimide was not mutagenic in *Salmonella typhimurium* strains TA97, TA98, TA100, or TA1535, with or without rat or hamster liver S9 activation enzymes. *In vivo*, there was a small but significant increase in the frequency of micronucleated normochromatic erythrocytes in male and female B6C3F$_1$ mice after 13 weeks of dermal exposure to dicyclohexylcarbodiimide. Negative results were obtained, however, in an acute three-injection micronucleus study in bone marrow of male F344/N rats.

CONCLUSIONS

Under the conditions of this 27-week dermal study, there was *no evidence of carcinogenic activity** of dicyclohexylcarbodiimide in female p53 haploinsufficient mice administered 0.75, 1.5, 3, 6, or 12 mg/kg in ethanol.

Female Tg.AC hemizygous mice dermally dosed with dicyclohexylcarbodiimide for 20 weeks had significantly increased incidences of squamous cell papilloma of the skin at the site of application.

Nonneoplastic lesions noted at the site of application included chronic active inflammation and epidermal hyperplasia in female p53 haploinsufficient mice and female Tg.AC hemizygous mice.

---

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 9. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Report appears on page 11.

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

Concentrations in ethanol	0, 0.75, 1.5, 3, 6, or 12 mg/kg
Body weights	Dosed groups similar to the vehicle control group
Survival rates	7/10, 7/10, 10/10, 6/10, 8/10, 9/10
Nonneoplastic effects	Skin (site of application): chronic active inflammation of the dermis (0/10, 0/10, 3/10, 5/10, 8/10, 0/10); epidermal hyperplasia (0/10, 0/10, 0/10, 6/10, 7/10, 1/10)
Neoplastic effects	Skin (site of application): squamous cell papilloma (0/10, 0/10, 1/10, 3/10, 6/10, 8/10)

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

Concentrations in ethanol	0, 0.75, 1.5, 3, 6, or 12 mg/kg
Body weights	Dosed groups similar to the vehicle control group
Survival rates	15/15, 15/15, 15/15, 12/15, 14/15, 7/15
Nonneoplastic effects	Skin (site of application): focal epidermal hyperplasia (0/15, 1/15, 5/15, 11/15, 1/15, 8/15); focal chronic active inflammation of the dermis (1/15, 0/15, 0/15, 13/15, 2/15, 9/15); focal ulcer (0/15, 0/15, 0/15, 2/15, 1/15, 8/15); focal chronic active inflammation of the subcutaneous tissue (0/15, 0/15, 0/15, 2/15, 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 the Technical 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 Technical 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 dicyclohexylcarbodiimide on August 28, 2006, 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 Technical 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.

Charlene A. McQueen, Ph.D., Chairperson
    College of Pharmacy
    University of Arizona
    Tucson, AZ

Diane F. Birt, Ph.D., Principal Reviewer
    Department of Food Science and Human Nutrition
    Iowa State University
    Ames, IA

Christopher Bradfield, Ph.D.*
    McArdle Laboratory for Cancer Research
    University of Wisconsin
    Madison, WI

Kenny Crump, Ph.D.*
    Environ International
    Ruston, LA

Prescott Deininger, Ph.D.
    Tulane University Medical Center
    New Orleans, LA

John P. Giesy, Jr., Ph.D.
    Department of Zoology
    Michigan State University
    East Lansing, MI

Nancy Kerkvliet, Ph.D.*
    Department of Environmental and Molecular Toxicology
    Oregon State University
    Corvallis, OR

Jon Mirsalis, Ph.D.
    SRI International
    Menlo Park, CA

Harish Sikka, Ph.D.
    Environmental Toxicology and Chemistry Laboratory
    State University of New York College at Buffalo
    Buffalo, NY

Keith Soper, Ph.D., Principal Reviewer
    Merck Research Laboratories
    West Point, PA

Vernon Walker, D.V.M., Ph.D., Principal Reviewer
    Lovelace Respiratory Institute
    Albuquerque, NM

_____

* Did not attend

SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On August 28, 2006, the draft GMM Report on the toxicology and carcinogenesis studies of dicyclohexylcarbodiimide 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. J.E. French, NIEHS, provided an overview of the NTP's development of an alternative test model using genetically modified mice. Dr. R.S. Chhabra, NIEHS, introduced the toxicology and carcinogenesis studies of dicyclohexylcarbodiimide by discussing the uses of the chemical, the rationale for the study, and the experimental design; reporting on survival and body weight effects and compound-related neoplasms and nonneoplastic lesions in the 27- and 20-week studies in p53 and Tg.AC transgenic mice, respectively; and comparing these results to clinical pathology, reproductive, and histopathology findings in the 3-month studies in standard rodent models. The proposed conclusions were *no evidence of carcinogenic activity* of dicyclohexylcarbodiimide in female p53 haploinsufficient mice administered 0.75, 1.5, 3, 6, or 12 mg/kg in ethanol for 27 weeks. There were significantly increased incidences of squamous cell papilloma of the skin at the site of application in female Tg.AC hemizygous mice administered 0.75, 1.5, 3, 6, or 12 mg/kg for 20 weeks. Nonneoplastic lesions at the site of application included chronic active inflammation and epidermal hyperplasia in female p53 haploinsufficient mice and female Tg.AC hemizygous mice.

Dr. Walker, the first principal reviewer, noted that in Tg.AC mice, forestomach papillomas occurred in every treated group but not in the controls. He thought this was worthy of mention in the body of the report even though the incidences were not statistically significant.

Dr. Birt, the second principal reviewer, asked that clarification be made of the potential impact to study interpretation due to the administration of lower dose concentrations for a time during the study; she also suggested that information about potential human exposure levels be added to the introduction.

Dr. Soper, the third principal reviewer, agreed with the proposed conclusions, noting the contrast of the response in skin papillomas between the p53 and Tg.AC mouse models.

Dr. Chhabra said that comments would be added explaining why the forestomach papillomas in Tg.AC mice were discounted. Regarding the low concentrations administered during the study, he explained that the dosing formulations were initially stored at room temperature, and the occasional loss of chemical by evaporation was remedied when the formulations were refrigerated. He said that while no quantitative measures of workplace exposure in humans are in the literature, the chemical is a strong irritant and there have been a number of incidents of severe dermatitis resulting from exposure. In mice, skin irritation resulted from concentrations as low as 0.006%.

Dr. Birt moved, and Dr. Walker seconded, that the conclusions be accepted as written. The motion was approved unanimously with seven votes.

INTRODUCTION
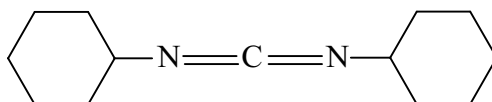
DICYCLOHEXYLCARBODIIMIDE

CAS No. 538-75-0

Chemical Formula: $C_{13}H_{22}N_2$ Molecular Weight: 206.32

Synonyms: Carbodicyclohexylimide; DCC; DCCI; *N,N′*-methanetetraylbis(cyclohexanamine)

CHEMICAL AND PHYSICAL PROPERTIES

Dicyclohexylcarbodiimide, a colorless crystalline solid with a heavy sweet odor, is a key chemical of the carbodiimide class of chemicals. Dicyclohexylcarbodiimide has a melting point of 35° to 36° C; boiling point ranges of 98° to 100° C (0.5 mm Hg), 138° to 140° C (2 mm Hg), and 154° to 156° C (11 mm Hg); and a flash point greater than 110° C (Sax and Lewis 1987; *Merck*, 1989; *Aldrich*, 1990). Dicyclohexylcarbodiimide is soluble in organic solvents, moisture sensitive, corrosive to tissue, incompatible with acids and oxidizers, and emits toxic fumes when combusted (Sax and Lewis, 1987; Kuney, 1990).

Dicyclohexylcarbodiimide is available in purities ranging from 99% to 99.5% in quantities up to 1,000 kg (Kuney, 1990; Van, 1990). The main impurities are unreacted isocyanates and polymerized carbodiimides (Chadwick and Cleveland, 1979).

PRODUCTION, USE, AND HUMAN EXPOSURE

Dicyclohexylcarbodiimide is manufactured by heating cyclohexyl isocyanate. The use of a catalyst accelerates the reaction and makes the production of dicyclohexyl-carbodiimide practical. Phospholine oxides are particularly effective catalysts, although simple trialkyl phosphine oxides or triethyl phosphate may also be used. The dimer is not an intermediate in the uncatalyzed reaction; in the presence of a phosphine oxide catalyst, a four-membered ring intermediate is highly probable (Chadwick and Cleveland, 1979). Alternative manufacturing methods have been suggested: 1) *N,N′*-dicylclo-hexylthiourea is reacted with cyanuric chloride in dichloromethane to yield an oily product that can be hydrolyzed with sodium hydroxide and heated, producing dicyclohexylcarbodiimide and trithiocyanuric acid (Furumoto, 1971a), or 2) *N,N′*-dicyclohexylthiourea is treated with dichlorodicyanobenzoquinone in dichloromethane to produce a mixture that can be evaporated and heated in sodium hydroxide to yield dicyclohexylcar-bodiimide (Furumoto, 1971b).

Although carbodiimides were discovered in 1873, it was not until the early 1950s that they were used in industry. The 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, carbodiimides and dicyclohexylcarbodiimide in particular have been widely used in the synthesis of *ortho*- and pyrophosphate esters, nucleotides, cyclic phosphates, oligoribonucleotides, polynucleotides, nucleoside-5′-phosphoroamidates, and mixed anhydrides (Azzi *et al*., 1984). The application of dicyclohexylcarbodiimide-induced formation of an amide bond has been used for many other purposes, among which is the chemical synthesis of peptides. It has been used particularly in the synthesis of polypeptides with high molecular weights from low-molecular weight polypeptides. As the manufacture of polypeptides is a rapidly growing segment of the chemical and pharmaceutical industries, the use of dicyclohexylcarbodiimide is expected to increase. This technique has also been applied in the preparation of gels for affinity chromatography and in a method for the quantitative modification and estimation of carboxylic groups in proteins. In addition, dicyclohexylcarbodiimide is increasingly used in the emerging industry of recombinant DNA as a coupling agent in protein synthesis (Azzi *et al*., 1984; Hoffman and Adams, 1989). Dicyclohexylcarbodiimide has been used as a coupling agent to produce natural lubricating oils by combining the free carboxyl and amino groups in the oil to neutral amide groups (Azzi *et al.*, 1984), and it has been used to synthesize two protected peptide proteins of scorpion neurotoxin II, steroidal esters of carboxylic derivatives of *N,N*-bis(2-chloroethyl)aniline, and acylureas (Orlowska *et al*., 1983; Pairas *et al*., 1985; Sabatier *et al*., 1987; Slebioda *et al*., 1990).

Since the discovery in 1967 that dicyclohexylcarbodiimide inhibits proton-translocating ATPase in mitochondria, it has been widely used in the field of bioenergetics. Studies have used this inhibitory effect on ATPases to determine the structure and function of membrane transport systems and enzyme complexes. These have included proton ATPases in mitochondria, chloroplasts, bacteria, and chromaffin granules (the catecholamine vesicles of the adrenal medulla); Ca^{2+}-ATPase of sarcoplasmic reticulum; mitochondrial nicotinamide nucleotide transhydrogenase; cytochrome c oxidase (the terminal electron-transporting enzyme of the respiratory chain of mitochondria and bacteria); and the cytochrome bc_1 complex of the mitochondrial respiratory chain (Azzi *et al*., 1984).

Human exposure to dicyclohexylcarbodiimide 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

The NTP performed absorption, distribution, metabolism, and excretion studies of dicyclohexylcarbodiimide in F344/N rats and B6C3F$_1$ mice (Appendix J). When radiolabeled dicyclohexylcarbodiimide was administered intravenously, rats excreted approximately 50% in urine, 20% to 30% in feces, and less than 1% in breath as volatile organics and CO_2; mice excreted approximately 50% in urine, 25% in feces, and 1% in breath. Following dermal application of radiolabeled dicyclohexylcarbodiimide, rats absorbed approximately 25% of the administered dose, with most of the dose remaining in the skin at the site of application; mice absorbed approximately 30% to 40% of the administered dose and excreted 11% to 22% in urine and 10% to 14% in feces. Seventy-two hours after dermal application of dicyclohexylcarbodiimide, negligible radioactivity was detected in collected tissues of both rats and mice.

TOXICITY

Experimental Animals

In rats, the oral LD_{50} for dicyclohexylcarbodiimide is 400 mg/kg, the intraperitoneal LD_{50} is 10 mg/kg, and the inhalation LC_{50} is 159 mg/m^3 per 6 hours; in mice, the LD_{50} by oral and intraperitoneal routes exceeds 800 mg/kg; in guinea pigs, the dermal LD_{50} is 10 mL/kg (NTIS, 2004). Dicyclohexylcarbodiimide has been identified as an irritant and contact sensitizer when topically applied to female B6C3F$_1$ mice at a concentration as low as 0.006% (w/v) (Hayes *et al*., 1998). The contact hypersensitivity of dicyclohexylcarbodiimide may be dependent on haplotype, however, as different responses have been observed in multiple strains of mice (Kato *et al*., 2002). In response to sensitization at a minimally irritating dose, different mouse strains with different histocompatibility-2 locus (H2) haplotypes (C57BL/6, H2b; DBA/1, H2q; BALB/c, H2d) responded to dicyclohexylcarbodiimide challenge, but some did not (C3H/HeJ, H2k). Both DBA/1 and FVB/N have the same haplotype (H2q) and, thus, FVB/N (H2q) and B6C3F$_1$ mice (H2b/k), which carry both responsive (b) and nonresponsive (k) alleles, should also respond to contact sensitization exposure. A mechanistic

relationship between mutagenicity, skin sensitization, and carcinogenicity has been proposed (Ashby *et al.*, 1993) based on reactive intermediate metabolites of mutagenic carcinogens adducting both protein (forming hapten) and DNA. However, contact sensitizers that adduct protein through Michael's substitution reactions to form hapten are not likely carcinogens. In the FVB/N-Tg.AC (v-Ha-*ras*) mouse used in these studies, tumorigenicity has been attributed to cytotoxicity and not contact sensitization (Albert *et al.*, 1996). The irritancy of dicyclohexylcarbodiimide to the skin of BALB/c mice has been reported to be exacerbated by concurrent stress (Flint *et al*., 2003).

Bioenergetic studies have indicated that dicyclohexylcarbodiimide is associated with a variety of adverse biological effects, many of which are due to the time-dependent, irreversible inhibitory effect of the chemical on proton translocation in ATPases and enzyme complexes of the respiratory chain. However, it has also been reported that, in the mucosa of the pig intestine, dicyclohexylcarbodiimide inhibits proline-β-naphthylamidase in a reversible and noncompetitive manner, indicating binding at a site other than its substrate binding site (Takahashi and Takahashi, 1990). Dicyclohexylcarbodiimide readily inactivates *Escherichia coli* BF1-ATPase at 0.05 mM, while the related chemical, diisopropylcarbodiimide, shows almost no inactivation at this concentration (Satre *et al*., 1979).

The metabolic effects of dicyclohexylcarbodiimide in suspensions of dog nephron segments has been investigated (Tejedor *et al*., 1987). In this study, dicyclohexylcarbodiimide inhibited a ouabain-insensitive activity responsible for respiration in proximal tubules but not in the thick ascending limbs. However, measurement of cellular ATP, ADP, and AMP demonstrated that dicyclohexylcarbodiimide interfered with phosphorylation but not respiration in aerobic tissues, probably at the functional oxygenase unit of mitochondrial H$^+$-ATPase. It was also found that the effect of dicyclohexylcarbodiimide on ATP turnover in papillary connecting ducts may not reflect direct inhibition of a membrane proton pump.

It has been reported that dicyclohexylcarbodiimide elicited a sharp increase in the rate of oxygen consumption in guinea pig peritoneal neutrophils; the respiratory burst consisted of a lag phase during which NADPH oxidase underwent activation and a superoxide-producing phase (Aviram and Aviram, 1983).

Inactivation of hormone receptors in purified rat ovarian plasma membranes by dicyclohexylcarbodiimide has been reported (Azulai and Salomon, 1979). In these studies, preincubation with 0.5 mM dicyclohexylcarbodiimide reduced human chorionic gonadotrophin binding by 50%, completely abolished hormone-sensitive adenylate cyclase (AC) activity stimulated by lutropin, follotropin, or human chorionic gonadotrophin, but did not affect basal AC activity and activity stimulated by sodium fluoride or guanyl-5′-imidodiphosphate.

Humans

Occupational contact dermatitis to dicyclohexylcarbodiimide has been reported in research laboratory workers since the late 1950s. Zschunke and Folesky (1975) reported about three research laboratory workers with contact dermatitis in 1959 and seven cases of contact dermatitis in pharmaceutical workers in 1975. Subsequent case reports of skin sensitization have since appeared (Simpson, 1979; White and MacDonald, 1979; Davies, 1983; Funfstuck *et al*., 1986; Lang and Hensel, 1987; Poesen *et al*., 1995; Vente *et al*., 1999). Hoffman and Adams (1989) reported two cases of contact allergic dermatitis to dicyclohexylcarbodiimide used in protein synthesis. A 33-year-old research chemist developed severe, blistering dermatitis of the hands, forearms, neck, and face after working with dicyclohexylcarbodiimide while synthesizing a precursor of an experimental drug. Patch testing of dicyclohexylcarbodiimide at 0.1% and 0.05% concentration in petrolatum elicited strong positive reactions. A 29-year-old chemist who had been working for several months with dicyclohexylcarbodiimide developed acute vesiculobullous dermatitis after contact with crystalline dicyclohexylcarbodiimide. Patch testing revealed strong positive reactions to 0.1% and 0.05% dicyclohexylcarbodiimide in acetone.

Dicyclohexylcarbodiimide vapor has been reported to cause four cases of acute chemical ophthalmitis (Changji, 1983). A related alkylcarbodiimide, diisopropylcarbodiimide, has been reported to have caused delayed, temporary blindness following an acute occupational exposure to diisopropylcarbodiimide vapor in a worker who cleaned up a 1 L spill 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 was restored over a 2-week period (Moyer, 1990). 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
A single oral reproductive study in rats of the related chemical carbodiimide has been reported (RTECS, 1991). In this study, a dose of 2,450 mg carbodiimide/kg body weight caused preimplantation mortality, 1,750 mg/kg caused paternal effects (testes, epididymis, sperm duct, prostate, seminal vesicle, Cowper's gland, and accessory glands), and 208 mg/kg affected postimplantation mortality and the live birth index. In the same study, 2,600 mg/kg affected the live birth index and growth statistics in newborn rats.

Humans
No information on the reproductive or developmental toxicity of dicyclohexylcarbodiimide in humans was found in the published literature.

CARCINOGENICITY
No information on the carcinogenicity of dicyclohexylcarbodiimide in animals or epidemiology studies or case reports associating dicyclohexylcarbodiimide with a cancer risk in humans were found in the published literature.

GENETIC TOXICITY
Witt *et al.* (1999) described the results of a series of *in vivo* mutagenicity tests with dicyclohexylcarbodiimide and the related compound diisopropylcarbodiimide. Both chemicals were shown to induce micronuclei in erythrocytes of male and female B6C3F$_1$ mice treated by skin painting for a period of 13 weeks, although the response induced by dicyclohexylcarbodiimide was weak. An additional acute bone marrow micronucleus test with dicyclohexylcarbodiimide in

male F344/N rats using intraperitoneal injection as the route of administration gave negative results.

A second subchronic micronucleus study was conducted with diisopropylcarbodiimide in male B6C3F$_1$ mice, in which the chemical was administered by skin painting for 18 weeks and weekly or biweekly counts of micronucleated polychromatic erythrocytes (PCEs), micronucleated normochromatic erythrocytes, and the percentage of PCEs were obtained (Witt *et al.*, 1999). Results of this study confirmed the activity of diisopropylcarbodiimide that was observed in the 13-week skin painting study. Similar to what was seen with dicyclohexylcarbodiimide, however, results of short-term tests with diisopropylcarbodiimide in both F344/N rats and B6C3F$_1$ mice showed no clear evidence of micronucleus induction in bone marrow PCEs.

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 dicyclohexylcarbodiimide exposure. These mouse models are susceptible to the rapid development of cancer. The Tg.AC hemizygous and p53 haploinsufficient mice 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
Tg.AC mice are hemizygous for a mutant V-Ha-*ras* transgene. 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. Because the inducible zeta-globin promoter drives the expression of a mutated v-Ha-*ras* oncogene,

the Tg.AC mouse is regarded as a genetically initiated model.

The Tg.AC 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). With the exception of bone marrow, constitutive expression of the transgene cannot be detected in adult tissues. The transgene is 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). The Tg.AC hemizygous mouse develops a high incidence of skin papillomas in response to topical application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and TPA has been used as a positive control in NIEHS Tg.AC mouse studies (Spalding *et al.*, 1993). TPA has been used as a positive control in NIEHS Tg.AC mouse studies to confirm the mice are responsive to carcinogens because it has been found that a subset of Tg.AC mice may revert and become nonresponsive to a tumor promoter (Honchel *et al*, 2001). 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 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 mice and lead to squamous cell papillomas and/or carcinomas of the forestomach. 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 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 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 papil-

lomas, cutaneous papillomas, alveolar-bronchiolar adenomas, salivary gland duct carcinomas, and erythroleukemia (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 the studies of dicyclohexylcarbodiimide 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)^tmlBrd(+/−) 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 G_0 to G_1, as well as G_2 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). Mutation in p53 may also increase the protein half-life and alter the functions that may contribute to transformation and development of the malignant phenotype. p53 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 tumor suppressor gene is one of the most common sites for mutations and gene alterations in human cancer (Harris, 1996a,b,c).

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 AND DESIGN

Dicyclohexylcarbodiimide and diisopropylcarbodiimide were nominated by the National Cancer Institute for toxicity and carcinogenicity studies as representatives of the carbodiimide chemical class. The results of the diiso-propylcarbodiimide studies were presented in separate Reports (NTP, 2007a,b).

Carbodiimides are known to be irritating to the skin, eyes, and respiratory tract. Despite a lack of adequate carcinogenicity data, dicyclohexylcarbodiimide is widely used in industry as a stabilizing agent, coupling agent, and condensing agent. Because of increasing use of dicyclohexylcarbodiimide in the synthesis of polypeptides in the chemical, pharmaceutical, and recombinant DNA industries, the potential for low-level human exposure has grown. The dermal route of exposure was chosen for the studies in this Report to mimic the primary route of human exposure.

Two of the studies in the current Report were performed in genetically modified mice. These studies in Tg.AC hemizygous and p53 haploinsufficient mouse models were performed as part of the NTP effort to evaluate alternative cancer models that require shorter exposure times, are less expensive to study, and use fewer animals when compared to the traditional 2-year study design.

MATERIALS AND METHODS

Procurement and Characterization

Dicyclohexylcarbodiimide

Dicyclohexylcarbodiimide, lot 00929TZ, was obtained from Aldrich Chemical Company (Milwaukee, WI) and was used in the 3-, 13-, and 20-week studies; lot 60104-1 was obtained from Chem-Impex International (Wood Dale, IL) and was used in the 27-week study. Identity and purity analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO), and by the study laboratories, Microbiological Associates, Inc. (Bethesda, MD; 3-, 13-, and 20-week studies), and BioReliance Corporation (Rockville, MD; 27-week study) (Appendix I). Reports on analyses performed in support of the dicyclohexylcarbodiimide studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a colorless crystalline material, was identified as dicyclohexylcarbodiimide by the study laboratories using infrared spectroscopy (lots 00929TZ and 60104-1) and by the analytical chemistry laboratory using infrared and proton nuclear magnetic resonance (NMR) spectroscopy (lot 60104-1). All spectra were consistent with the literature spectra (*Aldrich*, 1981a, 1985, 1993).

The purity of lot 00929TZ was determined by the study laboratory using gas chromatography (GC). The purity of lot 60104-1 was determined by the analytical chemistry laboratory using high-performance liquid chromatography (HPLC), and purity relative to a frozen reference standard of the same lot was measured by the study laboratory using HPLC.

For lot 00929TZ, GC indicated one major peak, three major impurities (ranging from 0.08% to 0.27% of the total peak area), and two minor impurities. The overall purity of lot 00929TZ was determined to be 99.5%. For lot 60104-1, HPLC indicated a major peak and six impurity peaks with a combined area of 1.69% relative to the total peak area. The overall purity of lot 60104-1 was estimated to be greater than 98%.

To ensure stability, the bulk chemical was stored at room temperature, under a nitrogen head space, protected from light. Periodic analyses of the bulk chemical were performed by the study laboratories during the studies using GC (lot 00929TZ) and HPLC (lot 60104-1); no degradation of the bulk chemical was detected.

Anhydrous Ethanol

Anhydrous ethanol was obtained from Pharmco Products, Inc. (Brookfield, CT). Identity and purity analyses of all lots used in the 27-week study were conducted by the study laboratory. The chemical, a clear liquid, was identified as ethanol using IR spectroscopy; the sample spectra were consistent with a literature spectrum (*Aldrich*, 1981b). The purity of each lot was analyzed using GC. The overall purity of each analyzed lot was determined to be greater than 99.9%.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared twice for the 3-week studies, at least every 2 weeks for the 13-week studies, every 3 weeks for the 20-week study, or monthly for the 27-week study by mixing dicyclohexylcarbodiimide and anhydrous ethanol to give the required concentration. The dose formulations were stored in sealed vials under a headspace of inert gas for up to 28 days (3- and 13-week studies) or 35 days (20- and 27-week studies) at room temperature for the 3-, 13-, and 20-week studies, and until April 1, 1999 (week 13), for the 27-week study; subsequent storage for the 27-week study was at –20° C.

Because the dose formulations were true solutions of the test article in ethanol, homogeneity studies were not performed. Stability studies of 0.38, 2, and 7 mg/mL dose formulations of lot 00929TZ were conducted by the study laboratory using GC. Stability was confirmed for up to 35 days for dose formulations stored at room temperature in sealed containers under a nitrogen headspace and for up to 3 hours when exposed to light and air at room temperature.

Periodic analyses of the dose formulations of dicyclo-hexylcarbodiimide were conducted by the study labora-tories using GC. Dose formulations were analyzed once for the 3-week studies; all five dose formulations were within 10% of the target concentrations (Table I4). Animal room samples were also analyzed; all 10 animal room samples for rats and mice were within 10% of the target concentrations. During the 13-week studies, the dose formulations were analyzed at the beginning, mid-point, and end of the studies; animal room samples of these dose formulations were also analyzed (Table I5). Of the dose formulations analyzed, all 15 for rats and all 15 for mice were within 10% of the target concentration; all 13 animal room samples for rats and 12 of 13 for mice were within 10% of the target concentrations. One aber-rant mouse animal room sample was determined to be due to evaporation from an inadequately sealed vial. Dose formulations were analyzed at the beginning, mid-point, and end of the 20-week study, and animal room samples of these dose formulations were also analyzed; all 15 dose formulations and all 13 animal room samples were within 10% of the target concentrations (Table I6). During the 27-week study, the dose formulations were analyzed four times; animal room samples were also analyzed. All 14 dose formulations analyzed were within 10% of the target concentrations; 8 of 14 animal room samples were not within 10% of target concentra-tions (Table I7). Unusual degradation was observed in the animal room samples from the formulations prepared on February 24, 1999, and March 24, 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. Starting on April 2, 1999, all dose formulations were stored at –20° C as a further precaution against degradation.

3-WEEK STUDIES
IN F344/N RATS AND B6C3F$_1$ MICE

Male and female F334/N rats and B6C3F$_1$ mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY). On receipt, the animals were approximately 5 weeks old. Rats were quarantined for 26 days and were 9 weeks old on the first day of the study. Mice were quarantined for 20 days and were 8 weeks old on the first day of the study. Before the studies began, two male and two female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. Groups of 5 male and 5 female rats and mice received dermal applications of 0, 2, 6, 17, 50, or 150 mg dicyclohexylcarbodiimide per mL of ethanol, 5 days per week for 16 days. Fixed

volumes of ethanol (rats, 0.3 mL; mice, 0.1 mL) were applied to the clipped dorsal skin, from the posterior of the scapulae to the base of the tail, resulting in daily doses of 0, 0.6, 1.8, 5.1, 15, or 45 mg dicyclohexylcar-bodiimide per rat and 0, 0.2, 0.6, 1.7, 5, or 15 mg per mouse. Rats and mice were housed individually, with feed and water available *ad libitum*. The animals were weighed and clinical findings were recorded initially, on day 8, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

Necropsies were performed on all rats and mice. The adrenal gland, heart, right kidney, liver, lung, right testis, and thymus were weighed. Histopathologic examina-tions were performed on rats administered 0, 0.6, 1.8, and 5.1 mg/animal and mice administered 0, 0.2, 0.6, and 1.7 mg/animal. Table 1 lists the tissues and organs examined.

13-WEEK STUDIES
IN F344/N RATS AND B6C3F$_1$ MICE

Male and female F334/N rats and B6C3F$_1$ mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY). On receipt, the animals were approximately 5 weeks old. Animals were quarantined for 12 to 15 days and were 7 weeks old on the first day of the studies. Before initiation of the studies, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evi-dence of disease. Blood was collected from five male and five female vehicle control rats and sentinel 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.

Groups of 10 male and 10 female core study rats received dermal applications of 0, 0.75, 1.5, 3, 6, or 12 mg dicyclohexylcarbodiimide/kg body weight in ethanol, 5 days per week for 13 weeks; groups of 10 male and 10 female clinical pathology rats were given the same doses for 22 days. Groups of 10 male and 10 female mice received dermal applications of 0, 1.5, 3, 6, 12, or 24 mg/kg in ethanol, 5 days per week for 13 weeks. Dosing volumes of 0.5 and 2 mL/kg for rats and mice, respectively, were applied to a clipped dorsal area from the posterior of the scapulae to the base of the tail. Rats and mice were housed individually, with feed and water available *ad libitum*. Body weights and clini-cal findings were recorded initially, weekly, and at the end of the studies for core study animals. Details of the

study design and animal maintenance are summarized in Table 1.

Blood was collected for hematology and clinical chemistry analyses from clinical pathology study rats on days 3 and 22 and from core study rats at study termination. Blood was collected for hematology analyses from mice at study termination. At all time points, the animals were anesthetized with a 70% CO_2/30% O_2 mixture and blood was collected from the retroorbital sinus. Blood for hematology analyses was placed in tubes containing EDTA as the anticoagulant. Erythrocyte, platelet, and leukocyte counts, automated hematocrit values, hemoglobin concentration, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration were determined using a Serono-Baker System 9010 hematology analyzer (Serono-Baker Diagnostics, Allentown, PA). Manual hematocrit determinations were performed using an Adams Microhematocrit Centrifuge, Model CT2900 (Becton, Dickinson and Company, Franklin Lakes, NJ). Differential leukocyte counts and erythrocyte and leukocyte morphologies were determined microscopically from blood smears stained with a modified Wright's stain on a Hema-Tek slide stainer (Miles Laboratory, Ames Division, Elkhart, IN). Reticulocytes were stained with new methylene blue and counted microscopically. For clinical chemistry analyses, blood samples were placed into untreated serum separator tubes, centrifuged, and the serum samples were analyzed using a Hitachi 717 chemistry analyzer (Boehringer Mannheim, Indianapolis, IN) and commercially available reagents. The parameters measured are listed in Table 1.

At the end of the 13-week studies, samples were collected for sperm motility and vaginal cytology evaluations on 0, 1.5, 3, and 6 mg/kg core study rats and 0, 3, 6, and 12 mg/kg mice. The parameters evaluated are listed in Table 1. For 12 consecutive days prior to scheduled terminal sacrifice, the vaginal vaults of the females were moistened with saline, if necessary, and samples of vaginal fluid and cells were collected and stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus). Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides and a small incision

was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65° C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

Necropsies were performed on all core study animals. The heart, right kidney, liver, lung, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed on 0, 6, and 12 mg/kg core study rats and 0, 12, and 24 mg/kg mice. Selected tissues were examined in the remaining dosed groups. 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).

20-WEEK STUDY
IN FEMALE TG.AC HEMIZYGOUS MICE

Female FVB/N-TgN(v-Ha-*ras*)Led (Tg.AC) hemizygous mice were obtained from Taconic Laboratory Animals and Services, Inc. (Germantown, NY). On receipt, the mice were 4 weeks old. Animals were quarantined for 14 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 and from one mouse that was sacrificed moribund during the study. The sera were analyzed for antibody titers to rodent viruses; all results were negative.

Doses for the 20-week study in Tg.AC hemizygous mice were based on the results of 13-week toxicity studies performed in B6C3F$_1$ mice. Groups of 10 female mice received dermal applications of 0, 0.75, 1.5, 3, 6, or 12 mg/kg in ethanol, 5 days per week for up to 20 weeks. Due to the severity of skin lesions observed in 12 mg/kg animals, the application of dicyclohexylcarbodiimide was discontinued after eight dermal applications in this group. A dosing volume of 2 mL ethanol per kg body weight 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. From June 26, 1995, doses were applied anterior or posterior to the original site if lesions were observed at the original site. Animals were housed individually, with feed and water available *ad libitum*. Body weights and clinical findings were recorded initially, weekly, and at the end of the study. 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. 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. Complete histopathologic examinations were performed on all vehicle control mice, all 12 mg/kg mice, and all mice that died before the end of the study. In addition, the skin was examined in all remaining dosed groups. 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.

27-Week Study in Female p53 Haploinsufficient Mice

Female B6.129-*Trp53*tm1Brd (N5) haploinsufficient mice were obtained from Taconic Farms, Inc. (Germantown, NY). On receipt, the animals were approximately 6 weeks old. Animals were quarantined for 14 days and were 8 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.

Doses for the 27-week study in p53 haploinsufficient mice were based on the results of 13-week toxicity studies performed in B6C3F$_1$ mice. Groups of 15 female mice received dermal applications of 0, 0.75, 1.5, 3, 6, or 12 mg/kg in ethanol, 5 days per week for up to 27 weeks. Dosing of the 6 and 12 mg/kg groups was discontinued after 11 and 8 days, respectively, because of the severity of skin lesions at the site of application. The dosing volume of 2 mL/kg was applied to a shaved dorsal area posterior of the scapulae to the base of the tail. Mice were housed individually, with feed and water available *ad libitum*. Body weights and clinical findings were recorded initially, weekly, and at the end of the study. Details of the study design and animal maintenance are summarized in Table 1.

Necropsies were performed on all animals. 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. Histopathologic examinations were performed on all animals. 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. The final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, reviewing pathologist, NTP Pathology Working Group chairperson, and the NTP pathologist.

ABLE 1
Experimental Design and Materials and Methods in the Dermal Studies of Dicyclohexylcarbodiimide

3-Week Studies	13-Week Studies	20-Week Study	27-Week Study
Study Laboratory			
Microbiological Associates, Inc. (Bethesda, MD)	Microbiological Associates, Inc. (Bethesda, MD)	Microbiological Associates, Inc. (Bethesda, MD)	BioReliance Corporation (Rockville, MD)
Strain and Species			
F344/N rats B6C3F$_1$ mice	F344/N rats B6C3F$_1$ mice	FVB/N-TgN(v-Ha-*ras*)Led (Tg.AC) hemizygous mice	B6.129-*Trp53*[tm1Brd] (N5) haploinsufficient mice
Animal Source			
Taconic Laboratory Animals and Services (Germantown, NY)	Taconic Laboratory Animals and Services (Germantown, NY)	Taconic Laboratory Animals and Services (Germantown, NY)	Taconic Laboratory Animals and Services (Germantown, NY)
Time Held Before Studies			
Rats: 26 days Mice: 20 days	Rats: 12 days (males) or 13 days (females) Mice: 14 days (males) or 15 days (females)	14 days	14 days
Average Age When Studies Began			
Rats: 9 weeks Mice: 8 weeks	7 weeks	6 weeks	8 weeks
Date of First Dose			
Rats: June 28, 1994 Mice: June 29, 1994	Rats: September 20 (males) or September 21 (females), 1994 Mice: September 22 (males) or September 23 (females), 1994	June 15, 1995	January 5, 1999
Duration of Dosing			
5 doses per week for 16 days	5 doses per week for 13 weeks	5 doses per week for 2 (12 mg/kg) or 20 weeks	5 doses per week for 2 weeks (12 mg/kg), 3 weeks (6 mg/kg), or 27 weeks
Date of Last Dose			
Rats: July 13, 1994 Mice: July 14, 1994	Rats: December 19 (males) or December 20 (females), 1994 Mice: December 21 (males) or December 22 (females), 1994	June 26 (12 mg/kg) or November 1, 1995	July 7-8, 1999
Necropsy Dates			
Rats: July 14, 1994 Mice: July 15, 1994	Rats: December 20 (males) or December 21 (females), 1994 Mice: December 22 (males) or December 23 (females), 1994	November 2, 1995	July 8-9, 1999
Average Age at Necropsy			
Rats: 11 weeks Mice: 10 weeks	Rats: 20 (males) or 20 (females) weeks Mice: 20 weeks	27 weeks	34 weeks
Size of Study Groups			
5 males and 5 females	10 males and 10 females	10 females	15 females

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

3-Week Studies	13-Week Studies	20-Week Study	27-Week Study
Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 3-week studies	Same as 3-week studies	Same as 3-week studies
Animals per Cage 1	1	1	1
Method of Animal Identification Tail tattoo	Tail tattoo	Tail tattoo	Tail tattoo
Diet NIH-07 open formula pelleted diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed weekly	Same as 3-week studies	Same as 3-week studies	Irradiated NTP-2000 open formula pelleted diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed weekly
Water Tap water (Washington Suburban Sanitary Commission Potomac Plant) via automatic watering system (Edstrom Industries; Waterford, WI) available *ad libitum*	Same as 3-week studies	Same as 3-week studies	Same as 3-week studies
Cages Polycarbonate (Lab Products, Inc., Seaford, DE), changed once weekly	Same as 3-week studies	Same as 3-week studies	Same as 3-week studies
Bedding Heat-treated Sani-chips® (P.J. Murphy Forest Products Corp., Montville, NJ) changed once weekly	Same as 3-week studies	Same as 3-week studies	Same as 3-week studies
Cage Filters Remay 2016 (Snow Filtration, West Chester, OH), changed once weekly	Same as 3-week studies	Same as 3-week studies	Same as 3-week studies
Racks Stainless steel (Lab Products, Inc., Seaford, DE), changed and rotated once every 2 weeks	Same as 3-week studies	Same as 3-week studies	Same as 3-week studies
Animal Room Environment Temperature: $72° \pm 3°$ F Relative humidity: $50\% \pm 15\%$ Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Temperature: $72° \pm 3°$ F Relative humidity: $50\% \pm 15\%$ Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Temperature: $72° \pm 3°$ F Relative humidity: $50\% \pm 15\%$ Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Temperature: $72° \pm 3°$ F Relative humidity: $50\% \pm 15\%$ Room fluorescent light: 12 hours/day Room air changes: at least 10/hour

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

3-Week Studies	13-Week Studies	20-Week Study	27-Week Study
Doses Rats: 0, 0.6, 1.8, 5.1, 15, or 45 mg/animal in 0.3 mL ethanol Mice: 0, 0.2, 0.6, 1.7, 5, or 15 mg/animal in 0.1 mL ethanol	Rats: 0, 0.75, 1.5, 3, 6, or 12 mg/kg body weight in 0.5 mL/kg ethanol Mice: 0, 1.5, 3, 6, 12, or 24 mg/kg in 2 mL/kg ethanol	Mice: 0, 0.75, 1.5, 3, 6, or 12 mg/kg body weight administered in ethanol (2 mL/kg) 5 days per week	Mice: 0, 0.75, 1.5, 3, 6, or 12 mg/kg body weight administered in ethanol (2 mL/kg) 5 days per week
Type and Frequency of Observation Observed twice daily; animals were weighed and clinical findings recorded initially, on day 8, and at the end of the studies.	Observed twice daily; animals were weighed and clinical findings recorded initially, weekly, and at the end of the studies.	Observed twice daily; animals were weighed and clinical findings recorded initially, weekly, and at the end of the study	Observed twice daily; animals were weighed and clinical findings recorded initially, weekly, and at the end of the study
Method of Sacrifice Carbon dioxide asphyxiation	Same as 3-week studies	Same as 3-week studies	Same as 3-week studies
Necropsy Necropsies were performed on all animals. Organs weighed were the adrenal gland, heart, right kidney, liver, lung, right testis, and thymus.	Necropsies were performed on all core study animals. Organs weighed were the heart, right kidney, liver, lung, right testis, and thymus.	Necropsies were performed on all animals. Organs weighed were the heart, right kidney, liver, lung, and thymus.	Necropsies were performed on all animals.
Clinical Pathology None	Blood was collected from the retroorbital sinus of special study rats on days 3 and 22 and from all core study rats and mice surviving to the end of the studies for hematology and clinical chemistry (rats only) determinations. *Hematology:* automated and manual hematocrit; erythrocyte, reticulocyte, nucleated erythrocyte, and platelet counts; hemoglobin concentration; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; leukocyte count and differentials *Clinical chemistry:* urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and bile acids.	None	None

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

3-Week Studies	13-Week Studies	20-Week Study	27-Week Study
Histopathology			
Histopathology was performed on vehicle control, 0.6, 1.8, and 5.1 mg/animal rats and vehicle control, 0.2, 0.6, and 1.7 mg/animal mice. In addition to gross lesions and tissue masses, the following tissues were examined: brain, kidney, liver, lung, skin, and spinal cord.	Complete histopathology was performed on vehicle control, 6, and 12 mg/kg core study rats, and vehicle control, 12, and 24 mg/kg mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, gallbladder (mice), heart with aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, uterus, and Zymbal's gland. In addition, the bone marrow of rats, spleen of male rats and male and female mice, and the skin of rats and mice were examined in all remaining dosed groups.	Complete histopathology was performed on all vehicle control and 12 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 with aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, salivary gland, skin, spleen, stomach (forestomach and glandular), thymus, thyroid gland, trachea, urinary bladder, and uterus. The skin at the site of application was also examined in all remaining dosed groups.	Histopathology was performed on all animals. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, kidney, liver, lung, lymph nodes (mandibular, mediastinal, and mesenteric), mammary gland, ovary, parathyroid gland, pituitary gland, skin, spleen, stomach (forestomach and glandular), thymus, thyroid gland, trachea, and uterus.

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

3-Week Studies	13-Week Studies	20-Week Study	27-Week Study
Sperm Motility and Vaginal Cytology			
None	At the end of the studies, sperm samples were collected from vehicle control, 1.5, 3, and 6 mg/kg core study male rats and vehicle control, 3, 6, and 12 mg/kg male mice for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, spermatid counts, and epididymal spermatozoal motility and concentration. The left cauda, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from vehicle control, 1.5, 3, and 6 mg/kg core study female rats and vehicle control, 3, 6, and 12 mg/kg female mice for vaginal cytology evaluations. The percentage of time spent in the various estrous cycle stages and the estrous cycle length were evaluated.	None	None

STATISTICAL METHODS

Calculation and Analysis of Lesion Incidences

The incidences of lesions are presented in Appendixes A, B, C, and D 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

Two approaches were employed to assess the significance of pairwise comparisons between dosed groups and vehicle control groups in the 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). Hematology, clinical chemistry, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) (as modified by Williams, 1986) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1957) were examined by NTP personnel, and implausible values were eliminated from the analysis. Average severity values were analyzed for significance with the Mann-Whitney U test (Hollander and Wolfe, 1973). Because vaginal cytology data are proportions (the proportion of the observation period that an animal was in a given estrous stage), an arcsine transformation was used to bring the data into closer conformance with a normality assumption. Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for simultaneous equality of measurements across exposure concentrations.

QUALITY ASSURANCE METHODS

The 3-, 13-, 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.

GENETIC TOXICOLOGY

Salmonella typhimurium Mutagenicity Test Protocol

Testing was performed as reported by Zeiger *et al.* (1992). Dicyclohexylcarbodiimide was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains TA97, TA98, TA100, and TA1535, either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following 2 days incubation at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and five doses of dicyclohexylcarbodiimide. The high dose was limited by toxicity. All trials were repeated at the same or a higher S9 fraction.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

Rat Bone Marrow
Micronucleus Test Protocol

The detailed protocol is described by Witt *et al*. (1999). Information from the 13-week study with dicyclohexylcarbodiimide was used to select the range of doses in this test. The standard three-injection protocol is described in detail by Shelby *et al*., 1993. Five male F344/N rats per dose group were injected intraperitoneally (three times at 24-hour intervals) with dicyclohexylcarbodiimide dissolved in corn oil. Vehicle control animals were injected with corn oil alone. The positive control for each trial was cyclophosphamide administered by intraperitoneal injection at a dose of 25 mg/kg. The rats were killed 24 hours after the third injection, and blood smears were prepared from bone barrow cells obtained from femurs. Air-dried smears were fixed in absolute methanol and stained with acridine orange (Tice *et al*., 1990). Two thousand polychromatic erythrocytes (PCEs) were scored for the frequency of micronucleated cells in two to five rats per dose group. In addition, the percentage of PCEs among the total erythrocyte population was scored for each dose group as a measure of toxicity.

The results were tabulated as the mean of the pooled results from all animals within a treatment group, plus or minus the standard error of the mean. The frequency of micronucleated cells among PCEs was analyzed by a statistical software package that tested for increasing trend over dose groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dosed group and the control group (ILS, 1990). In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single dosed group is less than or equal to 0.025 divided by the number of dosed groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, reproducibility of any effects observed, and the magnitudes of those effects.

Mouse Peripheral Blood
Micronucleus Test Protocol

A detailed discussion of this assay is presented by Witt *et al.* (1999). At the end of the 13-week study, peripheral blood samples were obtained from male and female B6C3F$_1$ mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned at 1,000× magnification using epi-illuminated fluorescence microscopy to determine the frequency of micronuclei in 1,000 normochromatic erthyrocytes (NCEs) in each of 10 mice per dose group per sex. The percentage of PCEs among total erythrocytes was also determined.

The results were tabulated as described for PCEs in the bone marrow micronucleus test protocol. Results of the 13-week study were accepted without repeat tests because additional test data could not be obtained.

Evaluation Protocol

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple aliquots of a chemical were tested in the same assay, and different results were obtained among aliquots and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The summary tables in the Abstract of this Report present a result that represents a scientific judgement of the overall evidence for activity of the chemical in an assay.

RESULTS

3-WEEK STUDY IN F344/N RATS

All males and females in the 15 and 45 mg groups, four 5.1 mg males, and all 5.1 mg females died before the end of the study (Table 2). Of the surviving groups, final mean body weights were similar to those of the vehicle controls, although the single surviving 5.1 mg male rat lost weight during the study. Clinical findings included skin irritation at the site of application at doses of 1.8 mg or greater. Clinical signs of toxicity in 5.1 mg or greater groups included lethargy, ruffled fur, abnormal breathing, coma, and thinness. Organ weights of surviving groups of dosed rats were generally similar to those of the vehicle control groups (Table G1).

Histologic examination of rats dosed with 5.1 mg dicyclohexylcarbodiimide or less revealed test article-related lesions of the skin at the site of application including epidermal hyperplasia, epidermal necrosis, or chronic active inflammation in the dermis (data not shown).

Dose Selection Rationale: In the 3-week rat study, the 1.8 mg/animal approximately corresponds to 12 mg/kg body weight. The doses above 1.8 mg/animal were considered to be prohibitive for 13-week studies due to mortality and clinical signs of toxicity. Therefore, for the 13-week rat study, doses selected were 0.75, 1.5, 3, 6, and 12 mg/kg in ethanol.

TABLE 2
Survival and Body Weights of F344/N Rats in the 3-Week Dermal Study of Dicyclohexylcarbodiimide

Dose (mg/animal)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	207 ± 6	258 ± 9	51 ± 2	
0.6	5/5	209 ± 5	261 ± 8	52 ± 3	101
1.8	5/5	210 ± 7	254 ± 9	45 ± 3	99
5.1	1/5[c]	209 ± 6	191	–23	74
15	0/5[d]	208 ± 5	—	—	
45	0/5[e]	211 ± 9	—	—	
Female					
0	5/5	144 ± 4	165 ± 3	21 ± 2	
0.6	5/5	142 ± 5	164 ± 8	22 ± 3	99
1.8	5/5	143 ± 3	155 ± 5	12 ± 3	94
5.1	0/5[f]	143 ± 6	—	—	
15	0/5[d]	144 ± 4	—	—	
45	0/5[e]	141 ± 4	—	—	

[a] Number of animals surviving at 3 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] Day of death: 10, 11, 11, 11
[d] Day of death: 3 or 4
[e] Day of deaths: 2
[f] Day of death: 9, 9, 9, 10, 11

13-WEEK STUDY IN F344/N RATS

All 12 mg/kg male and female rats died or were found moribund and sacrificed prior to day 45 (Table 3). Final mean body weight and body weight gain of 6 mg/kg males were significantly less than those of the vehicle controls (Table 3 and Figure 1).   Clinical findings in 12 mg/kg males and females included skin irritation at the site of application, lethargy, tremors, ataxia, ruffled fur, a thin appearance, nasal/eye discharge, and head tilt. All 3 and 6 mg/kg males and females had irritation of the skin at the site of application.

TABLE 3
Survival and Body Weights of F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	121 ± 3	330 ± 5	209 ± 5	
0.75	10/10	120 ± 2	337 ± 6	216 ± 6	102
1.5	10/10	120 ± 3	334 ± 6	213 ± 5	101
3	10/10	120 ± 3	325 ± 7	205 ± 6	99
6	10/10	118 ± 2	300 ± 4**	182 ± 3**	91
12	0/10[c]	118 ± 3	—	—	
Female					
0	10/10	102 ± 2	186 ± 3	84 ± 2	
0.75	10/10	102 ± 2	191 ± 3	89 ± 2	103
1.5	10/10	104 ± 2	190 ± 4	86 ± 3	102
3	10/10	104 ± 2	193 ± 3	89 ± 2	104
6	10/10	103 ± 2	192 ± 4	89 ± 3	103
12	0/10[d]	103 ± 2	—	—	

** Significantly different (P≤0.01) from the vehicle control group by Williams' test
[a] Number of animals surviving at 13 weeks/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Week of death: 4, 5, 5, 6, 6, 6, 6, 6, 7, 7
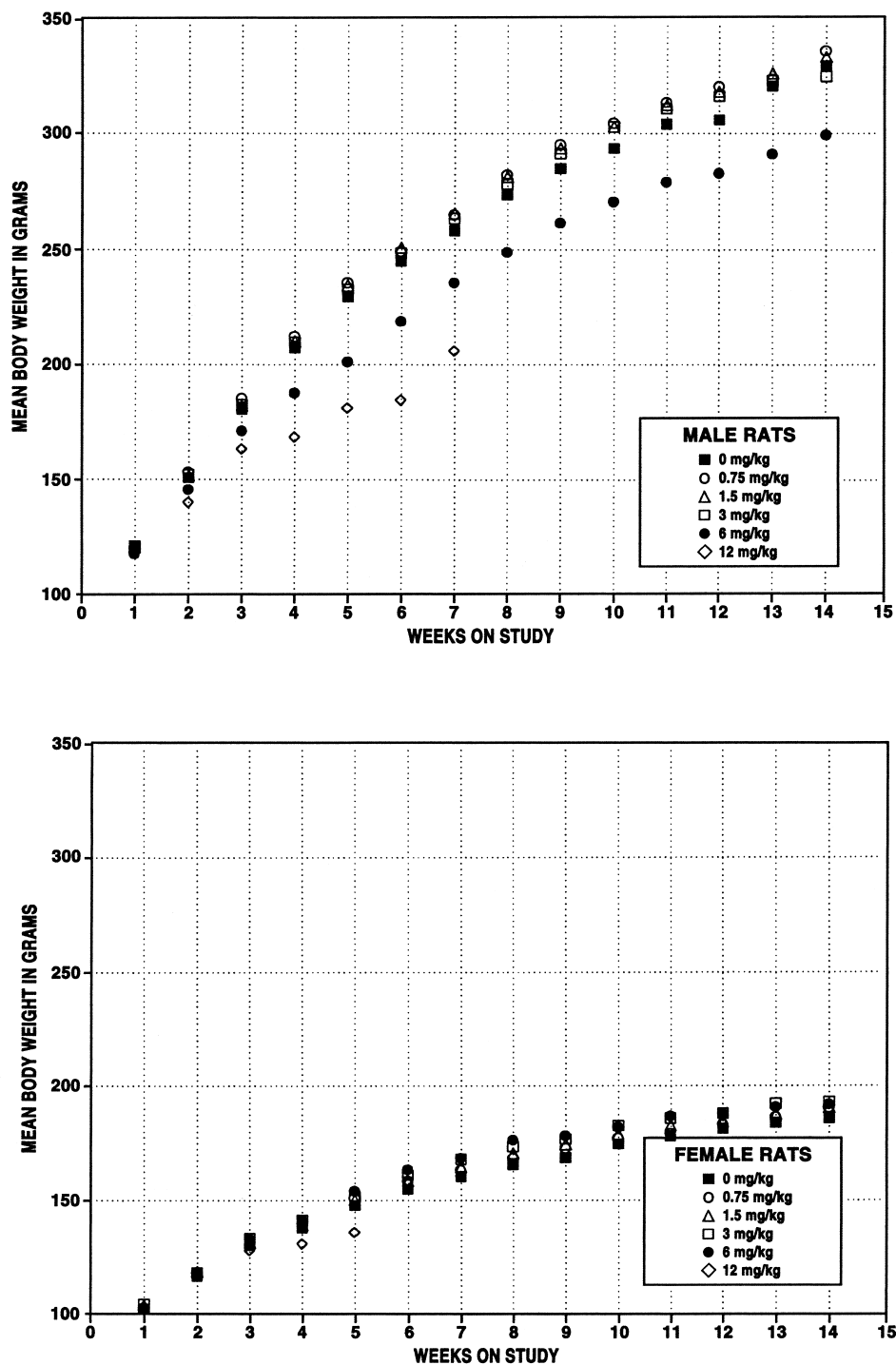[d] Week of death: 4, 4, 4, 5, 5, 5, 5, 6, 7, 7

FIGURE 1
Growth Curves for Male and Female F344/N Rats
Administered Dicyclohexylcarbodiimide Dermally for 13 Weeks

The hematology and clinical chemistry data for rats are presented in Table F1. Evidence of an inflammatory process, indicated by increases in leukocytes, was present at all timepoints. On day 3, a neutrophilia (increased neutrophil counts) was evident in 6 mg/kg or greater males and females. On day 22, a dose-related inflammatory leukogram with a left shift, characterized by increases in leukocyte, neutrophil, and band neutrophil counts, occurred in 3 mg/kg males and 6 mg/kg or greater males and females; monocyte and eosinophil counts were also increased. However, by week 13, the inflammatory leukogram ameliorated and statistically increased neutrophil counts were observed only in 6 mg/kg females. These leukocyte changes would be consistent with the progression of an inflammatory process from the acute to chronic phases and would be a response consistent with the development of skin necrosis and chronic active inflammation observed morphologically. On day 22 and at week 13, minimal decreases in the erythron (<6%), characterized by decreases in hematocrit, hemoglobin and mean cell volume values, and erythrocyte counts, occurred in 6 mg/kg males and 12 mg/kg males and females (day 22) and 6 mg/kg females (week 13). This minimal decrease in the erythron would be consistent with erythron decreases that can develop in response to chronic inflammation and would be considered a secondary response to skin inflammation. Small decreases in albumin concentration (<20%) occurred in 3 mg/kg or greater males and 6 mg/kg or greater females on day 22 and at week 13. The albumin decrease could be a secondary response to chronic inflammation (Kaneko, 1989). Small, transient increases in serum alanine aminotransferase (ALT) activity occurred on days 3 and 22 in treated males and females; the effect abrogated by week 13, and no other marker of hepatocellular injury was affected. The cause was unknown. Because no other marker was affected and the response was transient, a transient induction of ALT production by the liver could be considered (Rosen *et al.*, 1959a,b).

The absolute and relative weights of the heart, kidney, and liver of 3 and 6 mg/kg females were generally greater than those of the vehicle controls (Table G2). Relative weights of the heart and kidney of 3 and 6 mg/kg males were significantly greater than those of the vehicle controls. Dermal administration of dicyclohexylcarbodiimide did not affect reproductive tissue parameters in males or vaginal cytology parameters in females (Tables H1 and H2).

Test article-related gross lesions of the skin at the site of application included crusts or lesions observed in all animals dosed with 3 mg/kg or greater. At the site of application, epidermal hyperplasia occurred in all treated groups, and the incidences of this lesion were significantly increased in 3 mg/kg or greater males and 1.5 mg/kg or greater females; the incidences and severities of the lesion increased in a dose-related fashion (Tables 4, A1, and A2). In addition, chronic active inflammation occurred in most of the dosed groups, and the incidences of this lesion were significantly increased in 6 and 12 mg/kg males and 1.5 mg/kg or greater females. The incidences of sebaceous gland hyperplasia were significantly increased in 3 mg/kg or greater males and 6 and 12 mg/kg females, and the incidence of epidermal necrosis was significantly increased in male rats administered 12 mg/kg. Incidences of sebaceous gland hyperplasia were considered to be a secondary effect of inflammation rather than a direct compound effect. On the surface of the skin there was necrosis with marked acute inflammation consisting of polymorphonuclear cells with edema and coagulated serum admixed with cell debris (crusts). Deeper in the dermis, mononuclear cells mixed with proliferating fibroblasts were present, which suggested a more chronic feature of the inflammatory lesion.

Similar nonneoplastic lesions occurred in control site skin of both sexes of dosed rats, including epidermal necrosis (male: vehicle control, 0/10; 0.75 mg/kg, 0/10; 1.5 mg/kg, 0/10; 3 mg/kg, 0/10; 6 mg/kg, 1/10; 12 mg/kg, 1/10; female: 0/10, 0/10, 0/10, 0/10, 0/10, 1/10), chronic active inflammation (male: 0/10, 0/10, 0/10, 0/10, 3/10, 2/10; female: 0/10, 0/10, 0/10, 1/10, 1/10, 2/10), and epidermal hyperplasia (male: 0/10, 0/10, 0/10, 0/10, 5/10, 1/10; female: 0/10, 1/10, 1/10, 0/10, 1/10, 1/10) (Tables A1 and A2). These lesions were microscopically similar to the same lesions seen at the site of application, although most were milder in severity.

Slightly increased incidences of minimal hematopoietic cell proliferation occurred in the spleen of dosed groups of male rats (0/10, 1/10, 1/10, 1/10, 2/10, 1/10; Table A1). Increased hematopoiesis can occur with inflammatory conditions due to the physiological need for more white blood cells. Therefore, this finding was considered secondary to the skin lesions at the site of application. The incidences of diffuse bone marrow hyperplasia in 12 mg/kg males (vehicle control, 0/10;

0.75 mg/kg, 0/10; 1.5 mg/kg, 0/10; 3 mg/kg, 0/10; 6 mg/kg, 0/10; 12 mg/kg, 10/10; Table A1) and 12 mg/kg females (0/10, 0/10, 0/10, 0/10, 1/10, 9/10; Table A2) were significantly greater than those in the vehicle controls; the average severity grades for this lesion were 2.0 for 6 mg/kg females and 2.3 for 12 mg/kg males and females. As noted above, incidences of increased hematopoiesis can occur with inflammatory conditions due to the physiological need for more white blood cells, and therefore this finding was also considered secondary to the skin lesions at the site of application.

TABLE 4
Incidences of Nonneoplastic Lesions of the Skin (Site of Application) in F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Male						
Number Examined Microscopically	10	10	10	10	10	10
Epidermis, Hyperplasia[a]	0	1 (1.0)[b]	3 (1.0)	9** (1.1)	10** (1.5)	10** (2.8)
Epidermis, Necrosis	0	0	0	0	0	6** (1.8)
Sebaceous Gland, Hyperplasia	0	1 (1.0)	1 (1.0)	4* (1.5)	7** (1.6)	6** (2.0)
Inflammation, Chronic Active	0	1 (1.0)	0	1 (2.0)	7** (1.0)	9** (2.9)
Female						
Number Examined Microscopically	10	10	10	10	10	10
Epidermis, Hyperplasia	0	3 (1.0)	7** (1.0)	7** (1.1)	10** (1.8)	10** (2.8)
Epidermis, Necrosis	0	0	0	0	1 (2.0)	3 (1.7)
Sebaceous Gland, Hyperplasia	0	1 (1.0)	0	0	5* (1.0)	7** (1.4)
Inflammation, Chronic Active	0	0	4* (1.0)	4* (1.0)	9** (1.4)	9** (2.4)

*   Significantly different (P≤0.05) from the vehicle control group by the Fisher exact test
** P≤0.01
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

3-WEEK STUDY IN B6C3F$_1$ MICE

One 0.6 mg female mouse and all mice in the 1.7, 5, and 15 mg groups died before the end of the study (Table 5). Final mean body weights of the 0.6 mg groups were significantly less than those of the vehicle controls, and animals in these groups generally lost weight during the study. Clinical findings included skin irritation at the site of application in all dosed groups and clinical signs of toxicity at doses of 0.6 mg or greater. Heart weights of 0.6 mg groups of mice were significantly greater than those of the vehicle controls (Table G3); thymus weights in these groups were significantly less than those of the vehicle controls.

Histopathologic examination of mice dosed with 1.7 mg dicyclohexylcarbodiimide or less revealed lesions of the skin at the site of application including epidermal hyperplasia, epidermal necrosis, and acute or chronic active dermal inflammation (data not shown).

Dose Selection Rationale: In the 3-week mouse study, 0.6 mg/animal approximately corresponds to 24 mg/kg body weight. The doses above 0.6 mg/animal were considered to be prohibitive for 13-week studies due to mortality and clinical signs of toxicity. Therefore, for the 13-week mouse study, doses selected were 1.5, 3, 6, 12, and 24 mg/kg in ethanol.

TABLE 5
Survival and Body Weights of B6C3F$_1$ Mice in the 3-Week Dermal Study of Dicyclohexylcarbodiimide

Dose (mg/animal)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	27.0 ± 1.1	28.8 ± 1.1	1.8 ± 0.0	
0.2	5/5	26.7 ± 0.7	28.3 ± 0.5	1.6 ± 0.3	98
0.6	5/5	27.0 ± 0.5	26.0 ± 0.5*	−1.0 ± 0.4**	90
1.7	0/5[c]	26.5 ± 0.6	—	—	
5	0/5[d]	26.6 ± 0.7	—	—	
15	0/5[e]	26.5 ± 0.7	—	—	
Female					
0	5/5	20.6 ± 0.6	24.0 ± 0.5	3.4 ± 0.3	
0.2	5/5	20.4 ± 0.5	23.9 ± 0.4	3.5 ± 0.4	100
0.6	4/5[f]	20.8 ± 0.7	21.3 ± 0.8**	−0.1 ± 0.5**	89
1.7	0/5[g]	21.0 ± 0.3	—	—	
5	0/5[d]	20.8 ± 0.3	—	—	
15	0/5[e]	20.6 ± 0.6	—	—	

*   Significantly different (P≤0.05) from the vehicle control group by Dunnett's or Williams' test
** P≤0.01
[a] Number of animals surviving at 3 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.
[c] Day of death: 4, 4, 4, 4, 6
[d] Day of deaths: 2 or 3
[e] Day of deaths: 2
[f] Day of death: 15
[g] Day of deaths: 3

13-WEEK STUDY IN B6C3F$_1$ MICE

All 24 mg/kg male and female mice died or were found moribund and sacrificed prior to day 16 (Table 6). Final mean body weights of 6 and 12 mg/kg males and mean body weight gains of 6 and 12 mg/kg males and females were significantly less than those of the vehicle controls (Table 6 and Figure 2). With the exception of two males and six females in the 24 mg/kg group, all animals administered 6 mg/kg or greater had skin irritation at the site of application. Additional clinical findings noted in 24 mg/kg animals included ataxia, lethargy, thinness, abnormal posture, ruffled fur, and head tilt.

TABLE 6
Survival and Body Weights of B6C3F$_1$ Mice in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	24.8 ± 0.6	33.7 ± 0.6	8.9 ± 0.4	
1.5	10/10	24.8 ± 0.3	33.1 ± 0.7	8.3 ± 0.4	98
3	10/10	24.7 ± 0.4	33.0 ± 0.5	8.3 ± 0.2	98
6	10/10	24.9 ± 0.4	31.5 ± 0.5**	6.6 ± 0.3**	93
12	10/10	24.2 ± 0.5	30.4 ± 0.5**	6.2 ± 0.4**	90
24	0/10[c]	24.5 ± 0.5	—	—	
Female					
0	10/10	19.6 ± 0.3	28.8 ± 0.9	9.2 ± 0.7	
1.5	10/10	19.1 ± 0.5	28.3 ± 1.1	9.2 ± 0.8	98
3	10/10	19.1 ± 0.4	27.8 ± 0.5	8.7 ± 0.5	97
6	10/10	20.3 ± 0.2	27.7 ± 0.3	7.3 ± 0.3*	96
12	10/10	19.5 ± 0.3	26.5 ± 0.4	7.0 ± 0.3**	92
24	0/10[d]	19.5 ± 0.4	—	—	

*   Significantly different (P≤0.05) from the vehicle control group by Williams' test
** P≤0.01
[a]   Number of animals surviving at 13 weeks/number initially in group
[b]   Weights and weight changes are given as mean ± standard error.
[c]   Week of deaths: 2
[d]   Week of death: 2 or 3
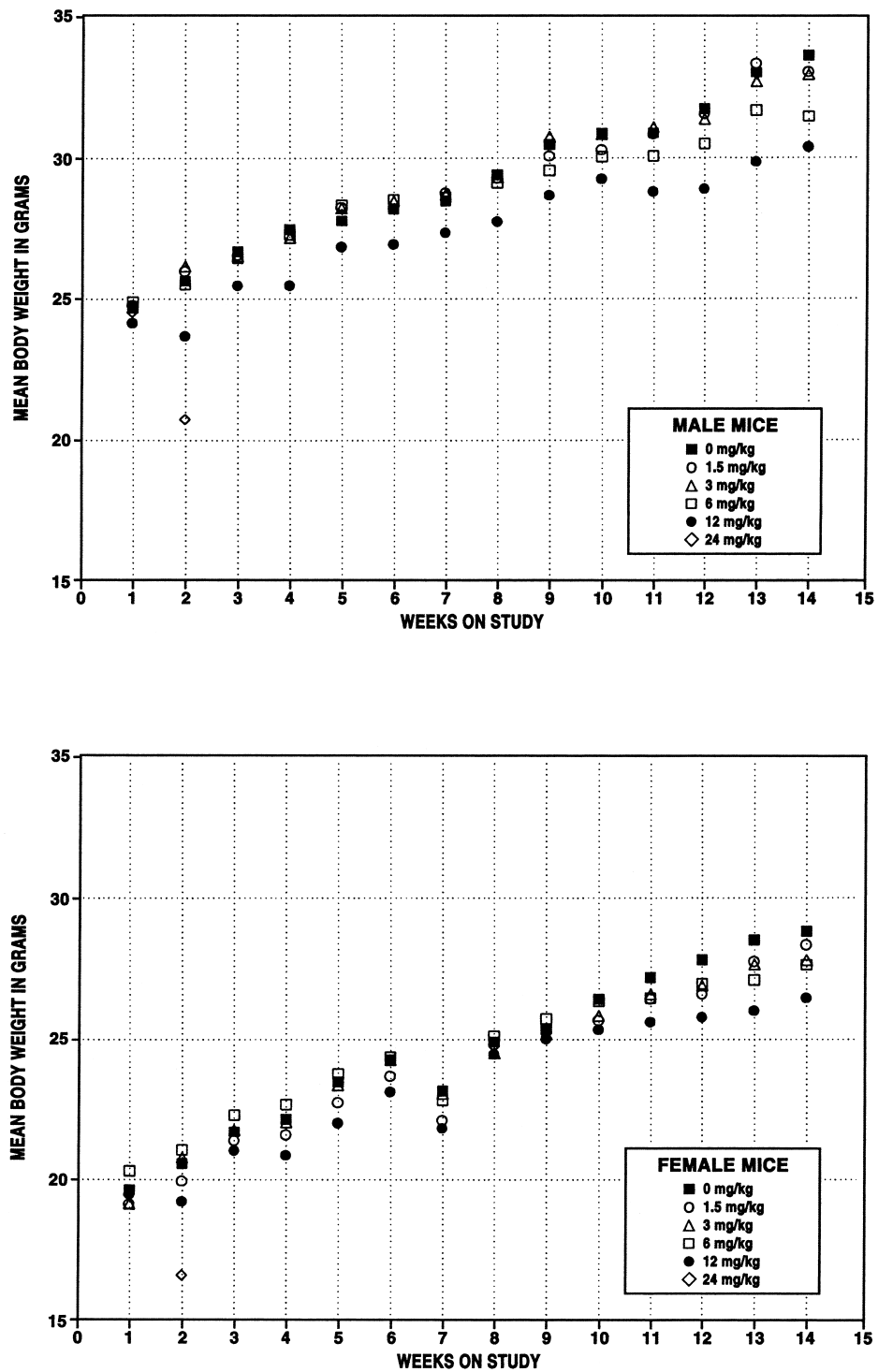
FIGURE 2
Growth Curves for Male and Female B6C3F$_1$ Mice
Administered Dicyclohexylcarbodiimide Dermally for 13 Weeks

The hematology data for mice are presented in Table F2. Similar to results from the rat study, evidence of an inflammatory process was indicated by increased leukocytes at week 13. The inflammatory leukogram, characterized by increased leukocyte and neutrophil counts, occurred in 12 mg/kg males and females. These leukocyte changes would be a response consistent with the development of the skin necrosis and chronic active inflammation observed morphologically. As in rats, minimal decreases in the erythron (<10%), characterized by decreases in hematocrit and hemoglobin values and erythrocyte counts, occurred in 1.5 mg/kg or greater males and 12 mg/kg females. This minimal decrease in erythron would be consistent with the erythron decreases that can develop in response to chronic inflammation and would be considered a secondary response to skin inflammation.

Absolute and relative lung weights of 3 mg/kg or greater male mice were generally less than those of the vehicle controls, and absolute and relative heart and liver weights of 6 and 12 mg/kg female mice were generally greater than those of the vehicle controls (Table G4).

Dermal administration of dicyclohexylcarbodiimide significantly decreased the weight of the epididymis in 6 and 12 mg/kg males and significantly decreased epididymal spermatozoal motility in 6 mg/kg males (Table H3). Vaginal cytology parameters in female mice were not affected by treatment with dicyclohexylcarbodiimide (Table H4).

Chemical-related gross lesions of the skin at the site of application consisted of pink lesions, tan crusts, or areas of mottled irritation observed in all 12 mg/kg males and females, eight 24 mg/kg males, and five 24 mg/kg females. At the site of application, the incidences of epidermal hyperplasia were significantly increased in all dosed groups of males and females except those administered 24 mg/kg (not increased in this group because of early mortality) (Tables 7, B1, and B2). The incidences of chronic active inflammation were significantly increased in all dosed groups except 1.5 mg/kg females, and the incidences of epidermal necrosis were significantly increased in 24 mg/kg males and females. Epidermal necrosis also occurred in several animals administered 6 or 12 mg/kg dicylohexylcarbodiimide.

TABLE 7
Incidences of Nonneoplastic Lesions of the Skin (Site of Application) in B6C3F$_1$ Mice in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg	24 mg/kg
Male						
Number Examined Microscopically	10	10	10	10	10	10
Epidermis, Hyperplasia[a]	0	7** (1.0)[b]	10** (1.0)	10** (1.4)	10** (2.3)	0
Epidermis, Necrosis	1 (2.0)	0	0	0	2 (2.0)	10** (3.8)
Inflammation, Chronic Active	0	4* (1.0)	10** (1.1)	10** (1.5)	10** (1.8)	10** (2.6)
Female						
Number Examined Microscopically	10	10	10	10	10	10
Epidermis, Hyperplasia	0	5* (1.0)	9** (1.3)	10** (1.8)	10** (2.7)	3 (3.0)
Epidermis, Necrosis	0	0	0	2 (2.0)	3 (1.7)	10** (3.5)
Inflammation, Chronic Active	0	1 (1.0)	9** (1.1)	10** (1.7)	10** (1.9)	10** (2.8)

* Significantly different (P≤0.05) from the vehicle control group by the Fisher exact test
** P≤0.01
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

The severities of epidermal hyperplasia and chronic active inflammation generally increased in a dose-related fashion for both sexes. The inflammation on the surface was acute, consisting of polymorphonuclear cells and cell debris mixed with coagulated serum (crusts). Deeper in the dermis, a few mononuclear cells mixed with small numbers of proliferating fibroblasts were present, which suggested a more chronic feature of the inflammatory lesion.

In control site skin, epidermal hyperplasia (male: vehicle control, 0/10; 1.5 mg/kg, 0/10; 3 mg/kg, 1/10; 6 mg/kg, 0/10; 12 mg/kg, 0/10; 24 mg/kg, 0/10; female: 0/10, 0/10, 0/10, 0/10, 3/10, 0/10)) and inflammation and/or chronic active inflammation (male: 0/10, 1/10, 1/10, 1/10, 4/10, 0/10; female: 2/10, 0/10, 3/10, 5/10, 5/10, 1/10) occurred in some of the dosed male and female mice (Tables B1 and B2). Three of the 10 male mice administered 12 mg/kg also had epidermal necrosis or diffuse epidermal necrosis in control site skin. These lesions were microscopically similar to lesions seen at the site of application, although most were milder in severity and were due to spread of the test article by excessive grooming of the site of application.

Incidences of hematopoietic cell proliferation in the spleen were increased in most dosed groups of males (0/10, 5/10, 2/10, 7/10, 6/10, 6/10; Table B1) and females (2/10, 2/10, 7/10, 9/10, 5/10, 3/10; Table B2). Increased hematopoiesis can occur with inflammatory conditions due to the physiological need for more white blood cells. Therefore, this finding was considered secondary to the skin lesions at the site of application.

Other nonneoplastic lesions occurred primarily in the 24 mg/kg groups and included bilateral atrophy of the preputial (4/10) and clitoral glands (1/10), atrophy of the thymus (8/8 in each sex), and hemorrhage of the brain (2/10 in males, 6/10 in females) (Tables B1 and B2). These lesions occurred in mice that died prior to 16 days on study and were considered secondary effects of ination and moribundity.

20-WEEK STUDY
IN FEMALE TG.AC HEMIZYGOUS MICE
Survival

There were no deaths considered related to dicyclo-hexylcarbodiimide administration. Thirteen animals died or were sacrificed moribund prior to the end of the study: three each from the vehicle control and 0.75 mg/kg groups, four from the 3 mg/kg group, two from the 6 mg/kg group, and one from the 12 mg/kg group (Table 8). Overall, the survival was within the range known (70% to 100%) for the Tg.AC hemizygous mouse.

Body Weights, Clinical Findings, and Organ Weights

Mean body weights of dosed groups of mice were similar to those of the vehicle controls (Table 8 and Figure 3). Clinical findings consisted of dose-related skin irritation at the site of application in groups treated with 1.5 mg/kg or greater. Due to the severity of skin lesions observed in 12 mg/kg animals, application of the test article was discontinued after eight dermal applications in this dose group. The absolute and relative weights of the liver in 1.5 mg/kg or greater mice were significantly greater than those in the vehicle controls (Table G5).

TABLE 8
Survival and Body Weights of Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Dicyclohexylcarbodiimide

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
0	7/10[c]	20.6 ± 0.4	26.8 ± 1.5	6.0 ± 1.2	
0.75	7/10[d]	20.9 ± 0.3	27.7 ± 1.0	7.1 ± 1.0	103
1.5	10/10	21.0 ± 0.4	26.7 ± 0.7	5.7 ± 0.7	100
3	6/10[e]	20.8 ± 0.3	27.5 ± 0.8	6.3 ± 0.8	103
6	8/10[f]	20.5 ± 0.4	28.8 ± 0.9	8.3 ± 0.7	108
12[g]	9/10[h]	20.5 ± 0.4	27.4 ± 0.9	6.9 ± 0.9	102

[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: 9, 12, 15
[d] Week of death: 7, 12, 19
[e] Week of death: 8, 12, 14, 17
[f] Week of death: 11, 18
[g] Treatment was discontinued after 8 days because of the severity of skin lesions at the site of application.
[h] Week of death: 11

FIGURE 3
Growth Curves for Female Tg.AC Hemizygous Mice
Administered Dicyclohexylcarbodiimide Dermally for 20 Weeks

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and/or nonneoplastic lesions of the skin (site of application) and forestomach. Summaries of the incidences of neoplasms and nonneoplastic lesions in female Tg.AC hemizygous mice are presented in Tables C1 and C2, respectively.

Skin (Site of Application): Test article-related gross lesions of the skin at the site of application included irritation (0/10, 0/10, 1/10, 1/10, 2/10, 0/10) and masses and/or nodules (papillomas) (0/10, 0/10, 1/10, 4/10, 6/10, 9/10); incidences of these gross lesions increased in a dose-related fashion. At the site of application, the incidences of chronic active inflammation of the dermis

and epidermal hyperplasia were significantly increased in mice administered 3 or 6 mg/kg and the incidences of squamous cell papilloma were significantly increased in mice administered 6 or 12 mg/kg (Tables 9, C1, and C2). The incidences of squamous cell papilloma and multiplicity of squamous cell papilloma increased in a dose-related manner. One squamous cell carcinoma occurred at the site of application in a 6 mg/kg mouse.

Forestomach: Six treated mice each had a grossly detectable papilloma of the forestomach (0/10, 2/10, 2/10, 1/10, 1/10, 0/10). Because the neoplasms did not increase in a dose-related manner, were not multiple, and were within historical control incidence rates for this model, the papillomas are not considered a treatment effect.

TABLE 9
**Incidences of Neoplasms and Nonneoplastic Lesions of the Skin (Site of Application)
in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Dicyclohexylcarbodiimide**

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg[a]
Number Examined Microscopically	10	10	10	10	10	10
Dermis, Inflammation, Chronic Active[b]	0	0	3 (1.7)[c]	5* (1.8)	8** (2.0)	0
Epidermis, Hyperplasia	0	0	0	6** (1.8)	7** (2.0)	1 (2.0)
Squamous Cell Papilloma, Multiple	0	0	1	0	3	5*
Squamous Cell Papilloma (includes multiple)	0	0	1	3	6**	8**
Squamous Cell Carcinoma	0	0	0	0	1	0

*   Significantly different (P≤0.05) from the vehicle control group by the Fisher exact test
** P≤0.01
a   Treatment was discontinued after 8 days because of the severity of skin lesions at the site of application.
b   Number of animals with lesion
c   Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

27-WEEK STUDY IN
FEMALE p53 HAPLOINSUFFICIENT MICE
Survival

Twelve animals died or were sacrificed moribund prior to the end of the study; three from the 3 mg/kg group, one from the 6 mg/kg group, and eight from the 12 mg/kg group (Table 10).

Body Weights and Clinical Findings

Mean body weights of dosed groups of mice were similar to those of the vehicle controls (Table 10 and Figure 4). Clinical findings related to the administration of dicyclohexylcarbodiimide included irritation and ulceration of the skin at the site of application and thinness, primarily in 6 and 12 mg/kg animals. Dosing of the 6 and 12 mg/kg groups was discontinued after 11 and 8 days, respectively, because of the severity of skin lesions at the site of application.

TABLE 10
Survival and Body Weights of Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Dicyclohexylcarbodiimide

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
0	15/15	18.6 ± 0.2	33.8 ± 1.2	15.3 ± 1.1	
0.75	15/15	18.2 ± 0.3	32.1 ± 1.1	13.9 ± 1.1	95
1.5	15/15	18.3 ± 0.3	35.5 ± 1.2	17.2 ± 1.1	105
3	12/15[c]	18.5 ± 0.3	32.3 ± 1.3	13.8 ± 1.2	96
6[d]	14/15[e]	18.0 ± 0.3	32.0 ± 1.4	14.0 ± 1.2	95
12[f]	7/15[g]	18.1 ± 0.3	33.6 ± 2.4	15.5 ± 2.2	99

[a] Number of animals surviving at 27 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: 7, 10, 24
[d] Treatment was discontinued after 11 days because of the severity of skin lesions at the site of application.
[e] Week of death: 5
[f] Treatment was discontinued after 8 days because of the severity of skin lesions at the site of application.
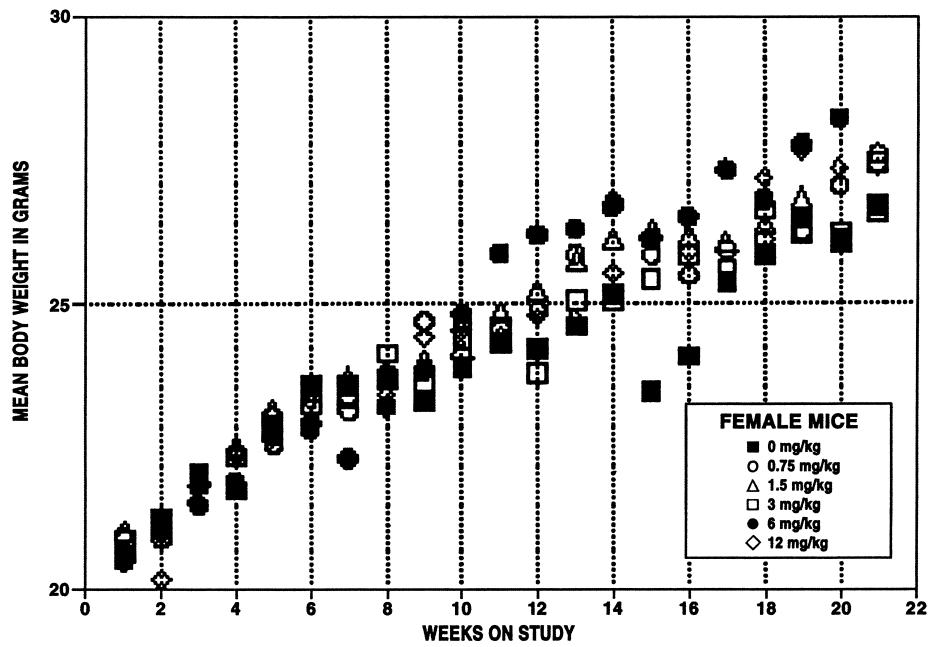[g] Week of death: 8, 9, 9, 9, 9, 9, 9, 10
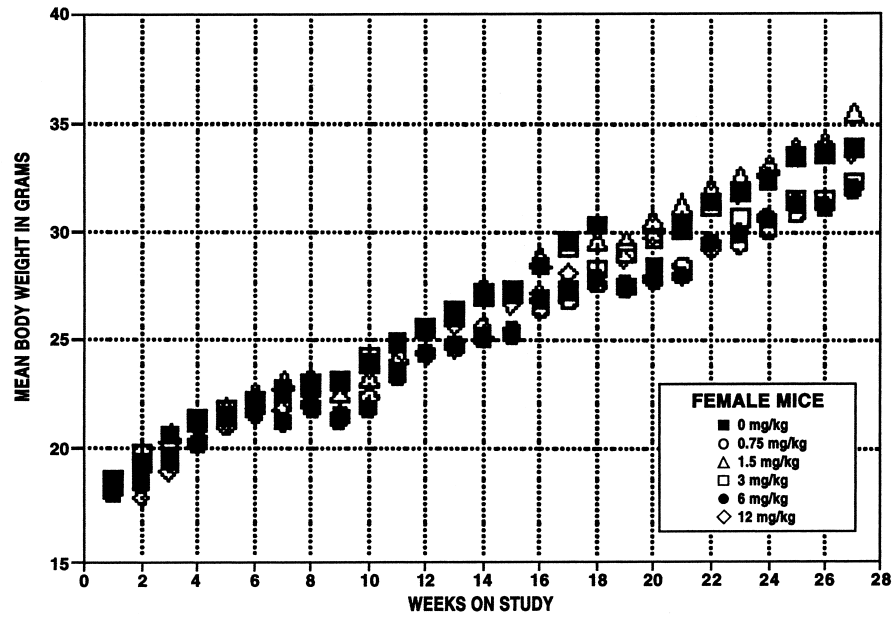
FIGURE 4
Growth Curves for Female p53 Haploinsufficient Mice
Administered Dicyclohexylcarbodiimide Dermally for 27 Weeks

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of nonneoplastic lesions of the skin (site of application and control site) and bone marrow. Summaries of the incidences of neoplasms and nonneoplastic lesions in female p53 haploinsufficient mice are presented in Tables D1 and D2, respectively.

Skin (Site of Application): Chemical-related gross lesions of the skin at the site of application included reddening (0/15, 0/15, 0/15, 0/15, 0/15, 5/15), red scars (0/15, 0/15, 0/15, 0/15, 0/15, 1/15), red crusts (0/15, 0/15, 0/15, 0/15, 0/15, 2/15), and ulcers (0/15, 0/15, 0/15, 2/15, 1/15, 2/15). At the site of application, the incidences of focal epidermal hyperplasia were significantly increased in mice administered 1.5, 3, or 12 mg/kg, and the severity of the lesion increased in a dose-related manner (Tables 11 and D2). In addition, incidences of focal chronic active inflammation of the dermis in 3 or 12 mg/kg mice and focal ulcer and focal chronic active inflammation of the subcutaneous tissue in 12 mg/kg mice were significantly increased. Focal hyperplasia of the sebaceous gland occurred in mice administered 3 or 12 mg/kg. Decreased incidences of these skin lesions in the 6 mg/kg animals compared to 3 mg/kg animals are most likely due to the decreased

number of dosing days for the 6 mg/kg group (11 days) compared to the 3 mg/kg group (27 weeks). Incidences of minimal to mild focal sebaceous gland hyperplasia were considered a secondary effect of inflammation at the site of application.

Skin (Control Site): Incidences of focal dermal chronic active inflammation (0/15, 0/15, 0/15, 3/15, 1/15, 2/15), focal subcutaneous chronic active inflammation (0/15, 0/15, 0/15, 1/15, 0/15, 2/15), focal epidermal hyperplasia (0/15, 0/15, 0/15, 2/15, 0/15, 1/15), focal ulceration (0/15, 0/15, 0/15, 1/15, 0/15, 2/15), and focal sebaceous gland hyperplasia (0/15, 0/15, 0/15, 1/15, 0/15, 1/15) in control site skin were less than those seen at the site of application and were less severe (Table D2).

Bone Marrow: Myeloid cell hyperplasia of the bone marrow occurred in the 3 mg/kg or greater groups (0/15, 0/15, 0/15, 3/15, 1/14, 8/15), and the incidence of this lesion was significantly increased in the 12 mg/kg group (Table D2). Increased hematopoiesis can occur with inflammatory conditions due to the physiological need for more white blood cells. Therefore, the increased incidence of myeloid cell hyperplasia is considered a reactive change in response to the skin lesions at the site of application.

TABLE 11
Incidences of Nonneoplastic Lesions of the Skin (Site of Application)
in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg[a]	12 mg/kg[b]
Number Microscopically Examined	15	15	15	15	15	15
Dermis, Inflammation, Chronic Active, Focal[c]	1 (1.0)[d]	0	0	13**(1.3)	2 (1.0)	9** (3.3)
Epidermis, Hyperplasia, Focal	0	1 (1.0)	5* (1.0)	11** (1.5)	1 (2.0)	8** (3.0)
Sebaceous Gland, Hyperplasia, Focal	0	0	0	3 (1.7)	0	3 (2.0)
Ulcer, focal	0	0	0	2 (4.0)	1 (4.0)	8** (3.9)
Subcutaneous Tissue, Inflammation, Chronic Active, Focal	0	0	0	2 (3.0)	0	8** (3.6)

* Significantly different (P≤0.05) from the vehicle control group by the Fisher exact test
** P≤0.01
[a] Treatment was discontinued after 11 days because of the severity of skin lesions at the site of application.
[b] Treatment was discontinued after 8 days because of the severity of skin lesions at the site of application.
[c] Number of animals with lesion
[d] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

GENETIC TOXICOLOGY

Dicyclohexylcarbodiimide was not mutagenic in *Salmonella typhimurium* strains TA97, TA98, TA100, or TA1535, with or without induced rat or hamster liver S9 activation enzymes (Table E1). Results of *in vivo* tests for chromosomal effects in F344/N rats and B6C3F$_1$ mice are discussed in detail by Witt *et al.* (1999), and the data are presented in Tables E2 and E3, respectively. Negative results were obtained in an acute exposure bone marrow micronucleus test in male F344/N rats using an intraperitoneal injection route of chemical administration (Table E2). Dicyclohexylcarbodiimide, administered dermally for 13 weeks, induced significant increases in the frequency of micronucleated normochromatic erythrocytes (NCEs) in peripheral blood of male B6C3F$_1$ mice, based on results of a trend test analysis (P=0.003), although none of the treated groups showed a significant increase in the frequency of micronucleated NCEs compared to the vehicle control group (Table E3). In female B6C3F$_1$ mice, the frequencies of micronucleated NCEs in all treated groups were greater than that in the vehicle controls, but the increase was significant only in the 3 mg/kg group, and the trend was not significant; results in female B6C3F$_1$ mice were concluded to be weakly positive. There was no effect of treatment on the percentage of polychromatic erythrocytes in peripheral blood of male or female B6C3F$_1$ mice, suggesting no measurable dicyclohexylcarbodiimide-induced toxicity to the bone marrow.

DISCUSSION AND CONCLUSIONS

Dicyclohexylcarbodiimide and diisopropylcarbodiimide are representatives of the carbodiimide chemical class that are widely used reagents in the chemical and pharmaceutical industries, and their use is increasing in the field of bioenergetics. Dicyclohexylcarbodiimide and diisopropylcarbodiimide are used as coupling and condensation agents (especially in peptide synthesis) and as stabilizing agents. Dicyclohexylcarbodiimide currently has the greater usage, but this might change because the reaction side products of diisopropylcarbodiimide are easier to remove than those of dicyclohexylcarbodiimide. Dicyclohexylcarbodiimide and diisopropylcarbodiimide are listed in the TSCAPP database (1991), but information on specific production volumes is not available. Occupational exposure to these chemicals occurs primarily by dermal contact or inhalation. The NTP has completed dermal toxicology and carcinogenicity studies of diisopropylcarbodiimide in traditional (F344/N rats and B6C3F$_1$ mice) and genetically modified (Tg.AC hemizygous mice and p53 haploinsufficient mice) animal models and reported the results in two Reports (NTP 2007a,b). In these studies, there were no treatment-related increased incidences of neoplasms in traditional or genetically modified models. In the current studies, the same genetically altered mouse models with either a loss of heterozygosity in a critical cancer suppressor gene (p53 haploinsufficient) or a gain of oncogene function (Tg.AC hemizygous) were used to determine how these animals would respond to dicyclohexylcarbodiimide exposure. The studies in these genetically modified mouse models were preceded by 3- and 13-week dermal toxicity studies in F344/N rats and B6C3F$_1$ mice that were performed to characterize the toxicity of dicyclohexylcarbodiimide and select the doses for the subsequent evaluations in genetically modified animals.

Because of the paucity of acute toxicity data for dicyclohexylcarbodiimide in the literature, the doses selected for the 3-week dermal studies in F344/N rats and B6C3F$_1$ mice were based on very limited information. The highest three doses (rats: 5.1, 15, 45 mg; mice: 1.7, 5, 15 mg) used in the 3-week studies were lethal to the animals. Clinical findings in the rats and mice included skin irritation at the site of application, and clinical signs of severe toxicity also occurred. The skin lesions at the

site of application included epidermal hyperplasia, necrosis, and chronic inflammation in the dermis. Results of the 13-week studies suggested that B6C3F$_1$ mice are generally more sensitive than F344/N rats to dicyclohexylcarbodiimide toxicity, with the major target organ being the skin at the site of application in both species. Dose-related increased incidences of inflammation and hyperplasia at the site of application were observed in both F344/N rats and B6C3F$_1$ mice. The severity of these lesions was generally more pronounced in B6C3F$_1$ mice than in F344/N rats. These results are consistent with published reports of the irritancy of dicyclohexylcarbodiimide; the compound has been shown to be both a skin (Zschunke and Folesky, 1975) and eye irritant in humans after occupational exposure (Changji, 1983).

In the NTP studies, the severities of acute toxicity (clinical signs of toxicity and mortality) and skin lesions at the site of application were much greater in the traditional animal models (F344/N rats and B6C3F$_1$ mice) following dermal administration of dicyclohexylcarbodiimide than diisopropylcarbodiimide. The difference could be due to differences in the disposition of these chemicals after dermal application. Kinetic studies conducted by the NTP in male F344/N rats and male B6C3F$_1$ mice showed that dicyclohexylcarbodiimide is partially absorbed (Appendix J); approximately 25% and 41% of dermally applied dicyclohexylcarbodiimide was absorbed by rats and mice, respectively. Seventy-two hours after dermal application, only a small percentage of the dose was found in selected tissues and the residual carcass. In contrast, kinetic studies of diisopropylcarbodiimide showed only minimal absorption ranging from 1.1% to 6.6% in F344/N rats and about 2.3% in male B6C3F$_1$ mice (NTP, 2007a). Thus, increased bioavailability of dicyclohexylcarbodiimide may be responsible for its greater acute toxicity compared to that observed with diisopropylcarbodiimide.

For the studies using genetically modified mouse models, a regimen of five doses was selected to assure the availability of animals for evaluation, should there be losses in the high dose groups due to overt toxicity as seen in the F344/N rat and B6C3F$_1$ mouse studies.

During the p53 mouse studies, the concentrations of dicyclohexylcarbodiimide may have been less than target concentrations for approximately 4 to 6 weeks due to possible degradation of the dose formulations in the animal room. Dermal application of dicyclohexylcarbodiimide to female p53 haploinsufficient mice resulted in chemical-related clinical signs of toxicity and histopathologic findings at the site of application that included irritation, epidermal hyperplasia, chronic active inflammation, and sebaceous gland hyperplasia. However, no incidences of neoplasms were found in any examined tissue.

In female Tg.AC hemizygous mice, nonneoplastic histopathologic findings at the site of application were similar to those seen with the p53 haploinsufficient model, but induction of dose-related incidences of squamous cell papilloma also occurred. In addition, multiplicity of papilloma increased in a dose-related manner. The dose-related increased incidences of papilloma were accompanied by skin effects such as irritation, inflammation, and/or hyperplasia at the site of application. Due to the severity of skin lesions observed in 12 mg/kg animals, application of dicyclohexylcarbodiimide was discontinued after eight applications; nevertheless, the dermal lesions persisted, and this group had the greatest incidence of papilloma.   This suggests that acute or short-term exposure to highly reactive chemicals like dicyclohexylcarbodiimide may be sufficient to trigger serious adverse effects that appear later in life.

The relationship of chemical-induced skin irritation, inflammation, and hyperplasia to tumor formation is not clearly understood in either conventional or Tg.AC mouse models. A number of studies have established that induction of sustained cellular hyperplasia as seen in Tg.AC hemizygous mice treated with dicyclohexylcarbodiimide correlates well with skin tumor induction by various tumor promoting agents such as phorbol esters, several peroxides, and chrysarobin (Argyris, 1981; Hennings *et al.*, 1993; DiGiovanni, 1995). In the current studies, it is not possible to determine how dicyclohexylcarbodiimide induced papilloma with a dose response that was similar to that of irritation, inflammation, and hyperplasia at the site of application. However, co-occurrence of these lesions is not always the case. For example, in the NTP dermal carcinogenicity studies on rotenone in Tg.AC hemizygous mice (Eastin *et al.*, 1998), no papillomas were observed despite extensive hyperplasia and inflammation in the skin at the site of application. Another example is the 2-year dermal study of two polyfunctional acrylates that were both negative

for carcinogenic activity in male C3H/HeNHsd mice despite the occurrence of proliferative effects in the skin at the site of application (Van Miller *et al.*, 2003).

The p53 haploinsufficient mouse model has been determined to be particularly useful for *in vivo* testing of mutagenic carcinogens (Donehower *et al.*, 1992; French *et al.*, 2001a,b).   In general, positive carcinogenicity results in the p53 haploinsufficient mouse model correlate better with strongly positive results in *in vivo* micronuclei tests than with positive results in *Salmonella* genotoxicity assays (Pritchard, *et al.*, 2003; French, 2004). The Tg.AC hemizygous mouse model responds to both genotoxic and nongenotoxic carcinogens (Spalding, *et al*. 1999, 2000; Tennant *et al.,* 2001). Because dicyclohexylcarbodiimide is not mutagenic in the *Salmonella typhimurium* gene mutation assay, the chemical was expected to be inactive in the p53 haploinsufficient strain.   In male and female $B6C3F_1$ mice treated dermally with dicyclohexylcarbodiimide for 13 weeks, increased frequencies of micronucleated normochromatic erythrocytes occurred in peripheral blood, suggesting that dicyclohexylcarbodiimide has a capacity for producing structural or numerical chromosomal alterations. Erythrocyte micronucleus frequencies were not determined in either of the genetically modified mouse models used in the current studies however, so no direct correlation can be made between the positive micronucleus results in $B6C3F_1$ mice and the increased incidences of papilloma in dicyclohexylcarbodiimide-treated Tg.AC hemizygous mice.

The NTP studies on dicyclohexylcarbodiimide and diisopropylcarbodiimide are part of a database being established to help develop more rapid and economical carcinogenicity assays and to reduce dependency on the 2-year bioassay. These two studies and two earlier NTP studies on trimethylolpropane triacrylate (NTP, 2005a) and pentaerythritol triacrylate (NTP, 2005b) are part of a set of prospective studies performed in Tg.AC hemizygous mice (Doi *et al.*, 2005). A tiered testing approach has been proposed recommending that agents positive for carcinogenicity in this model should be considered carcinogens, and negative studies should prompt further evaluations (Bucher and Portier, 2004). This approach was used for diisopropylcarbodiimide, which was negative in the Tg.AC hemizygous mouse and was also negative when tested by the traditional NTP 2-year study protocol (NTP, 2007a,b). Both trimethylolpropane triacrylate and pentaerythritol triacrylate were positive for carcinogenicity in the Tg.AC hemizygous mouse model, but the tiered approach was not followed for these

chemicals. This is because the Tg.AC hemizygous mouse model is not yet accepted by the scientific community as definitive for identification of chemical carcinogens due to the limited information that is available on the mechanism(s) for tumor formation in this model (Bucher and Portier, 2004). As a consequence, the NTP is currently testing trimethylolpropane triacrylate in a 2-year bioassay. The characterization of a positive study in the Tg.AC hemizygous mouse as definitive for carcinogenicity as reported here with dicyclohexylcarbodiimide may be justified by the fact that some regulatory agencies have begun accepting dermal carcinogenicity studies in the Tg.AC hemizygous model in lieu of a 2-year bioassay in mice, especially for pharmaceuticals (MacDonald *et al.*, 2004).

CONCLUSIONS

Under the conditions of this 27-week dermal study, there was *no evidence of carcinogenic activity** of dicyclohexylcarbodiimide in female p53 haploinsufficient mice administered 0.75, 1.5, 3, 6, or 12 mg/kg in ethanol.

Female Tg.AC hemizygous mice dermally dosed with dicyclohexylcarbodiimide for 20 weeks had significantly increased incidences of squamous cell papilloma of the skin at the site of application.

Nonneoplastic lesions noted at the site of application included chronic active inflammation and epidermal hyperplasia in female p53 haploinsufficient mice and female Tg.AC hemizygous mice.

————

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 9. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Report appears on page 11.

REFERENCES

Albert, R.E., French, J.E., Maronpot, R., Spalding, J., and Tennant, R. (1996). Mechanism of skin tumorigenesis by contact sensitizers: The effect of the corticosteroid fluocinolone acetonide on inflammation and tumor induction by 2,4-dinitro-1-fluorobenzene in the skin of the TG.AC (v-Ha-ras) mouse. *Environ. Health Perspect.* **104**, 1062-1068.

The Aldrich Library of Infrared Spectra (1981a). 3rd ed. (C.J. Pouchert, Ed.), Vol. 1, Spectrum 524C. Aldrich Chemical Co., Inc., Milwaukee, WI.

The Aldrich Library of Infrared Spectra (1981b). 3rd ed. (C.J. Pouchert, Ed.), Vol. 1, Spectrum 66B. Aldrich Chemical Co., Inc., Milwaukee, WI.

The Aldrich Library of FT-IR Spectra (1985). 1st ed. (C.J. Pouchert, Ed.), Vol. 1, p. 876(C). Aldrich Chemical Co., Inc., Milwaukee, WI.

Aldrich Catalog of Fine Chemicals (1990-1991) (1990). p. 442. Aldrich Chemical Co., Inc., Milwaukee, WI.

The Aldrich Library of ^{13}C and ^{1}H FT-NMR Spectra (1993). 1st ed., p. 1413(B). Aldrich Chemical Co., Inc., Milwaukee, WI.

Argyris, T.S. (1981). The nature of the epidermal growth produced by the first application of 12-*O*-tetradecanoyl-phorbol-13-acetate on the skin of mice initiated with dimethylbenzanthracene. *J. Invest. Dermatol.* **77**, 230-234.

Ashby, J., Hilton, J., Dearman, R.J., Callander, R.D., and Kimber, I. (1993). Mechanistic relationship among mutagenicity, skin sensitization, and skin carcinogenicity. *Environ. Health Perspect.* **101**, 62-67.

Aviram, A., and Aviram, I. (1983). Activation of the respiratory burst of guinea pig neutrophils by dicyclohexylcarbodiimide. *FEBS Lett*. **155**, 205-208.

Azulai, R., and Salomon, Y. (1979). Selective inactivation of hormone receptors in rat ovarian plasma membranes by N,N′-dicyclohexylcarbodiimide and light radiation. *Israel J. Med. Sci*. **15**, 82.

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.

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.

Bucher, J.R., and Portier, C. (2004). Human carcinogenic risk evaluation, Part V: The National Toxicology Program vision for assessing the human carcinogenic hazard of chemicals. *Toxicol. Sci.* **82**, 363-366.

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.

Changji, L. (1983). Acute chemical ophthalmitis caused by N,N′-dicyclohexyl carbodiimide vapor. *Acta Acad. Med. Sichuan* **14**, 180-182.

Code of Federal Regulations (CFR) **21**, Part 58.

Davies, M.G. (1983). Contact allergy to dicyclohexyl-carbodiimide. *Contact Dermatitis* **9**, 318.

DiGiovanni, J. (1995). Genetic factors controlling responsiveness to skin tumor promotion in mice. *Prog. Clin. Biol. Res.* **391**, 195-212.

Dixon, W.J., and Massey, F.J., Jr. (1957). *Introduction to Statistical Analysis*, 2nd ed., pp. 276-278, 412. McGraw-Hill Book Company, Inc., New York.

Doi, A.M., Hailey, J.R., Hejtmancik, M., Toft, J.D., II, Vallant, M., and Chhabra, R.S. (2005). Topical application of representative multifunctional acrylates produced proliferative and inflammatory lesions in F344/N rats and B6C3F1 mice, and squamous cell neoplasms in Tg.AC mice. *Toxicol. Pathol.* **33**, 631-640.

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.

Dunn, O.J. (1964). Multiple comparisons using rank sums. *Technometrics* **6**, 241-252.

Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* **50**, 1096-1121.

Eastin, W.C., Haseman, J.K., Mahler, J.F., and Bucher, J.R. (1998). The National Toxicology Program evaluation of genetically altered mice as predictive models for identifying carcinogens. *Toxicol. Pathol.* **26**, 461-473.

Ellis, H.V. (1991). Treatment for eye irritants. *Chem. Eng. News* **69**, 2.

Flint, M.S., Salmen, R.R., Brumbaugh, K., and Tinkle, S.S. (2003). Acute stress modulates the irritant component of sensitizers in allergic contact dermatitis: Implications for exposure assessment. *Toxicol. Appl. Pharmacol.* **188**, 50-58.

French, J.E. (2004). Identification and characterization of potential human carcinogens using B6.129tm1Trp53 heterozygous null mice and loss of heterozygosity at the Trp53 locus. *IARC Sci. Publ.* **157**, 271-287.

French, J.E., Lacks, G.D., Trempus, C., Dunnick, J.K., Foley, J., Mahler, J., Tice, R.R., and Tennant, R.W. (2001a). Loss of heterozygosity frequency at the Trp53 locus in p53-deficient (+/-) mouse tumors is carcinogen- and tissue-dependent. *Carcinogenesis* **22**, 98-106.

French, J., Storer, R.D., and Donehower, L.A. (2001b). The nature of the heterozygous Trp53 knockout model for identification of mutagenic carcinogens. *Toxicol. Pathol.* **29** (Suppl.), 24-29.

Funfstuck, V., Knopf, B., and Hipler, C. (1986). Contact allergy to dicyclohexylcarbodiimide [in German]. *Derm. Beruf. Umwelt.* **34**, 110-111.

Furumoto, S. (1971a). Novel method for the synthesis of carbodiimide. *Nippon Kagaku Zasshi* **92**, 1005-1007.

Furumoto, S. (1971b). Novel method for the synthesis of carbodiimides. *Nippon Kagaku Zasshi* **92**, 357-360.

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.

Hayes, B.B., Gerber, P.C., Griffey, S.S., and Meade, B.J. (1998). Contact hypersensitivity to dicyclohexylcarbodiimide and diisopropylcarbodiimide in female B6C3F1 mice. *Drug Chem. Toxicol.* **2**, 195-206.

Hennings, H., Glick, A.B., Greenhalgh, D.A., Morgan, D.L., Strickland, J.E., Tennenbaum, T., and Yuspa, S.H. (1993). Critical aspects of initiation, promotion, and progression in multistage epidermal carcinogenesis. *Proc. Soc. Exp. Biol. Med.* **202**, 1-8.

Hoffman, T.E., and Adams, R.M. (1989). Contact allergic dermatitis to dicyclohexylcarbodiimide used in protein synthesis. *J. Am. Acad. Dermatol.* **21**, 436-437.

Hollander, M., and Wolfe, D.A. (1973). *Nonparametric Statistical Methods*, pp. 120-123. John Wiley and Sons, New York.

Honchel, R., Rosenzweig, B.A., Thompson, K.L., Blanchard, K.T., Furst, S.M., Stoll, R.E., and Sistare, F.D. (2001). Loss of palindromic symmetry in Tg.AC mice with a nonresponder phenotype. *Mol. Carcinog.* **30**, 99-110.

Integrated Laboratory Systems (ILS) (1990). *Micronucleus Data Management and Statistical Analysis Software*, *Version 1.4*. ILS, Inc., P.O. Box 13501, Research Triangle Park, NC 27707.

Jonckheere, A.R. (1954). A distribution-free *k*-sample test against ordered alternatives. *Biometrika* **41**, 133-145.

Kaneko, J.J.. (1989). Serum proteins and the dysproteinemias. In *Clinical Biochemistry of Domestic Animals* (J.J. Kaneko, Ed.), 4th ed., pp. 142-165. Academic Press, Inc., San Diego, CA.

Kato, H., Hayashi, M., Fukumori, Y., and Kaneko, H. (2002). MHC restriction in contact hypersensitivity to dicyclohexylcarbodiimide. *Food Chem. Toxicol.* **40**, 1713-1718.

Kuney, J.H., Ed. (1990). *Chemcyclopedia 91: The Manual of Commercially Available Chemicals*, p. 408. American Chemical Society, Washington, DC.

Lang, M.H., and Hensel, O. (1987). Contact allergy to dicyclohexylcarbodiimide and N-hydroxyphthalimide [in German]. *Derm. Beruf. Umwelt.* **6**, 212-213.

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.

MacDonald, J., French, J.E., Gerson, R.J., Goodman, J., Inoue, T., Jacobs, A., Kasper, P., Keller, D., Lavin, A., Long, G., McCullough, B., Sistare, F.D., van der Laan, J.W. (2004). The utility of genetically modified mouse assays for identifying human carcinogens: A basic understanding and path forward. The Alternatives to Carcinogenicity Testing Committee ILSI HESI. *Toxicol. Sci.* **77**, 188-194.

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.

The Merck Index (1989). 11th ed. (S. Budavari, Ed.), p. 488. Merck and Company, Rahway, NJ.

Morrison, D.F. (1976). *Multivariate Statistical Methods*, 2nd ed., pp. 170-179. McGraw-Hill Book Company, New York.

Moyer, R.C. (1990). A carbodiimide eye irritant. *Chem. Eng. News* **68**, 2.

National Technical Information Service (NTIS) (2004). Vol. 0TS0555962. U.S. National Library of Medicine, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, tehip@teh.nlm.nih.gov.

National Toxicology Program (NTP) (2005a). Toxicology Studies of Trimethylolpropane Triacrylate (Technical Grade) (CAS No. 15625-89-5) in F344/N Rats, B6C3F$_1$ Mice, and Genetically Modified (FVB Tg.AC Hemizygous) Mice (Dermal Studies). Genetically Modified Mouse Report Series No. 3. NIH Publication No. 06-4450. National Institutes of Health, Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC.

National Toxicology Program (NTP) (2005b). Toxicology Studies of Pentaerythritol Triacrylate (Technical Grade) (CAS No. 3524-68-3) in F344/N Rats, B6C3F$_1$ Mice, and Genetically Modified (FVB Tg.AC Hemizygous) Mice (Dermal Studies). Genetically Modified Mouse Report Series No. 4. NIH Publication No. 06-4451. National Institutes of Health, Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC.

National Toxicology Program (NTP) (2007a). Toxicology and Carcinogenesis Studies of Diisopropylcarbodiimide (CAS No. 693-13-0) in F344/N Rats and B6C3F$_1$ 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.

National Toxicology Program (NTP) (2007b). 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). GMM Report Series No. 10, NIH Publication No. 07-4427. National Institutes of Health, Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC.

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.

Pairas, G., Camoutsis, C., and Catsoulacos, P. (1985). A new route for the synthesis of steroidal esters of carboxylic derivatives of N,N-bis(2-chloroethyl)aniline with DCC. *Eur. J. Med. Chem-Chem. Ther*. **20**, 287-288.

Poesen, N., de Moor, A., Busschots, A., and Dooms-Goossens, A. (1995). Contact allergy to dicyclohexyl carbodiimide and diisopropyl carbodiimide. *Contact Dermatitis* **32**, 368-369.

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] (1991). Carbodiimide, diisopropyl. Bethesda, MD: National Institute for Occupational Safety and Health; 1971 to present. Updated quarterly. Available from the National Library of Medicine.

Rosen, F., Roberts, N.R., Budnick, L.E., and Nichol, C.A. (1959a). Corticosteroids and transaminase activity: The specificity of the glutamic pyruvic transaminase response. *Endocrinology* **65**, 256-264.

Rosen, F., Roberts, N.R., and Nichol, C.A. (1959b). Glucocorticosteroids and transaminase activity. I. Increased activity of glutamicpyruvic transaminase in four conditions associated with gluconeogenesis. *J. Biol. Chem.* **234**, 476-480.

Sabatier, M.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* **24,** 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.

Sax, N.I., and Lewis, R.J., Sr. (1987). *Hawley's Condensed Chemical Dictionary*, 11th ed., p. 383. Van Nostrand Reinhold Co., New York.

Shelby, M.D., Erexson, G.L., Hook, G.J., and Tice, R.R. (1993). Evaluation of a three-exposure mouse bone marrow micronucleus protocol: Results with 49 chemicals. *Environ. Mol. Mutagen*. **21**, 160-179.

Shirley, E. (1977). A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. *Biometrics* **33**, 386-389.

Simpson, J.R. (1979). Contact dermatitis due to dicyclohexylcarbodiimide. *Contact Dermatitis* **5**, 333-334.

Slebioda, M., Wodecki, Z., and Kolodziejczyk, A.M. (1990). Formation of optically pure N-acyl-N,N′-dicyclohexylurea in N,N′-dicyclohexyl-carbodiimide-mediated peptide synthesis. *Int. J. Pept. Protein. Res.* **6**, 539-541.

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.

Spalding, J.W., French, J.E., Stasiewicz, S., Furedi-Machacek, M., Conner, F., Tice, R.R., and Tennant, R.W. (2000). Responses of transgenic mouse lines p53(+/–) and Tg.AC to agents tested in conventional carcinogenicity bioassays. *Toxicol. Sci.* **53**, 213-223.

Takahashi, T., and Takahashi, K. (1990). N,N′-Dicyclohexylcarbodiimide is a specific, reversible inhibitor of proline-β-naphthylamidase. *Biochem. Biophys. Res. Commun.* **3**, 1001-1006.

Tejedor, A., Noel, J., Vinay, P., and Gougoux, A. (1987). Metabolic effects of DCCD in suspensions of dog nephron segments. *Kidney Int*. **1**, 403.

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.

Tennant, R.W., Stasiewicz, S., Eastin, W.C., Mennear, J.H., and Spalding, J.W. (2001). The Tg.AC (v-Ha-ras) transgenic mouse: Nature of the model. *Toxicol. Pathol.* **29**, 51-59.

Tice, R.R., Erexson, G.L., Hilliard, C.J., Huston, J.L., Boehm, R.M., Gulati, D., and Shelby, M.D. (1990). Effect of treatment protocol and sample time on the frequencies of micronucleated polychromatic erythrocytes in mouse bone marrow and peripheral blood. *Mutagenesis* **5**, 313-321.

Trempus, 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.

TSCAPP (1991). *TSCA Plant and Production Database, Chemical Information System Database*, September, 1991.

Van, H., Ed. (1990). *OPD Chemical Buyers Directory*, 78th Annual ed., p. 424. Schnell Publishing Co., Inc., New York.

Van Miller, J.P., Garman, R.H., Hermansky, S.J., Mirsalis, J.C., and Frederick, C.B. (2003). Skin irritation, basal epithelial cell proliferation, and carcinogenicity evaluations of a representative specialty acrylate and methacrylate. *Regul. Toxicol. Pharmacol.* **37**, 54-65.

Vente, C., Menzel, S., Gutgesell, C., and Fuchs, T. (1999). Acute dermatitis after contact with dicyclohexylcarbodiimide. *Am. J. Contact Dermat.* **10**, 51-52.

White, I.R., and MacDonald, D.M. (1979). Dicyclohexyl carbodiimide sensitivity. *Contact Dermatitis* **4**, 275-276.

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.

Williams, D.A. (1986). A note on Shirley's nonparametric test for comparing several dose levels with a zero-dose control. *Biometrics* **42**, 183-186.

Witt, K., 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.* **1**, 65-74.

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.

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., and Mortelmans, K. (1992). Salmonella mutagenicity tests: V. Results from the testing of 311 chemicals. *Environ. Mol. Mutagen.* **19** (Suppl. 21), 2-141.

Zschunke, E., and Folesky, H. (1975). Some effects of dicyclohexylcarbodiimide on human skin. *Contact Dermatitis* **3**, 188.

APPENDIX A
SUMMARY OF LESIONS IN F344/N RATS
IN THE 13-WEEK DERMAL STUDY
OF DICYCLOHEXYLCARBODIIMIDE

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Moribund						8
Natural deaths						2
Survivors						
Terminal sacrifice	10	10	10	10	10	
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)				(10)	(10)
Hepatodiaphragmatic nodule	1 (10%)					
Inflammation, chronic active						1 (10%)
Stomach, forestomach	(10)				(10)	(10)
Hyperplasia, atypical, focal					1 (10%)	
Inflammation, acute, focal					1 (10%)	
Cardiovascular System						
Heart	(10)				(10)	(10)
Cardiomyopathy	1 (10%)				3 (30%)	2 (20%)
Endocrine System						
None						
General Body System						
None						
Genital System						
Preputial gland	(10)				(10)	(10)
Inflammation, chronic, focal	1 (10%)				1 (10%)	
Inflammation, chronic active, focal	1 (10%)					
Hematopoietic System						
Bone marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hyperplasia, diffuse						10 (100%)
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Depletion cellular, diffuse						1 (10%)
Hematopoietic cell proliferation, focal		1 (10%)	1 (10%)	1 (10%)	2 (20%)	1 (10%)
Thymus	(10)				(10)	(10)
Atrophy						1 (10%)
Necrosis, diffuse						2 (20%)

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

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Integumentary System						
Skin	(10)	(10)	(10)	(10)	(10)	(10)
Control, inflammation, chronic active					3 (30%)	2 (20%)
Epidermis, control, hyperplasia					5 (50%)	1 (10%)
Epidermis, control, necrosis					1 (10%)	1 (10%)
Epidermis, skin, site of application, hyperplasia		1 (10%)	3 (30%)	9 (90%)	10 (100%)	10 (100%)
Epidermis, skin, site of application, hyperplasia, focal		1 (10%)				
Epidermis, skin, site of application, necrosis						6 (60%)
Sebaceous gland, skin, site of application, hyperplasia		1 (10%)	1 (10%)	4 (40%)	7 (70%)	6 (60%)
Skin, site of application, inflammation, acute, diffuse						1 (10%)
Skin, site of application, inflammation, chronic active		1 (10%)		1 (10%)	7 (70%)	9 (90%)
Skin, site of application, inflammation, granulomatous						1 (10%)
Musculoskeletal System						
None						
Nervous System						
Brain	(10)	(10)	(10)	(10)	(10)	(10)
Hemorrhage						1 (10%)
Respiratory System						
Lung	(10)	(10)	(10)	(8)	(10)	(10)
Congestion, diffuse						2 (20%)
Inflammation, chronic active, focal	8 (80%)	8 (80%)	7 (70%)	7 (88%)	9 (90%)	3 (30%)
Special Senses System						
None						
Urinary System						
Kidney	(10)				(10)	(10)
Nephropathy	6 (60%)				5 (50%)	2 (20%)
Renal tubule, degeneration, focal	1 (10%)					
Urinary bladder	(10)				(10)	(10)
Hemorrhage						1 (10%)

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Accidental deaths	2					
Moribund						10
Survivors						
Terminal sacrifice	8	10	10	10	10	
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)			(1)	(10)	(10)
Hepatodiaphragmatic nodule				1 (100%)		
Cardiovascular System						
Heart	(10)				(10)	(10)
Cardiomyopathy	1 (10%)				1 (10%)	4 (40%)
Endocrine System						
Pituitary gland	(10)				(10)	(9)
Pars distalis, cyst						1 (11%)
General Body System						
None						
Genital System						
Clitoral gland	(10)				(9)	(10)
Inflammation, chronic active, focal					1 (11%)	
Uterus	(10)		(1)	(1)	(10)	(10)
Hydrometra			1 (100%)	1 (100%)	2 (20%)	
Bilateral, hydrometra	1 (10%)					
Hematopoietic System						
Bone marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hyperplasia, diffuse					1 (10%)	9 (90%)
Hyperplasia, focal, histiocytic	4 (40%)		4 (40%)	2 (20%)	1 (10%)	
Lymph node					(1)	
Hyperplasia, diffuse, lymphoid					1 (100%)	

[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 F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Integumentary System						
Skin	(10)	(10)	(10)	(10)	(10)	(10)
Control, inflammation, chronic active				1 (10%)	1 (10%)	2 (20%)
Dermis, skin, site of application, edema, focal						1 (10%)
Dermis, skin, site of application, inflammation, chronic active						1 (10%)
Epidermis, control, hyperplasia		1 (10%)	1 (10%)		1 (10%)	1 (10%)
Epidermis, control, necrosis						1 (10%)
Epidermis, skin, site of application, hyperplasia		3 (30%)	7 (70%)	7 (70%)	10 (100%)	10 (100%)
Epidermis, skin, site of application, necrosis					1 (10%)	3 (30%)
Epidermis, skin, site of application, necrosis, focal						1 (10%)
Sebaceous gland, skin, site of application, hyperplasia		1 (10%)			5 (50%)	7 (70%)
Skin, site of application, inflammation, acute					1 (10%)	
Skin, site of application, inflammation, chronic active			4 (40%)	4 (40%)	9 (90%)	9 (90%)
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)	(9)	(10)	(9)	(10)	(10)
Inflammation, chronic active, focal	8 (80%)	9 (100%)	6 (60%)	7 (78%)	7 (70%)	2 (20%)
Special Senses System						
None						
Urinary System						
Kidney	(10)				(10)	(10)
Nephropathy	2 (20%)				1 (10%)	4 (40%)

APPENDIX B
SUMMARY OF LESIONS IN B6C3F$_1$ MICE
IN THE 13-WEEK DERMAL STUDY
OF DICYCLOHEXYLCARBODIIMIDE

TABLE B1
**Summary of the Incidence of Nonneoplastic Lesions in Male B6C3F$_1$ Mice
in the 13-Week Dermal Study of Dicyclohexylcarbodiimide[a]**

	Vehicle Control	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg	24 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Accidental deaths	1	1	2			
Moribund						9
Natural death						1
Survivors						
Terminal sacrifice	9	9	8	10	10	
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)				(10)	(10)
Inflammation, focal					1 (10%)	
Cardiovascular System						
None						
Endocrine System						
Adrenal cortex	(10)				(10)	(9)
Bilateral, capsule, hyperplasia	1 (10%)					
Capsule, hyperplasia	1 (10%)				3 (30%)	
General Body System						
None						
Genital System						
Preputial gland	(9)	(10)	(10)	(10)	(10)	(10)
Atrophy				1 (10%)		
Bilateral, atrophy						4 (40%)
Hematopoietic System						
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Hematopoietic cell proliferation		5 (50%)	2 (20%)	7 (70%)	6 (60%)	6 (60%)
Thymus	(9)	(10)	(9)	(9)	(10)	(8)
Atrophy						8 (100%)
Thymocyte, necrosis					1 (10%)	

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

Summary of the Incidence of Nonneoplastic Lesions in Male B6C3F$_1$ Mice in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg	24 mg/kg
Integumentary System						
Skin	(10)	(10)	(10)	(10)	(10)	(10)
Hyperplasia		1 (10%)				
Control, inflammation					1 (10%)	
Control, inflammation, chronic active		1 (10%)	1 (10%)	1 (10%)	3 (30%)	
Epidermis, control, hyperplasia			1 (10%)			
Epidermis, control, necrosis					2 (20%)	
Epidermis, control, necrosis, diffuse					1 (10%)	
Epidermis, skin, site of application, hyperplasia		7 (70%)	10 (100%)	10 (100%)	10 (100%)	
Epidermis, skin, site of application, necrosis	1 (10%)				2 (20%)	10 (100%)
Epidermis, skin, site of application, necrosis, focal				2 (20%)		
Skin, site of application, inflammation, chronic active		4 (40%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Musculoskeletal System						
None						
Nervous System						
Brain	(10)	(10)	(10)	(10)	(10)	(10)
Pons, hemorrhage						2 (20%)
Respiratory System						
None						
Special Senses System						
None						
Urinary System						
None						

TABLE B2
Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Female B63CF$_1$ Mice
in the 13-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg	24 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Accidental death		1				
Moribund						10
Survivors						
Terminal sacrifice	10	9	10	10	10	
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)				(10)	(10)
Inflammation, focal	1 (10%)					
Cardiovascular System						
None						
Endocrine System						
Adrenal cortex	(10)				(10)	(10)
Bilateral, capsule, hyperplasia	5 (50%)				7 (70%)	
Capsule, hyperplasia	2 (20%)				2 (20%)	
Pituitary gland	(9)				(9)	(8)
Pars distalis, cyst						1 (13%)
General Body System						
None						
Genital System						
Clitoral gland	(8)	(7)	(5)	(9)	(7)	(10)
Bilateral, atrophy						1 (10%)
Hematopoietic System						
Lymph node					(1)	
Inguinal, hyperplasia					1 (100%)	
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Hematopoietic cell proliferation	2 (20%)	2 (20%)	7 (70%)	9 (90%)	5 (50%)	3 (30%)
Thymus	(10)	(10)	(10)	(9)	(10)	(8)
Atrophy						8 (100%)
Thymocyte, necrosis						1 (13%)

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

TABLE B2
**Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Female B63CF$_1$ Mice
in the 13-Week Dermal Study of Dicyclohexylcarbodiimide**

	Vehicle Control	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg	24 mg/kg
Integumentary System						
Skin	(10)	(10)	(10)	(10)	(10)	(10)
Control, inflammation	1 (10%)					1 (10%)
Control, inflammation, chronic active	1 (10%)		3 (30%)	5 (50%)	5 (50%)	
Epidermis, control, hyperplasia					3 (30%)	
Epidermis, skin, site of application, hyperplasia		5 (50%)	9 (90%)	10 (100%)	10 (100%)	3 (30%)
Epidermis, skin, site of application, necrosis				2 (20%)	3 (30%)	10 (100%)
Epidermis, skin, site of application, necrosis, focal				1 (10%)		
Skin, site of application, inflammation, chronic active		1 (10%)	9 (90%)	10 (100%)	10 (100%)	10 (100%)
Musculoskeletal System						
None						
Nervous System						
Brain	(10)	(10)	(10)	(10)	(10)	(10)
Lipoma				1 (10%)		
Pons, hemorrhage						6 (60%)
Respiratory System						
None						
Special Senses System						
None						
Urinary System						
None						

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

TABLE C1
Summary of the Incidence of Neoplasms in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Accidental death				1		
Natural deaths	1	1		1		1
Moribund	2	2		2	2	
Survivors						
Terminal sacrifice	7	7	10	6	8	9
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)	(6)	(6)	(7)	(10)	(10)
Leukemia erythrocytic	1 (10%)		2 (33%)		1 (10%)	
Pancreas	(10)	(3)		(4)	(2)	(10)
Leukemia erythrocytic					1 (50%)	
Stomach, forestomach	(10)	(4)	(2)	(5)	(4)	(10)
Squamous cell papilloma		2 (50%)	2 (100%)	1 (20%)	1 (25%)	
Tooth	(4)	(5)	(6)	(4)	(5)	(5)
Odontoma	3 (75%)	5 (100%)	6 (100%)	3 (75%)	5 (100%)	5 (100%)
Endocrine System						
Thyroid gland	(9)	(4)		(4)	(2)	(10)
C-cell, adenoma		1 (25%)				
Hematopoietic System						
Lymph node		(1)			(1)	
Leukemia erythrocytic					1 (100%)	
Spleen	(10)	(5)	(5)	(4)	(4)	(10)
Leukemia erythrocytic			2 (40%)		1 (25%)	
Thymus	(9)	(3)		(3)	(2)	(10)
Lymphoma malignant	1 (11%)					
Integumentary System						
Skin	(10)	(10)	(10)	(10)	(10)	(10)
Squamous cell papilloma			1 (10%)			1 (10%)
Skin, site of application, squamous cell carcinoma					1 (10%)	
Skin, site of application, squamous cell papilloma				3 (30%)	3 (30%)	3 (30%)
Skin, site of application, squamous cell papilloma, multiple			1 (10%)		3 (30%)	5 (50%)
Systemic Lesions						
Multiple organs[b]	(10)	(10)	(10)	(10)	(10)	(10)
Leukemia erythrocytic	1 (10%)		2 (20%)		1 (10%)	
Lymphoma malignant	1 (10%)					

TABLE C1

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

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Systems Examined with No Neoplasms Observed						
Cardiovascular System						
General Body System						
Genital System						
Musculoskeletal System						
Nervous System						
Respiratory System						
Special Senses System						
Urinary System						
Neoplasm Summary						
Total animals with primary neoplasms[c]	5	6	9	6	8	10
Total primary neoplasms	5	8	12	7	14	14
Total animals with benign neoplasms	3	6	8	6	8	10
Total benign neoplasms	3	8	10	7	12	14
Total animals with malignant neoplasms	2		2		2	
Total malignant neoplasms	2		2		2	

[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 C2
Summary of the Incidence of Nonneoplastic Lesions in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Accidental death				1		
Natural deaths	1	1		1		1
Moribund	2	2		2	2	
Survivors						
Terminal sacrifice	7	7	10	6	8	9
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Gallbladder	(9)	(3)		(3)	(2)	(7)
Inflammation		1 (33%)				1 (14%)
Intestine large, rectum	(9)	(3)		(4)	(2)	(9)
Anus, inflammation					1 (50%)	
Liver	(10)	(6)	(6)	(7)	(10)	(10)
Fibrosis		1 (17%)				1 (10%)
Hematopoietic cell proliferation	1 (10%)	1 (17%)			2 (20%)	1 (10%)
Inflammation, chronic active, focal		3 (50%)	3 (50%)	4 (57%)	6 (60%)	2 (20%)
Inflammation, focal	3 (30%)					5 (50%)
Necrosis		1 (17%)	1 (17%)	2 (29%)	1 (10%)	
Necrosis, focal	1 (10%)					1 (10%)
Stomach, forestomach	(10)	(4)	(2)	(5)	(4)	(10)
Inflammation		1 (25%)				
Stomach, glandular	(10)	(3)		(4)	(2)	(10)
Inflammation	1 (10%)					1 (10%)
Serosa, fibrosis						1 (10%)
Tooth	(4)	(5)	(6)	(4)	(5)	(5)
Inflammation, chronic active						1 (20%)
Cardiovascular System						
Heart	(10)	(3)		(4)	(2)	(10)
Inflammation, acute						1 (10%)
Mineralization, focal						1 (10%)
Endocrine System						
Pituitary gland	(10)	(2)		(3)	(1)	(8)
Pars distalis, necrosis, focal					1 (100%)	
General Body System						
None						
Genital System						
Clitoral gland	(8)	(1)		(4)	(1)	(9)
Inflammation						1 (11%)
Uterus	(10)	(3)		(4)	(2)	(10)
Cyst	1 (10%)					1 (10%)
Bilateral, cyst						1 (10%)

TABLE C2
Summary of the Incidence of Nonneoplastic Lesions in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Hematopoietic System						
Bone marrow	(10)	(3)		(4)	(2)	(10)
Hyperplasia	3 (30%)			2 (50%)	1 (50%)	2 (20%)
Myeloid cell, hyperplasia		1 (33%)				
Lymph node		(1)			(1)	
Bronchial, infiltration cellular, plasma cell		1 (100%)				
Lymph node, mandibular	(10)	(3)	(1)	(4)	(3)	(10)
Hematopoietic cell proliferation		1 (33%)				
Hemorrhage				1 (25%)		
Infiltration cellular, plasma cell			1 (100%)			1 (10%)
Infiltration cellular, histiocyte	2 (20%)					1 (10%)
Lymph node, mesenteric	(9)	(3)		(4)	(2)	(9)
Hematopoietic cell proliferation		1 (33%)				
Infiltration cellular, histiocyte	1 (11%)					
Necrosis, focal	1 (11%)			1 (25%)		
Spleen	(10)	(5)	(5)	(4)	(4)	(10)
Fibrosis						1 (10%)
Hematopoietic cell proliferation	8 (80%)	4 (80%)	4 (80%)	3 (75%)	3 (75%)	9 (90%)
Lymphoid follicle, depletion cellular						1 (10%)
Thymus	(9)	(3)		(3)	(2)	(10)
Atrophy	1 (11%)	2 (67%)		1 (33%)	2 (100%)	1 (10%)
Hemorrhage						1 (10%)
Integumentary System						
Skin	(10)	(10)	(10)	(10)	(10)	(10)
Hemorrhage				1 (10%)		
Hyperplasia						1 (10%)
Inflammation, chronic active			1 (10%)			
Dermis, inflammation					1 (10%)	
Dermis, inflammation, chronic active				1 (10%)		
Dermis, skin, site of application, inflammation, chronic active			3 (30%)	5 (50%)	8 (80%)	
Epidermis, skin, site of application, hyperplasia				6 (60%)	7 (70%)	1 (10%)
Musculoskeletal System						
Bone	(10)	(3)		(4)	(2)	(10)
Synovial tissue, inflammation	1 (10%)					
Nervous System						
Brain	(10)	(3)		(4)	(2)	(10)
Hemorrhage, acute				1 (25%)		
Respiratory System						
Larynx						(1)
Inflammation						1 (100%)
Lung	(10)	(3)		(4)	(2)	(10)
Hemorrhage, acute				1 (25%)		
Inflammation, chronic active						4 (40%)
Nose	(10)	(3)		(4)	(5)	(10)
Inflammation					3 (60%)	1 (10%)
Respiratory epithelium, inflammation						1 (10%)

TABLE C2

Summary of the Incidence of Nonneoplastic Lesions in Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Special Senses System						
None						
Urinary System						
Kidney	(10)	(3)		(4)	(2)	(10)
Inflammation, chronic active		1 (33%)				
Bilateral, inflammation						1 (10%)
Cortex, cyst	1 (10%)					

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

APPENDIX D
SUMMARY OF LESIONS
IN FEMALE p53 HAPLOINSUFFICIENT MICE
IN THE 27-WEEK DERMAL STUDY
OF DICYCLOHEXYLCARBODIIMIDE

TABLE D1
Summary of the Incidence of Neoplasms in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Disposition Summary						
Animals initially in study	15	15	15	15	15	15
Early deaths						
Natural deaths				1	1	1
Moribund				2		7
Survivors						
Terminal sacrifice	15	15	15	12	14	7
Animals examined microscopically	15	15	15	15	15	15
Alimentary System						
Mesentery				(1)		
Lymphoma malignant				1 (100%)		
Hematopoietic System						
Lymph node, mandibular	(15)	(15)	(14)	(14)	(14)	(14)
Histiocytic sarcoma					1 (7%)	
Lymph node, mesenteric	(14)	(15)	(15)	(15)	(14)	(14)
Histiocytic sarcoma					1 (7%)	
Lymph node, mediastinal	(14)	(15)	(15)	(12)	(13)	(13)
Histiocytic sarcoma					1 (8%)	
Lymphoma malignant				1 (8%)		
Thymus	(15)	(15)	(15)	(15)	(14)	(13)
Histiocytic sarcoma					1 (7%)	
Lymphoma malignant				1 (7%)		
Respiratory System						
Lung	(15)	(15)	(15)	(15)	(14)	(15)
Lymphoma malignant				1 (7%)		
Systemic Lesions						
Multiple organs[b]	(15)	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma					1 (7%)	
Lymphoma malignant				1 (7%)		

Systems Examined with No Neoplasms Observed
Cardiovascular System
Endocrine System
General Body System
Genital System
Integumentary System
Musculoskeletal System
Nervous System
Special Senses System
Urinary System

TABLE D1
Summary of the Incidence of Neoplasms in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Neoplasm Summary						
Total animals with primary neoplasms[c]				1	1	
Total primary neoplasms				1	1	
Total animals with malignant neoplasms				1	1	
Total malignant neoplasms				1	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 D2
Summary of the Incidence of Nonneoplastic Lesions in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Disposition Summary						
Animals initially in study	15	15	15	15	15	15
Early deaths						
Natural deaths				1	1	1
Moribund				2		7
Survivors						
Terminal sacrifice	15	15	15	12	14	7
Animals examined microscopically	15	15	15	15	15	15
Alimentary System						
Liver	(15)	(15)	(15)	(15)	(14)	(15)
Hematopoietic cell proliferation						1 (7%)
Hematopoietic cell proliferation, focal						1 (7%)
Infiltration cellular, focal, lymphocyte		1 (7%)		4 (27%)	1 (7%)	2 (13%)
Inflammation, chronic active, focal						1 (7%)
Hepatocyte, necrosis, focal	4 (27%)	6 (40%)	5 (33%)	4 (27%)	2 (14%)	1 (7%)
Periportal, vacuolization cytoplasmic, focal				1 (7%)		
Stomach, glandular	(15)	(15)	(15)	(15)	(14)	(14)
Infiltration cellular, focal, lymphocyte				1 (7%)		
Cardiovascular System						
None						
Endocrine System						
Adrenal cortex	(15)	(15)	(15)	(15)	(14)	(13)
Subcapsular, hyperplasia						1 (8%)
Subcapsular, hyperplasia, focal	13 (87%)	13 (87%)	12 (80%)	13 (87%)	14 (100%)	9 (69%)
Thyroid gland	(13)	(15)	(15)	(14)	(13)	(13)
Ectopic thymus		2 (13%)	3 (20%)	1 (7%)	1 (8%)	
Inflammation, chronic active, focal	1 (8%)					
General Body System						
None						
Genital System						
Ovary	(15)	(15)	(15)	(15)	(14)	(14)
Atrophy			1 (7%)			1 (7%)
Uterus	(15)	(15)	(15)	(15)	(14)	(14)
Endometrium, hyperplasia, cystic	14 (93%)	15 (100%)	15 (100%)	13 (87%)	13 (93%)	7 (50%)

TABLE D2
Summary of the Incidence of Nonneoplastic Lesions in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Hematopoietic System						
Bone marrow	(15)	(15)	(15)	(15)	(14)	(15)
Myeloid cell, hyperplasia				3 (20%)	1 (7%)	8 (53%)
Lymph node				(1)		
Inguinal, hyperplasia, lymphoid				1 (100%)		
Lymph node, mandibular	(15)	(15)	(14)	(14)	(14)	(14)
Hyperplasia, lymphoid	1 (7%)			2 (14%)	3 (21%)	
Lymph node, mediastinal	(14)	(15)	(15)	(12)	(13)	(13)
Hyperplasia, lymphoid			1 (7%)			
Spleen	(15)	(15)	(15)	(15)	(14)	(14)
Hematopoietic cell proliferation	15 (100%)	15 (100%)	15 (100%)	15 (100%)	13 (93%)	13 (93%)
Pigmentation						1 (7%)
Thymus	(15)	(15)	(15)	(15)	(14)	(13)
Atrophy, diffuse						2 (15%)
Atrophy, focal		2 (13%)	1 (7%)			
Integumentary System						
Mammary gland	(15)	(15)	(15)	(15)	(14)	(14)
Inflammation, chronic active, focal						1 (7%)
Skin	(15)	(15)	(15)	(15)	(15)	(15)
Control, ulcer, focal				1 (7%)		2 (13%)
Dermis, epidermis, hyperplasia, focal						1 (7%)
Dermis, control, inflammation, chronic active, focal				3 (20%)	1 (7%)	2 (13%)
Dermis, skin, site of application, fibrosis						1 (7%)
Dermis, skin, site of application, inflammation, chronic active, focal	1 (7%)			13 (87%)	2 (13%)	9 (60%)
Epidermis, control, hyperplasia, focal				2 (13%)		1 (7%)
Epidermis, skin, site of application, hyperplasia, diffuse			1 (7%)	2 (13%)		
Epidermis, skin, site of application, hyperplasia, focal		1 (7%)	5 (33%)	11 (73%)	1 (7%)	8 (53%)
Sebaceous gland, control, hyperplasia, focal				1 (7%)		1 (7%)
Sebaceous gland, skin, site of application, hyperplasia, focal				3 (20%)		3 (20%)
Skin, site of application, hyperkeratosis, diffuse				1 (7%)	1 (7%)	
Skin, site of application, parakeratosis, focal				1 (7%)	1 (7%)	
Skin, site of application, ulcer, focal				2 (13%)	1 (7%)	8 (53%)
Subcutaneous tissue, control, inflammation, chronic active, focal				1 (7%)		2 (13%)
Subcutaneous tissue, skin, site of application, inflammation, chronic, focal					1 (7%)	
Subcutaneous tissue, skin, site of application, inflammation, chronic active, focal				2 (13%)		8 (53%)

Musculoskeletal System
None

Nervous System
None

TABLE D2
Summary of the Incidence of Nonneoplastic Lesions in Female p53 Haploinsufficient Mice in the 27-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Respiratory System						
Lung	(15)	(15)	(15)	(15)	(14)	(15)
Alveolar epithelium, hyperplasia, focal			1 (7%)			
Alveolar epithelium, inflammation, chronic active, focal		1 (7%)				
Alveolus, inflammation, chronic active, focal	1 (7%)				1 (7%)	1 (7%)
Perivascular, infiltration cellular, focal, lymphocyte	1 (7%)				2 (14%)	
Special Senses System						
None						
Urinary System						
Kidney	(15)	(15)	(15)	(15)	(14)	(14)
Infiltration cellular, focal, lymphocyte		1 (7%)				
Pelvis, infiltration cellular, focal, lymphocyte				1 (7%)		

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

APPENDIX E
GENETIC TOXICOLOGY

TABLE E1
Mutagenicity of Dicyclohexylcarbodiimide in *Salmonella typhimurium*[a]

| Strain | Dose | Revertants/Plate[b] | | | | | |
| | | −S9 | | + hamster S9 | | + rat S9 | |
	(µg/plate)	Trial 1	Trial 2	10%	30%	10%	30%
TA100	0	84 ± 1.0	97 ± 5.5	105 ± 8.1	92 ± 1.9	98 ± 7.5	107 ± 5.4
	0.1		87 ± 3.8				
	0.3	86 ± 3.7	97 ± 6.7				
	1	85 ± 2.3	100 ± 3.8				
	3	88 ± 6.8	92 ± 6.1	95 ± 1.5		103 ± 2.9	
	10	85 ± 0.0	62 ± 3.8[c]	89 ± 1.2	101 ± 0.3	98 ± 4.3	102 ± 5.2
	33	Toxic		88 ± 7.9	90 ± 0.3	94 ± 4.3	112 ± 6.7
	100			94 ± 3.2	88 ± 5.8	83 ± 2.5	97 ± 2.8
	333			57 ± 5.4[c]	87 ± 0.9	22 ± 11.7[c]	86 ± 1.9
	666				8 ± 5.7[c]		39 ± 5.8[c]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[d]		829 ± 11.4	792 ± 19.7	522 ± 26.4	612 ± 11.8	428 ± 13.5	492 ± 35.4
TA1535	0	8 ± 2.3	12 ± 2.3	11 ± 1.5	10 ± 0.9	15 ± 1.5	12 ± 1.8
	0.1	10 ± 0.9	11 ± 1.8				
	0.3	8 ± 0.9	11 ± 1.5				
	1	10 ± 0.3	10 ± 3.3				
	3	11 ± 2.5	13 ± 2.1	10 ± 1.9	9 ± 2.6	12 ± 2.7	10 ± 1.0
	10	8 ± 1.2	6 ± 0.9	9 ± 0.3	9 ± 1.5	10 ± 1.9	11 ± 2.2
	33			9 ± 1.8	10 ± 1.9	12 ± 2.0	10 ± 2.3
	100			7 ± 0.6	8 ± 2.2	7 ± 0.6	15 ± 2.3
	333			4 ± 0.9[c]	6 ± 0.6	9 ± 1.5	9 ± 1.5
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		651 ± 20.4	443 ± 6.4	101 ± 7.3	263 ± 18.0	100 ± 6.1	119 ± 3.4
TA97	0	98 ± 9.0	115 ± 9.8	128 ± 6.3	151 ± 4.3	132 ± 15.3	162 ± 4.9
	0.1	112 ± 5.4	122 ± 6.7				
	0.3	115 ± 4.8	133 ± 5.4				
	1	119 ± 4.3	123 ± 10.7				
	3	112 ± 9.4	141 ± 10.5	124 ± 3.6	166 ± 6.0	141 ± 8.1	154 ± 3.6
	10	118 ± 10.7	105 ± 7.8	144 ± 7.4	172 ± 0.9	154 ± 4.4	159 ± 3.8
	33			157 ± 3.8	165 ± 3.5	153 ± 8.2	165 ± 3.2
	100			158 ± 6.6	153 ± 3.5	152 ± 13.5	150 ± 2.8
	333			118 ± 9.7	149 ± 7.2	116 ± 7.4	155 ± 6.0
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		341 ± 22.0	370 ± 32.7	447 ± 13.1	439 ± 22.2	325 ± 11.0	323 ± 20.0

TABLE E1
Mutagenicity of Dicyclohexylcarbodiimide in *Salmonella typhimurium*

| Strain | Dose | Revertants/Plate | | | | | |
| | | −S9 | | + hamster S9 | | + rat S9 | |
	(µg/plate)	Trial 1	Trial 2	10%	30%	10%	30%
TA98	0	13 ± 1.2	12 ± 2.9	20 ± 1.2	17 ± 3.6	13 ± 1.0	17 ± 1.5
	0.1		13 ± 1.5				
	0.3	13 ± 1.8	15 ± 3.3				
	1	16 ± 4.2	11 ± 0.6				
	3	13 ± 2.2	9 ± 1.2	16 ± 1.0		16 ± 0.9	
	10	11 ± 1.2	11 ± 2.8	11 ± 1.0	15 ± 2.4	12 ± 1.0	14 ± 0.6
	33	9 ± 0.3[c]		13 ± 1.3	14 ± 1.2	13 ± 3.2	13 ± 1.5
	100			10 ± 2.5[c]	13 ± 2.6	15 ± 2.3	14 ± 0.3
	333			8 ± 3.3[c]	12 ± 2.1[c]	9 ± 0.7[c]	11 ± 1.5[c]
	666				13 ± 1.5[c]		7 ± 0.9[c]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		287 ± 25.2	298 ± 21.1	349 ± 4.7	265 ± 17.2	226 ± 5.0	223 ± 13.1

[a] Study was performed at SRI International. The detailed protocol is presented by Zeiger *et al*. (1992). 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] Slight toxicity
[d] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA97), and
4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE E2
Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male F344/N Rats
Treated with Dicyclohexylcarbodiimide by Intraperitoneal Injection[a]

Compound	Dose (mg/kg)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]	PCEs[b] (%)	P Value[c]
Trial 1						
Corn oil[d]	0	5	0.4 ± 0.19		52.1 ± 1.10	
Dicyclohexylcarbodiimide	5	5	0.1 ± 0.10	0.910	34.5 ± 3.03	0.003
	10	4	0.5 ± 0.20	0.376	33.9 ± 2.18	0.002
	15	2[e]	0.3 ± 0.25	—	30.3 ± 7.75	—
	20	3	1.3 ± 0.67	0.018	33.0 ± 4.31	0.050
			P=0.006[f]		P<0.001[g]	
Cyclophosphamide[h]	25	5	15.9 ± 1.64	<0.001	2.3 ± 0.41	<0.001
Trial 2						
Corn oil	0	5	0.6 ± 0.37		55.2 ± 4.62	
Dicyclohexylcarbodiimide	10	5	0.7 ± 0.20	0.391	51.0 ± 6.07	0.599
	15	5	1.0 ± 0.45	0.159	34.2 ± 3.29	0.008
	20	5	0.7 ± 0.20	0.391	44.4 ± 7.37	0.255
			P=0.289		P=0.079	
Cyclophosphamide	25	5	31.3 ± 8.47	<0.001	2.8 ± 0.96	<0.001

[a]   Study was performed at ILS, Inc. The detailed protocol and these data are presented by Witt *et al.* (1999).
[b]   PCE=polychromatic erythrocyte
[c]   Mean ± standard error
[d]   Pairwise comparison with the vehicle control; significant at P≤0.008 (ILS, 1990)
[e]   Vehicle control
[f]   These data were not included in the overall statistical evaluation due to insufficient animals.
[g]   Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)
[h]   ANOVA using individual animal data, significant at P≤0.05
     Positive control

TABLE E3
Frequency of Micronuclei in Peripheral Blood Erythrocytes of B6C3F$_1$ Mice
Following Dermal Administration of Dicyclohexylcarbodiimide for 13 Weeks[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	P Value[c]	PCEs[b] (%)	P Value[c]
Male						
Ethanol[d]	0	10	3.6 ± 0.43		4.8 ± 0.25	
Dicyclohexylcarbodiimide	1.5	10	3.1 ± 0.48	0.730	4.3 ± 0.08	0.114
	3	10	5.3 ± 0.50	0.035	4.4 ± 0.11	0.264
	6	10	5.7 ± 0.70	0.015	4.1 ± 0.14	0.048
	12	10	5.7 ± 0.68	0.015	4.3 ± 0.14	0.107
			P=0.003[e]		P=0.071[f]	
Female						
Ethanol	0	10	2.7 ± 0.63		4.3 ± 0.19	
Dicyclohexylcarbodiimide	1.5	10	3.7 ± 0.58	0.105	4.9 ± 0.14	0.009
	3	10	5.1 ± 0.67	0.003	4.6 ± 0.16	0.085
	6	10	4.3 ± 0.68	0.028	4.9 ± 0.22	0.015
	12	10	4.4 ± 0.50	0.022	4.5 ± 0.22	0.191
			P=0.078		P=0.096	

[a] Study was performed at Microbiological Associates, Inc. The detailed protocol and these data are presented by Witt *et al*. (1999). NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte

[b] Mean ± standard error

[c] Pairwise comparison with the vehicle control; significant at P≤0.006 (ILS, 1990)

[d] Vehicle control

[e] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)

[f] ANOVA using individual animal data, significant at P≤0.05

APPENDIX F
CLINICAL PATHOLOGY RESULTS

TABLE F1
Hematology and Clinical Chemistry Data for F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
n	10	10	10	10	10	10

Male

Hematology

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Automated hematocrit (%)						
Day 3	40.9 ± 0.4	41.1 ± 0.7	42.2 ± 0.4	41.2 ± 0.6	40.4 ± 0.4	41.5 ± 0.8
Day 22	45.9 ± 0.4	45.0 ± 0.5	45.4 ± 0.6	44.6 ± 0.4	43.7 ± 0.3**	44.5 ± 0.5*
Week 13	44.1 ± 0.4	43.9 ± 0.7	44.4 ± 0.4	44.5 ± 0.3	44.5 ± 0.4	—[b]
Manual hematocrit (%)						
Day 3	44.3 ± 0.4	44.7 ± 0.8	45.4 ± 0.5	45.1 ± 0.6	45.5 ± 0.5	46.0 ± 0.7
Day 22	50.6 ± 0.6	49.8 ± 0.8	50.1 ± 0.6	48.8 ± 0.6	49.2 ± 0.4	49.5 ± 0.5
Week 13	46.8 ± 0.3	45.9 ± 0.6	46.6 ± 0.5	46.9 ± 0.4	46.4 ± 0.3	—
Hemoglobin (g/dL)						
Day 3	14.6 ± 0.1	14.7 ± 0.2	14.8 ± 0.1	14.7 ± 0.2	14.5 ± 0.2	14.6 ± 0.2
Day 22	16.2 ± 0.2	16.1 ± 0.2	16.1 ± 0.1	15.9 ± 0.1	15.5 ± 0.1**	15.6 ± 0.2**
Week 13	15.7 ± 0.1	15.5 ± 0.2	15.6 ± 0.1	15.7 ± 0.1	15.6 ± 0.1	—
Erythrocytes (10^6/μL)						
Day 3	6.55 ± 0.08	6.66 ± 0.11	6.72 ± 0.05	6.66 ± 0.09	6.50 ± 0.08	6.63 ± 0.11
Day 22	7.66 ± 0.09	7.56 ± 0.08	7.60 ± 0.08	7.54 ± 0.08	7.38 ± 0.06*	7.63 ± 0.08
Week 13	8.70 ± 0.09	8.73 ± 0.12	8.81 ± 0.07	8.77 ± 0.06	8.83 ± 0.06	—
Reticulocytes (10^6/μL)						
Day 3	0.41 ± 0.03	0.36 ± 0.03	0.33 ± 0.03	0.36 ± 0.03	0.36 ± 0.03	0.36 ± 0.03
Day 22	0.19 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.18 ± 0.02	0.14 ± 0.02
Week 13	0.18 ± 0.02	0.18 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	—
Nucleated erythrocytes (10^3/μL)						
Day 3	1.70 ± 0.42	0.30 ± 0.15*	1.10 ± 0.31	0.50 ± 0.31	0.90 ± 0.35	0.80 ± 0.20
Day 22	0.60 ± 0.31	0.70 ± 0.34	0.70 ± 0.21	0.10 ± 0.10	0.60 ± 0.31	0.30 ± 0.30
Week 13	0.50 ± 0.27	0.20 ± 0.13	0.20 ± 0.13	0.40 ± 0.22	0.50 ± 0.22	—
Mean cell volume (fL)						
Day 3	62.5 ± 0.4	61.7 ± 0.4	62.8 ± 0.3	61.9 ± 0.4	62.2 ± 0.5	62.5 ± 0.4
Day 22	59.9 ± 0.4	59.6 ± 0.2	59.8 ± 0.3	59.2 ± 0.5	59.3 ± 0.3	58.4 ± 0.2**
Week 13	50.7 ± 0.2	50.3 ± 0.2	50.4 ± 0.2	50.7 ± 0.1	50.4 ± 0.1	—
Mean cell hemoglobin (pg)						
Day 3	22.3 ± 0.1	22.1 ± 0.1	22.0 ± 0.1	22.1 ± 0.2	22.3 ± 0.1	22.0 ± 0.2
Day 22	21.1 ± 0.2	21.3 ± 0.1	21.2 ± 0.2	21.1 ± 0.1	21.0 ± 0.1	20.4 ± 0.1**
Week 13	18.0 ± 0.1	17.8 ± 0.1	17.7 ± 0.1	17.9 ± 0.1	17.7 ± 0.1	—
Mean cell hemoglobin concentration (g/dL)						
Day 3	35.7 ± 0.2	35.8 ± 0.3	35.0 ± 0.2	35.7 ± 0.3	35.9 ± 0.2	35.3 ± 0.3
Day 22	35.2 ± 0.2	35.7 ± 0.2	35.4 ± 0.3	35.6 ± 0.2	35.4 ± 0.2	35.0 ± 0.2
Week 13	35.5 ± 0.2	35.3 ± 0.3	35.1 ± 0.2	35.4 ± 0.2	35.2 ± 0.2	—
Platelets (10^3/μL)						
Day 3	896.1 ± 37.5	826.5 ± 30.3	795.2 ± 24.9	841.3 ± 39.1	827.5 ± 23.1	815.1 ± 23.8
Day 22	766.3 ± 20.5	775.1 ± 14.2	778.5 ± 18.2	761.6 ± 39.1	804.2 ± 15.3	788.0 ± 26.9
Week 13	759.8 ± 17.2	720.2 ± 26.6	708.4 ± 22.6	730.5 ± 27.5	777.2 ± 22.2	—
Leukocytes (10^3/μL)						
Day 3	10.85 ± 0.23	10.06 ± 0.38	9.87 ± 0.34	10.24 ± 0.47	10.82 ± 0.27	12.35 ± 0.35
Day 22	9.39 ± 0.24	8.76 ± 0.61	9.88 ± 0.67	10.80 ± 0.83	19.94 ± 1.54**	24.65 ± 1.77**
Week 13	8.26 ± 0.60	7.88 ± 0.45	7.20 ± 0.59	9.19 ± 0.93	9.63 ± 0.52	—
Segmented neutrophils (10^3/μL)						
Day 3	1.05 ± 0.13	0.97 ± 0.08	1.16 ± 0.11	0.99 ± 0.13	1.74 ± 0.21*	2.47 ± 0.18**
Day 22	0.96 ± 0.09	1.24 ± 0.14	1.15 ± 0.12	2.14 ± 0.31**	9.71 ± 1.00**	14.24 ± 1.24**
Week 13	1.18 ± 0.10	1.14 ± 0.18	1.02 ± 0.16	1.87 ± 0.65	1.80 ± 0.25	—

Hematology and Clinical Chemistry Data for F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
n	10	10	10	10	10	10
Male (continued)						
Hematology (continued)						
Bands ($10^3/\mu L$)						
Day 3	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.05 ± 0.02
Day 22	0.00 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.13 ± 0.05**	0.73 ± 0.12**	0.75 ± 0.06**
Week 13	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.01	0.02 ± 0.02	—
Lymphocytes ($10^3/\mu L$)						
Day 3	9.53 ± 0.16	8.93 ± 0.37	8.38 ± 0.30	9.05 ± 0.45	8.81 ± 0.24	9.50 ± 0.42
Day 22	8.16 ± 0.25	7.20 ± 0.51	8.41 ± 0.65	8.24 ± 0.65	8.85 ± 0.66	8.78 ± 1.15
Week 13	6.80 ± 0.57	6.39 ± 0.30	5.90 ± 0.47	7.13 ± 0.51	7.49 ± 0.33	—
Atypical lymphocytes ($10^3/\mu L$)						
Day 3	0.02 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.02	0.00 ± 0.00	0.01 ± 0.01
Day 22	0.01 ± 0.01[c]	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.03 ± 0.03
Week 13	0.03 ± 0.01	0.04 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.02	—
Monocytes ($10^3/\mu L$)						
Day 3	0.22 ± 0.04	0.12 ± 0.03	0.25 ± 0.04	0.16 ± 0.06	0.23 ± 0.05	0.29 ± 0.06
Day 22	0.22 ± 0.03	0.24 ± 0.04	0.24 ± 0.04	0.22 ± 0.05	0.58 ± 0.21	0.62 ± 0.11**
Week 13	0.19 ± 0.04	0.22 ± 0.07	0.15 ± 0.06	0.16 ± 0.04	0.19 ± 0.05	—
Eosinophils ($10^3/\mu L$)						
Day 3	0.03 ± 0.02	0.03 ± 0.02	0.06 ± 0.02	0.02 ± 0.01	0.03 ± 0.02	0.04 ± 0.02
Day 22	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.02	0.06 ± 0.03	0.07 ± 0.04	0.24 ± 0.06**
Week 13	0.06 ± 0.02	0.08 ± 0.03	0.11 ± 0.04	0.02 ± 0.02	0.08 ± 0.04	—
Clinical Chemistry						
Urea nitrogen (mg/dL)						
Day 3	23.9 ± 0.9	22.9 ± 0.6	22.8 ± 0.6	23.7 ± 0.7	22.1 ± 0.4	21.4 ± 0.7
Day 22	23.2 ± 0.6	22.9 ± 0.7	22.8 ± 0.6	23.4 ± 0.5	21.4 ± 0.5	21.1 ± 0.7
Week 13	19.9 ± 0.5	19.1 ± 0.3	19.7 ± 0.4	18.8 ± 0.9	19.8 ± 0.3	—
Creatinine (mg/dL)						
Day 3	0.26 ± 0.02	0.27 ± 0.02	0.24 ± 0.02	0.28 ± 0.02	0.23 ± 0.02	0.23 ± 0.02
Day 22	0.33 ± 0.02	0.28 ± 0.01	0.28 ± 0.01	0.26 ± 0.02*	0.30 ± 0.02	0.28 ± 0.01
Week 13	0.36 ± 0.02	0.38 ± 0.02	0.35 ± 0.02	0.35 ± 0.02	0.36 ± 0.02	—
Total protein (g/dL)						
Day 3	5.7 ± 0.1	5.7 ± 0.1	5.8 ± 0.1	5.8 ± 0.1	5.6 ± 0.0	5.6 ± 0.1
Day 22	6.2 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	5.8 ± 0.1**
Week 13	6.9 ± 0.1	6.9 ± 0.1	7.1 ± 0.1	6.6 ± 0.3	6.8 ± 0.1	—
Albumin (g/dL)						
Day 3	4.6 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.0	4.6 ± 0.0	4.5 ± 0.0
Day 22	4.8 ± 0.0	4.8 ± 0.0	4.7 ± 0.1	4.6 ± 0.1*	4.3 ± 0.0**	4.0 ± 0.1**
Week 13	5.0 ± 0.1	4.9 ± 0.1	5.0 ± 0.0	4.5 ± 0.2*	4.7 ± 0.1**	—
Alanine aminotransferase (IU/L)						
Day 3	51 ± 1	49 ± 1	53 ± 1	53 ± 1	61 ± 2**	76 ± 3**
Day 22	53 ± 2	54 ± 1	52 ± 2	62 ± 1**	68 ± 3**	77 ± 4**
Week 13	57 ± 3	60 ± 2	63 ± 3	63 ± 5	68 ± 4	—
Alkaline phosphatase (IU/L)						
Day 3	762 ± 26	752 ± 20	761 ± 22	767 ± 11	736 ± 16	728 ± 12
Day 22	520 ± 9	532 ± 10	501 ± 13	527 ± 11	462 ± 12**	442 ± 15**
Week 13	262 ± 5	276 ± 12	275 ± 3	259 ± 20	290 ± 6*	—

TABLE F1
Hematology and Clinical Chemistry Data for F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
n	10	10	10	10	10	10

Male (continued)

Clinical chemistry (continued)

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Creatine kinase (IU/L)						
Day 3	581 ± 54	665 ± 63	565 ± 82	602 ± 42	570 ± 56	582 ± 66
Day 22	535 ± 76	476 ± 105	504 ± 95	284 ± 25*[c]	347 ± 49	360 ± 42
Week 13	304 ± 49	192 ± 32	268 ± 55	236 ± 46	265 ± 38	—
Sorbitol dehydrogenase (IU/L)						
Day 3	21 ± 1	20 ± 2	23 ± 2	23 ± 1	20 ± 2	22 ± 2
Day 22	24 ± 2	28 ± 2	22 ± 2	31 ± 1	26 ± 2	24 ± 2
Week 13	22 ± 1	22 ± 2	24 ± 1	22 ± 1	20 ± 1	—
Bile acids (µmol/L)						
Day 3	25.0 ± 3.2	23.2 ± 3.1	31.8 ± 6.0	23.4 ± 2.6	37.0 ± 6.3	45.2 ± 5.3
Day 22	26.9 ± 3.4	22.8 ± 2.0	26.6 ± 2.8	25.9 ± 2.5	26.2 ± 4.3	20.3 ± 1.8
Week 13	22.0 ± 2.9	15.8 ± 1.7	17.2 ± 0.9	18.4 ± 1.2	20.2 ± 2.3	—

Female

Hematology

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Automated hematocrit (%)						
Day 3	42.8 ± 0.6	43.2 ± 0.4	43.3 ± 0.4	43.3 ± 0.5	43.7 ± 0.3	43.3 ± 0.5
Day 22	46.7 ± 0.4	46.9 ± 0.3	47.2 ± 0.3	47.1 ± 0.8	45.5 ± 0.3	46.1 ± 0.5
Week 13	45.6 ± 0.3	44.6 ± 0.6	45.2 ± 0.4	43.3 ± 0.4**	43.0 ± 0.5**	—
Manual hematocrit (%)						
Day 3	45.6 ± 0.9	45.3 ± 0.5	46.5 ± 0.5	46.5 ± 0.7	46.3 ± 0.6	46.0 ± 0.7
Day 22	48.5 ± 0.4	49.3 ± 0.4	49.3 ± 0.5	49.1 ± 1.0	48.3 ± 0.4	48.7 ± 0.4
Week 13	46.7 ± 0.3	45.9 ± 0.5	46.8 ± 0.2	45.7 ± 0.4	45.1 ± 0.5*	—
Hemoglobin (g/dL)						
Day 3	15.2 ± 0.2	15.3 ± 0.1	15.3 ± 0.1	15.3 ± 0.1	15.5 ± 0.1	15.3 ± 0.2
Day 22	16.2 ± 0.2	16.5 ± 0.1	16.4 ± 0.1	16.3 ± 0.3	15.9 ± 0.1	15.7 ± 0.1*
Week 13	15.8 ± 0.1	15.6 ± 0.2	15.8 ± 0.1	15.3 ± 0.2**	15.1 ± 0.2**	—
Erythrocytes (10^6/µL)						
Day 3	6.94 ± 0.09	7.02 ± 0.06	7.03 ± 0.07	7.03 ± 0.08	7.09 ± 0.07	7.01 ± 0.09
Day 22	7.60 ± 0.06	7.64 ± 0.05	7.66 ± 0.06	7.69 ± 0.13	7.52 ± 0.05	7.67 ± 0.12
Week 13	8.08 ± 0.06	7.92 ± 0.10	8.03 ± 0.06	7.68 ± 0.07**	7.74 ± 0.09**	—
Reticulocytes (10^6/µL)						
Day 3	0.25 ± 0.03	0.33 ± 0.03	0.29 ± 0.03	0.29 ± 0.03	0.32 ± 0.03	0.24 ± 0.03
Day 22	0.20 ± 0.02	0.13 ± 0.01**	0.12 ± 0.01**	0.14 ± 0.01	0.17 ± 0.02	0.17 ± 0.02
Week 13	0.16 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	—
Nucleated erythrocytes (10^3/µL)						
Day 3	0.70 ± 0.21	0.60 ± 0.22	0.60 ± 0.22	0.20 ± 0.13	0.70 ± 0.26	0.80 ± 0.13
Day 22	0.70 ± 0.21	0.70 ± 0.30	0.40 ± 0.16	0.50 ± 0.17	0.40 ± 0.22	0.10 ± 0.10
Week 13	1.10 ± 0.28	0.70 ± 0.34	0.70 ± 0.34	0.80 ± 0.36	0.10 ± 0.10*	—
Mean cell volume (fL)						
Day 3	61.6 ± 0.2	61.5 ± 0.1	61.6 ± 0.2	61.6 ± 0.2	61.6 ± 0.2	61.8 ± 0.3
Day 22	61.4 ± 0.3	61.4 ± 0.2	61.7 ± 0.2	61.2 ± 0.2	60.6 ± 0.2*	60.1 ± 0.3**
Week 13	56.3 ± 0.1	56.3 ± 0.1	56.3 ± 0.1	56.4 ± 0.1	55.6 ± 0.2*	—

Hematology and Clinical Chemistry Data for F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
n	10	10	10	10	10	10

Female (continued)

Hematology (continued)

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
Mean cell hemoglobin (pg)						
Day 3	21.9 ± 0.1	21.7 ± 0.1	21.8 ± 0.1	21.7 ± 0.1	21.8 ± 0.1	21.9 ±0.1
Day 22	21.3 ± 0.1	21.6 ± 0.1	21.5 ± 0.1	21.2 ± 0.1	21.1 ± 0.1	20.5 ± 0.2**
Week 13	19.6 ± 0.1	19.7 ± 0.1	19.7 ± 0.1	19.9 ± 0.1	19.5 ± 0.1	—
Mean cell hemoglobin concentration (g/dL)						
Day 3	35.5 ± 0.1	35.3 ± 0.2	35.4 ± 0.1	35.3 ± 0.1	35.4 ± 0.2	35.4 ± 0.2
Day 22	34.8 ± 0.2	35.2 ± 0.1	34.8 ± 0.1	34.7 ± 0.2	34.8 ± 0.2	34.2 ± 0.2
Week 13	34.8 ± 0.2	35.0 ± 0.2	35.0 ± 0.2	35.3 ± 0.2	35.1 ± 0.2	—
Platelets (10^3/µL)						
Day 3	785.9 ± 18.3	797.6 ± 18.7	788.3 ± 21.5	781.4 ± 26.3	833.7 ± 32.6	808.1 ± 19.5
Day 22	731.2 ± 21.5	786.7 ± 19.0	747.8 ± 22.5	673.8 ± 36.3	701.1 ± 16.4	707.3 ± 21.4
Week 13	649.0 ± 25.6	653.7 ± 14.2	631.7 ± 29.4	710.4 ± 26.7*	718.9 ± 25.1*	—
Leukocytes (10^3/µL)						
Day 3	10.79 ± 0.49	11.49 ± 0.33	11.33 ± 0.41	11.44 ± 0.34	11.78 ± 0.46	13.31 ± 0.62**
Day 22	7.62 ± 0.18	8.44 ± 0.67	8.07 ± 0.47	8.61 ± 0.75	14.50 ± 0.82**	20.13 ± 1.22**
Week 13	6.60 ± 0.31	6.37 ± 0.82	7.63 ± 0.73	6.74 ± 0.61	9.79 ± 1.18	—
Segmented neutrophils (10^3/µL)						
Day 3	0.90 ± 0.11	1.34 ± 0.11*	1.11 ± 0.15	1.21 ± 0.16	1.77 ± 0.25**	3.21 ± 0.30**
Day 22	1.12 ± 0.12	1.05 ± 0.16	1.26 ± 0.19	1.50 ± 0.27	6.06 ± 0.62**	9.75 ± 0.86**
Week 13	1.07 ± 0.15	0.91 ± 0.18	1.08 ± 0.22	1.17 ± 0.21	2.37 ± 0.46*	—
Bands (10^3/µL)						
Day 3	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.02	0.01 ± 0.01
Day 22	0.03 ± 0.01	0.03 ± 0.02	0.00 ± 0.00	0.05 ± 0.02	0.16 ± 0.07	0.19 ± 0.06
Week 13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	—
Lymphocytes (10^3/µL)						
Day 3	9.72 ± 0.45	9.92 ± 0.31	9.87 ± 0.36	9.99 ± 0.31	9.70 ± 0.45	9.71 ± 0.50
Day 22	6.33 ± 0.21	7.14 ± 0.58	6.64 ± 0.38	6.68 ± 0.52	7.80 ± 0.24**	9.59 ± 0.70**
Week 13	5.29 ± 0.26	5.18 ± 0.62	6.23 ± 0.61	5.39 ± 0.56	7.07 ± 0.81	—
Atypical lymphocytes (10^3/µL)						
Day 3	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.02
Day 22	0.02 ± 0.01	0.04 ± 0.01	0.01 ± 0.01	0.08 ± 0.03	0.08 ± 0.04	0.06 ± 0.03
Week 13	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	—
Monocytes (10^3/µL)						
Day 3	0.14 ± 0.03	0.18 ± 0.06	0.26 ± 0.05	0.21 ± 0.04	0.25 ± 0.05	0.26 ± 0.06
Day 22	0.09 ± 0.02	0.08 ± 0.02	0.11 ± 0.03	0.15 ± 0.04	0.25 ± 0.06*	0.31 ± 0.10
Week 13	0.20 ± 0.04	0.23 ± 0.08	0.28 ± 0.04	0.15 ± 0.03	0.25 ± 0.08	—
Eosinophils (10^3/µL)						
Day 3	0.02 ± 0.02	0.03 ± 0.02	0.08 ± 0.03	0.04 ± 0.03	0.04 ± 0.02	0.10 ± 0.04
Day 22	0.03 ± 0.02	0.10 ± 0.02*	0.05 ± 0.03	0.15 ± 0.05**	0.16 ± 0.05*	0.23 ± 0.07*
Week 13	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.02 ± 0.01	0.09 ± 0.02	—
Clinical Chemistry						
Urea nitrogen (mg/dL)						
Day 3	24.6 ± 0.9	23.1 ± 0.5	24.4 ± 0.6	23.5 ± 1.0	24.2 ± 0.7	24.1 ± 0.7
Day 22	26.5 ± 0.8	25.9 ± 0.6	25.5 ± 0.6	25.3 ± 0.8	24.4 ± 0.6*	24.3 ± 0.6
Week 13	23.4 ± 0.7	23.0 ± 0.5	23.1 ± 0.7	22.1 ± 0.7	22.0 ± 0.4	—

Hematology and Clinical Chemistry Data for F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
n	10	10	10	10	10	10

Female (continued**)**

Clinical chemistry (continued)

Creatinine (mg/dL)						
Day 3	0.25 ± 0.02	0.24 ± 0.02	0.28 ± 0.01	0.28 ± 0.01	0.30 ± 0.00*	0.29 ± 0.02*
Day 22	0.27 ± 0.02	0.26 ± 0.02	0.28 ± 0.01	0.24 ± 0.02	0.29 ± 0.01	0.22 ± 0.01
Week 13	0.32 ± 0.01	0.30 ± 0.00	0.32 ± 0.01	0.31 ± 0.01	0.30 ± 0.00	—
Total protein (g/dL)						
Day 3	5.7 ± 0.1	5.7 ± 0.1	5.7 ± 0.1	5.7 ± 0.1	5.8 ± 0.1	5.8 ± 0.1
Day 22	5.8 ± 0.1	5.8 ± 0.1	5.8 ± 0.1	5.8 ± 0.1	5.6 ± 0.1	5.5 ± 0.1
Week 13	7.0 ± 0.1	6.6 ± 0.1	6.7 ± 0.1	6.8 ± 0.1	6.9 ± 0.1	—
Albumin (g/dL)						
Day 3	4.8 ± 0.0	4.8 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.1
Day 22	4.7 ± 0.0	4.7 ± 0.1	4.6 ± 0.0	4.7 ± 0.1	4.1 ± 0.1**	3.8 ± 0.1**
Week 13	4.8 ± 0.1	4.8 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.5 ± 0.1**	—
Alanine aminotransferase (IU/L)						
Day 3	46 ± 1	45 ± 1	47 ± 1	44 ± 1	56 ± 2**	67 ± 4**
Day 22	42 ± 1	44 ± 2	47 ± 2*	49 ± 2*	56 ± 2**	62 ± 2**
Week 13	55 ± 4	59 ± 4	63 ± 4	59 ± 3	64 ± 6	—
Alkaline phosphatase (IU/L)						
Day 3	643 ± 17	649 ± 9	633 ± 11	628 ± 9	638 ± 15	571 ± 53
Day 22	483 ± 13	489 ± 9	446 ± 8*	473 ± 15	404 ± 12**	384 ± 22**
Week 13	301 ± 8	292 ± 8	311 ± 8	293 ± 4	311 ± 13	—
Creatine kinase (IU/L)						
Day 3	343 ± 51	352 ± 35	332 ± 46	288 ± 42	309 ± 32	351 ± 38
Day 22	361 ± 57	261 ± 17	476 ± 85	284 ± 27	395 ± 46	337 ± 26
Week 13	294 ± 37	256 ± 35	318 ± 61	245 ± 36	223 ± 30	—
Sorbitol dehydrogenase (IU/L)						
Day 3	22 ± 1	23 ± 1	21 ± 1	22 ± 1	22 ± 1	22 ± 1
Day 22	24 ± 1	26 ± 1	24 ± 1	23 ± 1	22 ± 1	19 ± 1**
Week 13	25 ± 2	27 ± 1	26 ± 1	26 ± 1	24 ± 1	—
Bile acids (μmol/L)						
Day 3	15.2 ± 2.4	25.1 ± 5.5	20.3 ± 5.6	18.7 ± 3.3	26.1 ± 5.4	20.0 ± 4.0
Day 22	15.5 ± 1.2	19.3 ± 2.5	24.2 ± 2.7	17.6 ± 2.4	22.0 ± 3.4	23.3 ± 8.1
Week 13	18.1 ± 1.8	15.6 ± 1.4	23.8 ± 5.4	17.8 ± 2.9	19.0 ± 2.4	—

*   Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test
** P≤0.01
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] No data available due to 100% mortality
[c] n=9

TABLE F2
Hematology Data for B6C3F$_1$ Mice in the 13-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
n	10	10	10	10	10
Male					
Automated hematocrit (%)	52.9 ± 0.7	52.0 ± 0.7	51.1 ± 0.8	50.0 ± 0.6*	47.8 ± 0.6**
Manual hematocrit (%)	53.0 ± 0.6	51.4 ± 0.5*	50.4 ± 0.6**	50.2 ± 0.6**	48.3 ± 0.7**
Hemoglobin (g/dL)	16.9 ± 0.1	16.6 ± 0.2	16.4 ± 0.2*	16.1 ± 0.1**	15.3 ± 0.2**
Erythrocytes ($10^6/\mu$L)	10.39 ± 0.13	10.23 ± 0.15	10.03 ± 0.15	9.89 ± 0.15	9.44 ± 0.11**
Reticulocytes ($10^6/\mu$L)	0.20 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.18 ± 0.02
Nucleated erythrocytes ($10^3/\mu$L)	0.10 ± 0.10	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	51.0 ± 0.2	50.9 ± 0.2	50.9 ± 0.2	50.6 ± 0.3	50.6 ± 0.2
Mean cell hemoglobin (pg)	16.3 ± 0.2	16.3 ± 0.1	16.3 ± 0.1	16.3 ± 0.1	16.2 ± 0.1
Mean cell hemoglobin concentration (g/dL)	31.9 ± 0.3	32.0 ± 0.2	32.1 ± 0.2	32.2 ± 0.2	32.0 ± 0.2
Platelets ($10^3/\mu$L)	637.2 ± 25.7	708.9 ± 36.1	664.6 ± 26.3	737.2 ± 15.0*	830.8 ± 25.3**
Leukocytes ($10^3/\mu$L)	4.73 ± 0.45	5.98 ± 0.64	4.99 ± 0.39	6.11 ± 0.69	7.80 ± 0.63**
Segmented neutrophils ($10^3/\mu$L)	0.41 ± 0.05	0.88 ± 0.25	0.52 ± 0.10	0.74 ± 0.17	1.27 ± 0.22**
Bands ($10^3/\mu$L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.01*
Lymphocytes ($10^3/\mu$L)	4.24 ± 0.41	4.99 ± 0.46	4.38 ± 0.32	5.22 ± 0.55	6.34 ± 0.51**
Monocytes ($10^3/\mu$L)	0.05 ± 0.01	0.08 ± 0.02	0.06 ± 0.02	0.11 ± 0.03	0.16 ± 0.05
Eosinophils ($10^3/\mu$L)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.01
Female					
Automated hematocrit (%)	50.9 ± 0.7	50.8 ± 0.6	50.7 ± 0.6	48.7 ± 0.8	48.1 ± 0.6*
Manual hematocrit (%)	51.1 ± 0.7	51.2 ± 0.4	51.4 ± 0.6	49.0 ± 0.7	49.0 ± 0.4
Hemoglobin (g/dL)	16.3 ± 0.2	16.3 ± 0.1	16.3 ± 0.1	15.8 ± 0.2	15.7 ± 0.1*
Erythrocytes ($10^6/\mu$L)	9.96 ± 0.14	9.92 ± 0.12	9.93 ± 0.12	9.55 ± 0.17	9.40 ± 0.11*
Reticulocytes ($10^6/\mu$L)	0.19 ± 0.01	0.25 ± 0.02	0.20 ± 0.02	0.24 ± 0.02	0.20 ± 0.02
Nucleated erythrocytes ($10^3/\mu$L)	0.10 ± 0.10	0.00 ± 0.00	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	51.1 ± 0.1	51.3 ± 0.1	51.1 ± 0.2	51.0 ± 0.1	51.2 ± 0.2
Mean cell hemoglobin (pg)	16.4 ± 0.1	16.4 ± 0.1	16.4 ± 0.1	16.5 ± 0.1	16.7 ± 0.1
Mean cell hemoglobin concentration (g/dL)	32.0 ± 0.2	32.1 ± 0.2	32.1 ± 0.1	32.4 ± 0.2	32.6 ± 0.2
Platelets ($10^3/\mu$L)	607.9 ± 34.2	637.2 ± 25.6	616.5 ± 31.1	686.9 ± 36.9	751.2 ± 39.4*
Leukocytes ($10^3/\mu$L)	4.05 ± 0.39	4.16 ± 0.26	4.38 ± 0.28	4.42 ± 0.25	5.76 ± 0.45**
Segmented neutrophils ($10^3/\mu$L)	0.36 ± 0.03	0.43 ± 0.05	0.35 ± 0.04	0.50 ± 0.08	1.05 ± 0.23**
Bands ($10^3/\mu$L)	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.01
Lymphocytes ($10^3/\mu$L)	3.59 ± 0.36	3.64 ± 0.26	3.92 ± 0.27	3.80 ± 0.25	4.52 ± 0.26
Atypical lymphocytes ($10^3/\mu$L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.01
Monocytes ($10^3/\mu$L)	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.11 ± 0.02
Eosinophils ($10^3/\mu$L)	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	0.05 ± 0.01

* Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test
** Significantly different (P≤0.01) from the vehicle control group by Shirley's test
[a] Mean ± standard error. Statistical tests were performed on unrounded data. No data available for the 24 mg/kg groups due to 100% mortality.

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

TABLE G1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 3-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.6 mg/animal	1.8 mg/animal	5.1 mg/animal
n	5	5	5	1
Male				
Necropsy body wt	258 ± 9	261 ± 8	254 ± 9	191
Adrenal gland				
Absolute	0.052 ± 0.002	0.047 ± 0.002	0.052 ± 0.004	0.071
Relative	0.203 ± 0.009	0.183 ± 0.011	0.206 ± 0.016	0.372
Heart				
Absolute	0.818 ± 0.018	0.827 ± 0.025	0.858 ± 0.036	0.803
Relative	3.176 ± 0.067	3.170 ± 0.052	3.373 ± 0.059	4.204
R. Kidney				
Absolute	1.132 ± 0.035	1.189 ± 0.034	1.210 ± 0.045	1.013
Relative	4.389 ± 0.047	4.559 ± 0.034	4.765 ± 0.109**	5.304
Liver				
Absolute	13.625 ± 0.542	13.584 ± 0.527	14.335 ± 0.461	
Relative	52.792 ± 0.905	52.032 ± 0.938	56.560 ± 1.818	
Lung				
Absolute	1.239 ± 0.047	1.361 ± 0.039	1.298 ± 0.061	
Relative	4.801 ± 0.086	5.229 ± 0.171	5.112 ± 0.195	
R. Testis				
Absolute	1.374 ± 0.021	1.330 ± 0.032	1.349 ± 0.038	
Relative	5.339 ± 0.108	5.101 ± 0.060	5.316 ± 0.060	
Thymus				
Absolute	0.463 ± 0.032	0.482 ± 0.042	0.404 ± 0.024	
Relative	1.787 ± 0.067	1.845 ± 0.141	1.596 ± 0.103	
Female				
Necropsy body wt	165 ± 3	164 ± 8	155 ± 5	
Adrenal gland				
Absolute	0.054 ± 0.001	0.055 ± 0.002	0.056 ± 0.006	
Relative	0.327 ± 0.012	0.335 ± 0.018	0.365 ± 0.045	
Heart				
Absolute	0.614 ± 0.018	0.608 ± 0.032	0.613 ± 0.019	
Relative	3.721 ± 0.097	3.700 ± 0.072	3.967 ± 0.087	
R. Kidney				
Absolute	0.748 ± 0.020	0.763 ± 0.025	0.751 ± 0.014	
Relative	4.533 ± 0.080	4.668 ± 0.131	4.876 ± 0.174	
Liver				
Absolute	7.962 ± 0.131	7.346 ± 0.198	7.495 ± 0.199	
Relative	48.313 ± 1.002	44.962 ± 1.225	48.528 ± 0.999	
Lung				
Absolute	1.027 ± 0.021	1.084 ± 0.076	0.989 ± 0.020	
Relative	6.232 ± 0.174	6.659 ± 0.539	6.427 ± 0.269	
Thymus				
Absolute	0.378 ± 0.017	0.373 ± 0.032	0.334 ± 0.015	
Relative	2.290 ± 0.090	2.258 ± 0.107	2.171 ± 0.124	

** Significantly different ($P \leq 0.01$) from the vehicle control group by Williams' test
[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); no data available for the 5.1 (female), 15, and 45 mg/animal groups due to 100% mortality.

TABLE G2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 13-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3mg/kg	6 mg/kg
n	10	10	10	10	10
Male					
Necropsy body wt	330 ± 5	337 ± 6	334 ± 6	325 ± 7	300 ± 4**
Heart					
Absolute	1.008 ± 0.021	1.068 ± 0.013	0.995 ± 0.019	1.076 ± 0.034	0.998 ± 0.025
Relative	3.059 ± 0.057	3.179 ± 0.047	2.983 ± 0.036	3.308 ± 0.067**	3.330 ± 0.060**
R. Kidney					
Absolute	1.247 ± 0.012	1.302 ± 0.022	1.277 ± 0.026	1.296 ± 0.025	1.203 ± 0.018
Relative	3.785 ± 0.044	3.873 ± 0.043	3.828 ± 0.049	3.991 ± 0.034**	4.016 ± 0.039**
Liver					
Absolute	12.450 ± 0.286	13.334 ± 0.278	13.146 ± 0.404	12.937 ± 0.336	11.713 ± 0.209
Relative	37.761 ± 0.720	39.631 ± 0.396	39.347 ± 0.719	39.777 ± 0.421	39.104 ± 0.564
Lung					
Absolute	1.784 ± 0.126	1.925 ± 0.104	1.819 ± 0.106	1.747 ± 0.076	1.711 ± 0.099
Relative	5.412 ± 0.378	5.717 ± 0.288	5.452 ± 0.303	5.362 ± 0.167	5.702 ± 0.309
R. Testis					
Absolute	1.411 ± 0.011	1.441 ± 0.022	1.444 ± 0.029	1.447 ± 0.028	1.386 ± 0.020
Relative	4.290 ± 0.087	4.291 ± 0.072	4.328 ± 0.055	4.457 ± 0.054	4.630 ± 0.062**
Thymus					
Absolute	0.388 ± 0.035	0.369 ± 0.021	0.331 ± 0.012	0.311 ± 0.015*	0.314 ± 0.024*
Relative	1.179 ± 0.111	1.099 ± 0.068	0.997 ± 0.047	0.954 ± 0.033	1.046 ± 0.079
Female					
Necropsy body wt	186 ± 3	191 ± 3	190 ± 4	193 ± 3	192 ± 4
Heart					
Absolute	0.729 ± 0.015	0.734 ± 0.013	0.755 ± 0.018	0.790 ± 0.017**	0.786 ± 0.008**
Relative	3.927 ± 0.066	3.849 ± 0.044	3.963 ± 0.030	4.090 ± 0.034*	4.101 ± 0.060*
R. Kidney					
Absolute	0.747 ± 0.011	0.747 ± 0.015	0.791 ± 0.022	0.807 ± 0.019*	0.816 ± 0.011**
Relative	4.023 ± 0.043	3.922 ± 0.074	4.152 ± 0.059	4.177 ± 0.058	4.254 ± 0.041**
Liver					
Absolute	6.845 ± 0.140	6.904 ± 0.198	7.138 ± 0.253	7.469 ± 0.213*	7.479 ± 0.168*
Relative	36.878 ± 0.775	36.162 ± 0.683	37.391 ± 0.606	38.633 ± 0.581	38.943 ± 0.476*
Lung					
Absolute	1.164 ± 0.051	1.166 ± 0.062	1.267 ± 0.063	1.270 ± 0.069	1.255 ± 0.060
Relative	6.287 ± 0.321	6.101 ± 0.275	6.640 ± 0.256	6.571 ± 0.324	6.540 ± 0.311
Thymus					
Absolute	0.260 ± 0.014	0.260 ± 0.017	0.285 ± 0.012	0.248 ± 0.014	0.259 ± 0.013
Relative	1.403 ± 0.082	1.364 ± 0.082	1.495 ± 0.054	1.293 ± 0.085	1.349 ± 0.061

* Significantly different (P≤0.05) from the vehicle control group by Williams' test
** P≤0.01
[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); no data available for the 12 mg/kg groups due to 100% mortality.

TABLE G3

Organ Weights and Organ-Weight-to-Body-Weight Ratios for B6C3F₁ Mice in the 3-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.2 mg/animal	0.6 mg/animal
Male			
n	5	5	5
Necropsy body wt	28.8 ± 1.1	28.3 ± 0.5	26.0 ± 0.5*
Adrenal gland			
Absolute	0.010 ± 0.001	0.011 ± 0.001	0.024 ± 0.015
Relative	0.348 ± 0.032	0.402 ± 0.026	0.959 ± 0.590
Heart			
Absolute	0.141 ± 0.004	0.144 ± 0.005	0.161 ± 0.004**
Relative	4.912 ± 0.108	5.073 ± 0.127	6.194 ± 0.139**
R. Kidney			
Absolute	0.299 ± 0.010	0.291 ± 0.008	0.269 ± 0.005*
Relative	10.405 ± 0.291	10.291 ± 0.217	10.371 ± 0.275
Liver			
Absolute	1.586 ± 0.048	1.668 ± 0.058	1.599 ± 0.040
Relative	55.203 ± 0.835	58.891 ± 1.104*	61.584 ± 1.327**
Lung			
Absolute	0.177 ± 0.006	0.191 ± 0.011	0.185 ± 0.005
Relative	6.163 ± 0.268	6.749 ± 0.320	7.126 ± 0.252
R. Testis			
Absolute	0.125 ± 0.007	0.112 ± 0.005	0.109 ± 0.003
Relative	4.343 ± 0.264	3.962 ± 0.148	4.187 ± 0.148
Thymus			
Absolute	0.050 ± 0.004	0.047 ± 0.003	0.028 ± 0.004**
Relative	1.760 ± 0.173	1.649 ± 0.119	1.073 ± 0.162*
Female			
n	5	5	4
Necropsy body wt	24.0 ± 0.5	23.9 ± 0.4	21.3 ± 0.8**
Adrenal gland			
Absolute	0.014 ± 0.001	0.013 ± 0.002	0.011 ± 0.001
Relative	0.576 ± 0.041	0.532 ± 0.060	0.526 ± 0.038
Heart			
Absolute	0.132 ± 0.001	0.126 ± 0.003	0.148 ± 0.007*
Relative	5.491 ± 0.094	5.265 ± 0.126	6.957 ± 0.212**
R. Kidney			
Absolute	0.213 ± 0.010	0.215 ± 0.006	0.209 ± 0.013
Relative	8.880 ± 0.287	9.004 ± 0.281	9.790 ± 0.273
Liver			
Absolute	1.408 ± 0.040	1.378 ± 0.034	1.370 ± 0.098
Relative	58.649 ± 0.708	57.585 ± 1.229	64.180 ± 2.380*
Lung			
Absolute	0.188 ± 0.006	0.194 ± 0.010	0.171 ± 0.009
Relative	7.844 ± 0.351	8.132 ± 0.452	8.048 ± 0.531
Thymus			
Absolute	0.070 ± 0.003	0.080 ± 0.009	0.024 ± 0.005**
Relative	2.906 ± 0.136	3.356 ± 0.371	1.099 ± 0.205**

*   Significantly different (P≤0.05) from the vehicle control group by Williams' or Dunnett's test
** P≤0.01
[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); no data available for the 1.7, 5, and 15 mg/animal groups due to 100% mortality.

TABLE **G4**

Organ Weights and Organ-Weight-to-Body-Weight Ratios for B6C3F$_1$ Mice in the 13-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
n	10	10	10	10	10
Male					
Necropsy body wt	33.7 ± 0.6	33.1 ± 0.7	33.0 ± 0.5	31.5 ± 0.5**	30.4 ± 0.5**
Heart					
Absolute	0.154 ± 0.004	0.151 ± 0.006	0.161 ± 0.005	0.152 ± 0.003	0.158 ± 0.004
Relative	4.577 ± 0.065	4.563 ± 0.135	4.884 ± 0.141	4.840 ± 0.091	5.191 ± 0.075**
R. Kidney					
Absolute	0.308 ± 0.005	0.322 ± 0.005	0.318 ± 0.010	0.281 ± 0.017	0.287 ± 0.010
Relative	9.179 ± 0.193	9.788 ± 0.246	9.647 ± 0.280	8.937 ± 0.524	9.423 ± 0.234
Liver					
Absolute	1.578 ± 0.037	1.595 ± 0.061	1.645 ± 0.051	1.450 ± 0.034	1.566 ± 0.044
Relative	46.954 ± 1.032	48.179 ± 1.401	49.919 ± 1.417	46.055 ± 0.435	51.567 ± 1.115*
Lung					
Absolute	0.311 ± 0.013	0.290 ± 0.014	0.264 ± 0.020*	0.241 ± 0.012**	0.206 ± 0.010**
Relative	9.239 ± 0.361	8.754 ± 0.376	8.024 ± 0.615	7.677 ± 0.418*	6.759 ± 0.269**
R. Testis					
Absolute	0.121 ± 0.003	0.116 ± 0.003	0.124 ± 0.003	0.107 ± 0.010	0.117 ± 0.003
Relative	3.606 ± 0.087	3.510 ± 0.081	3.780 ± 0.097	3.417 ± 0.333	3.858 ± 0.080
Thymus					
Absolute	0.043 ± 0.003	0.046 ± 0.003	0.043 ± 0.004	0.040 ± 0.002	0.040 ± 0.003
Relative	1.289 ± 0.096	1.375 ± 0.085	1.286 ± 0.126	1.261 ± 0.083	1.321 ± 0.091
Female					
Necropsy body wt	28.8 ± 0.9	28.3 ± 1.1	27.8 ± 0.5	27.7 ± 0.3	26.5 ± 0.4
Heart					
Absolute	0.135 ± 0.003	0.134 ± 0.004	0.135 ± 0.002	0.149 ± 0.004**	0.148 ± 0.003**
Relative	4.712 ± 0.126	4.745 ± 0.081	4.869 ± 0.079	5.391 ± 0.108**	5.591 ± 0.106**
R. Kidney					
Absolute	0.205 ± 0.004	0.202 ± 0.006	0.198 ± 0.004	0.204 ± 0.005	0.212 ± 0.004
Relative	7.158 ± 0.143	7.177 ± 0.176	7.115 ± 0.159	7.383 ± 0.108	8.026 ± 0.140**
Liver					
Absolute	1.291 ± 0.015	1.366 ± 0.047	1.339 ± 0.033	1.380 ± 0.023	1.416 ± 0.027**
Relative	45.012 ± 0.904	48.533 ± 1.516*	48.143 ± 0.813*	49.919 ± 0.739**	53.470 ± 0.612**
Lung					
Absolute	0.267 ± 0.018	0.268 ± 0.018	0.267 ± 0.012	0.279 ± 0.023	0.227 ± 0.017
Relative	9.245 ± 0.474	9.422 ± 0.483	9.584 ± 0.392	10.077 ± 0.798	8.514 ± 0.488
Thymus					
Absolute	0.059 ± 0.002	0.059 ± 0.003	0.053 ± 0.005	0.048 ± 0.003	0.051 ± 0.004
Relative	2.057 ± 0.104	2.104 ± 0.130	1.911 ± 0.175	1.733 ± 0.122	1.946 ± 0.181

* Significantly different (P≤0.05) from the vehicle control group by Williams' or Dunnett's test
** Significantly different (P≤0.01) from the vehicle control group by Williams' test
[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); no data available for the 24 mg/kg groups due to 100% mortality.

TABLE G5

Organ Weights and Organ-Weight-to-Body-Weight Ratios for Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	0.75 mg/kg	1.5 mg/kg	3 mg/kg	6 mg/kg	12 mg/kg
n	7	7	10	6	8	9
Necropsy body wt	26.8 ± 1.5	27.7 ± 1.0	26.7 ± 0.7	27.5 ± 0.8	28.8 ± 0.9	27.4 ± 0.9
Heart						
Absolute	0.127 ± 0.005	0.121 ± 0.005	0.125 ± 0.003	0.126 ± 0.004	0.136 ± 0.004	0.139 ± 0.004
Relative	4.778 ± 0.125	4.381 ± 0.087	4.702 ± 0.105	4.587 ± 0.093	4.719 ± 0.080	5.087 ± 0.198
R. Kidney						
Absolute	0.214 ± 0.010	0.207 ± 0.006	0.210 ± 0.005	0.221 ± 0.007	0.238 ± 0.008	0.251 ± 0.017*
Relative	8.047 ± 0.210	7.500 ± 0.224	7.919 ± 0.217	8.045 ± 0.172	8.286 ± 0.249	9.158 ± 0.580
Liver						
Absolute	1.316 ± 0.091	1.444 ± 0.050	1.539 ± 0.084*	1.562 ± 0.075*	1.636 ± 0.064**	1.638 ± 0.044**
Relative	49.012 ± 1.366	52.350 ± 1.771	58.099 ± 3.589*	56.844 ± 1.992*	56.888 ± 2.098*	59.911 ± 1.459**
Lung						
Absolute	0.211 ± 0.009	0.199 ± 0.014	0.204 ± 0.007	0.201 ± 0.010	0.197 ± 0.010	0.195 ± 0.007
Relative	8.068 ± 0.632	7.188 ± 0.364	7.662 ± 0.299	7.330 ± 0.402	6.835 ± 0.284	7.139 ± 0.286
Thymus						
Absolute	0.037 ± 0.004[b]	0.043 ± 0.003	0.040 ± 0.002	0.040 ± 0.003	0.042 ± 0.005	0.040 ± 0.004
Relative	1.464 ± 0.224[b]	1.564 ± 0.052	1.485 ± 0.053	1.467 ± 0.119	1.470 ± 0.167	1.472 ± 0.155

*   Significantly different (P≤0.05) from the vehicle control group by Williams' or Dunnett's test
** (P≤0.01)
[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).
[b] n = 6

APPENDIX H
REPRODUCTIVE TISSUE EVALUATIONS
AND ESTROUS CYCLE CHARACTERIZATION

TABLE H1
**Summary of Reproductive Tissue Evaluations for Male F344/N Rats
in the 13-Week Dermal Study of Dicyclohexylcarbodiimide**[a]

	Vehicle Control	1.5 mg/kg	3 mg/kg	6 mg/kg
n	10	10	10	10
Weights (g)				
Necropsy body wt	330 ± 5	334 ± 6	325 ± 7	300 ± 4**
L. Cauda epididymis	0.1421 ± 0.0035	0.1437 ± 0.0042	0.1493 ± 0.0045	0.1474 ± 0.0039
L. Epididymis	0.4774 ± 0.0110	0.4690 ± 0.0100	0.4723 ± 0.0037	0.4641 ± 0.0081
L. Testis	1.4409 ± 0.0204	1.4478 ± 0.0208	1.5049 ± 0.0267	1.4022 ± 0.0194
Spermatid measurements				
Spermatid heads (10^7/g testis)	9.76 ± 0.30	9.43 ± 0.22	9.14 ± 0.21	10.11 ± 0.38
Spermatid heads (10^7/testis)	14.06 ± 0.48	13.63 ± 0.28	13.73 ± 0.28	14.16 ± 0.52
Spermatid count				
(mean/10^{-4} mL suspension)	70.30 ± 2.42	68.15 ± 1.40	68.65 ± 1.37	70.78 ± 2.59
Epididymal spermatozoal measurements				
Motility (%)	86.93 ± 0.36	86.79 ± 0.61	86.69 ± 0.47	87.28 ± 1.12
Concentration				
(10^6/g cauda epididymal tissue)	513 ± 14	515 ± 67	404 ± 48	404 ± 34

** Significantly different (P≤0.01) from the vehicle control group by Williams' test
[a] Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (tissue weights) or Dunn's test (spermatid and epididymal spermatozoal measurements).

TABLE H2
**Estrous Cycle Characterization for Female F344/N Rats
in the 13-Week Dermal Study of Dicyclohexylcarbodiimide**[a]

	Vehicle Control	1.5 mg/kg	3 mg/kg	6 mg/kg
n	10	10	10	10
Necropsy body wt (g)	186 ± 3	190 ± 4	193 ± 3	192 ± 4
Estrous cycle length (days)	4.95 ± 0.05	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00
Estrous stages (% of cycle)				
Diestrus	40.8	44.2	45.0	45.0
Proestrus	19.2	17.5	18.3	18.3
Estrus	21.7	20.8	20.8	18.3
Metestrus	18.3	17.5	15.8	18.3

[a] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle length). By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages.

TABLE H3
Summary of Reproductive Tissue Evaluations for Male B6C3F$_1$ Mice in the 13-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	3 mg/kg	6 mg/kg	12 mg/kg
n	9	8	10	10
Weights (g)				
Necropsy body wt	33.8 ± 0.6	32.9 ± 0.6	31.5 ± 0.5**	30.4 ± 0.5**
L. Cauda epididymis	0.0189 ± 0.0011	0.0182 ± 0.0013	0.0174 ± 0.0008	0.0163 ± 0.0007
L. Epididymis	0.0539 ± 0.0012	0.0515 ± 0.0016	0.0492 ± 0.0013*	0.0467 ± 0.0018**
L. Testis	0.1128 ± 0.0034	0.1138 ± 0.0025	0.1150 ± 0.0023	0.1095 ± 0.0026
Spermatid measurements				
Spermatid heads (10^7/g testis)	16.16 ± 0.33	16.09 ± 0.52	15.90 ± 0.47	15.82 ± 0.41
Spermatid heads (10^7/testis)	1.82 ± 0.05	1.83 ± 0.06	1.82 ± 0.05	1.73 ± 0.04
Spermatid count (mean/10^{-4} mL suspension)	56.83 ± 1.64	57.13 ± 1.81	56.98 ± 1.55	53.93 ± 1.16
Epididymal spermatozoal measurements				
Motility (%)	88.98 ± 0.24	88.35 ± 0.19	87.32 ± 0.38**	88.34 ± 0.33
Concentration (10^6/g cauda epididymal tissue)	918 ± 77	921 ± 85	937 ± 56	1,052 ± 65

* Significantly different (P≤0.05) from the vehicle control group by Williams' test
** Significantly different (P≤0.01) from the vehicle control group by Williams' test (body and left epididymal weights) or Dunn's test (epididymal spermatozoal motility)
[a] Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (left cauda epididymal and testis weights) or Dunn's test (spermatid measurements and epididymal spermatozoal concentration).

TABLE H4
Estrous Cycle Characterization for Female B6C3F$_1$ Mice in the 13-Week Dermal Study of Dicyclohexylcarbodiimide[a]

	Vehicle Control	3 mg/kg	6 mg/kg	12 mg/kg
n	10	10	10	10
Necropsy body wt (g)	28.8 ± 0.9	27.8 ± 0.5	27.7 ± 0.3	26.5 ± 0.4*
Estrous cycle length (days)	4.00 ± 0.00	4.50 ± 0.50	4.05 ± 0.05	4.25 ± 0.21
Estrous stages (% of cycle)				
Diestrus	30.0	29.2	28.3	26.7
Proestrus	21.7	20.8	15.8	20.0
Estrus	24.2	25.0	31.7	28.3
Metestrus	24.2	25.0	23.3	25.0
Uncertain diagnoses	0.0	0.0	0.8	0.0

* Significantly different (P≤0.05) from the vehicle control group by Dunnett's test
[a] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group for estrous cycle length are not significant by Dunn's test. By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages.

APPENDIX I
CHEMICAL CHARACTERIZATION
AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION

Dicyclohexylcarbodiimide

Dicyclohexylcarbodiimide, lot 00929TZ, was obtained from Aldrich Chemical Company (Milwaukee, WI) and was used in the 3-, 13-, and 20-week studies; lot 60104-1 was obtained from Chem-Impex International (Wood Dale, IL) and was used in the 27-week study. Identity and purity analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO), and by the study laboratories, Microbiological Associates, Inc. (Bethesda, MD; 3-, 13-, and 20-week studies) and BioReliance Corporation (Rockville, MD; 27-week study). Reports on analyses performed in support of the dicyclohexylcarbodiimide studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a colorless crystalline material, was identified as dicyclohexylcarbodiimide by the study laboratories using infrared (IR) spectroscopy (lots 00929TZ and 60104-1) and by the analytical chemistry laboratory using IR and proton nuclear magnetic resonance (NMR) spectroscopy (lot 60104-1). All spectra were consistent with the literature spectra (*Aldrich*, 1981a, 1985, 1993). Representative IR and NMR spectra are presented in Figures I1 and I2.

The purity of lot 00929TZ was determined by the study laboratory using gas chromatography (GC) by system A (Table I1). The purity of lot 60104-1 was determined by the analytical chemistry laboratory using high-performance liquid chromatography (HPLC) by system 1, and purity relative to a frozen reference standard of the same lot was measured by the study laboratory using HPLC by system 2 (Table I2).

For lot 00929TZ, GC by system A indicated one major peak, three major impurities (ranging from 0.08% to 0.27% of the total peak area), and two minor impurities. The overall purity of lot 00929TZ was determined to be 99.5%. For lot 60104-1, HPLC by system 1 indicated a major peak and six impurity peaks with a combined area of 1.69% relative to the total peak area. The overall purity of lot 60104-1 was estimated to be greater than 98%.

To ensure stability, the bulk chemical was stored at room temperature, under a nitrogen head space, protected from light as recommended by the manufacturers. Periodic analyses of the bulk chemical were performed by the study laboratories using GC by system A for the 3-, 13-, and 20 week studies (lot 00929TZ) and HPLC by system 2 for the 27-week studies (lot 60104-1). No degradation of the bulk chemical was detected.

Anhydrous Ethanol

Anhydrous ethanol was obtained from Pharmco Products, Inc. (Brookfield, CT). Identity and purity analyses of all lots used in the 27-week study were conducted by the study laboratory. The chemical, a clear liquid, was identified as ethanol by IR spectroscopy; the sample spectra were consistent with a literature spectrum (*Aldrich*, 1981b). The purity of each lot was analyzed using GC by system B. The overall purity of each analyzed lot was determined to be greater than 99.9%.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

Dose formulations were prepared twice (3-week studies), approximately every 2 weeks (13-week studies), every 3 weeks (20-week study), and monthly (27-week study) by mixing dicyclohexylcarbodiimide with anhydrous ethanol to give the required concentration (Table I3). The dose formulations were stored in sealed vials under a headspace of inert gas for up to 28 days (3- and 13-week studies) or 35 days (20- and 27-week studies) at room temperature for the 3-, 13-, and 20-week studies, and until April 1, 1999 (week 13), for the 27-week study; subsequent storage for the 27-week study was at –20° C.

Because the dose formulations were true solutions of the test article in ethanol, homogeneity studies were not performed. Stability studies of 0.38, 2, and 7 mg/mL formulations of lot 00929TZ were conducted by the study laboratory using GC by system C. Stability was confirmed for up to 35 days for dose formulations stored at room temperature in sealed containers under a nitrogen headspace and for up to 3 hours when exposed to light and air at room temperature.

Periodic analyses of the dose formulations of dicyclohexylcarbodiimide were conducted by the study laboratories using GC by system C. During the 3-week studies, the dose formulations were analyzed once; all five dose formulations were within 10% of the target concentrations (Table I4). Animal room samples of these dose formulations were also analyzed; all 10 animal room samples for rats and mice were within 10% of the target concentrations. During the 13-week studies, the dose formulations were analyzed at the beginning, midpoint, and end of the studies; animal room samples of these dose formulations were also analyzed (Table I5). Of the dose formulations analyzed, all 15 for rats and all 15 for mice were within 10% of the target concentrations; all 13 animal room samples for rats and 12 of 13 for mice were within 10% of the target concentrations. One aberrant mouse animal room sample was determined to be due to evaporation from an inadequately sealed vial. Dose formulations were analyzed at the beginning, midpoint, and end of the 20-week study; animal room samples of these dose formulations were also analyzed (Table I6). All 15 dose formulations and all 13 animal room samples were within 10% of the target concentrations. Dose formulations in the initial set and every other set thereafter were analyzed in the 27-week study; animal room samples of these dose formulations were also analyzed (Table I7). All 14 dose formulations analyzed were within 10% of the target concentrations; 8 of 14 animal room samples were not within 10% of target concentrations. Unusual degradation was observed in the animal room samples from the formulations prepared on February 24, 1999, and March 24, 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. Starting on April 2, 1999, all dose formulations were stored at –20° C as an additional precaution against degradation.
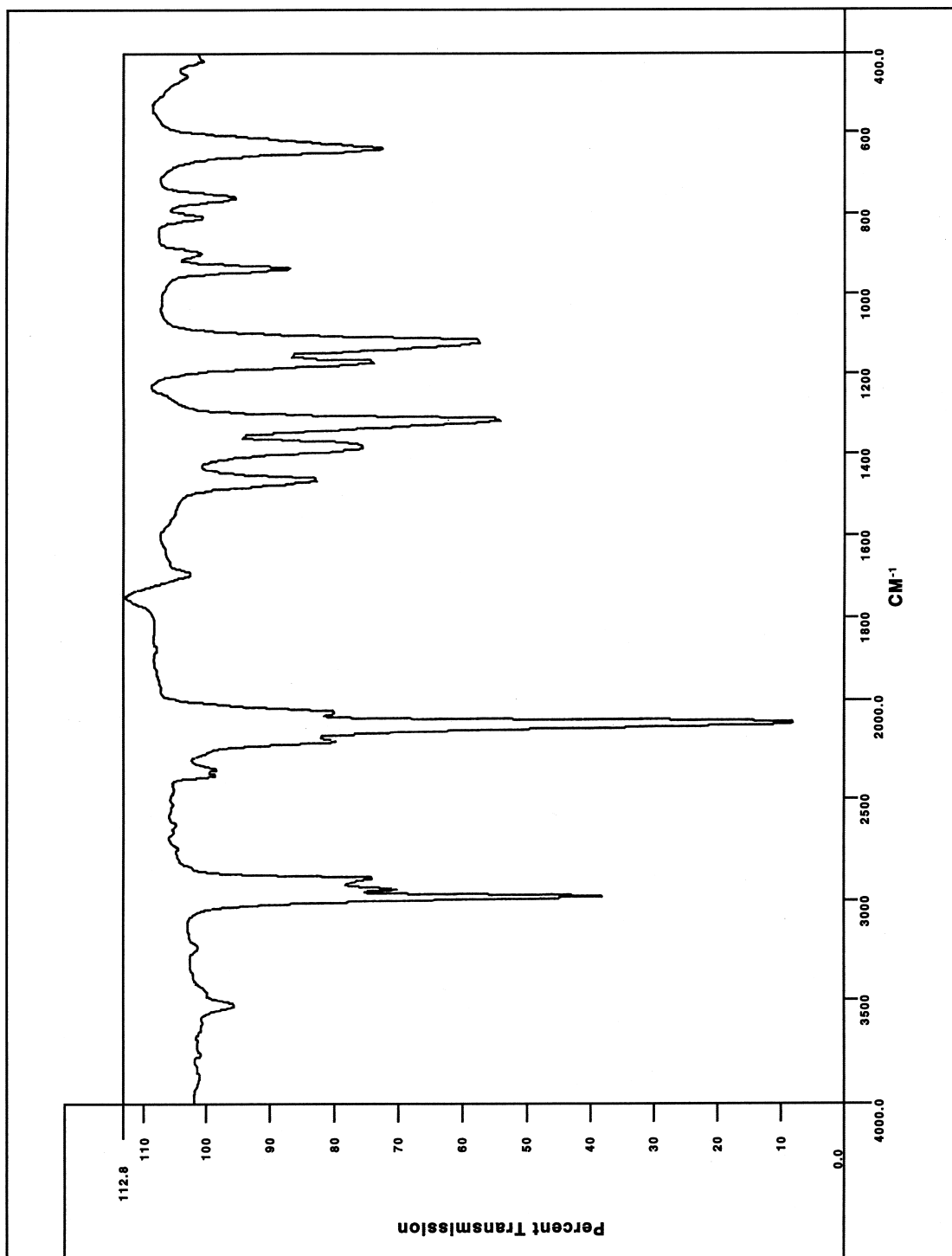
FIGURE I1
Infrared Absorption Spectrum of Dicyclohexylcarbodiimide

FIGURE I2
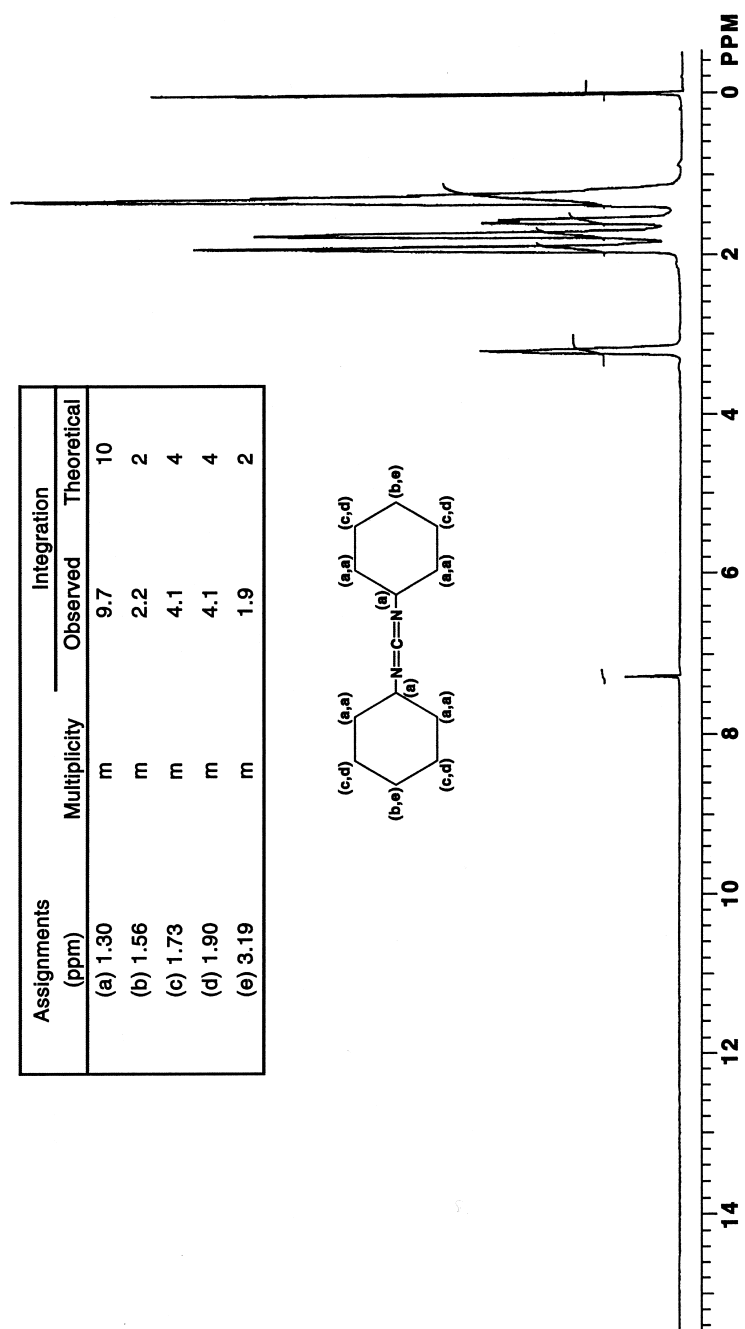**¹H Nuclear Magnetic Resonance Spectrum of Dicyclohexylcarbodiimide**

TABLE I1
Gas Chromatography Systems Used in the Dermal Studies of Dicyclohexylcarbodiimide[a]

Detection System	Column	Carrier Gas	Oven Temperature Program
System A			
Flame ionization	HP-1, 5 m × 0.53 mm (Hewlett-Packard Company, Palo Alto, CA)	Nitrogen at 17.5 mL/minute	135° C to 235° C at 10° C/minute, held for 10 minutes
System B			
Flame ionization	Alltech Econo-Cap Carbowax, 30 m × 0.53 mm, 1.2-µm film (Alltech, Deerfield, IL)	Nitrogen at 6 mL/minute	Isothermal at 62° C
System C			
Flame ionization	HP-1, 5 m × 0.53 mm, 2.65-µm film (Hewlett-Packard Company)	Nitrogen at 17.5 mL/minute	Isothermal at 140° C

[a]   All gas chromatographs were manufactured by Hewlett-Packard Company (Palo Alto, CA)

TABLE I2
High-Performance Liquid Chromatography Systems Used in the Dermal Studies of Dicyclohexylcarbodiimide[a]

Detection System	Column	Solvent System
System 1		
Ultraviolet (233 nm) light	Alltech Alltima C18, 250 mm × 3.2 mm, 5 µm, heated to 40° C (Alltech, Deerfield, IL)	Acetonitrile:water (50:50), isocratic flow rate 1.0 mL/minute
System 2		
Ultraviolet (233 nm) light	Alltech Alltima C18, 250 mm × 3.2 mm, 5 µm, heated to 40° C (Alltech)	Acetonitrile:water (80:20), isocratic flow rate 1.0 mL/minute

[a]   The high-performance liquid chromatographs were manufactured by Waters Corp. (Milford, MA) (system 1) or Hewlett-Packard Company (Palo Alto, CA) (system 2).

TABLE I3
Preparation and Storage of Dose Formulations in the Dermal Studies of Dicyclohexylcarbodiimide

3-Week Studies	**13-Week Studies**	**20-Week Study**	**27-Week Study**
Preparation			
A weighed amount of dicyclohexylcarbodiimide was dissolved in absolute ethanol to prepare the highest dose concentration formulation; lower dose formulations were prepared by diluting aliquots of this preparation with absolute ethanol. Dose formulations were prepared twice.	Same as 3-week studies except that all dose formulations were prepared at least every 2 weeks.	Same as 3-week studies except that all dose formulations were prepared every 3 weeks.	A weighed amount of dicyclohexylcarbodiimide was dissolved in absolute ethanol to obtain the appropriate concentration for each dose formulation. Dose formulations were prepared monthly.
Chemical Lot Number			
00929TZ	00929TZ	00929TZ	60104-1
Maximum Storage Time			
28 days	28 days	35 days	35 days
Storage Conditions			
Stored at room temperature, under nitrogen headspace, protected from light	Stored at room temperature, under nitrogen headspace, protected from light	Stored at room temperature, under nitrogen headspace, protected from light	Stored at room temperature from December 30, 1998, to April 1, 1999, then stored at –20° C, under nitrogen headspace, protected from light
Study Laboratory			
Microbiological Associates, Inc. (Bethesda, MD)	Microbiological Associates, Inc. (Bethesda, MD)	Microbiological Associates, Inc. (Bethesda, MD)	BioReliance Corporation (Rockville, MD)

TABLE I4
Results of Analyses of Dose Formulations Administered to F344/N Rats and B6C3F$_1$ Mice
in the 3-Week Dermal Studies of Dicyclohexylcarbodiimide

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
June 23, 1994	June 23, 1994	2	1.95	−3
		6	6.02	0
		17	16.3	−4
		50	49.2	−2
		150	147	−2
	July 6, 1994[b]	2	1.91	−5
		6	6.05	+1
		17	16.0	−6
		50	47.7	−5
		150	135	−10
	July 6, 1994[c]	2	1.92	−4
		6	6.10	+2
		17	16.3	−4
	July 7, 1994[c]	50	46.6	−7
		150	141	−6

[a] Results of duplicate analyses. For rats, dosing volume=0.3 mL; 2 mg/mL=0.6 mg, 6 mg/mL=1.8 mg, 17 mg/mL=5.1 mg,
       50 mg/mL=15 mg, 150 mg/mL=45 mg. For mice, dosing volume = 0.1 mL; 2 mg/mL=0.2 mg, 6 mg/mL=0.6 mg,
       17 mg/mL=1.7 mg, 50 mg/mL=5 mg, 150 mg/mL=15 mg.
[b] Animal room samples for rats
[c] Animal room samples for mice

**Results of Analyses of Dose Formulations Administered to F344/N Rats and B6C3F$_1$ Mice
in the 13-Week Dermal Studies of Dicyclohexylcarbodiimide**

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Rats				
September 14, 1994	September 14, 1994	1.5	1.47	−2
		3	2.91	−3
		6	5.91	−2
		12	11.6	−3
		24	23.7	−1
	October 3, 1994[b]	1.5	1.45	−3
		3	2.89	−4
		6	5.97	−1
		12	11.8	−2
		24	23.6	−2
October 19, 1994	October 19, 1994	1.5	1.47	−2
		3	2.91	−3
		6	5.64	−6
		12	11.6	−3
		24	23.8	−1
	November 9-10, 1994[b]	1.5	1.49	−1
		3	3.02	+1
		6	5.90	−2
		12	12.1	+1
November 30, 1994	November 30, 1994	1.5	1.47	−2
		3	3.02	+1
		6	6.00	0
		12	12.0	0
		24	22.8	−5
	December 29, 1994[b]	1.5	1.46	−3
		3	2.88	−4
		6	5.94	−1
		12	11.6	−3
Mice				
September 14, 1994	September 14, 1994	1.5	1.47	−2
		3	2.91	−3
		6	5.91	−2
		12	11.6	−3
	October 3, 1994[b]	1.5	1.46	−3
		3	2.97	−1
		6	5.91	−2
		12	11.8	−2
September 19, 1994	September 19, 1994	0.75	0.743	−1
	October 3, 1994[b]	0.75	0.761	+1

TABLE I5
**Results of Analyses of Dose Formulations Administered to F344/N Rats and B6C3F$_1$ Mice
in the 13-Week Dermal Studies of Dicyclohexylcarbodiimide**

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Mice (continued)				
October 19, 1994	October 19, 1994	0.75	0.747	0
		1.5	1.47	−2
		3	2.91	−3
		6	5.64	−6
		12	11.6	−3
	November 9, 1994[b]	0.75	0.762	+2
		1.5	1.53	+2
		3	3.04	+1
		6	5.98	0
November 30, 1994	November 30, 1994	1.5	1.47	−2
		3	3.02	+1
		6	6.00	0
		12	12.0	0
	December 29, 1994[b]	1.5	1.47	−2
		3	3.79[c]	+26
		6	6.09	+2
December 1, 1994	December 1, 1994	0.75	0.771	+3
	December 29, 1994[b]	0.75	0.757	+1

[a] Results of duplicate analyses. For rats, dosing volume=0.5 mL/kg; 1.5 mg/mL=0.75 mg/kg, 3.0 mg/mL=1.5 mg/kg,
     6.0 mg/mL=3 mg/kg, 12 mg/m =6 mg/kg, 24 mg/mL=12 mg/kg. For mice, dosing volume=2.0 mL/kg; 0.75 mg/mL=1.5 mg/kg,
     1.5 mg/mL=3 mg/kg, 3.0 mg/mL=6 mg/kg, 6.0 mg/mL=12 mg/kg, 12 mg/mL=24 mg/kg
[b] Animal room samples
[c] High value due to evaporation of vehicle from inadequately sealed vial

TABLE I6
Results of Analyses of Dose Formulations Administered to Female Tg.AC Hemizygous Mice in the 20-Week Dermal Study of Dicyclohexylcarbodiimide

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
June 6, 1995	June 7, 1995	0.38	0.384	+1
		0.75	0.770	+3
		1.5	1.50	0
		3	3.06	+2
		6	5.99	0
	July 10, 1995[b]	0.38	0.364	−4
		0.75	0.705	−6
		1.5	1.51	+1
		3	3.07	+2
		6	5.89	−2
July 18, 1995	July 19, 1995	0.38	0.380	0
		0.75	0.708	−6
		1.5	1.54	+3
		3	3.03	+1
		6	5.98	0
	August 18, 1995[b]	0.38	0.368	−3
		0.75	0.741	−1
		1.5	1.52	+1
		3	3.00	0
		6	—[c]	—
August 29, 1995	August 29, 1995	0.38	0.386	+2
		0.75	0.754	+1
		1.5	1.52	+1
		3	2.90	−3
		6	6.04	+1
	September 29, 1995[b]	0.38	0.361	−5
		0.75	0.729	−3
		1.5	1.46	−3
		3	2.84	−5
		6	—	—

[a] Results of duplicate analyses. Dosing volume=2.0 mL/kg; 0.38 mg/mL=0.75 mg/kg, 0.75 mg/mL=1.5 mg/kg, 1.5 mg/mL=3 mg/kg, 3.0 mg/mL=6 mg/kg, 6.0 mg/mL=12 mg/kg
[b] Animal room samples
[c] Due to the severity of skin lesions, application of the test article was discontinued after 8 days.

TABLE I7
**Results of Analyses of Dose Formulations Administered to Female p53 Haploinsufficient Mice
in the 27-Week Dermal Study of Dicyclohexylcarbodiimide**

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
December 30, 1998	December 30, 1998	0.38	0.383	+1
		0.75	0.806	+7
		1.5	1.55	+3
		3	3.06	+2
		6	6.16	+3
	February 4, 1999[b]	0.38	0.397	+4
		0.75	0.888	+18
		1.5	1.61	+7
		3	3.08	+3
		6	6.39	+7
February 24, 1999	February 24, 1999	0.38	0.386	+2
		0.75	0.760	+1
		1.5	1.54	+3
	April 1, 1999[b]	0.38	0.182	−52
		0.75	0.423	−44
		1.50	0.780	−48
March 24, 1999	April 8, 1999[b]	0.38	0.259	−32
		0.75	0.513	−32
		1.5	1.15	−23
April 12, 1999	April 12, 1999	0.38	0.415	+9
		0.75	0.717	−4
		1.5	1.53	+2
June 14, 1999	June 14, 1999	0.38	0.414	+9
		0.75	0.684	−9
		1.5	1.41	−6
	July 15, 1999[b]	0.38	0.407	+7
		0.75	0.653	−13
		1.5	1.45	−3

[a]   Results of duplicate analyses. Dosing volume=2.0 mL/kg; 0.38 mg/mL=0.75 mg/kg, 0.75 mg/mL=1.5 mg/kg, 1.5 mg/mL=3 mg/kg, 3.0 mg/mL=6 mg/kg, 6.0 mg/mL=12 mg/kg
[b]   Animal room samples

APPENDIX J
ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

INTRODUCTION

Dicyclohexylcarbodiimide is a highly reactive compound used widely as a condensing agent in the preparation of peptides and nucleotides and as a stabilizing agent for polymers. Dicyclohexylcarbodiimide is a white crystalline solid with a melting point of 35° C. It is soluble in most organic solvents and reacts with most nucleophilic species including water, alcohols, amines, thiols, carboxylic acids, and phosphates. The potential for human exposure to dicyclohexylcarbodiimide exists during its use in the synthesis of polypeptides in the chemical and pharmaceutical industries, as well as during protein synthesis in the recombinant DNA industry.

Carbodiimides are known to be irritating to the skin, eyes, and respiratory tract. Occupational contact dermatitis due to dicyclohexylcarbodiimide exposure has been reported in laboratory workers since the later 1950s, and dicyclohexylcarbodiimide vapor has been reported to cause acute ophthalmitis. It is believed that dicyclohexylcarbodiimide may function as an alkylating agent and, therefore, is a potential vesicant and/or carcinogen.

As presented elsewhere in this Report, the NTP has conducted 13-week dermal toxicity studies of dicyclohexylcarbodiimide at doses up to 12 mg/kg in F344/N rats and 24 mg/kg in B6C3F$_1$ mice. In these studies, the dose application sites were not covered. All of the animals in the highest dose groups for both species died, possibly due to acute central nervous system toxicity. Nonneoplastic lesions including epidermal hyperplasia, inflammation, or necrosis were observed in the skin at the site of application in most of the dosed groups in both species. During 3-week repeated-dose dermal toxicity studies in F344/N rats and B6C3F$_1$ mice similarly treated with dicyclohexylcarbodiimide, similar dose-related clinical signs of neurotoxicity were reported.

The carcinogenic potential of dicyclohexylcarbodiimide has been studied by the NTP in Tg.AC hemizygous mice as also presented elsewhere in this Report, using uncovered dermal applications of the chemical for 20 weeks at doses of 0, 0.75, 1.5, 3, 6, or 12 mg/kg. Increased incidences of epidermal hyperplasia and dermal inflammation occurred in the skin at the site of application in groups administered 3 or 6 mg/kg dicyclohexylcarbodiimide, and the chemical was found to have carcinogenic potential in these animals, as squamous cell papilloma of the skin at the site of application occurred in animals dosed with 1.5 mg/kg or greater.

The objectives of the studies described here were to: a) determine the absorption and rates and routes of excretion of radiolabeled compounds following dermal exposure to [^{14}C]-dicyclohexylcarbodiimide, b) determine the rates and routes of excretion of radiolabeled compounds following intravenous administration of [^{14}C]-dicyclohexylcarbodiimide, c) determine the timecourse of radioactivity in blood following intravenous administration, and d) characterize, to the extent possible, the metabolites of [^{14}C]dicyclohexylcarbodiimide in blood, urine, and selected tissues.

MATERIALS AND METHODS

Young adult male F344/N rats and B6C3F$_1$ mice were obtained from Charles River Laboratories (Raleigh, NC). Animals were quarantined for at least 1 week prior to being used in a study. Animals used for excreta collection were acclimated to the metabolism chambers 1 day prior to dosing. Animals were fed certified Purina Rodent Chow (#5002) and furnished tap water *ad libitum*. Prior to study initiation, animals within the required weight range were randomized to ensure unbiased choice of study animals. Animals were housed in polycarbonate cages with stainless steel bar lids accommodating pelleted feed and water. Cage sizes were 19" × 10.5" × 8" high for rats, and 11.5" × 7.5" × 5" high for mice. Bedding was either Ab-Sorb-Dri® hardwood chips (Lab Products, Maywood, NJ) or Sani-Chips® (P. J. Murphy Forest Products Corp., Montville, NJ). Room temperature was

maintained at 64° to 79° F, and relative humidity ranged from 30% to 70%. Light/darkness was cycled at 12-hour intervals. Animals used for the collection of excreta were housed individually in all-glass, Roth-type metabolism chambers that provided for separate collection of urine, feces, carbon dioxide (CO_2), and expired volatiles during the collection interval.

Anesthesia of the animals was used to minimize pain or distress. Rats were anesthetized with an intramuscular injection of ketamine:xylazine (7:1, approximately 60 mg/kg or to effect). Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg or to effect). Anesthetized rats were euthanized by cutting the diaphragm after final blood collection or by CO_2 asphyxiation at the end of the study. Anesthetized mice were euthanized by cervical dislocation, cutting the diaphragm, or CO_2 asphyxiation after final blood collection.

Rats were surgically fitted with indwelling jugular cannulae for serial blood sampling. The cannula design was similar to that of Harms and Ojeda (1974) as modified by McKenna and Bieri (1984). Rats were anesthetized, and access to the right external jugular vein was gained by a ventral incision of approximately 2 cm at the level of the clavicle approximately 0.5 cm from the midline. The vessel was blunt dissected, and the cannula (filled with sterile saline containing 20 IU/mL of sodium heparin) was inserted to a depth of approximately 28 mm. Placement in the atrium was confirmed by patency. The cannula was secured to the vessel and at the site of insertion by sutures to the surrounding musculature and was then exteriorized by passing the distal end subcutaneously around the neck to exit the dorsal surface via a small incision between the scapulae. The ventral incision was closed with sutures, and the distal end of the cannula was coiled and sutured to the base of the neck between the scapulae. Animals were allowed to recover in their metabolism chambers for approximately 24 hours prior to dosing.

Approximately 24 hours prior to dermal dose administration, animals were anesthetized and the fur on their backs was removed with a No. 40 animal clipper (Oster® Professional Products, Milwaukee, WI). The clipped area was washed with soapy water, rinsed with water, dried, and wiped with acetone. The clipped area was not wiped with acetone for the 0.7 and 6.2 mg/kg definitive dermal studies in mice. The clipped area was examined for nicks or breaks in the skin; any animal with broken skin was excluded. A dose application site was inscribed within the clipped area using a permanent felt tip marker for rats (12 cm²) and mice (2.8 cm²). The animals were placed in individual glass metabolism chambers to recover prior to dosing.

Nonradiolabeled dicyclohexylcarbodiimide (purity 99%) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI), in one lot (PG06319KG). The identity of the nonradiolabeled dicyclohexylcarbodiimide was confirmed by nuclear magnetic resonance (NMR) spectroscopy and gas chromatography/mass spectrometry.

Dicyclohexylcarbodiimide, uniformly labeled with carbon-14 in the cyclohexyl ring (2 µCi/µL in hexanes, 11.14 µg/µL, 37.04 mCi/mmol, 10 mCi total), was received from Wizard Laboratories, Inc. (West Sacramento, CA), at a stated radiochemical purity of 98.8% determined by thin layer chromatography. The radiochemical purity of [14C]-dicyclohexylcarbodiimide was determined by high performance liquid chromatography (HPLC). The HPLC system consisted of a Waters model 510 pump (Waters Corp., Milford, MA), a Rheodyne model 7125 injector (Rheodyne LLC, Rohnert Park, CA), a Supelcogel® TPR-100 column (15 cm × 0.46 cm) (Supelco, Bellefonte, PA), and a β-Ram radioactivity detector equipped with a 100 µL solid glass scintillator flow cell (IN/US Systems Inc., Tampa, FL). The mobile phase was acetonitrile at a flow rate of 0.75 mL/minute.
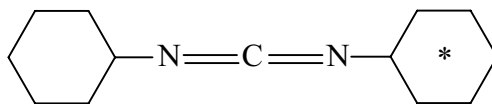
*Position of the [14]C Radiolabel

Chemical Structure of Dicyclohexylcarbodiimide

1,3-Dicyclohexylurea was prepared from dicyclohexylcarbodiimide by acid hydrolysis. A total of 1.04 g of dicyclohexylcarbodiimide (nonradiolabeled dicyclohexylcarbodiimide spiked with 2.6 µCi of [^{14}C]-dicyclohexylcarbodiimide) was dissolved in 2.95 mL of acetonitrile, and 25 mL of 0.1 N hydrochloric acid was added drop-wise with stirring. The reaction mixture was heated at reflux overnight, cooled, and neutralized with 1 N sodium hydroxide. The white precipitate was collected using suction filtration and was rinsed with water. The precipitate was dried and transferred to a scintillation vial for storage. Both ^{13}C- and ^{1}H-NMR spectra (in dimethyl-d$_6$ sulfoxide) were obtained to confirm the identity of the product as 1-3-dicyclohexylurea.

To determine the stability of dicyclohexylcarbodiimide in whole blood from rats, 100 µL of a solution of 0.2 mg [^{14}C]-dicyclohexylcarbodiimide/mL of propylene glycol was added to 2 mL of blood, mixed, and then incubated at 37° C. Following incubation for approximately 1, 5, or 15 minutes, 1 mL of hexanes was added to each sample. The samples were vortexed, then centrifuged for 12 minutes at approximately 1,500 × g. Weighed aliquots of the hexanes extracts and the pellets (following solubilization) were analyzed by liquid scintillation spectrometry (LSS), and the total radioactivity extracted from blood was calculated. The stability of dicyclohexylcarbodiimide in whole blood from mice was assessed using a procedure similar to that described above for rats. A 10 µL solution of 0.2 mg [^{14}C]-dicyclohexylcarbodiimide/mL of propylene glycol was added to 0.25 mL of mouse blood, mixed, and incubated at 37° C for up to 30 minutes. At individual time points, the samples were extracted and analyzed for radiochemical content as described previously.

Intravenous dose formulations contained sufficient quantities of [^{14}C]-dicyclohexylcarbodiimide and nonradiolabeled dicyclohexylcarbodiimide dissolved in propylene glycol [containing up to 2% (v:v) ethanol] to deliver doses of 0.3 to 3 mg/kg to rats and 0.4 to 0.6 mg/kg to mice in a dose volume of 1 to 2.5 mL/kg. Individual dose formulations were prepared the day prior to administration and stored at approximately –20° C.

Dermal dose formulations contained sufficient quantities of [^{14}C]-labeled and nonlabeled dicyclohexylcarbodiimide dissolved in hexanes to deliver doses of 0.3 to 3.1 mg/kg (approximately 0.005 to 0.4 mg/cm^2) to rats and 0.7 to 6.2 mg/kg (approximately 0.005 to 0.05 mg/cm^2) to mice in a dose volume of approximately 1.5 to 5.0 mL/kg.

To determine the rate of sublimation of dicyclohexylcarbodiimide, a solution of [^{14}C]-dicyclohexylcarbodiimide in hexanes, equivalent to a dermal dose formulation, was applied to the internal base of a 20 mL glass scintillation vial. The vial was fitted with a Teflon® septum cap, and a 1-dram glass vial was filled with dry ice and inserted into the scintillation vial below the septum. The base of the scintillation vial was heated in a water bath at approximately 30° to 32° C. After warming for approximately 3 hours, the apparatus was quickly disassembled. The scintillation vial was filled with 2 mL of ethanol and 12 mL of Ultima Gold™ (PerkinElmer, Inc., Boston, MA) scintillation fluor. The 1-dram vial and the Teflon® septum were placed into separate scintillation vials that were also filled with ethanol and Ultima Gold™, and all of the samples were analyzed by LSS.

To determine the extraction efficiency of dicyclohexylcarbodiimide from the charcoal cover used at the site of dermal administration, a solution of [^{14}C]-dicyclohexylcarbodiimide in hexanes (approximately 25 µL), equivalent to a dermal dose formulation, was applied to three pieces of charcoal-impregnated filter similar in size to the appliance covers used for mouse dermal experiments. After approximately 20 minutes, each filter was cut into four pieces and oxidized using a Packard Model 306C Oxidizer (Packard Instrument Co., Inc., Downers Grove,

IL). $^{14}CO_2$ was trapped using Carbosorb™ (PerkinElmer, Inc.) and analyzed by LSS using PermaFluor™ (PerkinElmer, Inc.) as the scintillation cocktail.

For intravenous administration, the dose formulation was mixed by vortexing and the dosing solution was drawn into a glass syringe (1 mL for rats, 250 µL for mice) equipped with a Teflon®-tipped plunger (Hamilton Co., Reno, NV) and an appropriately sized needle. Prior to weighing the filled dosing apparatus, excess dose formulation was wiped from the needle. Intravenous dosing of rats was conducted via an indwelling jugular cannula; for mice, the doses were injected into a lateral tail vein. The dose per animal (µCi) was calculated as the difference in the weight of the syringe/needle filled (S_B) and empty (S_A) multiplied by the concentration of ^{14}C in the dosing formulation (A_D) minus the ^{14}C removed from the needle wipe after dosing (NW) as shown:

$$[\mu Ci/animal = (S_B - S_A)\, A_D\ - NW]$$

For dermal application in rats, protective appliances used to cover the dose application site were prepared from 6.5 cm × 6.5 cm × 1 cm (length × width × thickness) sections of Reston® self-adhesive foam pads (3M Company, St. Paul, MN). A 4 cm × 4 cm window was cut in the center of each foam section creating a frame. Rubber cement was then applied to the nonadhesive side of one frame and attached to the adhesive side of a second frame (total thickness approximately 2 cm). Additional rubber cement was spread over the nonadhesive side of the double-thickness appliance. At least 24 hours later, strips of double-sided carpet tape (approximately 6 mm wide) were attached to the top (nonadhesive side) of the frame so that no gaps in the tape were present.

Prior to dosing each rat, the skin at the dose application site was reexamined for breaks in the skin. If none were found, a protective appliance was attached to the back of each rat with Hollister® medical adhesive (Hollister, Inc., Libertyville, IL) so that the inside edge of the appliance was approximately 0.5 cm outside of the area where the dose was to be applied. The appliance was secured to the animal by firmly grasping the appliance and underlying skin between the thumb and fingers and gently squeezing. The dose formulations were vortexed before being drawn into the dosing apparatus that consisted of a ball-tipped needle attached to a 500 µL glass syringe. The syringe was filled to the appropriate mark (300 to 360 µL); excess dose formulation was removed from the outside of the needle with a Kimwipe®, and the filled apparatus was weighed. The dose was applied to the premarked area of the rat's back; the syringe tip was wiped with a Kimwipe®, and the apparatus reweighed. The Kimwipe® was placed into a scintillation vial containing 2 mL of ethanol and analyzed by LSS after the addition of scintillation cocktail. After dosing, a piece of 50/50 polyester/cotton sheeting was affixed to the appliance frame using carpet tape strips, and a metal appliance shield was taped onto the animal's back using Elastoplast® (BSN-JOBST, Inc., Charlotte, NC) adhesive bandage prior to returning the rat to its cage.

For dermal studies in mice, the dose formulation was vortexed, drawn into a 50 µL Hamilton syringe, the excess was wiped off the apparatus, the apparatus was weighed, and the formulation was applied to the premarked dose application site. After dosing, the apparatus was wiped again with a Kimwipe® and reweighed. The Kimwipe® was placed into a scintillation vial containing approximately 2 mL ethanol and analyzed by LSS after the addition of scintillation cocktail. After dosing, a nonocclusive protective appliance was glued over the dose application site using Duro Quick Gel® (Loctite Corporation, Cleveland, OH). For high dose dermal studies, the protective appliance was a wire mesh tissue capsule (Shandon Lipshaw, Inc., Pittsburgh, PA). A metal protective shield was also placed over the capsule and attached to the animal using Duro Quick Gel® and Elastoplast® adhesive bandage.

The dermal doses for rats and mice (in µCi) were calculated using the formula described above for the intravenous injection studies in rats and mice.

Urine and feces were collected separately into containers cooled with dry ice. Urine was collected 0, 8, 24, 48, and 72 hours after dosing except for the 0.3 mg/kg pilot intravenous study in rats and the tissue distribution study in mice. Urine collection flasks were also rinsed with methanol; the methanol rinses were analyzed separately. At sacrifice, urine was collected directly from the bladder and added to the last urine collection. Feces were collected once daily. Urine and feces samples were stored in the dark at approximately –20° C until analyzed. An additional

cage rinse using hexanes was performed for dermal studies in mice in an effort to recover sublimed dicyclohexylcarbodiimide.

Exhaled volatile organics and CO_2 were collected during the dermal and intravenous studies in rats and mice. Radiolabeled components in breath were collected by passing air sequentially through the metabolism cage (flow rate=200 to 550 mL/minute), through two cold traps, each containing approximately 60 mL of ethanol, and through a series of two traps each containing approximately 500 mL of 1 N NaOH. The first cold trap was maintained at 4° C using an ice/water bath, and the second was maintained at –60° C using an isopropanol/dry ice bath. The traps were analyzed at 0 hours and changed at 6, 24, 48, and 72 hours for rat studies and at 8, 24, 48, and 72 hours for mouse studies. For some of the dermal studies in mice, in addition to the traps mentioned above, the radiolabeled components in breath were also collected by passing air from the metabolism cage through a charcoal sorbent tube (SKC, Inc., Eighty Four, PA). The charcoal trap was located between the cryogenic and CO_2 traps and was changed at 4, 8, 24, 48, and 72 hours.

Serial blood collections (approximately 300 µL) were taken at multiple times postdosing. Serial blood samples were collected into heparinized, disposable 1 mL glass syringes (Glaspak™, Becton, Dickinson and Company, Franklin Lakes, NJ). For all studies, blood was collected at sacrifice by cardiac puncture using a heparinized, disposable 10 mL glass syringe for rats and a 1 mL glass syringe for mice. After aliquots were taken for LSS analysis, a 1 mL aliquot of hexanes was added to each of the blood collections. The samples were vortexed and centrifuged for 5 to 10 minutes. The pellet was frozen using a dry ice/acetone bath, and the hexanes layer was transferred to a tared 1/2 dram vial.

At the termination of the dermal studies in rats (72 hours after dose application), the animals were anesthetized and the metal appliance shield was removed and swiped for radioactivity. The polyester/cotton fabric cover was then removed from the foam appliance and placed into a scintillation vial containing 2 mL of ethanol. The dose application site was washed with cotton gauzes soaked with soapy water (approximately 30 mL Liquid Ivory/L). The skin was rinsed with cotton gauzes soaked with water and dried. Each gauze was placed in a separate 20 mL scintillation vial containing 2 mL of water. With the protective appliance still attached, the dose application site was removed from the rat by blunt dissection. Care was taken not to remove any muscle or adipose from under the skin. The excised skin was placed into a Nalgene® bottle containing approximately 60 mL of 2 N ethanolic sodium hydroxide. The appliance was cut into 16 pieces, and each piece was placed into a separate 20 mL scintillation vial containing 2 mL of ethanol. The shield swipes, dose application site wash gauzes, and the appliance pieces were analyzed by LSS.

For the dermal studies in mice, the animals were anesthetized and the dose application site (appliance intact) and surrounding skin were carefully removed by blunt dissection. The charcoal-impregnated filter was removed from the wire mesh tissue capsule. This filter was cut into four pieces and placed into separate scintillation vials to be oxidized. The skin was then excised and attached to a glass funnel with the metal appliance intact. The metal tissue capsule and the skin were rinsed with hexanes and the rinses collected. The metal appliance was then removed from the skin and placed in a jar containing approximately 40 mL of hexanes and 20 mL of acetone. The dose application site was washed with gauzes soaked with hexanes, soapy water, and water. The rinses and gauzes were analyzed by LSS. The excised skin was digested in approximately 10 mL of 2 N ethanolic sodium hydroxide.

Samples of adipose, muscle, skin, kidney, liver, lung, spleen, heart, testes, bladder, and brain were excised from the animals and assayed for radiochemical content. For the intravenous studies in rats, the jugular vein was also excised and assayed for radiochemical content. For the 6 mg/kg intravenous study, the entire brain, heart, kidney, liver, and lung were excised from the animals and stored frozen. Carcasses were digested in 2 N ethanolic sodium hydroxide using approximately 500 mL for each rat and 75 mL for each mouse. For the intravenous studies, the tails were removed from all animals at sacrifice and digested in an appropriate volume of 2 N ethanolic sodium hydroxide.

All samples were assayed for total radioactivity by LSS either after dissolution in scintillation cocktail or following digestion in an organic tissue solubilizer or 2 N ethanolic sodium hydroxide. Darkened samples were neutralized and bleached (perchloric acid/hydrogen peroxide) before analysis. After the addition of scintillation cocktail, samples containing bases were placed in the dark for approximately 6 hours prior to analysis to minimize nonspecific chemiluminescence. Samples were assayed for ^{14}C in a Packard Model 1900TR scintillation spectrometer (Packard Instrument Co., Inc.), which was checked monthly for counting efficiency and calibrated for quench correction using the external standard method.

For pharmacokinetic analysis, a standard noncompartmental model number 21 (WinNonlin® Version 1.5, Scientific Consulting, Inc., Cary, NC) was used to analyze the concentration of total radioactivity in blood versus time for the 0.3 mg/kg intravenous study in rats and to calculate the elimination half-life ($t_{1/2}$) of dicyclohexylcarbodiimide.

RESULTS

The radiochemical purity of lot PG06319KG of [^{14}C]-dicyclohexylcarbodiimide was determined to be approximately 96%, and it contained an impurity (approximately 3%) with the same retention time as 1,3-dicyclohexylurea. The identity of a synthetic standard of 1,3-dicyclohexylurea was confirmed by carbon-13 and proton NMR spectroscopy using DMSO-d$_6$ as the solvent; both spectra were in agreement with the literature spectra (*Aldrich,* 1993) for this compound, with slight differences due to the solvent.

[^{14}C]-Dicyclohexylcarbodiimide was stable for up to 73 hours in ethanol when stored at room temperature, with approximately 93% [^{14}C]-dicyclohexylcarbodiimide and 6% [^{14}C]-1,3-dicyclohexylurea being measured at this timepoint. [^{14}C]-Dicyclohexylcarbodiimide was stable for up to 96 hours in propylene glycol; at analysis, 83% to 90% of the radioactivity was associated with [^{14}C]-dicyclohexylcarbodiimide and 9% to 15% was associated with [^{14}C]-1,3-dicyclohexylurea. Charcoal-impregnated filters retained approximately 90.9% of a representative radiolabeled dose.

To check the sublimation properties of the chemical, a representative dose of [^{14}C]-dicyclohexylcarbodiimide was applied to a glass surface warmed to a near physiologic temperature for 3 hours; approximately 26.3% of the radiolabel sublimed, with an overall recovery of 99.6% of the applied dose.

When rat whole blood samples were spiked with [^{14}C]-dicyclohexylcarbodiimide in propylene glycol at 37° C and extracted almost immediately with hexanes, approximately 87% of the radiolabel was recovered. HPLC analysis indicated that 92% of the radiolabel was [^{14}C]-dicyclohexylcarbodiimide and 5% was [^{14}C]-1,3-dicyclohexylurea. When mouse whole blood samples were spiked with [^{14}C]-dicyclohexylcarbodiimide in propylene glycol and incubated for 1 minute at 37° C, only 50% of the radiolabel was extracted by hexanes; extraction efficiency dropped below 40% when samples were incubated for 30 minutes before extraction.

[^{14}C]-Dicyclohexylcarbodiimide was not stable in rat plasma samples; recovered radiolabel consisted of 76.9% [^{14}C]-dicyclohexylcarbodiimide and 20.3% [^{14}C]-1,3-dicyclohexylurea for samples containing hexanes, and 61.0% [^{14}C]-dicyclohexylcarbodiimide and 36.3% [^{14}C]-1,3-dicyclohexylurea when samples were dried under nitrogen. HPLC analysis indicated that the process of drying the hexanes off the samples may have increased the production of [^{14}C]-1,3-dicyclohexylurea.

Intravenous Studies
Rats
Male F344/N rats received single intravenous injections of 0.3 (two studies) or 3 mg [^{14}C]-dicyclohexylcarbodiimide/kg body weight (approximately 12.1 µCi/animal); after 72 hours, one of the groups received an additional 5.9 mg/kg (13.1 µCi/animal). For all dose groups, 52% to 54% of the administered

radioactivity was excreted in urine and 20% to 30% in feces through 48 hours after administration (Table J1). Minimal amounts of radioactivity were excreted (<0.2%) in breath as volatile organics or CO_2 in rats administered 0.3 mg/kg. Analysis to determine the timecourse of [14C]-dicyclohexylcarbodiimide in the blood of rats given a single intravenous injection of 0.3 mg/kg showed a gradual decrease in dicyclohexylcarbodiimide equivalents through 48 hours after administration (Table J2). The elimination half-life of total radioactivity (i.e., parent compound and/or degradation products and metabolites) in blood was determined to be approximately 8.7 hours. In both 0.3 mg/kg intravenous injection studies, less than 0.01% of the radioactivity in blood was extracted with hexanes. Analyses of tissue concentrations of dicyclohexylcarbodiimide equivalents 48 or 72 hours after dosing indicated that brain, heart, kidney, liver, lung, jugular vein, spleen, and adipose had tissue:blood ratios greater than 10 (Table J3). For the three single-dose intravenous studies in rats, the average total recoveries of radiolabel were 88.8%, 89.2%, and 98.9% (Tables J1 and J3).

Mice
Seventy-two hours after a single intravenous injection of 0.6 mg/kg (2.5 µCi/animal), male $B6C3F_1$ mice excreted approximately 50% of the radioactivity in urine, 25% in feces, and 0.2% and 0.5% as volatile organic compounds and CO_2 in breath, respectively (Table J4). Approximately 75% of the total dose was excreted within 72 hours of dosing. Tissue analysis indicated tissue:blood ratios greater than 10 for dicyclohexylcarbodiimide equivalents in adipose and heart, and approximately 2% and 6% of the administered radiolabel was detected in the carcass and tail, respectively (Table J5). The mean total recovery of radioactivity was approximately 92% (Tables J4 and J5).

A timecourse study of total radiolabel in the blood of mice following a single intravenous injection of 0.4 mg/kg (1.57 µCi/animal) indicated that approximately 11% of the administered dose was in blood 1 minute after dosing, and the mean concentration of dicyclohexylcarbodiimide equivalents steadily decreased over 48 hours until less than 1% remained in blood (Table J6). Variability in concentration at each timepoint (due to each sample being drawn from a separate animal) made pharmacokinetic analysis impossible.

Dermal Studies
Rats
As shown in Tables J7 and J8, 72 hours after male F344/N rats were administered a single dermal dose of 0.3 or 3.1 mg/kg, approximately 6% or 2.9% of the radioactivity was excreted in urine, respectively; fecal excretion was 0.7% or 0.5%, respectively. For both dose groups, approximately 1% of the administered radioactivity was excreted as exhaled volatile organics. Tissue analysis indicated that 16% or 19% of the radioactivity remained in the application site skin of rats dosed with 0.3 or 3.1 mg/kg, respectively, and less than 2% or 1% were detected in the residual carcasses of the two groups, respectively. Total dermal absorption was approximately 25% of the administered dose in both studies. Although the concentrations of radiolabel in collected tissues and the residual carcass were low in both dosed groups of rats, the heart, kidney, and liver had tissue:blood ratios of dicyclohexylcarbodiimide equivalents greater than 10 (Table J9).

Mice
Seventy-two hours after male $B6C3F_1$ mice were administered a single dose of 0.7 mg/kg (2.9 µCi/animal; 0.005 mg/cm^2), the animals had excreted 11% of the applied radioactivity in urine and 14% in feces, with less than 1% of the radioactivity excreted as volatile organics and CO_2 in breath (Table J10); approximately 2% of the radioactivity remained in the application site skin, 1.5% in collected tissues, and less than 1% in the residual carcass (Table J11). Approximately 30% of the dose was absorbed during the 72-hour study; the average total recovery was 89% of the administered dose and this included 59% of the dose that was unabsorbed. Although tissue absorption of [14C]-dicyclohexylcarbodiimide was low in this study, a tissue:blood ratio of dicyclohexylcarbodiimide equivalents of approximately 22 was found in the liver (Table J12).

Seventy-two hours after male B6C3F$_1$ mice were administered a single dermal dose of 6.2 mg/kg (25.5 µCi/animal; 0.05 mg/cm^2), the animals had excreted approximately 22% of the radioactivity in urine, 10% in feces, and less than 2% as volatile organics and CO_2 (Table J10); approximately 3% of the radioactivity remained in the application site skin, 4.5% in collected tissues, and essentially none was detected in the residual carcass (Table J11). Approximately 41% of the dose was absorbed during the 72-hour study; the average total recovery was 84% of the administered dose and this included 43% of the dose that was unabsorbed.

DISCUSSION

Dose formulations of [^{14}C]-dicyclohexylcarbodiimide in 100% propylene glycol and hexanes were found to be appropriately stable for dose administration and subsequent analysis. Dicyclohexylcarbodiimide has considerable volatility and sublimes at room temperature. Because the dermal applications utilized a nonocclusive appliance to protect from oral ingestion of the applied dose and contamination of the excreta by sloughed skin, it was necessary to implement procedures for recovering the sublimed dose. These procedures included exhaustive cleaning of the metabolism cage and use of activated charcoal appliance covers. Several *in vitro* experiments were performed to assess the stability of [^{14}C]-dicyclohexylcarbodiimide in whole blood and to determine the recovery of radiolabel from whole blood extracted with hexanes. When rat and mouse whole blood samples were spiked with [^{14}C]-dicyclohexylcarbodiimide in propylene glycol and extracted, extraction efficiencies dropped rapidly as dicyclohexylcarbodiimide reacted to form solvent-insoluble products.

For the intravenous studies in male F344/N rats, the doses were administered through an indwelling jugular cannula. The rates and routes of excretion of [^{14}C]-dicyclohexylcarbodiimide were studied after single intravenous doses of 0.3 or 3 mg/kg. For both of these doses in rats, approximately 54% of the administered radioactivity was excreted in urine and 20% to 30% in feces through 48 hours postdosing. For 0.3 mg/kg rats, minimal amounts (<0.2%) of radioactivity were excreted either as volatile organics or CO_2. The timecourse of dicyclohexylcarbodiimide equivalents in blood following a single 0.3 mg/kg intravenous dose showed a gradual decrease throughout the 48-hour study. Less than 0.01% of the radioactivity in blood was extractable with hexanes, which suggests that blood radioactivity is associated with metabolites or reaction products of dicyclohexylcarbodiimide rather than dicyclohexylcarbodiimide itself. Pharmacokinetic analysis of the concentration of total radiolabel in blood versus time showed that the elimination half-life of [^{14}C]-dicyclohexylcarbodiimide is 8.7 hours.

For the intravenous studies in male B6C3F$_1$ mice, doses were administered through a lateral tail vein. The rates and routes of excretion of [^{14}C]-dicyclohexylcarbodiimide were studied after a single intravenous dose of 0.6 mg/kg. Approximately 50% of the radioactivity was excreted in urine and 25% in feces by 72 hours after dosing. Approximately 0.5% of the radioactivity was excreted as CO_2 and 0.2% as volatile organics in breath. This amount is less than that recovered in the volatile organic traps during the dermal study (approximately 1.5%) and suggests that the traps used in the dermal study may have trapped a small amount of the sublimed dose. A mean total of approximately 75% of the applied radioactivity was excreted during the 72-hour study, and approximately 92% of the dosed radioactivity was recovered; because similar results were obtained from the intravenous injection studies in rats, this suggests that the rates of excretion in rats and mice are similar. Adipose, heart, and liver had elevated tissue:blood ratios of dicyclohexylcarbodiimide equivalents; unlike the dermal study in mice, the skin:blood ratio was not elevated. Approximately 2% of the radiolabel remained in the residual carcass, while the tail contained approximately 6%, suggesting that [^{14}C]-dicyclohexylcarbodiimide may chemically react at or near the site of injection and not be entirely systemically available. This was also demonstrated by the timecourse of [^{14}C]-dicyclohexylcarbodiimide-derived radioactivity in blood; 1 minute after a single intravenous administration of 0.4 mg/kg, approximately 11% of the administered radiolabel was measured in the blood, and the percentage steadily decreased at each subsequent timepoint until less than 1% remained after 48 hours.

In studies not reported here, urine from intravenously dosed F344/N rats was analyzed by HPLC in an effort to develop a chromatographic method for the separation of what appeared to be numerous metabolites. Chromatographic resolution of all urinary metabolites was not achieved. However, the number of metabolites detected raised a question of whether [^{14}C]-dicyclohexylcarbodiimide had reacted with various endogenous materials to form radiolabeled components or simply formed [^{14}C]-1,3-dicyclohexylurea, which was excreted in the urine and chromatographed in close association with normal urine components. Samples of urine from an intravenous study were subjected to ultrafiltration; approximately 79% of the recovered radiolabel was in the filtrate. This result suggests that the urinary metabolites of dicyclohexylcarbodiimide are small molecules.

An experiment to test charcoal as a potential dermal appliance trap for volatilized [^{14}C]-dicyclohexylcarbodiimide demonstrated that approximately 91% of an applied radiolabeled dose could be recovered from a charcoal-impregnated filter by combustion. Single-dose dermal administration studies were conducted in male F344/N rats at 0.3 (0.005 mg/cm^2) and 3.1 mg/kg (0.05 mg/cm^2). The dose application sites were covered by nonocclusive appliances in these studies, and the rates and routes of excretion were found to be similar through 72 hours following application; 3.1 mg/kg rats excreted approximately 3% of administered radioactivity in urine and less than 1% in feces, and 0.3 mg/kg rats excreted approximately 6% of the applied radioactivity in urine and a minimal amount in feces over the same time interval. For both groups, approximately 1% of the applied radioactivity was recovered as volatile organic compounds in breath. For 3.1 mg/kg rats, approximately 19% of the applied radioactivity remained in the application site skin and less than 1% remained in the residual carcass; for 0.3 mg/kg rats, approximately 16% of the radioactivity remained in the application site skin and approximately 2% was found in the residual carcass. Total dermal absorption of dicyclohexylcarbodiimide was similar at the two doses. Although only a small percentage of the dose was found in collected tissues and the residual carcass of the rats at 72 hours, several tissues (heart, kidney, and liver) had elevated tissue:blood ratios of dicyclohexylcarbodiimide equivalents, but these ratios are not biologically relevant due to the low amount found in tissues and blood.

Single-dose dermal administration studies were conducted in male B6C3F$_1$ mice at 0.7 mg/kg (0.005 mg/cm^2) and 6.2 mg/kg (0.05 mg/cm^2) to determine the dermal absorption of [^{14}C]-dicyclohexylcarbodiimide, the rates and routes of excretion, and the terminal body burden. As in the dermal studies in rats, the dose application sites were covered by nonocclusive protective appliances, and the rates and routes of excretion were similar in the two dose groups 72 hours after administration; 6.2 mg/kg mice excreted approximately 22% of the administered radioactivity in urine and 10% in feces, and 0.7 mg/kg mice excreted approximately 11% of the applied radioactivity in urine and 14% in feces. In both dose groups, less than 2% of the applied radioactivity was excreted as volatile organics and CO$_2$ in breath. The application site skin contained approximately 2% to 3% of each dose, and the residual carcass contained less than 1%. Approximately 41% of the 6.2 mg/kg dose and 30% of the 0.7 mg/kg dose were absorbed by mice during the 72-hour exposures. The mean total recoveries of administered radioactivity were approximately 84% and 89% for the 6.2 and 0.6 mg/kg groups, respectively. Only small percentages of the applied doses were found in collected tissues and the residual carcass; however, elevated tissue:blood ratios of dicyclohexylcarbodiimide equivalents were observed for adipose, skin, heart, liver, and kidney for 6.2 mg/kg mice and liver only for 0.7 mg/kg mice. Since so little radioactivity was present in the blood, the tissue:blood ratios have limited biological relevance.

REFERENCES

The Aldrich Library of ^{13}C and ^{1}H FT-NMR Spectra (1993). 1st ed., p. 1310(B). Aldrich Chemical Co., Inc., Milwaukee, WI.

Harms, P.J., and Ojeda, S.R. (1974). A rapid and simple procedure for chronic cannulation of the rat jugular vein. *J. Appl. Physiol*. **36**, 391-392.

McKenna, M.C., and Bieri, J.G. (1984). Multilayer cannula for long-term infusion of unrestrained rats. *Lab. Anim. Sci.* **34**, 308-310.

TABLE J1
Cumulative Excretion of Radioactivity by Male F344/N Rats After a Single Intravenous Injection of [^{14}C]-Dicyclohexylcarbodiimide

End of Collection Period (hours)	Urine[a]	Cage Rinse	Feces	Volatile Organics	Exhaled CO_2	Total
			0.3 mg/kg[b]			
6	—[c]	—	—	0.04 ± 0.02	0.08 ± 0.01	0.12 ± 0.03
8	31.2 ± 7.2	—	—	0.04 ± 0.02	0.08 ± 0.01	31.3 ± 7.1
24	49.5 ± 4.2	—	16.5 ± 1.5	0.08 ± 0.03	0.14 ± 0.02	66.2 ± 5.2
48	52.4 ± 4.5	—	20.5 ± 0.9	0.11 ± 0.05	0.16 ± 0.02	73.1 ± 5.4
72	53.7 ± 4.7	0.4 ± 0.2	21.6 ± 1.4	0.12 ± 0.05	0.17 ± 0.02	75.9 ± 6.1
			0.3 mg/kg[b]			
8	33.1 ± 3.8	—	—	—	—	33.1 ± 3.8
24	51.7 ± 2.3	—	15.7 ± 2.5	—	—	67.4 ± 3.8
48	54.3 ± 2.4	1.0 ± 0.3	20.0 ± 3.9	—	—	75.6 ± 3.9
			3 mg/kg[d]			
8	34.5 ± 2.2	—	—	—	—	34.5 ± 2.2
24	51.2 ± 1.9	—	24.8 ± 12.9	—	—	76.8 ± 11.5
48	53.9 ± 1.6	—	30.3 ± 12.1	—	—	84.3 ± 10.8
72	55.0 ± 1.7	0.4 ± 0.2[e]	31.4 ± 12.0	—	—	86.4 ± 10.7[e]

[a] Urine analysis includes the methanol rinse of the urine flask for all samples except the 72-hour sample after the 3 mg/kg dose; for this sample, the methanol rinse is included in the total only.
[b] Data are presented as cumulative percentage of the dose (mean ± standard deviation) for five rats.
[c] No collection was scheduled for this time interval
[d] Data are presented as cumulative percentage of the dose (mean ± standard deviation) for six rats.
[e] n=3

TABLE J2
Concentrations of Radiolabel in Blood of Male F344/N Rats After a Single Intravenous Injection of 0.3 mg/kg [^{14}C]-Dicyclohexylcarbodiimide

Time After Dosing (hours)	Dicyclohexylcarbodiimide Equivalents in Blood (ng-Eq/g Blood)					
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean[a]
IAD	145	153	154	148	168	154 ± 8.8
0.08	141	149	155	135	156	147 ± 9.0
0.17	135	138	151	151	142	143 ± 7.4
0.33	130	138	151	153	135	141 ± 10.1
0.67	122	124	128	151	132	132 ± 11.8
1	110	109	118	118	112	113 ± 4.1
2	95.2	89.4	94.3	105	95.4	95.8 ± 5.6
4	68.2	77.2	83.2	108	77.7	82.9 ± 15.1
8	50.7	52.8	48.1	74.6	53.9	56.0 ± 10.6
24	25.9	16.8	24.6	47.8	22.6	27.5 ± 11.8
48	2.1	2.7	2.6	2.4	2.7	2.5 ± 0.3

IAD=Sample acquired immediately after dosing, approximately 2 to 4 minutes.
[a] Data are presented as mean ± standard deviation.

TABLE J3
Tissue Distribution of Radiolabel in Male F344/N Rats 48 or 72 Hours After a Single Intravenous Injection of [^{14}C]-Dicyclohexylcarbodiimide

Tissue[a]	Dicyclohexylcarbodiimide Equivalents in Tissue (ng-Eq/g Tissue)	Tissue:Blood Ratio	Dose in Total Tissue (%)[b]
		0.3 mg/kg[c] (72 hours)	
Adipose	221.0 ± 56.1	82.1 ± 23.4	5.2 ± 1.4
Bladder	27.3 ± 5.7	10.1 ± 1.6	0.004 ± 0.001
Blood	2.7 ± 0.2	Unity	0.047 ± 0.003
Brain	71.6 ± 33.4	26.7 ± 12.9	0.2 ± 0.1
Heart	186.0 ± 89.2	69.3 ± 34.2	0.20 ± 0.08
Jugular Vein	45.2 ± 17.5	16.9 ± 7.1	0.015 ± 0.003
Kidney	109.0 ± 16.0	40.3 ± 3.4	0.30 ± 0.03
Liver	23.4 ± 2.5	8.6 ± 0.5	0.321 ± 0.006
Lung	44.3 ± 27.7	16.6 ± 10.7	0.06 ± 0.03
Muscle	26.8 ± 7.5	9.9 ± 2.4	4.3 ± 1.2
Skin	21.5 ± 5.7	8.0 ± 2.0	1.2 ± 0.3
Spleen	34.4 ± 9.0	12.6 ± 2.6	0.03 ± 0.01
Testes	12.4 ± 1.8	4.6 ± 0.7	0.05 ± 0.01
Carcass	NA	NA	0.83 ± 1.1
		0.3 mg/kg[c] (48 hours)	
Adipose	221.0 ± 69.9	90.5 ± 33.5	5.1 ± 1.6
Bladder	23.7 ± 7.7	9.7 ± 3.6	0.004 ± 0.002
Blood	2.5 ± 0.2	Unity	0.043 ± 0.004
Brain	66.9 ± 4.0	26.9 ± 1.5	0.17 ± 0.01
Heart	157.0 ± 18.1	63.5 ± 11.3	0.17 ± 0.01
Jugular Vein	55.9 ± 9.7	22.9 ± 6.5	0.018 ± 0.004
Kidney	143.0 ± 5.6	58.0 ± 6.9	0.36 ± 0.03
Liver	35.5 ± 1.4	14.3 ± 1.5	0.45 ± 0.03
Lung	37.6 ± 2.9	15.2 ± 1.6	0.046 ± 0.003
Muscle	30.4 ± 3.1	12.2 ± 0.7	4.8 ± 0.5
Skin	26.3 ± 4.3	10.6 ± 2.1	1.5 ± 0.3
Spleen	44.4 ± 5.9	17.8 ± 1.6	0.04 ± 0.01
Testes	13.8 ± 0.6	5.6 ± 0.7	0.054 ± 0.003
Carcass	NA	NA	0.9 ± 1.3
		3 mg/kg[d] (72 hours)	
Blood	24.6 ± 3.1	Unity	0.044 ± 0.005
Brain	523.0 ± 23.2	21.4 ± 2.4	0.15 ± 0.01
Heart	1,400.0 ± 60.0	57.3 ± 7.5	0.14 ± 0.01
Kidney	934.0 ± 78.1	38.1 ± 2.9	0.25 ± 0.02
Liver	231.0 ± 4.4	9.5 ± 1.1	0.4 ± 0.1
Lung	285.0 ± 35.7	11.8 ± 3.1	0.037 ± 0.005
Carcass	NA	NA	11.5 ± 0.9

NA=Not applicable
[a] Carcass values are based on the residual digested carcass less the equivalents measured in skin, muscle, adipose, and blood.
[b] Percent dose was calculated using the following values for the mass of total tissue expressed as percent of body weight: adipose, 7.0%; blood, 5.2%; muscle, 48%; and skin, 17%.
[c] Data are presented as mean ± standard deviation for five rats.
[d] Data are presented as mean ± standard deviation for three rats.

TABLE J4
Cumulative Excretion of Radioactivity by Male B6C3F$_1$ Mice After a Single Intravenous Injection
of 0.6 mg/kg [^{14}C]-Dicyclohexylcarbodiimide[a]

End of Collection Period (hours)	Urine	Feces	Volatile Organics	Exhaled CO$_2$	Total
8	13.5 ± 17.0	—[b]	0.05 ± 0.03	0.28 ± 0.08	13.9 ± 17.1
24	30.4 ± 16.1[c]	15.9 ± 2.9	0.12 ± 0.03	0.32 ± 0.10	46.8 ± 14.7
48	37.1 ± 13.7[c]	21.8 ± 4.7	0.18 ± 0.06	0.37 ± 0.08	59.4 ± 13.4
72	49.5 ± 7.8[d]	24.6 ± 4.9	0.24 ± 0.10	0.46 ± 0.08	74.9 ± 5.0

[a]   Data are presented as cumulative percentage of the dose (mean ± standard deviation) for five mice.
[b]   No collection was scheduled for this time interval.
[c]   Includes methanol rinse of the urine flask
[d]   Cage rinse is included.

TABLE J5
Tissue Distribution of Radiolabel in Male B6C3F$_1$ Mice 72 hours After a Single Intravenous Injection
of 0.6 mg/kg [^{14}C]-Dicyclohexylcarbodiimide[a]

Tissue	Dicyclohexylcarbodiimide Equivalents in Tissue (ng-Eq/g Tissue)	Tissue:Blood Ratio	Dose in Total Tissue (%)[b]
Adipose	234.0 ± 166.0	25.8 ± 20.2	3.72 ± 2.64
Bladder	40.0 ± 22.8	4.25 ± 2.48	0.007 ± 0.004
Blood	10.1 ± 2.36	Unity	0.127 ± 0.031
Brain	60.6 ± 22.4	6.53 ± 2.98	0.149 ± 0.061
Heart	189.0 ± 66.7	20.2 ± 8.68	0.134 ± 0.050
Kidney	66.4 ± 11.6	6.95 ± 2.15	0.175 ± 0.027
Liver	91.8 ± 13.4	9.39 ± 2.07	0.609 ± 0.058
Lung	49.6 ± 20.2	5.20 ± 2.27	0.047 ± 0.020
Muscle	46.0 ± 19.2	4.96 ± 2.35	3.45 ± 1.41
Skin	19.9 ± 2.09	2.02 ± 0.28	0.483 ± 0.043
Spleen	26.1 ± 4.49	2.75 ± 0.93	0.0098 ± 0.0027
Tail	76.8 ± 42.3	7.30 ± 3.12	6.29 ± 3.68
Testes	24.9 ± 6.06	2.65 ± 1.00	0.032 ± 0.009
Carcass[c]	NA	NA	2.12 ± 3.95

NA=Not applicable
[a]   Data are presented as mean ± standard deviation for five mice.
[b]   Percent dose was calculated using the following values for the mass of total tissue expressed as percent of body weight: adipose, 9.6%;
        blood, 7.6%; muscle, 45.2%; and skin, 14.4%.
[c]   Carcass value is based on the residual digested carcass less the equivalents measured in skin, muscle, adipose, and blood.

TABLE J6
Concentrations of Radiolabel in Blood of Male B6C3F$_1$ Mice
After a Single Intravenous Injection of 0.4 mg/kg [^{14}C]-Dicyclohexylcarbodiimide

Time After Dosing	Dicyclohexylcarbodiimide Equivalents in Blood (ng-Eq/g Blood)				
	Mouse 1	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mean[a]**
1 minute	6.84	740	358	746	463 ± 354
4 minutes	270	205	227	231	233 ± 27
8 minutes	220	187	166	234	202 ± 30.7
20 minutes	173	131	145	137	147 ± 18.5
1 hour	93.1	43.2	80.0	29.5	61.5 ± 30.0
2 hours	24.5	36.6	49.2	35.0	36.3 ± 10.1
8 hours	56.3	18.1	12.8	25.1	28.1 ± 19.5
24 hours	6.36	15.1	16.0	9.86	11.8 ± 4.53
48 hours	7.69	6.95	9.55	6.93	7.78 ± 1.23
	% Dose in Blood				
1 minute	0.266	15.7	9.71	16.8	10.6 ± 7.56
4 minutes	3.62	4.80	3.70	4.17	4.07 ± 0.544
8 minutes	3.53	3.62	3.44	3.84	3.61 ± 0.172
20 minutes	3.25	3.44	3.52	2.70	3.23 ± 0.367
1 hour	1.76	1.48	1.66	1.08	1.50 ± 0.301
2 hours	0.760	0.754	1.20	0.933	0.912 ± 0.210
8 hours	0.734	0.255	0.343	0.501	0.459 ± 0.210
24 hours	0.162	0.333	0.500	0.223	0.304 ± 0.148
48 hours	0.125	0.143	0.163	0.140	0.143 ± 0.016

[a] Data are presented as mean ± standard deviation.

TABLE J7
Cumulative Excretion of Radioactivity by Male F344/N Rats After a Single Dermal Application of [^{14}C]-Dicyclohexylcarbodiimide

End of Collection Period (hours)	Urine	Feces	Volatile Organics	Exhaled CO$_2$	Total
			0.3 mg/kg[a]		
6	—[b]	—	0.6 ± 0.2	0.01 ± 0.01	0.6 ± 0.2
8	0.6 ± 0.2	—	0.6 ± 0.2	0.01 ± 0.01	1.2 ± 0.1
24	2.5 ± 0.7	0.2 ± 0.1	0.9 ± 0.2	0.01 ± 0.01	3.6 ± 0.7
48	4.1 ± 1.1	0.5 ± 0.2	0.9 ± 0.2	0.02 ± 0.01	5.6 ± 1.3
72	5.8 ± 1.6[c]	0.7 ± 0.2	1.0 ± 0.2	0.02 ± 0.01	7.5 ± 1.8
			3.1 mg/kg[d]		
6	—	—	0.8 ± 0.1	0.01 ± 0.01	0.8 ± 0.1
8	0.7 ± 0.3	—	0.8 ± 0.1	0.01 ± 0.01	1.6 ± 0.3
24	1.8 ± 0.5	0.2 ± 0.1	1.1 ± 0.1	0.01 ± 0.01	3.2 ± 0.6
48	2.4 ± 0.7	0.4 ± 0.1	1.2 ± 0.1	0.01 ± 0.01	4.0 ± 0.9
72	2.9 ± 0.9[c]	0.5 ± 0.2	1.2 ± 0.1	0.02 ± 0.01	4.6 ± 1.1

[a] Data are presented as cumulative percentage of the dose (mean ± standard deviation) for three rats.
[b] No collection was scheduled for this time interval.
[c] Cage rinse is included.
[d] Data are presented as cumulative percentage of the dose (mean ± standard deviation) for five rats.

TABLE J8
Distribution of Radioactivity in Male F344/N Rats 72 Hours After a Single Dermal Application of [^{14}C]-Dicyclohexylcarbodiimide

	0.3 mg/kg[a]	3.1 mg/kg[b]
Absorbed Dose		
Urine	5.8 ± 1.6	2.9 ± 0.9
Feces	0.7 ± 0.2	0.5 ± 0.2
Exhaled CO$_2$	0.02 ± 0.01	0.02 ± 0.01
Volatile Organics	1.0 ± 0.2	1.2 ± 0.1
Application Site Skin	16.0 ± 3.5	19.1 ± 5.3
Collected Tissues[c]	0.3 ± 0.1	0.07 ± 0.02
Residual Carcass[d]	1.7 ± 1.5	0.6 ± 0.4
Total Absorbed Dose	25.5 ± 6.1	24.4 ± 8.3
Total Unabsorbed Dose[e]	64.2 ± 6.1	65.4 ± 6.8
Total Dose Recovered	89.7 ± 1.0	89.8 ± 3.2

[a] Data are presented as percentage (mean ± standard deviation) for three rats.
[b] Data are presented as percentage (mean ± standard deviation) for five rats.
[c] Kidney, liver, lung, spleen, heart, testes, bladder, brain, and blood
[d] Dose recovered in the residual carcass less the dose measured in skin, muscle, adipose, and blood
[e] Total radioactivity in the appliance, skin, skin wash, etc.

TABLE J9

Tissue Distribution of Radiolabel in Male F344/N Rats 72 hours After a Single Dermal Application of [^{14}C]-Dicyclohexylcarbodiimide

Tissue	Dicyclohexylcarbodiimide Equivalents in Tissue (ng-Eq/g Tissue)	Tissue:Blood Ratio	Dose in Total Tissue (%)[a]
		0.3 mg/kg[b]	
Adipose	2.94 ± 0.64	3.18 ± 0.28	0.07 ± 0.02
Bladder	5.44 ± 2.04	5.85 ± 1.89	0.0008 ± 0.0004
Blood	0.92 ± 0.16	Unity	0.016 ± 0.003
Brain	3.66 ± 0.67	3.98 ± 0.24	0.007 ± 0.001
Heart	14.0 ± 2.7	15.2 ± 1.7	0.014 ± 0.003
Kidney	43.9 ± 9.7	47.4 ± 3.2	0.10 ± 0.02
Liver	11.7 ± 2.7	12.7 ± 1.4	0.14 ± 0.04
Lung	4.42 ± 0.79	4.80 ± 0.25	0.005 ± 0.001
Muscle	2.26 ± 0.61	2.43 ± 0.24	0.36 ± 0.11
Skin	2.24 ± 0.62	2.41 ± 0.39	0.13 ± 0.04
Spleen	3.70 ± 0.74	4.01 ± 0.23	0.002 ± 0.001
Testes	2.49 ± 0.30	2.72 ± 0.15	0.009 ± 0.001
		3.1 mg/kg[c]	
Adipose	18.2 ± 7.0	5.61 ± 2.05	0.04 ± 0.02
Bladder	10.7 ± 2.8	3.47 ± 1.80	0.00014 ± 0.00003
Blood	3.34 ± 0.78	Unity	0.006 ± 0.001
Brain	9.58 ± 2.22	2.95 ± 0.69	0.002 ± 0.001
Heart	33.5 ± 4.6	10.5 ± 3.2	0.003 ± 0.001
Kidney	81.7 ± 10.9	25.5 ± 6.6	0.018 ± 0.003
Liver	35.0 ± 8.6	10.7 ± 2.6	0.04 ± 0.01
Lung	13.0 ± 2.1	4.08 ± 1.21	0.0013 ± 0.0003
Muscle	5.60 ± 1.03	1.75 ± 0.51	0.09 ± 0.02
Skin	22.1 ± 19.6	7.31 ± 6.65	0.12 ± 0.11
Spleen	11.0 ± 1.9	3.47 ± 1.24	0.0006 ± 0.0001
Testes	6.10 ± 1.81	1.88 ± 0.58	0.002 ± 0.001

[a] Percent dose was calculated using the following values for the mass of total tissue expressed as percent of body weight: adipose, 7.0%; blood, 5.2%; muscle, 48%; and skin, 17%.

[b] Data are presented as mean ± standard deviation for three rats.

[c] Data are presented as mean ± standard deviation for five rats.

TABLE J10
Cumulative Excretion of Radioactivity by Male B6C3F$_1$ Mice After a Single Dermal Application of [^{14}C]-Dicyclohexylcarbodiimide

End of Collection Period (hours)	Urine[a]	Feces	Volatile Organics Trapped by Ethanol	Volatile Organics Trapped by Charcoal	Exhaled CO_2	Total
			0.7 mg/kg[b]			
4	—[c]	—	0.12 ± 0.03	0.007 ± 0.007	0.10 ± 0.04	0.23 ± 0.05
8	3.9 ± 3.9	—	0.14 ± 0.04	0.05 ± 0.04	0.12 ± 0.04	4.2 ± 4.0
24	7.0 ± 6.9	10.2 ± 6.8	0.20 ± 0.04	0.05 ± 0.04	0.14 ± 0.03	17.6 ± 4.5
48	9.0 ± 8.1	13.0 ± 7.5	0.23 ± 0.04	0.05 ± 0.04	0.15 ± 0.03	22.4 ± 3.9
72	11.4 ± 8.7[d]	14.0 ± 6.9	0.25 ± 0.04	0.05 ± 0.04	0.17 ± 0.03	26.0 ± 3.2
			6.2 mg/kg[e]			
8	NS	—	1.05 ± 0.43	NA	0.08 ± 0.03	1.14 ± 0.44
24	10.5 ± 1.6	4.51 ± 0.47	1.29 ± 0.49	NA	0.13 ± 0.04	16.4 ± 2.0
48	13.4 ± 1.8	6.57 ± 0.89	1.40 ± 0.55	NA	0.16 ± 0.05	21.5 ± 2.2
72	22.2 ± 2.4[d]	10.2 ± 1.3	1.51 ± 0.53	NA	0.18 ± 0.05	34.0 ± 3.8

NS=No sample was acquired for this time interval. NA=Not applicable.
[a] Includes methanol rinse of the urine flask.
[b] Data are presented as cumulative percentage of the dose (mean ± standard deviation) for four mice.
[c] No collection was scheduled for this time interval.
[d] Cage rinse is included.
[e] Data are presented as cumulative percentage of the dose (mean ± standard deviation) for five mice.

TABLE J11

Distribution of Radioactivity in Male B6C3F$_1$ Mice 72 Hours After a Single Dermal Application of [^{14}C]-Dicyclohexylcarbodiimide[a]

	0.7 mg/kg	6.2 mg/kg
Absorbed Dose		
Urine	11.4 ± 8.7	22.2 ± 2.4
Feces	14.0 ± 6.9	10.2 ± 1.3
Exhaled CO_2	0.17 ± 0.03	0.2 ± 0.1
Volatile Organics Trapped by Ethanol	0.25 ± 0.04	1.5 ± 0.5
Volatile Organics Trapped by Charcoal	0.05 ± 0.04	—[b]
Application Site Skin	2.0 ± 0.4	3.2 ± 1.5
Collected Tissues[c]	1.5 ± 0.12	4.5 ± 2.8
Residual Carcass[d]	0.5 ± 0.5	0.0 ± 0.0
Total Absorbed Dose	29.9 ± 3.3	41.2 ± 4.8
Total Unabsorbed Dose[e]	58.7 ± 4.2	42.9 ± 4.8
Total Dose Recovered	88.6 ± 5.7	84.1 ± 3.2

[a]   Data are presented as percentage (mean ± standard deviation) for five mice.
[b]   No sample was collected.
[c]   Skin, muscle, adipose, and blood
[d]   Dose recovered in the residual carcass less the dose measured in skin, muscle, adipose, and blood
[e]   Total radioactivity in the appliance, skin, skin wash, etc.

TABLE J12
Tissue Distribution of Radiolabel in Male B6C3F$_1$ Mice 72 Hours After a Single Dermal Application of [^{14}C]-Dicyclohexylcarbodiimide

Tissue[a]	Dicyclohexylcarbodiimide Equivalents in Tissue (ng-Eq/g Tissue)	Tissue:Blood Ratio	Dose in Total Tissue (%)[b]
		0.7 mg/kg[c]	
Adipose	26.7 ± 15.1	7.69 ± 5.84	0.33 ± 0.24
Bladder	21.2 ± 8.7	5.37 ± 2.59	0.004 ± 0.003
Blood	4.29 ± 1.57	Unity	0.04 ± 0.01
Brain	5.29 ± 0.42	1.33 ± 0.37	0.013 ± 0.001
Heart	28.3 ± 9.5	6.68 ± 0.93	0.022 ± 0.007
Kidney	34.2 ± 16.5	7.83 ± 1.03	0.07 ± 0.03
Liver	103.0 ± 78.1	22.1 ± 7.9	0.6 ± 0.3
Lung	6.98 ± 1.85	1.68 ± 0.30	0.006 ± 0.002
Muscle	5.14 ± 1.24	1.24 ± 0.16	0.30 ± 0.04
Skin	9.52 ± 1.69	2.37 ± 0.63	0.18 ± 0.04
Spleen	9.67 ± 5.66	2.17 ± 0.52	0.003 ± 0.002
Testes	5.91 ± 0.81	1.46 ± 0.31	0.0068 ± 0.0008
Carcass	NA	NA	0.5 ± 0.5
Application Site Skin	27.9 ± 5.2	6.85 ± 1.50	1.96 ± 0.43
		6.2 mg/kg[d]	
Adipose	542 ± 105	19.9 ± 3.7	0.8 ± 0.1
Bladder	210.0 ± 69.8	7.7 ± 2.6	0.0027 ± 0.0007
Blood	27.3 ± 1.1	Unity	0.033 ± 0.002
Brain	81.0 ± 9.3	3.0 ± 0.4	0.021 ± 0.002
Heart	352 ± 109	12.9 ± 3.7	0.020 ± 0.002
Kidney	253.0 ± 29.3	9.3 ± 0.9	0.057 ± 0.005
Liver	488.0 ± 36.2	17.9 ± 1.3	0.43 ± 0.07
Lung	84.3 ± 11.6	3.1 ± 0.4	0.008 ± 0.002
Muscle	80.0 ± 8.1	2.9 ± 0.3	0.59 ± 0.07
Skin	1,050 ± 1,130	37.9 ± 39.5	2.5 ± 2.8
Spleen	85.0 ± 14.5	3.1 ± 0.6	0.0025 ± 0.0003
Testes	83.7 ± 10.2	3.1 ± 0.3	0.009 ± 0.001
Carcass	NA	NA	0.0 ± 0.0
Application Site Skin	403 ± 183	14.9 ± 6.9	3.2 ± 1.5

NA=Not applicable
[a] Carcass values are based on the residual digested carcass less the equivalents measured in skin, muscle, adipose, and blood.
[b] Percent dose was calculated using the following values for the mass of total tissue expressed as percent of body weight: adipose, 9.6%; blood, 7.6%; muscle, 45.2%; and skin, 14.4%.
[c] Data are presented as mean ± standard deviation for four mice.
[d] Data are presented as mean ± standard deviation for five mice.