NTP
National Toxicology Program
U.S. Department of Health and Human Services

NTP Technical Report on the Toxicology and Carcinogenesis Studies of Isoeugenol (CAS No. 97-54-1) in F344/N Rats and B6C3F1 Mice (Gavage Studies)

NTP TR 551

SEPTEMBER 2010

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDIES OF ISOEUGENOL

(CAS NO. 97-54-1)

IN F344/N RATS AND B6C3F1 MICE

(GAVAGE STUDIES)

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

September 2010

NTP TR 551

NIH Publication No. 10-5892

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 Technical Report series began in 1976 with carcinogenesis studies conducted by the National Cancer Institute. In 1981, this bioassay program was transferred to the NTP. The studies described in the Technical Report series are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected substances in laboratory animals (usually two species, rats and mice). 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 Technical 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.

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CONTRIBUTORS

National Toxicology Program

Evaluated and interpreted results and reported findings

D.W. Bristol, Ph.D., Study Scientist
J.C. Peckham, D.V.M., M.S., Ph.D., Study Pathologist
Experimental Pathology Laboratories, Inc.
J.R. Bucher, Ph.D.
L.T. Burka, Ph.D.
R.S. Chhabra, Ph.D.
P.M. Foster, Ph.D.
R.A. Herbert, D.V.M., Ph.D.
M.J. Hooth, Ph.D.
A.P. King-Herbert, D.V.M.
G.E. Kissling, Ph.D.
D.E. Malarkey, D.V.M., Ph.D.
J.H. Roycroft, Ph.D.
J.M. Sanders, Ph.D.
C.S. Smith, Ph.D.
G.S. Travlos, D.V.M.
M.K. Vallant, B.S., M.T.
N.J. Walker, Ph.D.
K.L. Witt, M.S.

Battelle Columbus Operations

Conducted studies and evaluated pathology findings

M.R. Hejtmancik, Ph.D., Principal Investigator
S.L. Grumbein, D.V.M., Ph.D.
M.J. Ryan, D.V.M., Ph.D.
J.D. Toft, II, D.V.M., M.S.

Experimental Pathology Laboratories, Inc.

Provided pathology review

M.H. Hamlin, II, D.V.M., Principal Investigator
K.J. Cimon, D.V.M., M.S.
J.C. Peckham, D.V.M., M.S., Ph.D.
G.A. Willson, D.V.M., Ph.D.

Dynamac Corporation

Prepared quality assurance audits

S. Brecher, Ph.D., Principal Investigator
S. Iyer, B.S.
V.S. Tharakan, D.V.M.

NTP Pathology Working Group

Evaluated slides and contributed to pathology report on rats (March 14, 2006)

G.D. Hill, D.V.M., Ph.D., Coordinator
ILS, Inc.
M.F. Cesta, D.V.M.
ILS, Inc.
K.J. Cimon, D.V.M., M.S.
Experimental Pathology Laboratories, Inc.
S.A. Elmore, D.V.M., M.S.
National Toxicology Program
G.P. Flake, M.D.
National Toxicology Program
R.A. Herbert, D.V.M., Ph.D.
National Toxicology Program
D.E. Malarkey, D.V.M., Ph.D.
National Toxicology Program
J.B. Nold, D.V.M., Ph.D.
GlaxoSmithKline
J.C. Peckham, D.V.M., M.S., Ph.D.
Experimental Pathology Laboratories, Inc.
A.W. Suttie, B.V.Sc., Ph.D.
ILS, Inc.
L.M. Wancket, B.S., Observer
University of Illinois

Evaluated slides and contributed to pathology report on mice (March 21, 2006)

M.F. Cesta, D.V.M., Coordinator
ILS, Inc.
S.A. Elmore, D.V.M., M.S.
National Toxicology Program
G.P. Flake, M.D.
National Toxicology Program
R.A. Herbert, D.V.M., Ph.D.
National Toxicology Program
G.D. Hill, D.V.M., Ph.D.
ILS, Inc.
A.W. Suttie, B.V.Sc., Ph.D.
ILS, Inc.
G.A. Willson, D.V.M., Ph.D.
Experimental Pathology Laboratories, Inc.
L.M. Wancket, B.S., Observer
University of Illinois

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 Technical Report

S.R. Gunnels, M.A., Principal Investigator
L.M. Harper, B.S.
P.C. Rathman, B.S.E.
D.C. Serbus, Ph.D.
G.E. Simmons, M.A.

CONTENTS

SUMMARY

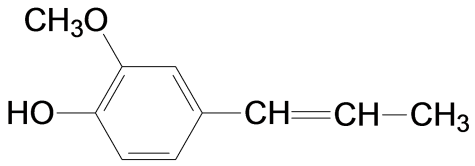
Background
Isoeugenol is a fragrant oil found in many plants including clove, nutmeg, sandalwood, dill seed, gardenia and petunia. It is used in cleaning products, perfumes, and foods and also as an anesthetic in fisheries. We studied the effects of isoeugenol on male and female rats and mice to identify potential toxic or cancer-related hazards.

Methods
We deposited solutions containing isoeugenol in corn oil directly into the stomach through a tube to groups of 50 male and female rats and mice for two years. Exposed animals received either 75, 150, or 300 milligrams of isoeugenol per kilogram of body weight. Control animals received corn oil with no chemical added by the same method. At the end of the study tissues from more than 40 sites were examined for every animal.

Results
There were increased rates of liver cancer (hepatocellular adenoma and hepatocellular carcinoma) in male mice exposed to isoeugenol. Two male rats given 300 mg/kg isoeugenol developed rare neoplasms of the thyroid gland and two others developed rare mammary gland carcinomas. There was an increased rate of histiocytic sarcomas in female mice exposed to isoeugenol. Atrophy, metaplasia, or degeneration of the olfactory epithelium of the nose was seen in all groups of male and female rats and mice exposed to isoeugenol.

Conclusions
We conclude that isoeugenol caused liver cancer in male mice. The occurrence of rare thyroid and mammary gland tumors in male rats and increased incidences of histiocytic sarcomas in female mice may have been associated with exposure to isoeugenol. Exposure to isoeugenol caused a variety of lesions of the olfactory epithelium of the nose in rats and mice.

ABSTRACT



ISOEUGENOL

(7:1 ratio of *trans:cis* isomers)

CAS No. 97-54-1

Molecular Formula: $C_{10}H_{12}O_2$ Molecular Weight: 164.22

Synonyms: 1-(4′-Hydroxy-3′-methoxyphenyl)propene; 4-hydroxy-3-methoxy-1-propenylbenzene; 1-(3-methoxy-4-hydroxyphenyl)-1-propene; 2-methoxy-4-prop-1-enylphenol (IUPAC); phenol, 2-methoxy-4-propenyl (8CI); phenol, 2-methoxy-4-(1-propenyl) (9CI); 4-propenylguaiacol

Isoeugenol is one of several structurally similar phenylpropenoid compounds produced by plants. It has been extracted from calamus, savory, basil, ylang-ylang, clove, tuberose, jonquil, nutmeg, tobacco, sandalwood, dill seed, mace, gardenia, petunia, and other flowers. Isoeugenol can also be produced by isomerization of eugenol, which occurs naturally in clove, pimento, bay leaf, and cinnamon. As a fragrance with a spicy, carnation-like odor, isoeugenol is incorporated into numerous household and personal hygiene products, including perfumes, cream lotions, soaps, and detergents. As a flavoring agent, isoeugenol is added to nonalcoholic drinks, baked foods, and chewing gums. Isoeugenol was nominated by the National Cancer Institute and was selected for carcinogenicity testing because of widespread human exposure through its use as a flavoring and fragrance agent and because of its structural similarity to phenylpropenoids such as safrole, isosafrole, eugenol, methyleugenol, estragole, and anethole, most of which are known rodent carcinogens. Male and female F344/N rats and B6C3F1 mice were administered isoeugenol (99% or greater pure) in corn oil by gavage for 3 months or 2 years. Genetic toxicity tests were conducted in *Salmonella typhimurium, Escherichia coli,* cultured Chinese hamster ovary cells, and mouse peripheral blood erythrocytes.

3-Month Study in Rats

Groups of 10 male and 10 female rats were exposed to isoeugenol in corn oil by gavage at doses of 0, 37.5, 75, 150, 300, or 600 mg/kg, 5 days per week for 14 weeks. All rats survived to the end of the study except one 600 mg/kg male and one 37.5 mg/kg female that were killed in dosing accidents. Mean body weights of all exposed groups of males were significantly less than that of the vehicle control group; however, only the decrease for the 600 mg/kg group exceeded 10% and was considered related to isoeugenol exposure. Liver weights were significantly increased in 300 and 600 mg/kg females. The incidences of minimal atrophy of the olfactory epithelium of the nose were significantly increased in 150 mg/kg or greater males and in 300 or 600 mg/kg females. The incidence of atrophy of olfactory nerve bundles was significantly increased in 600 mg/kg females. Minimal to mild periportal hepatocellular cytoplasmic alteration occurred in all 300 or 600 mg/kg females.

3-Month Study in Mice

Groups of 10 male and 10 female mice were exposed to isoeugenol in corn oil by gavage at doses of 0, 37.5, 75, 150, 300, or 600 mg/kg, 5 days per week for 14 weeks. All mice survived to the end of the study. The mean body weight of 600 mg/kg males was significantly less (12%) than that of the vehicle controls. Liver weights of 300 and 600 mg/kg males were significantly greater than those of the vehicle controls. Minimal to moderate atrophy of olfactory epithelial tissue and nerve bundles was observed in 600 mg/kg males and females.

2-Year Study in Rats

Groups of 50 male and 50 female rats were exposed to isoeugenol in corn oil by gavage at doses of 0, 75, 150, or 300 mg/kg, 5 days per week for 105 weeks. Survival rates of exposed male and female rats were similar to those of vehicle controls. Mean body weights of 300 mg/kg male rats were 9% greater than the vehicle controls at the end of the study. The general lack of toxicity and nonneoplastic lesions indicates that rats might have been able to tolerate higher doses.

Two male rats in the 300 mg/kg group had rare benign or malignant thymomas, while two other males in this group had rare mammary gland carcinomas. Low incidences of minimal atrophy and minimal to mild respiratory metaplasia of the olfactory epithelium were increased in 150 mg/kg males and 300 mg/kg males and females. Similar incidences of minimal to mild olfactory epithelial degeneration in 300 mg/kg males were also increased. Incidences of keratoacanthoma of the skin were decreased in 150 and 300 mg/kg males.

2-Year Study in Mice

Groups of 50 male and 50 female mice were exposed to isoeugenol in corn oil by gavage at doses of 0, 75, 150, or 300 mg/kg, 5 days per week for 104 (females) or 105 (males) weeks. Survival of 300 mg/kg males was significantly decreased compared to the vehicle controls. Mean body weights of 300 mg/kg male and female groups were less than those of vehicle controls at the end of the study, 10% and 15% less, respectively.

In all groups of exposed males, the incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined) were significantly greater than those in the vehicle control group; incidences of multiple hepatocellular adenoma were also significantly increased. Incidences of clear cell focus were significantly increased in 75 and 150 mg/kg male mice.

There was a significant positive trend in the incidences of histiocytic sarcoma in females, and this neoplasm occurred in multiple tissues.

Incidences of respiratory metaplasia in olfactory epithelium in all exposed groups and of atrophy and hyaline droplet accumulation in all exposed groups except 75 mg/kg females were significantly greater than those in corresponding vehicle control groups. Incidences of minimal to marked hyperplasia of Bowman's gland were increased significantly in all exposed groups. Incidences of minimal to mild necrosis of renal papilla and mild to moderate necrosis of renal tubules were increased significantly in 300 mg/kg females. Incidences of forestomach squamous hyperplasia, inflammation, and ulceration (males only) increased with exposure and were significant in the 300 mg/kg groups. The incidence of glandular stomach ulcers was low but significantly increased in the 300 mg/kg groups.

Genetic Toxicology

Isoeugenol was not mutagenic in two independent assays in bacteria (*S. typhimurium* and *E. coli*) conducted with and without exogenous metabolic activation (S9 liver enzymes). Neither did it induce chromosomal aberrations in cultured Chinese hamster ovary cells, with or without S9 activation. Frequencies of micronucleated erythrocytes were not increased in peripheral blood of

male mice exposed to isoeugenol by gavage for 3 months; however, an increasing trend and a threefold increase in the 600 mg/kg group indicate a positive result for this test in female mice.

Conclusions

Under the conditions of these 2-year gavage studies, there was *equivocal evidence of carcinogenic activity** of isoeugenol in male F344/N rats based on increased incidences of rarely occurring thymoma and mammary gland carcinoma. There was *no evidence of carcinogenic activity* of isoeugenol in female F344/N rats administered 75, 150, or 300 mg/kg. There was *clear evidence of carcinogenic activity* of isoeugenol in male B6C3F1 mice based on increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined). There was *equivocal evidence of carcinogenic activity* of iso-eugenol in female B6C3F1 mice based on increased incidences of histiocytic sarcoma.

Exposure to isoeugenol resulted in nonneoplastic lesions of the nose in male and female rats; of the nose, forestomach, and glandular stomach in male and female mice; and of the kidney in female mice.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 12. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 14.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Isoeugenol

	Male F344/N Rats	Female F344/N Rats	Male B6C3F1 Mice	Female B6C3F1 Mice
Doses in corn oil by gavage	0, 75, 150, or 300 mg/kg	0, 75, 150, or 300 mg/kg	0, 75, 150, or 300 mg/kg	0, 75, 150, or 300 mg/kg
Body weights	300 mg/kg group 9% greater than vehicle control group	Exposed groups similar to vehicle control group	300 mg/kg group 10% less than vehicle control group	300 mg/kg group 15% less than vehicle control group
Survival rates	35/50, 34/50, 33/50, 30/50	33/50, 35/50, 34/50, 31/50	39/50, 38/50, 36/50, 27/50	34/49, 39/50, 38/50, 33/50
Nonneoplastic effects	Nose: olfactory epithelium, atrophy (1/50, 5/48, 9/49, 13/49); olfactory epithelium, metaplasia, respiratory (4/50, 6/48, 10/49, 15/49); olfactory epithelium, degeneration (1/50, 0/48, 2/49, 6/49)	Nose: olfactory epithelium, atrophy (0/50, 0/49, 0/49, 4/49); olfactory epithelium, metaplasia, respiratory (5/50, 5/49, 9/49, 12/49)	Nose: olfactory epithelium, atrophy (5/50, 13/50, 36/50, 41/50); olfactory epithelium, metaplasia, respiratory (4/50, 31/50, 47/50, 49/50); olfactory epithelium, degeneration (1/50, 1/50, 7/50, 6/50); olfactory epithelium, accumulation, hyaline droplet (0/50, 6/50, 26/50, 19/50); glands, hyperplasia (3/50, 34/50, 49/50, 48/50) Forestomach: hyperplasia, squamous (7/50, 8/49, 8/50, 14/49); inflammation (5/50, 8/49, 9/50, 14/49); ulcer (1/50, 4/49, 4/50, 9/49) Glandular stomach: ulcer (0/50, 1/49, 4/49, 5/44)	Nose: olfactory epithelium, atrophy (3/48, 8/50, 36/50, 43/50); olfactory epithelium, metaplasia, respiratory (6/48, 37/50, 49/50, 50/50); olfactory epithelium, accumulation, hyaline droplet (0/48, 4/50, 18/50, 12/50); glands, hyperplasia (6/48, 38/50, 49/50, 49/50) Forestomach: hyperplasia, squamous (2/48, 8/50, 5/49, 8/50); inflammation (2/48, 8/50, 5/49, 8/50) Glandular stomach: ulcer (0/46, 1/48, 1/47, 7/48) Kidney: papilla, necrosis (including bilateral) (0/47, 1/50, 1/49, 18/49); renal tubule, necrosis (0/47, 1/50, 0/49, 6/49)
Neoplastic effects	None	None	Liver: hepatocellular adenoma (24/50, 35/50, 37/50, 33/50); hepatocellular carcinoma (8/50, 18/50, 19/50, 18/50); hepatocellular adenoma or carcinoma (28/50, 43/50, 43/50, 43/50)	None
Equivocal findings	Thymus: thymoma, benign or malignant (0/47, 0/43, 0/49, 2/48) Mammary gland: carcinoma (0/50, 0/50, 0/50, 2/50)	None	None	All organs: histiocytic sarcoma (0/49, 1/50, 1/50, 4/50)
Level of evidence of carcinogenic activity	Equivocal evidence	No evidence	Clear evidence	Equivocal evidence

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Isoeugenol

Male F344/N Rats	Female F344/N Rats	Male B6C3F1 Mice	Female B6C3F1 Mice
Genetic toxicology			
***Salmonella typhimurium* gene mutations:**	Negative in strains TA98, TA100, TA1535, TA1537 and in *Escherichia coli* strain WP2 uvra/pKM101with and without S9		
Chromosomal aberrations			
Cultured Chinese hamster ovary cells *in vitro*:	Negative with and without S9		
Micronucleated erythrocytes			
Mouse peripheral blood *in vivo*:	Negative in males; positive in females		

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 Technical Report on isoeugenol on February 28, 2008, 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.

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

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

Tracie E. Bunton, D.V.M., Ph.D.
Toxicology Consultant
Eicarte LLC
Mechanicsburg, PA

Russell C. Cattley, V.M.D., Ph.D.
Amgen
Thousand Oaks, CA

Kenny S. Crump, Ph.D., Principal Reviewer
ENVIRON International Corporation
Monroe, LA

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

Raymond F. Novak, Ph.D.
Institute of Environmental Health Sciences
Wayne State University
Detroit, MI

Michael V. Pino, D.V.M., Ph.D., Principal Reviewer
Drug Safety Evaluation
Sanofi-aventis
Bridgewater, NJ

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

SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On February 28, 2008, the draft Technical Report on the carcinogenesis studies of isoeugenol 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. D.W. Bristol, NIEHS, introduced the toxicology and carcinogenesis studies of isoeugenol by describing its natural occurrence in plants, its uses in fragrances and spices, the structures of the related chemicals in the phenylpropenoid family, the design of the short- and long-term NTP studies, the survival, body weights, nonneoplastic lesions observed in the studies, the occurrence of neoplasms in the long-term study, and an overall comparison of the results of the NTP studies of estragole, methyleugenol, and isoeugenol. The proposed conclusions were *equivocal evidence of carcinogenic activity* of isoeugenol in male F344/N rats, *no evidence of carcinogenic activity* of isoeugenol in female F344/N rats administered 75, 150, or 300 mg/kg, *clear evidence of carcinogenic activity* of isoeugenol in male B6C3F1 mice, and *equivocal evidence of carcinogenic activity* of isoeugenol in female B6C3F1 mice. Exposure to isoeugenol resulted in nonneoplastic lesions of the nose in male and female rats and the nose, forestomach, and glandular stomach in male and female mice.

Dr. Crump, the first principal reviewer, felt the study was well conducted, and he agreed with the proposed conclusions.

Dr. Pino, the second principal reviewer, suggested that body weight be included as part of the dose-setting rationale and asked for clarification of whether organ weight effects were primary or secondary. He thought the kidney effects were worth highlighting in the summary.

Dr. Bradfield, the third principal reviewer, agreed with the proposed conclusions.

Dr. Mirsalis suggested that the statistical significance of the micronucleus tests was sometimes misleading, given a low value for the control measure compared with historical controls.

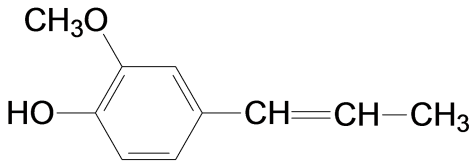
Dr. T. Goodrich, representing AQUI-S New Zealand, Ltd., contrasted the gas chromatography procedures used in the NTP study and by the manufacturer and suggested that the material used in the NTP study had exceeded its shelf life.

Dr. M. Toneby from Scan Aqua, the European representative of AQUI-S, presented results of a mouse micronucleus test showing no effect from isoeugenol and suggested that, considering other tests as well, isoeugenol was not genotoxic.

Dr. Bristol replied that a variety of other chemical assays that were also performed on the test material showed no significant polymerization of the test material and that some of Dr. Goodrich's information referred to material used in a much earlier NTP study.

Dr. Crump moved, and Dr. Soper seconded, to accept the proposed conclusions as written, with the inclusion of the nonneoplastic kidney lesions. The motion was carried with seven yes votes and one no vote (Dr. Pino).

INTRODUCTION



ISOEUGENOL

(7:1 ratio of *trans:cis* isomers)

CAS No. 97-54-1

Molecular Formula: $C_{10}H_{12}O_2$ Molecular Weight: 164.22

Synonyms: 1-(4′-Hydroxy-3′-methoxyphenyl)propene; 4-hydroxy-3-methoxy-1-propenylbenzene; 1-(3-methoxy-4-hydroxyphenyl)-1-propene; 2-methoxy-4-prop-1-enylphenol (IUPAC); phenol, 2-methoxy-4-propenyl (8CI); phenol, 2-methoxy-4-(1-propenyl) (9CI); 4-propenylguaiacol

Chemical and Physical Properties

Isoeugenol belongs to a group of plant-derived volatile compounds, the phenylpropenes, that are biosynthesized from phenylalanine (Pichersky *et al.*, 2006; Koeduka *et al.*, 2008) (Figure 1). At room temperature, it is an oily, yellow liquid with a sweet, spicy, floral odor (Buckley, 2007). Isoeugenol has a boiling point of 266° C, a melting point of –10° C, and a specific gravity of 1.080 at 25° C; it is slightly soluble in water and miscible in ethanol and ether (*Merck,* 1989).

Structurally, phenylpropenoid compounds all have a phenyl group that is attached to an olefinic propenyl group (Figure 1). They are alike in that the phenyl group bears one or more hydroxy, methoxy, or methylenedioxy substituents and is attached to a propene group. The structural feature that partitions them into sets of functionally different analogs, the allylic and propenylic benzenes, is the position that the phenyl ring occupies on the straight-chain, 3-carbon propenyl group. In allylbenzene compounds like eugenol, methyleugenol, estragole, safrole, and myristicin (Figure 1), the phenyl ring [Ph] is attached to the saturated methylene carbon of the propenyl group [Ph-CH_2-CH=CH_2]; alternatively this structure can also be represented as a benzyl group [Ph-CH_2-] attached to ethene [-CH=CH_2]. In contrast, in propenylbenzene compounds, such as isoeugenol, anethole, isosafrole, and asarone (Figure 1), the phenyl ring is attached to the terminal methylene carbon of the propenyl double bond [Ph-CH=CH-CH_3]. In propenylbenzenes, the phenyl and propenyl groups form a conjugated system, like that in β-methylstyrene. Propenylbenzenes are sometimes referred to as isoallylbenzenes.

The double bond of propenylbenzenes is asymmetric, so analogs exist in both *Z*- and *E*- (*cis*- and *trans*-, respectively) positional isomer forms. In both nature and commerce, isoeugenol is usually a mixture of *Z*- and *E*-isomers in the approximate ratio of 1:7, but the pure *E*-isomer is also available commercially. Unique names, structure representations, and other identifiers for each isoeugenol isomer and the corresponding mixture of isomers are presented in Table 1.

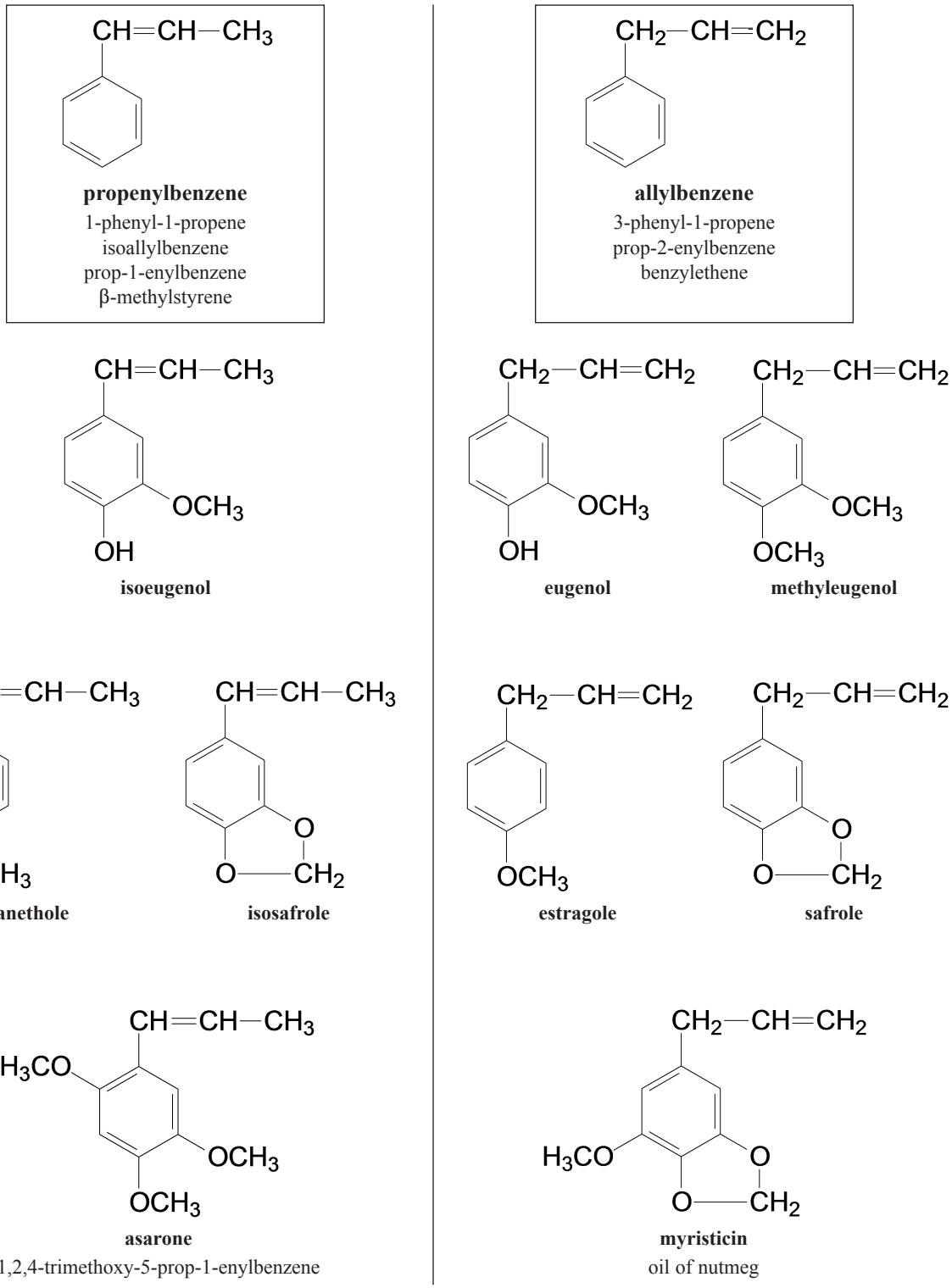
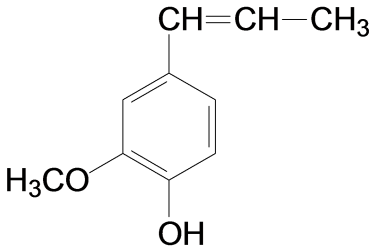


Figure 1
Phenylpropenoid Compounds Related to Isoeugenol

TABLE 1
Forms of Isoeugenol (PubChem, 2006)



Common Name: (Z and/or E)-isoeugenol

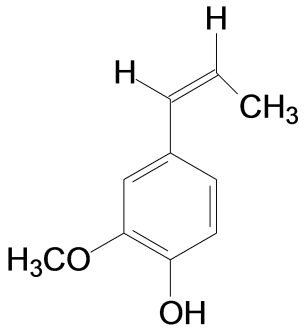
CAS No.: 97-54-1

IUPAC[a] Name: 2-methoxy-4-prop-1-enyl-phenol

Synonyms: phenol, 2-methoxy-4-(1-propenyl)- (9CI); 4-hydroxy-3-methoxy-1-propenylbenzene; 4-(1-propenyl)guaiacol

IUPAC International Chemical Identifier: InChI=1/C10H12O2/c1-3-4-8-5-6-9(11)10(7-8)12-2/h3-7,11H,1-2H3

Isomeric or Canonical SMILES[b]: CC=CC1=CC(=C(C=C1)O)OC



Common Name: (*Z*)-isoeugenol

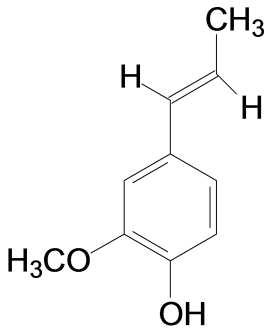
CAS No.: 5932-68-3

IUPAC Name: 2-methoxy-4-[(*Z*)-prop-1-enyl]phenol

Synonyms: *cis*-isoeugenol; (*Z*)-isoeugenol; isoeugenol *cis*-form; *cis*-4-propenylguaiacol; *cis*-2-methoxy-4-propenylphenol; phenol, 2-methoxy-4-propenyl-, (*Z*)-; EINECS 227-633-7

IUPAC International Chemical Identifier: InChI=1/C10H12O2/c1-3-4-8-5-6-9(11)10(7-8)12-2/h3-7,11H,1-2H3/b4-3+

Isomeric or Canonical SMILES: C\C=C\C1=CC(=C(C=C1)O)OC



Common Name: (*E*)-isoeugenol

CAS No.: 5912-86-7

IUPAC Name: 2-methoxy-4-[(*E*)-prop-1-enyl]phenol

Synonyms: *trans*-isoeugenol; (*E*)-isoeugenol; isoeugenol *trans*-form; 1-(3-methoxy-4-hydroxyphenyl)-1-propene; 4-hydroxy-3-methoxy-1(1-propenyl)benzene; *trans*-p-propenylquaiacol; CCRIS 744

IUPAC International Chemical Identifier: InChI=1/C10H12O2/c1-3-4-8-5-6-9(11)10(7-8)12-2/h3-7,11H,1-2H3/b4-3-

Isomeric or Canonical SMILES: C\C=C/C1=CC(=C(C=C1)O)OC

[a] International Union of Pure and Applied Chemistry
[b] Simplified Molecular Input Line Entry Specification

Production, Use, and Human Exposure

Isoeugenol is a fragrant essential oil found in many different plants. It has been extracted, admixed with eugenol and other plant volatiles, from calamus, savory, basil, ylang-ylang, clove, tuberose, jonquil, nutmeg, tobacco, sandalwood, dill seed, mace, gardenia, petunia, and other flowers (Wynder and Hoffmann, 1967; Opdyke, 1975; Demole *et al.*, 1976; Hattori *et al.*, 1978). The relative amounts of isoeugenol and eugenol found in plants vary widely. Recent studies show that, although both are produced by reductive deacylation of a common precursor, coniferyl acetate, their biosynthesis is governed by NADPH-dependent synthase enzymes that represent distinct protein lineages (Koeduka *et al.*, 2006, 2008; Dexter *et al.*, 2007). The structures of these enzymes have been determined, and binding-interaction studies indicate that the conversion of coniferyl acetate to eugenol and isoeugenol proceeds by a two-step pathway in which a quinone-methide is formed and then reduced by NADPH-derived hydride (Louie *et al.*, 2007; Koeduka *et al.*, 2008).

Isoeugenol is produced commercially by isomerization of eugenol (Figure 1), which occurs in oils isolated from clove, pimento, bay leaf, and cinnamon (*Remington's*, 1980). The synthetic conversion involves heating eugenol with caustic potash (*Hawley's*, 2001), with group VIII metals or their compounds, such as rhodium chloride (Cerveny *et al*., 1987), or with more environmentally friendly MgAl hydrotalcite (Kishore and Kannan, 2002). In 1983, approximately 21,000 pounds were produced, based on information from five producers. Imported isoeugenol ranged from 12,000 to 122,000 pounds according to four importers (USEPA, 1990). In 1992, the United States imported 330 metric tons (approximately 728,000 pounds) of eugenol/iso-eugenol (*Chemical Economics Handbook*, 1996). The total European usage of isoeugenol was estimated to be 26,000 kg/year, of which 60% was for household laundry and cleaning products, including laundry detergents, laundry pretreatment products, fabric softeners, hard-surface cleaners, hand dishwashing products, and toilet cleaners (HERA, 2005). In the future, isoeugenol use may increase as a renewable feedstock for the environmentally friendly production of other aromatic flavorings and fragrances. It was recently converted to vanilla in 81% yield and without over-oxidation to vanillic acid or other by-products by *Escherichia coli* cells that had been genetically engineered to overexpress isoeugenol monooxygenase isolated from *Pseudomonas putida* IE27 (Yamada *et al*., 2008). Isoeugenol has been identified in pulp and paper mill effluents (Nestmann and Lee, 1983).

The sweet, spicy floral fragrance of isoeugenol leads to its use in perfumes, cosmetics, personal hygiene products, household cleaning agents, and foods. Tasting like anise or licorice, isoeugenol is added to nonalcoholic drinks, baked foods, candy, and chewing gums. The concentration of isoeugenol in some edible products is approximately 4 ppm for beverages, 4 to 10 ppm for foods, and 0.3 to 1,000 ppm for gums (Opdyke, 1975). Recently, isoeugenol has been used as the active ingredient in AQUI-S®, a "zero withdrawal anesthetic" used in the culture and management of finfish and shellfish. AQUI-S® is approved for use in Australia, Chile, the Faeroe Islands, Korea, and New Zealand (Schnick, 1999, 2006). When used as an anesthetic, AQUI-S® is reported to prevent struggling and thereby maintain muscle quality during the "rested harvesting" of king salmon. Although measurable levels of isoeugenol are detected, they have no effect on levels of other volatile organic compounds that have been identified for possible use as markers of salmon freshness (Wierda *et al.*, 2006).

Human exposure to isoeugenol occurs as a result of its presence in the workplace and through a variety of consumer products. Between 1981 and 1983, the estimated number of workers exposed to isoeugenol was 35,000 (NIOSH, 1990). The estimated daily per capita intake of isoeugenol is approximately 120 µg in Europe and 40 µg in the United States, while the estimated daily per capita intake of isoeugenyl methyl ether is approximately 130 µg in Europe and the United States (WHO, 2004). Information for the latter chemical is included here because *O*-demethylation readily converts it to isoeugenol (Solheim and Scheline, 1976). As a fragrance, isoeugenol is incorporated into numerous household cleaning agents and personal hygiene products, including perfumes, cream lotions, soaps, and detergents (Opdyke, 1975).

Regulatory Status

Isoeugenol is Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) for use in foods as a synthetic flavoring substance and adjuvant (21 CFR, § 172.515). There is no permissible exposure limit, threshold limit value, or recommended exposure

limit for isoeugenol, but it is to be used in the minimum quantity to produce the intended effect. It may be used alone or in combination with other GRAS substances, which include isoeugenol esters (formate, acetate, and phenylacetate) and ethers (methyl, ethyl, and benzyl), that may be readily metabolized to the parent isoeugenol. The FDA Center for Veterinary Medicine granted four designations for aquacultural uses of isoeugenol under the Minor Use and Minor Species Animal Health Act (FDA, 2006). The National Institute of Occupational Safety and Health (NIOSH) registry number for isoeugenol is SL7875000. There is no additional Occupational Safety and Health Administration, American Conference of Governmental Industrial Hygienists, or NIOSH regulatory information for isoeugenol (ACGIH, 2005).

Absorption, Distribution, Metabolism, and Excretion

Experimental Animals

Much of the considerable effort directed toward identifying the metabolism of allylbenzene and propenylbenzene analogs has been reviewed by Scheline (1991). The allylbenzene analogs estragole, methyleugenol, and safrole are carcinogens in both rats and mice (Long *et al*., 1963; Hagan *et al*., 1965, 1967; Boberg *et al*., 1983; Miller *et al*., 1983; Wiseman *et al*., 1987; Miller, 1994a). Accordingly, their metabolism has been studied at length. The cytochrome P450 system catalyzes the metabolism of allylbenzene analogs by three competing pathways: hydroxylation at the methylene carbon of the allyl group to form the corresponding 1-hydroxy-allylbenzene; oxidation of the allylic double bond to form the corresponding 2,3-oxide; and *O*-dealkylation of a phenyl methoxy or methylenedioxy group. The hydroxylation pathway, thought to be the first step in activating allylbenzene analogs towards carcinogenesis, is catalyzed by CYP2E1 and probably CYP2C6 isozymes (Jeurissen *et al*., 2004, 2006, 2007). Sulfonation of the 1-hydroxy metabolite produces an electrophilic sulfonate ester, which reacts with various cellular nucleophiles (i.e., base sites in DNA, RNA, or proteins) to form covalently bound adducts, leading ultimately to carcinogenesis (Boberg *et al*., 1983; Miller, 1994a; Gardner *et al*., 1997). A fourth pathway exists for phenylpropenoid compounds like eugenol and isoeugenol that have a free phenolic group because they may be conjugated and excreted without first undergoing phase I metabolism.

Although the carcinogenic activity and metabolism of isoeugenol and its propenylbenzene analogs are less well characterized, similarly three pathways have been identified for their phase I metabolism: ω-hydroxylation of the terminal methyl group to form the corresponding 3-hydroxy-1-phenylpropene (note that the 3-hydroxy metabolite of isoeugenol is naturally occurring coniferyl alcohol), oxidation of the propenyl double bond to form the corresponding 1,2-oxide, and *O*-dealkylation (Scheline, 1991). Additionally, like allylbenzenes, propenylbenzenes that have a free phenolic group may form glucuronide or sulfonate conjugates and be excreted without undergoing phase I metabolism.

The importance of oxide formation and subsequent metabolism in phenylpropenoid toxicity is not entirely clear. Studies show that allylbenzenes form 2,3-oxide metabolites (Borchert *et al*., 1973; Stillwell *et al*., 1974; Solheim and Scheline, 1976; Delaforge *et al*., 1980), while propenylbenzenes such as isoeugenol, isosafrole, anethole, and asarone form 1,2-oxide metabolites (Klungsøyr and Scheline, 1982; Sangster *et al*., 1984; Wiseman *et al*., 1987; Luo and Guenthner, 1996). Synthetic anethole oxide and *trans*-asarone oxide are both mutagenic in *Salmonella* strains and carcinogenic in mice (Kim *et al*., 1999). Some reports indicate that only small amounts of 1,2-oxides are formed in rodents (Solheim and Scheline, 1976; Newberne *et al.,* 1999; Badger *et al*., 2002). However, recent studies indicate that microbes readily convert isoeugenol to vanilla in up to 71% yield (Yamada *et al*., 2008) by an epoxide/epoxide-diol pathway (Zhang *et al*., 2006; Hua *et al*., 2007; Xu *et al*., 2007).

Although isoeugenol is detoxified by phase II conjugation of its free phenolic group, direct single-electron oxidation is a fifth pathway that results in formation of the quinone-methide metabolite (Thompson *et al*., 1993, 1998; Bertrand *et al*., 1997; Burkey *et al*., 2000; Badger *et al*., 2002). The formation of quinone or quinone-methide metabolites is thought to be responsible for skin sensitization caused by both isoeugenol and eugenol (Thompson *et al*., 1993, 1998; Bertrand *et al*., 1997; Burkey *et al*., 2000) and could be responsible for other toxic responses. The formation of a quinone-methide metabolite is further supported by recent studies, which indicate that the biosynthesis of eugenol and isoeugenol proceeds by NADPH-dependent reduction of their quinone-methide, formed from coniferyl acetate (Louie *et al*., 2007; Koeduka *et al*., 2008). Note that eugenol, isoeugenol, and coniferyl alcohol form the same

quinone-methide and that presence of a phenolic hydroxyl group *para* to the propenyl group is essential for its formation.

The uptake and excretion of isoeugenol is both thorough and rapid. Following a single oral dose of ^{14}C-isoeugenol to male F344 rats (156 mg/kg; 50 μCi/kg), more than 85% was absorbed in 72 hours (Badger *et al.*, 2002). Excretion was mainly in the urine as sulfate or glucuronide conjugates. Approximately 10% was excreted in the feces (likely unabsorbed), and less than 0.1% was recovered as CO_2 or expired organics. No parent isoeugenol was detected in the blood at any time. Following intravenous administration, isoeugenol disappeared rapidly with a $t_{1/2}$ of 12 minutes and a Cl_s of 1.91 L/minute per kg. Excretion of the intravenous dose was similar to that following oral administration. The total amount of radioactivity remaining in the animal was less than 0.25% after 72 hours by either route of exposure. These results demonstrate that isoeugenol is rapidly metabolized and excreted in urine as phase II conjugates.

Metabolism studies of isosafrole, another propenylbenzene analog, in Wistar rats indicated that 89% of the gavage dose was excreted as 10 different urinary metabolites in 72 hours, with most being excreted in the first 24 hours (Klungsøyr and Scheline, 1982). *O*-demethylenation to 3,4-dihydroxyisosafrole was the most prominent pathway (92% of the urinary metabolites were demethylenated), but minor amounts of the corresponding 3-hydroxy and epoxide-diol metabolites were also detected. While 1.3% of the dose was recovered as 3-hydroxyisosafrole, no 1-hydroxysafrole was detected. The 3-hydroxy metabolite is rapidly converted to its 1-hydroxy isomer by strong acid *in vitro* (Peele and Oswald, 1977), but this has been observed to occur *in vivo* only in trace amounts or not at all, thus minimizing formation of the 1-sulfate ester conjugate, the putative ultimate carcinogen formed from allylbenzenes.

The predominant paths for metabolic activation of propenylbenzenes involve side-chain oxidation of the double bond to form the corresponding 1′,2′-oxide and hydroxylation of the terminal methyl group to form the corresponding 3′-hydroxypropenylbenzene. These primary products are further metabolized, setting up competition between activation and detoxification pathways. Isosafrole metabolism was studied in Wistar rats, where 89% of the dose was excreted as urinary metabolites in 72 hours (Klungsøyr and Scheline, 1982). *O*-Demethylation to 1,2-dihydroxy-4-(1′-propenyl) benzene was the most prominent reaction (92% of the urinary metabolites were demethylenated), but hydroxylation of the terminal methyl group and epoxide-diol formation also occurred in minor amounts. Although isoeugenol oxide itself was not detected, approximately 2% of the dose was recovered as its reduced 1,2-dihydrodiol metabolite. Only 1.3% of the dose was recovered as 3-hydroxyisosafrole and no 1-hydroxysafrole was detected. The 3-hydroxy metabolite can be converted to its 1-hydroxy isomer *in vitro* using harsh conditions (Peele and Oswald, 1977), but this isomerization has been observed *in vivo* only in trace amounts. Thus it is unlikely that propenylbenzenes form more than trace amounts, if any, of the 1-sulfoxy conjugate, the putative ultimate carcinogen formed from allylbenzenes. The fact that isoeugenol is formed in high yield when eugenol is heated with a catalyst (Cerveny *et al.*, 1987; *Hawley's*, 2001) indicates that the conjugated phenyl double bond system of propenylbenzenes is considerably more stable thermodynamically than the isolated phenyl group and double bond of allylbenzenes. Accordingly, 1- to 3-hydroxyl isomerization is unlikely unless facilitated enzymatically. In fact, the 3-hydroxy metabolite of isoeugenol is coniferyl alcohol, the stable, natural precursor for the biosynthesis of isoeugenol, eugenol, and many lignan compounds (Koeduka *et al.*, 2006, 2008; Dexter *et al.*, 2007).

Metabolism studies have been conducted with structural analogs of isoeugenol (Figure 1). Early studies showed that allylbenzene analogs are metabolized to allyl epoxides (Borchert *et al.*, 1973; Stillwell *et al.*, 1974; Delaforge, 1976; Solheim and Scheline, 1976) and phenols (Solheim and Scheline, 1976). Allylbenzenes have been found to induce cytochrome P450 enzyme systems (Ioannides *et al.*, 1981; Gardner *et al.*, 1997), and 1-hydroxylated metabolites are thought to play a role in the production of tumors. Miller *et al.* (1983) showed that the 1′-hydroxy metabolites of the allylbenzenes estragole, methyleugenol, safrole, and 1-allyl-4-methoxynaphthalene were hepatocarcinogenic to mice; however, 1-hydroxyallylbenzene and the 1-hydroxy metabolite of elemicin were inactive in the same assays. They also showed that the 3-hydroxy metabolite of the propenylbenzene anethole was also inactive. A variety of studies conducted over a 30-year period, primarily by E.C. and J.A. Miller and associates, provide strong evidence in support of their theory that metabolism of phenylpropenoid compounds, particularly the allylbenzene analogs, results in the formation of electrophilic

intermediates that are attacked by cellular nucleophiles, i.e. base sites present in proteins, DNA, RNA, etc. (Miller, 1994a,b). The precise reaction mechanism that produces covalently bound adducts is not known, but for at least one case, Smith *et al.* (1986) present evidence that the nucleophilic substitution is bimolecular.

Humans

Isoeugenol is absorbed into the systemic circulation after dermal application or ingestion. Application of 10 mM of ^{14}C-isoeugenol to human cadaver skin using various vehicles (ethanol:water, propylene glycol, liquid paraffin, lotions, white petrolatum, or macrogol ointment) resulted in penetration values ranging from 0.29% to 4% (water-based vehicles) and 0.05% to 11% (lotions and ointments) (Jimbo *et al.*, 1983).

TOXICITY

Experimental Animals

Isoeugenol is slightly toxic following acute exposure. Oral LD_{50} values range from 1,290 to 1,880 mg/kg for rats and 1,130 to 1,780 mg/kg for guinea pigs (Jenner *et al.*, 1964). Exposure of male and female Osborne-Mendel rats to 10,000 ppm isoeugenol in the diet (equivalent to 500 mg/kg) for 16 weeks had no effect on body weight, food intake, hematology, or organ weights; gross and microscopic examinations revealed no treatment-related lesions (Hagan *et al.*, 1967).

Isoeugenol causes dermal toxicity, which is exhibited primarily as irritation. Application of 0.1 g of isoeugenol per day for 3 days to the clipped skin of guinea pigs or albino rabbits resulted in a dose-related irritation response (Itoh, 1982). When 50 mg of isoeugenol was applied as a patch to the shaved skin of guinea pigs for 48 hours every 2 weeks followed by treatment with isoeugenol at concentrations of 0.1% or 1% for 48 hours, sensitization was observed in 40% and 80% of the 0.1% and 1% animals, respectively (Itoh, 1982). However, 0.05 g of isoeugenol applied to a patch for 48 hours to miniature pigs did not result in dermal toxicity (Motoyoshi *et al.*, 1979).

Both eugenol and isoeugenol are potent skin sensitizers in the mouse local lymph node assay, although isoeugenol is stronger (Loveless *et al*., 1996). Studies in mice led Bertrand *et al*. (1997) to suggest that the two chemicals form reactive quinone-methide haptens by different mechanistic pathways. The cytotoxic effect of *p*-alkylphenols and estragole have been linked to the formation of quinone-methide intermediates (Thompson *et al*., 1993, 1998). Eugenol may sensitize via primary demethylation followed by oxidation of the resulting pyrocatechol to produce an *o*-quinone metabolite and its tautomeric *p*-quinone-methide, while isoeugenol sensitization is consistent with direct oxidation to its *p*-quinone-methide without first undergoing demethylation. However, Thompson *et al*. (1993, 1998) have shown that eugenol can be oxidized directly to its quinone-methide metabolite. By analogy, isoeugenol, which also has a free phenolic hydroxyl group, can undergo a similar direct oxidation to form the identical quinone-methide. The difference in sensitizing strength may reflect the position of the propenyl double bond. The double bond of isoeugenol, being conjugated with the phenyl ring, may be oxidized faster or more completely to the quinone-methide.

Isoeugenol has been reported to induce UDP-glucuronide transferase activity in rats and guinea pigs (Boutin *et al.*, 1985), inhibit the growth of ascites sarcoma BP 8 cells and oxidative metabolism of hamster brown fat cells (Curvall *et al.*, 1984), and scavenge superoxide anions generated by the xanthine-xanthine oxidase system (Rajakumar and Rao, 1993).

Humans

Dermal exposure to isoeugenol may produce moderate irritation and contact dermatitis (Thompson *et al.*, 1983). Concern has grown in recent years and parallels the growing incidence of human allergic contact dermatitis from exposure to isoeugenol in cosmetics and cleaning agents (de Groot and Frosch, 1997; Buckley *et al.,* 2000; White *et al.*, 2007). This led the International Fragrance Association to recommend that the level for safe use of isoeugenol in consumer products be reduced from 0.2% to 0.02% (White *et al.*, 1999). Subsequently, the European Commission passed the 7th Amendment to the Cosmetics Directive (2003), which lists 26 allergenic fragrance chemicals, including isoeugenol and eugenol, that must be labeled on detergent packaging if added above a concentration of 0.01% weight/weight. This requirement alerts users to the presence of ingredients that may cause an allergic reaction and applies to detergents that are made for washing dishes, crockery, pots, pans, and kitchen utensils by hand. In spite of these interventions, the incidence of isoeugenol contact allergy increased during the 5-year period from 2001 to 2005

(White *et al.*, 2007). A recent study (Rastogi and Johansen, 2008) indicates that substantial amounts of isoeugenyl acetate are now present in some perfumed products, apparently to decrease the amount of isoeugenol needed to provide a desired fragrance; however, this substitution does not allay concern about isoeugenol exposure because skin may readily metabolize the acetate ester to isoeugenol, perhaps exerting concomitant contact allergy in sensitive individuals.

Reproductive and Developmental Toxicity

A multigenerational reproductive toxicity study of isoeugenol has been performed (Layton *et al.*, 2001; NTP, 2002). Groups of 20 adult male and female Sprague-Dawley rats were exposed to 0, 70, 230, or 700 mg isoeugenol/kg body weight per day by gavage in corn oil. The F_0 cohabitation period began on study day 8; subsequently, mating pairs produced three litters (F_{1a}, F_{1b}, and F_{1c}). Animals from the F_{1c} litters were first exposed to isoeugenol on postnatal day 21. On postnatal days 71 to 91, F_{1c} animals were assigned to mating pairs and produced three litters (F_{2a}, F_{2b}, and F_{2c}). The study evaluated body weight, feed consumption, clinical signs, number and weight of pups, anogenital distance, sperm parameters, vaginal cytology, organ weights, gross pathology, and microscopic pathology. Treatment-related general toxicity included mean body weight decreases in respective F_0 and F_1 generations of 230 and 700 mg/kg males (3% and 18%) and 700 mg/kg females (4% and 12%), while forestomach hyperkeratosis and hyperplasia increased in all exposed groups of F_0 and F_1 rats. Exposure to isoeugenol at 700 mg/kg per day resulted in mild reproductive toxicity, as evidenced by a decrease in the number of F_1 male pups per litter and decreases in F_2 male and female pup weights.

In an NTP developmental toxicity study (NTP, 1998; George *et al.*, 2001), timed-pregnant CD® outbred albino Sprague-Dawley rats were exposed to doses of 250, 500, or 1,000 mg/kg of isoeugenol by gavage in corn oil on gestational days 6 through 19; controls received 5 mL/kg corn oil. Isoeugenol exposure did not affect prenatal mortality (resorption or late fetal death). Average fetal body weight per litter was decreased by 7% (male) or 9% (female) in the 1,000 mg/kg group on gestation day 20. The incidence of unossified sternebra in fetuses from the 1,000 mg/kg group was increased; no other statistically significant fetal abnormalities were observed. Based on reduced body weight and gestational weight gain, 250 mg/kg per day was the lowest-observed-adverse-effect level (LOAEL) for maternal toxicity. Due to intrauterine growth retardation and mildly delayed skeletal ossification, the developmental toxicity LOAEL was 1,000 mg/kg per day.

Carcinogenicity

No studies were found in the literature regarding the chronic toxicity or carcinogenicity of isoeugenol in humans.

Isoeugenol shares structural features with two sets of phenylpropenoid analogs (Figure 1). The carcinogenicity of many of these compounds, their known metabolites, and their potentially active derivatives have been characterized in rodent bioassays.

Safrole, fed to male and female Osborne-Mendel rats at up to 5,000 ppm in feed for 2 years, induced significant increases in liver tumors (hepatocellular carcinoma, hepatocellular adenoma, hepatocholangiocarcinoma, and hepatocholangioma) (Long *et al*., 1963). Mice exposed to 464 mg/kg safrole by stomach tube from postnatal days 7 to 28 and then to 1,112 ppm safrole in feed developed liver neoplasms (Innes *et al*., 1969). Male B6C3F1 mice nursed by safrole-treated mothers also developed liver tumors (Vesselinovitch *et al*., 1979). Unlike safrole, isosafrole did not induce tumors at any site in Osborne-Mendel rats exposed to 5,000 ppm in feed for 2 years (Hagan *et al.*, 1965). Using the same protocol, while dihydrosafrole did not produce liver tumors, it induced 75% incidences of benign and malignant esophageal tumors in rats (Hagan *et al.*, 1965).

Miller *et al*. (1983) performed comprehensive sets of bioassays to characterize the carcinogenicity of phenylpropenoid compounds and their metabolites. When administered to CD-1 mice prior to weaning then evaluated for hepatic tumors about 1 year later, neither anethole nor eugenol exhibited carcinogenic activity at doses that induced incidences of 61% to 73% in male and female mice exposed to safrole and in male but not female mice exposed to estragole. Anethole elicited a weak response in male mice when the dose was doubled. The 2,3-oxides of safrole, estragole, and eugenol were all negative in this assay. Similar preweaning-exposure assays for the development of liver tumors in B6C3F1

male mice were positive for estragole, methyleugenol, and the 1-hydroxy metabolites of estragole, methyleugenol, and 2,3-dehydroestragole, but negative for anethole, 3-hydroxyanethole, elemicin, 1-hydroxyelemicin, myristicin, dill apiol, and parsley apiol. Female CD-1 mice developed hepatic tumors when exposed in the diet for 1 year to safrole, estragole, and 1-hydroxysafrole, but not to anethole or eugenol. CD-1 female mice developed benign epidermal papillomas and keratoacanthomas when exposed topically to the 2,3-oxides of safrole, 1-hydroxysafrole, estragole, 1-hydroxyestragole, and eugenol.

Wiseman *et al.* (1987) extended the work of Miller *et al*. (1983) to determine relative hepatocarcinogenic potencies for allylbenzene and propenylbenzene analogs administered to B6C3F1 mice prior to weaning. Some conclusions of the authors follow: 1-hydroxyestragole injected intraperitoneally at 12 days of age approximately doubled the hepatomas per liver induced by the same dose administered at 1 day of age; the acetylenic compounds 1-hydroxy-2,3-dehydroestragole and l-hydroxy-2,3-dehydrosafrole induced five- and 10-fold more hepatomas per liver than the corresponding parent compounds; estragole derivatives were two- to three-fold more potent than safrole derivatives; 1-hydroxyelemicin and l-oxoestragole induced weak but statistically significant hepatocarcinogenic responses; 1-propenyl analogs anethole, 3′-hydroxy-*trans*-anethole, isosafrole, and *trans*-cinnamaldehyde did not induce hepatic tumors, but *cis*- and *trans*-asarone were active; pentachlorophenol, a sulfotransferase inhibitor, reduced the hepatocarcinogenic response of estragole but not that of *cis*- or *trans*-asarone, indicating likely activation of asarone by epoxidation.

Eugenol 0, 3,000 or 6,000 ppm in feed for 2 years induced a weak response of hepatic tumors in male and female B6C3F1 mice, but no tumors in F344/N rats (NTP, 1983). In contrast, administration of methyleugenol to F344/N rats and B6C3F1 mice by gavage for 2 years induced liver neoplasms in both sexes of rats and mice, neuroendocrine tumors of the glandular stomach in rats and male mice, as well as kidney neoplasms, mesotheliomas, mammary gland fibroadenomas, and subcutaneous fibromas and fibrosarcomas in male rats (NTP, 2000). Methyleugenol increased the incidence of fundic mucosal atrophy, neuroendocrine hyperplasia, and benign and malignant neuroendocrine tumors of the glandular stomach (Johnson *et al.*, 2000; NTP, 2000). A possible explanation for this finding involves a cytotoxic loss of parietal cells, resulting in mucosal atrophy, decreased gastric secretion (hypochlorhydria), increased intragastric pH, and increased serum gastrin (hypergastrinemia). An increase in stomach pH leads to gastrin production. Thake *et al*. (1995) showed that long-term inhibition of gastric acid secretion induces enterochromaffin-like cell tumors. Thus, increased pH and gastrin secretion may stimulate hyperplasia of neuroendocrine cells, which may in turn ultimately lead to tumor formation. In light of the methyleugenol study results, intragastric pH and serum gastrin were measured during the subchronic isoeugenol rat study (Appendix K).

Most recently, carcinogenic activity of estragole has been observed in rats. Administration of 600 mg estragole/kg body weight in corn oil by gavage for 3 months induced hepatic cholangiocarcinomas and hepatocellular adenoma in male F344/N rats (NTP, 2010).

Genetic Toxicity

The available mutagenicity data for isoeugenol were reviewed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (WHO, 2004); the test results provided little evidence for isoeugenol mutagenicity. Briefly, isoeugenol was not mutagenic in any of several tester strains of *Salmonella typhimurium* or *Escherichia coli* strain WP2, with or without liver S9 activation enzymes (Douglas *et al.*, 1980; Florin *et al.*, 1980; Sekizawa and Shibamoto, 1982; Mortelmans *et al.*, 1986). However, positive results were reported for isoeugenol in the *Bacillus subtilis* DNA-repair test (Rec assay) conducted in the absence of S9 activation enzymes and using ethanol as the solvent (Sekizawa and Shibamoto, 1982); due to the variable growth rates observed in the *B. subtilis* tester strains in this Rec assay, the test results were judged to be inconclusive (WHO, 2004). Furthermore, negative results were reported in the *B. subtilis* Rec assay for isoeugenol when dimethyl sulfoxide was used as the solvent (Sekizawa and Shibamoto, 1982).

Isoeugenol did not induce chromatid breaks or sister chromatid exchanges in cultured Chinese hamster ovary cells exposed to isoeugenol concentrations over a range of 1.6 to 16 µg/mL in the absence of S9 (Sasaki *et al.*, 1989), but it was reported to induce sister chromatid

exchanges (indicators of DNA damage) in human lymphocytes treated with 82 µg/mL isoeugenol *in vitro* (Jansson *et al.*, 1986).

No induction of unscheduled DNA synthesis was observed after treatment of cultured primary hepatocytes obtained from B6C3F1 mice or F344 rats with 164 µg/mL isoeugenol (Burkey *et al.*, 2000).

Study Rationale

Isoeugenol was nominated by the National Cancer Institute and was selected for carcinogenicity testing because of widespread human exposure through its use as a flavoring and fragrance agent and because of its structural similarity to phenylpropenoid compounds such as safrole, isosafrole, methyleugenol, estragole, and anethole, some of which are carcinogenic. The oral route of exposure was chosen because it is the major route of human exposure, and gavage was chosen after preliminary studies showed that isoeugenol in feed was unpalatable to both rats and mice and the concentration in feed decreased when stored at room temperature. These effects were attributed to the relatively high vapor pressure of isoeugenol. Furthermore, most previous studies of phenylpropenoid analogs had been conducted by gavage or dosed feed routes of exposure. In commerce, isoeugenol is approximately a 1:7 mixture of *Z* and *E* isomers, so that was the form tested by the NTP.

MATERIALS AND METHODS

Procurement and Characterization

Isoeugenol

Isoeugenol was obtained from Penta International Corporation (Livingston, NJ) in one lot (60449) that was used in the 3-month and 2-year studies. Identity and purity analyses were performed by the analytical chemistry laboratory, Battelle Columbus Operations (Chemistry Support Services, Columbus, OH) and the study laboratory, Battelle Columbus Operations (Columbus, OH); Karl Fischer titration and elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN) (Appendix H). Reports on analyses performed in support of the isoeugenol studies are on file at the National Toxicology Program Archives.

The chemical, a yellow liquid, was identified as isoeugenol by the analytical chemistry laboratory using infrared spectral analysis and by both proton and carbon-13 nuclear magnetic resonance spectroscopy. The study laboratory confirmed the identity of the test article by infrared spectroscopy. The purity of lot 60449 was determined by the analytical chemistry and study laboratories using gas chromatography (GC) and by the analytical chemistry laboratory using high performance liquid chromatography (HPLC). Elemental analyses for carbon and hydrogen were in agreement with the theoretical values for isoeugenol. Karl Fischer titration indicated 0.57% water. GC by one system indicated one major peak and four smaller peaks. The major peak, 87% of the total peak area, was determined to be *E*-isoeugenol, and the second largest peak, 12% of the total peak area, was determined to be *Z*-isoeugenol. The identity of three minor peaks with a combined area of approximately 1% of the total area was not determined. HPLC indicated one major peak, believed to be the coelution of *Z*- and *E*-isoeugenol, and one impurity with an area of 0.5% relative to the total peak area. In agreement with the manufacturer, the overall purity of lot 60449 was determined to be 99% or greater.

To ensure stability, the bulk chemical was stored at or below –20° C, protected from light, in 1-L Teflon® bottles. The study laboratory monitored stability during the 3-month and 2-year studies by periodic analysis using GC. No degradation of the bulk chemical was detected. Isoeugenol dimers occur naturally in plants as lignans. They are also formed by photooxidation at or above room temperature when exposed to ultraviolet light (Dellagreca *et al.*, 2008). Isoeugenol dimers exhibit anti-inflammatory, potential anticancer, and skin sensitizing activities (Fujisawa *et al.*, 2002; Li and Yang, 2008; Takeyoshi *et al.*, 2008). The initial 99% purity of the isoeugenol used in these NTP studies was monitored by performing periodic GC analysis of the bulk chemical during the 3-month and 2-year studies. The periodic analysis utilized an internal standard to quantify purity, and results were compared with those obtained from concurrent analysis of a reference sample that had been prepared upon receipt and stored at or below –20° C (Appendix H). Accordingly, the series of stability analyses would detect any decrease in isoeugenol purity over time, whether or not isoeugenol dimers or other conversion products themselves were detected. The results of these stability analyses showed that isoeugenol purity remained unchanged.

Corn Oil

Corn oil was obtained in multiple lots from Spectrum Chemicals and Laboratory Products (Gardena, CA) for use during the 3-month and 2-year studies. The study laboratory determined peroxide levels prior to first use and every 2 months during the studies by potentiometric titration; all peroxide concentrations were below the acceptable limit of 3 mEq/kg.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared by mixing isoeugenol with corn oil to give the required concentrations (Table H1) and stored at room temperature in amber glass bottles with Teflon®-lined lids for up to 35 days. Homogeneity studies of 0.2 and 120 mg/mL formulations and stability studies of the 0.2 mg/mL formulation were performed by the analytical chemistry laboratory using GC on a different lot (46928) of isoeugenol obtained from Penta International Corporation. Homogeneity was confirmed, and the 120 mg/mL dose formulation was found to be suitable for gavage. Stability was confirmed for up to 35 days for dose formulations stored in amber glass bottles with Teflon®-lined lids at –20° C, 5° C, and room temperature, as well as for 3 hours under simulated animal room conditions.

Periodic analyses of the dose formulations of isoeugenol were conducted by the study laboratory using GC. During the 3-month studies, the dose formulations were analyzed three times; animal room samples were also analyzed. All 15 dose formulations analyzed for rats and mice were within 10% of the target concentrations; all 15 animal room samples for rats and 14 of 15 for mice were within 10% of the target concentrations (Table H2). During the 2-year studies, the dose formulations were analyzed approximately every 3 months; animal room samples were also analyzed (Table H3). All 27 dose formulations for rats and 27 of 28 for mice were within 10% of the target concentrations. All nine animal room samples analyzed for rats and mice were within 10% of the target concentrations.

3-Month Studies

The 3-month studies were conducted to evaluate the cumulative toxic effects of repeated exposure to isoeugenol and to determine appropriate doses for the 2-year studies.

Male and female F344/N rats and B6C3F1 mice were obtained from Taconic Farms, Inc. (Germantown, NY). On receipt, the rats and mice were 4 to 5 weeks old. Animals were quarantined for 11 to 14 days and were 6 to 7 weeks old on the first day of the studies. Before the studies began, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. At 1 month and at the end of the studies, serologic analyses were performed on five male and five female sentinel rats and mice using the protocols of the NTP Sentinel Animal Program (Appendix J).

Groups of 10 male and 10 female rats and mice were exposed to isoeugenol in corn oil by gavage at doses of 0, 37.5, 75, 150, 300, or 600 mg/kg, 5 days per week for 14 weeks. Additional special study groups of 10 male and 10 female rats received the same doses for 31 days. Feed and water were available *ad libitum*, except special study rats were fasted for 24 hours prior to blood collection on day 31. Rats and female mice were housed five per cage; male mice were housed individually. Animals were weighed and clinical observations were recorded initially, weekly, and at the end of the exposure phase. Details about materials and methods used in the study are summarized in Table 2.

Animals were anesthetized with carbon dioxide, and blood was collected from the retroorbital sinus of special study rats on days 4 and 23 and from core study rats and mice on day 93 for hematology and clinical chemistry (rats only) analysis. Blood samples for hematology analyses were placed in tubes containing potassium EDTA. Erythrocyte, leukocyte, and platelet counts; hemoglobin concentrations; mean cell volume; mean cell hemoglobin; and mean cell hemoglobin concentration were determined using a Cell-Dyn 3500 (Abbott Diagnostics, Abbott Park, IL). Manual hematocrit values were determined by microhematocrit centrifugation techniques. Blood samples for clinical chemistry analyses were placed in tubes containing separator gel and allowed to clot. After clot retraction occurred, the samples were centrifuged, and the serum was aliquoted for assay of serum chemistry analytes using a Hitachi 911 chemistry analyzer (Boehringer Mannheim, Indianapolis, IN). Table 2 lists the parameters measured.

On day 31, blood was collected from the retroorbital sinus of special study rats. Serum was separated and stored at –70° C until analysis. Serum gastrin levels were determined by radioimmunoassay (American Laboratory Products Company, Windham, NH). Following blood collection, special study rats were euthanized with carbon dioxide and the stomach was isolated for pH determination. The duodenum distal to the pylorus was incised; a Model Number PHR-146 pH electrode (Jenco Instruments, Inc., San Diego, CA) was inserted, and a Model Number 6250 pH meter (Jenco Instruments) was used to measure stomach pH. After recording the pH, the stomach was fixed, embedded, and stained for histopathologic examination.

In addition to stomach, liver samples were collected from special study rats, weighed, and stored at –70° C for cytochrome P450 determinations. Microsomal suspensions were prepared using the Pearce method (Pearce *et al.*, 1996). The concentration of protein in each suspension was determined using the microtiter plate method of the Coomassie® Plus Protein Assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard. Cytochrome P450 CYP1A1-associated 7-ethoxyresorufin-*O*-deethylase (EROD), CYP1A2-associated acetanilide-4-hydroxylase (A4H), and CYP2B-associated 7-pentoxyresorufin-*O*-deethylase (PROD) activities were determined in microsomal proteins. Data were reported as pmol/minute per mg microsomal protein (EROD and PROD) or nmol/minute per mg microsomal protein (A4H).

Necropsies were performed on all core study animals. The heart, right kidney, liver, lungs, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (eyes were fixed in Davidson's solution for up to 72 hours and then transferred to 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 vehicle control and 600 mg/kg rats and mice; tissues were examined to a no-effect level in the remaining dosed groups. Table 2 lists the tissues and organs routinely examined. After a review of the laboratory report and selected histopathology slides by a quality assessment pathologist, the findings and reviewed slides were submitted to the NTP Pathology Working Group (PWG) coordinator for a second independent review. Any inconsistencies in the diagnoses made by the laboratory and quality assessment pathologists were resolved. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, reviewing pathologist(s), and the PWG coordinator. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al*. (1985).

2-Year Studies

Study Design

Groups of 50 male and 50 female rats and mice were exposed to isoeugenol in corn oil by gavage at doses of 0, 75, 150, or 300 mg/kg, 5 days per week for 104 (female mice) or 105 (rats and male mice) weeks.

Source and Specification of Animals

Male and female F344/N rats and B6C3F1 mice were obtained from Taconic Farms, Inc. (Germantown, NY), for use in the 2-year studies. On receipt, the rats and mice were approximately 4 weeks old. Rats and mice were quarantined for 11 to 14 days before the beginning of the studies. Five male and five female rats and mice were randomly selected for parasite evaluation and gross observation of disease. Rats and mice were approximately 5 to 7 weeks old at the beginning of the studies. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix J).

Animal Maintenance

Rats were housed three (males) or five (females) per cage, and mice were housed individually (males) or five (females) per cage. Feed and water were available *ad libitum*. Cages were changed at least weekly (male mice) or twice weekly (rats and female mice); racks were changed every 2 weeks. Further details of animal maintenance are given in Table 2. Information on feed composition and contaminants is provided in Appendix I.

Clinical Examinations and Pathology

All animals were observed twice daily. Clinical findings were recorded during week 5, every 4 weeks thereafter, and at the end of the exposure phase. Rats and mice were weighed initially, weekly for the first 13 weeks, every 4 weeks thereafter, and at the end of the exposure phase.

Complete necropsies and microscopic examinations were performed on all rats and mice. At necropsy, all organs and tissues were examined for grossly visible lesions and all major tissues were fixed and preserved in 10% neutral buffered formalin (eyes were fixed in Davidson's solution for up to 72 hours and then transferred to 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 for microscopic examination. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. Tissues examined microscopically are listed in Table 2.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. 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 evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy; the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist evaluated slides from all tumors and all potential target organs, which included the glandular stomach and nose of rats and mice, pancreas of rats, liver of male rats and male and female mice, preputial gland of male rats, forestomach of mice, and kidney of female mice.

The quality assessment report and the reviewed slides were submitted to the NTP Pathology Working Group (PWG) coordinator, who reviewed the selected tissues and addressed any inconsistencies in the diagnoses made by the laboratory and quality assessment pathologists. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologists, or lesions of general interest were presented by the coordinator to the PWG for review. The PWG consisted of the quality assessment pathologist and other pathologists experienced in rodent toxicologic pathology. This group examined the tissues without any knowledge of dose groups or previously rendered diagnoses. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, reviewing pathologist(s), and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). For subsequent analyses of the pathology data, the decision of whether to evaluate the diagnosed lesions for each tissue type separately or combined was generally based on the guidelines of McConnell *et al.* (1986).

Table 2
Materials and Methods in the Gavage Studies of Isoeugenol

	3-Month Study	2-Year Studies
Study Laboratory	Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)
Strain and Species	F344/N rats B6C3F1 mice	F344/N rats B6C3F1 mice
Animal Source	Taconic Farms, Inc. (Germantown, NY)	Taconic Farms, Inc. (Germantown, NY)
Time Held Before Studies	Rats: 11 (males) or 12 (females) days Mice: 13 (females) or 14 (males) days	Rats: 11 (males) or 12 (females) days Mice: 13 (females) or 14 (males) days
Average Age When Studies Began	6 to 7 weeks	5 to 7 weeks
Date of First Dose	Rats: April 16 (males) or 17 (females), 2001 Mice: April 18 (females) or 19 (males), 2001	Rats: April 22 (males) or 23 (females), 2002 Mice: May 8 (females) or 9 (males), 2002
Duration of Dosing	5 days/week for 14 weeks	5 days/week for 104 (female mice) or 105 (rats and male mice) weeks
Date of Last Dose	Rats: July 16 (males) or 17 (females), 2001 Mice: July 18 (females) or 19 (males), 2001	Rats: April 20 (males) or 22 (females), 2004 Mice: May 4 (females) or 6 (males), 2004
Necropsy Dates	Rats: July 17 (males) or 18 (females), 2001 Mice: July 19 (females) or 20 (males), 2001	Rats: April 19-21 (males) or 21-23 (females), 2004 Mice: May 3-5 (females) or 5-7 (males), 2004
Average Age at Necropsy	19 to 20 weeks	110 weeks

Table 2
Materials and Methods in the Gavage Studies of Isoeugenol

	3-Month Study	2-Year Studies
Size of Study Groups	Rats: 10 males and 10 females (core study) 10 males and 10 females (special study) Mice: 10 males and 10 females	50 males and 50 females
Method of Distribution	Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 3-month studies
Animals per Cage	Rats: 5 Mice: 1 (males) or 5 (females)	Rats: 3 (males) or 5 (females) Mice: 1 (males) or 5 (females)
Method of Animal Identification	Tail tattoo	Tail tattoo
Diet	NTP-2000 irradiated wafer or pelleted diet (Zeigler Brothers, Inc., Gardners, PA); available *ad libitum* (except special study rats fasted 24 hours before day 31 blood collection) changed weekly	Same as 3-month studies
Water	Tap water (Columbus, OH, municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*	Same as 3-month studies
Cages	Polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly (male mice) or twice weekly (rats and female mice)	Same as 3-month studies
Bedding	Irradiated Sani-Chips® (P.J. Murphy Forest Products Corporation, Montville, NJ), changed weekly (male mice) or twice weekly (rats and female mice)	Same as 3-month studies
Rack Filters	Spun-bonded polyester (Snow Filtration Company, Cincinnati, OH), changed every 2 weeks	Same as 3-month studies
Racks	Stainless steel (Lab Products, Inc., Seaford, DE), changed every 2 weeks	Same as 3-month studies
Animal Room Environment	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: ≥ 10/hour	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: ≥ 10/hour
Doses	0, 37.5, 75, 150, 300, and 600 mg/kg (dosing volumes = 5 mL/kg body weight for rats or 10 mL/kg for mice)	0, 75, 150, and 300 mg/kg (dosing volumes = 5 mL/kg body weight for rats or 10 mL/kg for mice)
Type and Frequency of Observation	Observed twice daily; animals were weighed initially, then weekly, and at the end of the exposure phase; clinical findings for core study animals were recorded initially, weekly, and at the end of the exposure phase.	Observed twice daily; animals were weighed initially, weekly for the first 13 weeks, every 4 weeks thereafter, and at the end of the exposure phase; clinical findings were recorded during week 5, every 4 weeks thereafter, and at the end of the exposure phase.
Method of Sacrifice	Carbon dioxide asphyxiation	Same as 3-month studies
Necropsy	Necropsies were performed on all animals. Organs weighed were heart, right kidney, liver, lung, right testis, and thymus.	Necropsies were performed on all animals.

Table 2
Materials and Methods in the Gavage Studies of Isoeugenol

	3-Month Study	2-Year Studies
Clinical Pathology	Blood was collected from the retroorbital sinus of clinical pathology rats on days 4 and 23 and from core study rats and mice on day 93 of exposure for hematology and clinical chemistry (rats only). ***Hematology:*** hematocrit; hemoglobin; erythrocyte, reticulocyte, nucleated erythrocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte counts and differentials. ***Clinical chemistry:*** urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and bile acids	None
Histopathology	Complete histopathology was performed on core study vehicle control and 600 mg/kg rats and mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eyes, gallbladder (mice), Harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung (with mainstem bronchus), 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, and uterus. Tissues were examined in the remaining dosed groups to a no-effect level.	Complete histopathology was performed on all rats and mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eyes, gallbladder (mice), Harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, larynx, liver, lung (with mainstem bronchus), 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, and uterus.
Special Study	On day 31, blood was collected from the retroorbital sinus of special study rats for serum gastrin analysis; the stomach was isolated for pH determination, and liver samples were taken for determinations of hepatic tissue mass, microsomal protein, acetanilide-4-hydroxylase, 7-ethoxyresorufin-*O*-deethylase, and 7-pentoxyresorufin-*O*-deethylase. Stomachs from all animals were evaluated microscopically. See Appendix K.	None
Toxicokinetic Study	Groups of 21 male and female rats received a single intravenous injection of 17 mg isoeugenol/kg body weight or a single gavage dose of 17, 70, or 140 mg/kg. Groups of 42 male and female mice received a single intravenous injection of 35 mg/kg or a single gavage dose of 35, 70, or 140 mg/kg. After dosing, animals were anesthetized and blood was collected from the retroorbital sinus of rats and by cardiac puncture from mice. Plasma was analyzed using gas chromatography and mass spectrometry (Appendix L).	None

Statistical Methods

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation of Incidence

The incidences of neoplastic or nonneoplastic lesions are presented in Tables A1, A4, B1, B3, C1, C4, D1, and D4 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A2, B2, C2, and D2) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., Harderian gland, intestine, mammary gland, and skin) before microscopic evaluation, the denominators consist of the number of animals that had a gross abnormality. When neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed. Tables A2, B2, C2, and D2 also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm. This survival-adjusted rate (based on the Poly-3 method described below) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, only to site-specific, lesion-free animals that do not reach terminal sacrifice.

Analysis of Neoplastic and Nonneoplastic Lesion Incidences

The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997) was used to assess neoplasm and nonneoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to take survival differences into account. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk. For analysis of a given site, each animal is assigned a risk weight. This value is one if the animal had a lesion at that site or if it survived until terminal sacrifice; if the animal died prior to terminal sacrifice and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the kth power.

This method yields a lesion prevalence rate that depends only upon the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time (Bailer and Portier, 1988). Unless otherwise specified, a value of k=3 was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier (1988) following an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control F344 rats and B6C3F1 mice (Portier *et al.,* 1986). Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams (1993).

Tests of significance included pairwise comparisons of each dosed group with controls and a test for an overall dose-related trend. Continuity-corrected Poly-3 tests were used in the analysis of lesion incidence, and reported P values are one sided. The significance of lower incidences or decreasing trends in lesions is represented as 1– P with the letter N added (e.g., P = 0.99 is presented as P = 0.01N).

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and 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, serum gastrin, stomach pH, and cytochrome P450 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 the 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.

Historical Control Data

The concurrent control group represents the most valid comparison to the treated groups and is the only control group analyzed statistically in NTP bioassays. However, historical control data are often helpful in interpreting potential exposure-related effects, particularly for uncommon or rare neoplasm types. For meaningful comparisons, the conditions for studies in the historical database must be generally similar. One significant factor affecting the background incidence of neoplasms at a variety of sites is diet. In 1995, the NTP incorporated a new diet (NTP-2000) that contains less protein and more fiber and fat than the NIH-07 diet previously used in toxicity and carcinogenicity studies (Rao, 1996, 1997). The current NTP historical database contains all studies that use the NTP-2000 diet with histopathology findings completed within the most recent 5-year period. A second potential source of variability is route of administration. In general, the historical database for a given study will include studies using the same route of administration, and the overall incidences of neoplasms for all routes of administration are included for comparison, including the present study.

Quality Assurance Methods

The 3-month and 2-year studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 2-year studies were submitted to the NTP Archives, the corresponding laboratory reports were audited retrospectively by an independent quality assurance contractor. A separate audit covered completeness and accuracy of the changes to diagnoses incorporated into final pathology tables. This NTP Technical Report was audited before publication. Audit procedures and findings are presented in reports that are on file at NIEHS.

Genetic Toxicology

The genetic toxicity of isoeugenol was assessed by testing the ability of the chemical to induce mutations in various strains of *Salmonella typhimurium* and *Escherichia coli*, chromosomal aberrations in cultured Chinese hamster ovary cells, and increases in the frequency of micronucleated erythrocytes in mouse peripheral blood. Micronuclei (literally "small nuclei" or Howell-Jolly bodies) are biomarkers of induced structural or numerical chromosomal alterations and are formed when acentric fragments or whole chromosomes fail to incorporate into either of two daughter nuclei during cell division (Schmid, 1975; Heddle *et al.*, 1983). The protocols for these studies and the results are given in Appendix E.

The genetic toxicity studies have evolved from an earlier effort by the NTP to develop a comprehensive database permitting a critical anticipation of a chemical's carcinogenicity in experimental animals based on numerous considerations, including the molecular structure of the chemical and its observed effects in short-term *in vitro* and *in vivo* genetic toxicity tests (structure-activity relationships). The short-term tests were originally developed to clarify proposed mechanisms of chemical-induced DNA damage based on the relationship between electrophilicity and mutagenicity (Miller and Miller, 1977) and the somatic mutation theory of cancer (Straus, 1981; Crawford, 1985). However, it should be noted that not all cancers arise through genotoxic mechanisms.

DNA reactivity combined with *Salmonella* mutagenicity is highly correlated with induction of carcinogenicity in multiple species/sexes of rodents and at multiple tissue sites (Ashby and Tennant, 1991). A positive response in the *Salmonella* test was shown to be the most predictive *in vitro* indicator for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens) (Tennant *et al.,* 1987; Zeiger *et al.,* 1990). Additionally, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. However, these other tests can provide useful information on the types of DNA and chromosomal damage induced by the chemical under investigation.

The predictivity for carcinogenicity of a positive response in acute *in vivo* bone marrow chromosome aberration or micronucleus tests appears to be less than

that in the *Salmonella* test (Shelby *et al.,* 1993; Shelby and Witt, 1995). However, clearly positive results in long-term peripheral blood micronucleus tests have high predictivity for rodent carcinogenicity; a positive result in one sex or negative results in both sexes tested in this assay do not correlate well with either negative or positive results in rodent carcinogenicity studies (Witt *et al.*, 2000). Because of the theoretical and observed associations between induced genetic damage and adverse effects in somatic and germ cells, the determination of *in vivo* genetic effects is important to the overall understanding of the risks associated with exposure to a particular chemical. Most organic chemicals that are identified by the International Agency for Research on Cancer as human carcinogens, other than hormones, are genotoxic. The vast majority of these are detected by both the *Salmonella* assay and rodent bone marrow cytogenetics tests (Shelby, 1988; Shelby and Zeiger, 1990).

RESULTS

3-Month Study in Rats

Dosing accidents resulted in the early death of one 600 mg/kg male and one 37.5 mg/kg female (Table 3). Decreases in mean body weights and body weight gains of all dosed groups of males were statistically significant compared to those of the vehicle controls; however, only the decrease in the 600 mg/kg group was clearly related to isoeugenol exposure. Mean body weights of dosed females were similar to that of the vehicle control group. No clinical findings related to isoeugenol exposure were observed.

Results from the hematology and clinical chemistry analyses for rats are listed in Table F1. Minor changes occurred throughout the hematology and clinical chemistry variables in the rats. All changes were within physiological normal levels, and in general, there was no evidence of a dose relationship; they were not considered biologically important or toxicologically relevant.

Absolute and relative liver weights were significantly increased in 300 and 600 mg/kg female rats as were kidney weights in 600 mg/kg female rats (Table G1). No other changes in organ weights of male or female rats were related to isoeugenol exposure.

Incidences of minimal atrophy of the olfactory epithelium were increased in all exposed groups and were significantly increased in males administered 150 mg/kg or greater and females administered 300 or 600 mg/kg (Table 4). Minimal to mild atrophy of olfactory nerve

Table 3
Survival and Body Weights of Rats in the 3-Month Gavage Study of Isoeugenol

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight[c] Relative to Controls (%)
		Initial	Terminal	Change	
Male					
0	10/10	98 ± 3	352 ± 8	253 ± 9	
37.5	10/10	95 ± 2	325 ± 3*	229 ± 3*	92
75	10/10	97 ± 3	334 ± 6*	238 ± 5*	95
150	10/10	98 ± 3	336 ± 7*	238 ± 5*	95
300	10/10	96 ± 3	326 ± 6**	230 ± 7*	93
600	9/10[c]	95 ± 3	307 ± 7**	212 ± 7**	87
Female					
0	10/10	86 ± 2	188 ± 4	102 ± 5	
37.5	9/10[d]	86 ± 2	188 ± 3	101 ± 3	100
75	10/10	87 ± 2	192 ± 4	106 ± 4	102
150	10/10	87 ± 2	184 ± 2	96 ± 2	97
300	10/10	86 ± 2	193 ± 3	107 ± 3	103
600	10/10	87 ± 2	189 ± 4	103 ± 4	100

* Significantly different (P≤0.05) from the vehicle control group by Williams' test
** P≤0.01
[a] Number of animals surviving at 3 months/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Day 85
[d] Week of death: 4
[e] Week of death: 3

bundles was observed in all exposed groups of males and in females exposed to 150 mg/kg or greater; the incidence was significantly increased in 600 mg/kg females. Olfactory epithelial atrophy was characterized by loss of cilia, altered orientation of affected cells, and decreased numbers of epithelial cells that resulted in thinning of the olfactory epithelium. The regions affected included the ventral nasal septum at Level III and less frequently along the junction of the nasal septum with the dorsal wall of the nasal meatus in the middle nasal section, Level II. In a few males, atrophy of the olfactory epithelium was accompanied by chronic active inflammation. Glands under the affected olfactory epithelium were unremarkable. Atrophy of olfactory nerve bundles was observed in areas beneath the atrophic epithelium. Nerve atrophy with concomitant reductions in the number and size of nerve bundles was considered secondary to the atrophy of the overlying olfactory epithelium.

Incidences of minimal to mild periportal hepatocellular cytoplasmic alteration were significantly increased in females exposed to 300 or 600 mg/kg (Table 4). The liver alteration consisted of decreased eosinophilic cytoplasmic staining with increased microvacuolation and accentuated basophilic granulation of periportal hepatocytes, similar to what is commonly associated with glycogen depletion.

The special study results for serum gastrin and stomach pH assays are listed in Table K1. The only effect attributed to exposure was a small but significant decrease in stomach pH of female rats exposed to 150 mg/kg or greater. Microscopic examination of stomachs from special study rats exposed for 31 days did not show any isoeugenol-related changes (Table K3). Results of cytochrome P450 activities in hepatic microsomes from rats exposed to isoeugenol for 31 days are summarized in Table K2. These parameters were unaltered in exposed females. In male rats, dose-related decreases to 70% of EROD (CYP1A1) activity and to 72% of PROD (CYP2B) activity were observed in groups exposed to 75 mg/kg or greater. Although a small decrease in acetanilide-4-hydroxylase (A4H) (CYP1A2) activity in the 600 mg/kg male group was statistically significant, the response across groups did not correlate with dose.

Dose Selection Rationale: Rats administered isoeugenol in corn oil by gavage for 3 months showed minimal toxicity. The olfactory epithelium was the most prominent site of toxicity in both males and females. In females, liver cytoplasmic alterations were noted as well in the 300 and 600 mg/kg groups. These nasal and liver lesions were not considered to affect survival in the subsequent 2-year study; however, the importance of olfaction in rodent feeding behavior increased concern that they might affect body weight gain. Because effects were

TABLE 4
Incidences of Selected Nonneoplastic Lesions in the 3-Month Gavage Study of Isoeugenol in Rats

	Vehicle Control	37.5 mg/kg		75 mg/kg		150 mg/kg		300 mg/kg		600 mg/kg	
Male											
Nose[a]	10	10		10		10		10		10	
Olfactory Epithelium, Atrophy[b]	0	3	(1.0)[c]	3	(1.0)	4*	(1.0)	4*	(1.0)	5*	(2.0)
Nerve, Atrophy	0	1	(1.0)	1	(1.0)	3	(1.0)	2	(1.0)	3	(1.3)
Female											
Nose	10	10		10		10		10		10	
Olfactory Epithelium, Atrophy	0	1	(1.0)	2	(1.0)	2	(1.0)	5*	(1.0)	6**	(1.0)
Nerve, Atrophy	0	0		0		2	(1.0)	2	(1.0)	5*	(1.0)
Liver	10	10		10		10		10		10	
Periportal, Cytoplasmic Alteration	0	0		0		0		10**	(1.0)	10**	(1.4)

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

generally similar in magnitude in the 300 and 600 mg/kg groups of both sexes, 300 mg/kg was selected as the highest dose for the 2-year study. The dosing regimen for the 2-year study of isoeugenol in rats was 0, 75, 150, and 300 mg/kg.

2-Year Study in Rats

Survival

Estimates of 2-year survival probabilities for male and female rats are shown in Table 5 and in the Kaplan-Meier survival curves (Figure 2). Survival rates of dosed male and female rats were similar to those of vehicle controls.

Body Weights and Clinical Findings

The mean body weight of 300 mg/kg males was greater than that of the vehicle controls after week 64 of exposure, and their final mean body weight was 9% greater than that of the vehicle controls. The mean body weights of all other exposed groups were similar to those of the vehicle control groups throughout the study (Tables 6 and 7; Figure 3). No clinical findings related to the administration of isoeugenol were observed.

Table 5
Survival of Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Male				
Animals initially in study	50	50	50	50
Accidental deaths[a]	0	1	0	2
Moribund	14	9	14	11
Natural deaths	1	6	3	7
Animals surviving to study termination	35	34	33	30
Percent probability of survival at end of study[b]	70	69	66	63
Mean survival (days)[c]	702	685	660	676
Survival analysis[d]	P = 0.387	P = 1.000	P = 0.659	P = 0.501
Female				
Animals initially in study	50	50	50	50
Accidental death[a]	0	1	0	0
Moribund	15	8	9	9
Natural deaths	2	6	7	10
Animals surviving to study termination	33	35	34	31
Percent probability of survival at end of study	66	72	68	62
Mean survival (days)	699	674	693	640
Survival analysis	P = 0.360	P = 0.767N	P = 1.000N	P = 0.523

[a] Censored from survival analyses
[b] Kaplan-Meier determinations
[c] Mean of all deaths (uncensored, censored, and terminal sacrifice).
[d] The result of the life table trend test (Tarone, 1975) is in the vehicle control column, and the result of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. A lower mortality in a dosed group is indicated by **N**.

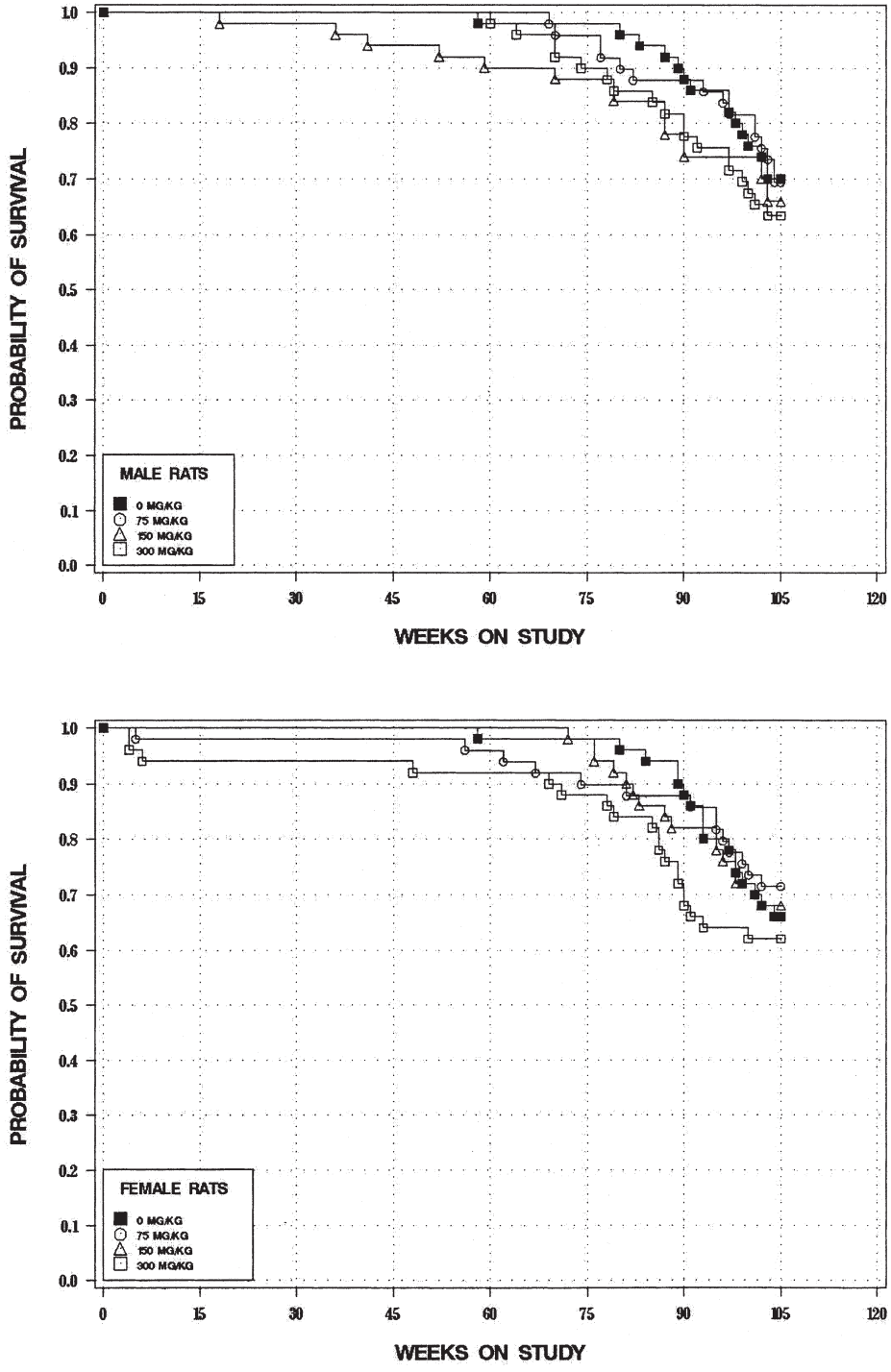


FIGURE 2
Kaplan-Meier Survival Curves for Male and Female Rats Administered Isoeugenol by Gavage for 2 Years

TABLE 6
Mean Body Weights and Survival of Male Rats in the 2-Year Gavage Study of Isoeugenol

Days on Study	Vehicle Control		75 mg/kg			150 mg/kg			300 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	94	50	94	100	50	94	100	50	94	100	50
8	126	50	127	101	50	126	100	50	126	100	50
15	158	50	161	102	50	159	101	50	159	100	50
22	192	50	196	102	49	195	101	50	194	101	50
29	219	50	223	102	49	221	101	50	217	100	50
36	242	50	246	102	49	244	101	50	241	100	50
43	258	50	262	102	49	261	101	50	256	99	50
50	272	50	276	102	49	272	100	50	270	99	50
57	286	50	289	101	49	284	99	50	282	99	50
64	302	50	304	101	49	299	99	50	297	98	50
71	314	50	317	101	49	310	99	50	310	99	50
78	327	50	330	101	49	323	99	50	324	99	50
85	338	50	341	101	49	336	99	50	332	98	50
113	373	50	377	101	49	371	100	50	371	100	50
141	395	50	396	100	49	390	99	49	388	98	50
169	419	50	422	101	49	419	100	49	417	100	50
197	432	50	436	101	49	434	100	49	433	100	50
225	443	50	444	100	49	445	100	49	453	102	50
253	455	50	456	100	49	461	101	48	462	102	50
281	468	50	465	100	49	470	101	48	472	101	50
309	476	50	475	100	49	480	101	47	483	101	50
337	486	50	484	100	49	491	101	47	492	101	50
365	498	50	493	99	49	503	101	46	506	102	50
393	506	50	501	99	49	506	100	46	507	100	50
421	514	49	509	99	49	514	100	45	515	100	49
449	517	49	510	99	49	518	100	45	528	102	48
477	518	49	513	99	48	521	101	45	531	103	48
505	522	49	510	98	47	520	100	44	536	103	45
533	521	49	513	98	45	521	100	44	541	104	44
561	518	48	510	98	44	523	101	42	542	105	42
589	514	47	502	98	43	515	100	42	538	105	42
617	504	45	499	99	43	517	103	39	535	106	40
645	501	43	494	99	43	514	103	37	539	108	37
673	491	43	492	100	41	508	104	37	535	109	37
701	495	38	492	99	39	502	101	37	541	109	32
Mean for weeks											
1-13	241		244	101		240	100		239	99	
14-52	438		439	100		440	100		441	101	
53-101	509		503	99		514	101		530	104	

TABLE 7
Mean Body Weights and Survival of Female Rats in the 2-Year Gavage Study of Isoeugenol

Days on Study	Vehicle Control		75 mg/kg			150 mg/kg			300 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	91	50	91	100	50	90	99	50	90	99	50
8	111	50	110	99	50	109	98	50	110	99	50
15	128	50	127	99	50	126	98	50	128	100	50
22	141	50	140	100	50	139	99	50	142	100	50
29	152	50	152	100	50	152	100	50	152	100	48
36	164	50	162	99	49	161	98	50	162	99	48
43	169	50	169	100	49	168	99	50	168	99	47
50	176	50	175	100	49	173	99	50	173	98	47
57	181	50	181	100	49	179	99	50	179	99	47
64	183	50	183	100	49	182	100	50	182	99	47
71	186	50	190	102	49	188	101	50	187	100	47
78	192	50	193	101	49	192	100	50	192	100	47
85	198	50	198	100	49	197	100	50	196	99	47
113	208	50	209	100	49	207	100	50	208	100	47
141	215	50	216	100	49	215	100	50	214	100	47
169	228	50	228	100	49	224	98	50	223	98	47
197	231	50	233	101	49	229	99	50	230	100	47
225	237	50	239	101	49	235	99	50	237	100	47
253	245	50	246	100	49	243	99	50	242	99	47
281	252	50	253	100	49	249	99	50	246	98	47
309	261	50	260	100	49	256	98	50	254	97	47
337	266	50	268	101	49	265	100	50	261	98	46
365	279	50	279	100	49	274	98	50	271	97	46
393	288	50	287	100	47	286	99	50	278	96	46
421	298	49	298	100	47	297	100	50	289	97	46
449	309	49	307	99	46	305	99	50	298	96	46
477	314	49	310	99	45	312	99	50	303	96	46
505	322	49	318	99	45	318	99	49	313	97	44
533	329	49	323	98	44	323	98	47	318	97	44
561	330	48	325	99	44	326	99	46	320	97	42
589	330	47	323	98	43	324	98	43	320	97	42
617	332	45	322	97	43	328	99	41	320	96	38
645	330	42	326	99	42	328	99	41	320	97	33
673	336	39	329	98	39	334	100	38	325	97	32
701	339	36	336	99	36	335	99	36	328	97	31
Mean for weeks											
1-13	159		159	100		158	99		158	99	
14-52	238		239	100		236	99		235	99	
53-101	318		314	99		315	99		308	97	

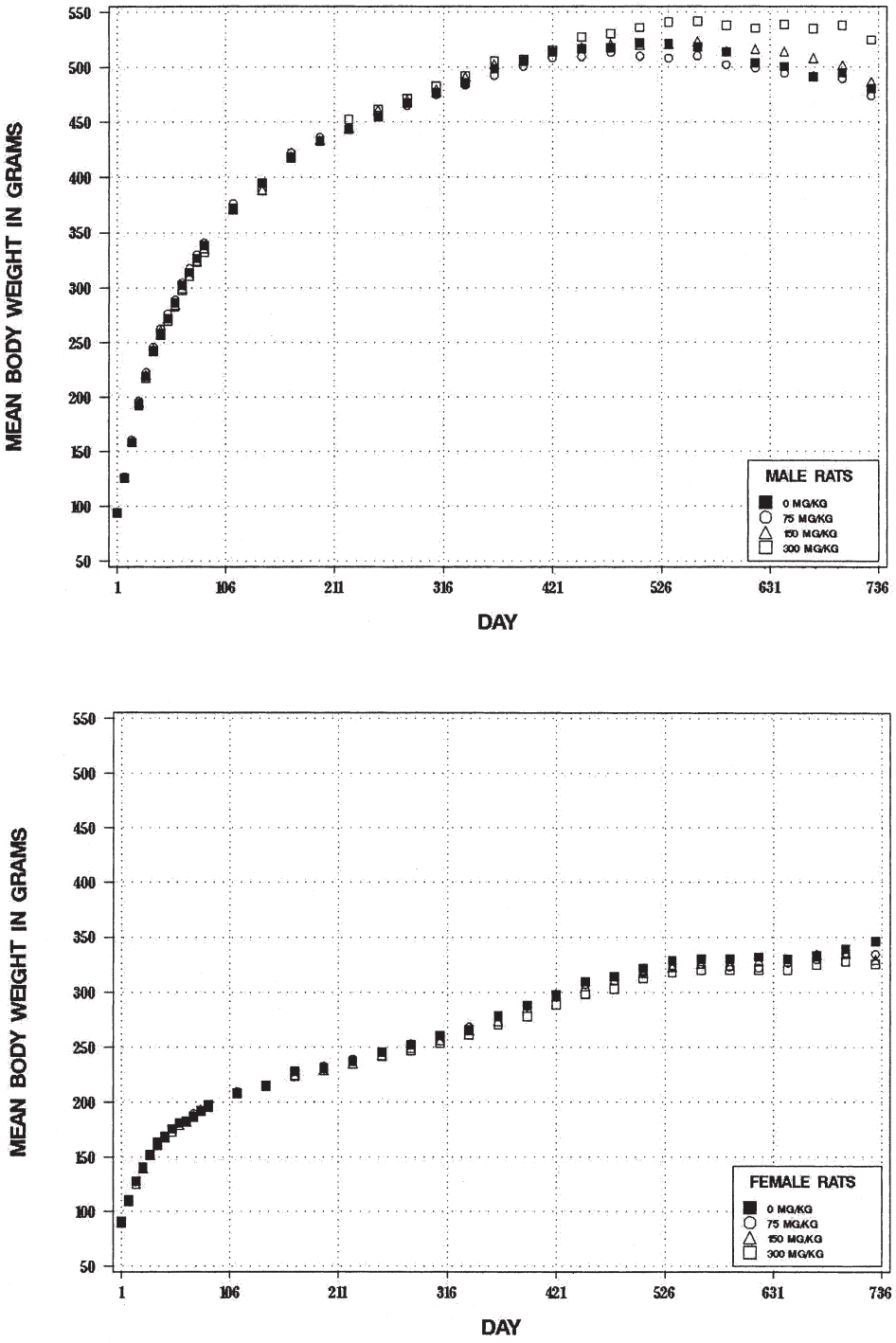


FIGURE 3
Growth Curves for Male and Female Rats Administered Isoeugenol by Gavage for 2 Years

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms or nonneoplastic lesions of the thymus, mammary gland, skin, nose, liver, pancreas, and testes. Summaries of the incidences of neoplasms and nonneoplastic lesions, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix A for male rats and Appendix B for female rats.

Thymus: The incidence of thymoma in exposed male rats was not statistically significant compared to vehicle controls, but the trend across all groups was significant (Tables 8 and A1). The incidence of benign or malignant thymoma (combined) in the 300 mg/kg males exceeded the historical range for vehicle controls in corn oil gavage studies and for controls by all routes (Tables 8 and A3a). The two thymomas were proliferative lesions consisting of neoplastic epithelial and lymphoid components. One was benign and the other was malignant. The benign neoplasm was a small, circumscribed, nodular mass composed of epithelioid cells forming bundles. These cells had abundant eosinophilic cytoplasm with a tendency toward spindle shapes. There were scattered lymphoid cells and vacuolated cells, probably macrophages. The nuclei of neoplastic cells were oval to round with little chromatin and had a few mitotic figures. The malignant neoplasm was a large invasive epithelial mass that replaced much of the thymus. The predominant neoplastic tissues formed solid sheets and tubules of various sizes. The neoplastic cells varied in appearance from well-differentiated epithelium to pleomorphic and anaplastic forms, often blending into poorly differentiated spindle areas. Numerous mitotic figures were present. Large cystic structures contained cellular debris. The neoplastic tissues extended to the mediastinum.

TABLE 8
Incidences of Thymoma of the Thymus in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Number Examined Microscopically	47	43	49	48
Thymoma, Benign[a]	0	0	0	1
Thymoma, Malignant[b]	0	0	0	1
Thymoma, Benign or Malignant[c]				
Overall rate[d]	0/47 (0%)	0/43 (0%)	0/49 (0%)	2/48 (4%)
Adjusted rate[e]	0.0%	0.0%	0.0%	4.9%
Terminal rate[f]	0/33 (0%)	0/27 (0%)	0/32 (0%)	1/30 (3%)
First incidence (days)	—[h]	—	—	719
Poly-3 test[g]	P = 0.047	—[i]	—	P = 0.230

[a] Historical incidence for 2-year gavage studies with corn oil vehicle control groups (mean ± standard deviation): 0/94; all routes: 2/1,146 (0.2% ± 0.6%), range 0%-2%
[b] Historical incidence for 2-year corn oil gavage studies: 0/94; all routes: 1/1,146 (0.1% ± 0.4%), range 0%-2%
[c] Historical incidence for 2-year corn oil gavage studies: 0/94; all routes: 3/1,146 (0.3% ± 0.7%), range 0%-2%
[d] Number of animals with neoplasm per number of animals with thymus examined microscopically
[e] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[f] Observed incidence at terminal kill
[g] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence is the P value corresponding to pairwise comparison between the vehicle controls and that dosed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.
[h] Not applicable; no neoplasms in animal group
[i] Value of statistic cannot be computed.

Mammary Gland: Rare, malignant carcinomas occurred in two 300 mg/kg male rats (Tables A1 and A2). The trend was statistically significant, no carcinomas occurred in corn oil vehicle controls in the current historical database, and the rate (4%) was equal to the highest rate for controls by all routes [8/1,199 (1% ± 1%), range 0%-4%; Table A3b]. These mammary gland carcinomas were not accompanied by increased incidences of adenoma or hyperplasia. In one animal, the mammary gland carcinoma consisted of a single highly cellular mass of neoplastic epithelial cells with small numbers of alveolar structures. Irregular cords of neoplastic cells were invading the adjacent tissues. Numerous mitotic figures were evident. The other animal had several nodules of malignant neoplastic glandular epithelium in multiple glands. The cellular patterns varied from nodule to nodule but generally had irregularly shaped tubular structures formed by multiple layers of densely packed, cuboidal cells. Mitotic figures were frequent. These cells were invading the adjacent tissues.

Skin: Keratoacanthoma occurred with a negative trend in male rats, and the incidences were significantly decreased in the 150 and 300 mg/kg groups (0 mg/kg, 7/50; 75 mg/kg, 5/50; 150 mg/kg, 1/50; 300 mg/kg, 1/50; Tables A1 and A2). The incidences in the 150 and 300 mg/kg groups were below the historical range for vehicle controls in corn oil gavage studies [9/100 (9% ± 7%), range 4%-14%] and at the lower end of the historical range for controls by all routes [71/1,199 (6% ± 5%), range 0%-20%] (Table A3c). The keratoacanthomas were benign neoplastic proliferations of basal and squamous cells of the epidermis that formed craters or invaginated into the dermis and subcutis. They were often cystic masses filled by layers of keratin and had characteristic thick walls of stratified squamous epithelium.

Nose: Incidences of minimal to mild atrophy of the olfactory epithelium and respiratory metaplasia of the olfactory epithelium were increased in 75 mg/kg males and significantly increased in 150 mg/kg males and 300 mg/kg males and females (Tables 9, A4, and B3). Olfactory epithelial atrophy was characterized by decreased numbers of epithelial cells that resulted in thinning of the olfactory epithelium. It primarily affected epithelium lining the dorsal meatus at nasal Level II. Atrophy of the olfactory epithelium was fre-

TABLE 9
Incidences of Nonneoplastic Lesions of the Nose in Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control		75 mg/kg		150 mg/kg		300 mg/kg	
Male								
Number Examined Microscopically	50		48		49		49	
Olfactory Epithelium, Atrophy[a]	1	(1.0)[b]	5	(1.2)	9**	(1.0)	13**	(1.0)
Olfactory Epithelium, Metaplasia, Respiratory	4	(1.0)	6	(1.3)	10**	(1.7)	15**	(1.9)
Olfactory Epithelium, Degeneration	1	(1.0)	0		2	(1.0)	6*	(1.2)
Female								
Number Examined Microscopically	50		49		49		49	
Olfactory Epithelium, Atrophy	0		0		0		4*	(1.0)
Olfactory Epithelium, Metaplasia, Respiratory	5	(1.6)	5	(1.8)	9	(1.8)	12*	(1.3)

* Significantly different (P≤0.05) from the vehicle control group by the the Poly-3 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

quently associated with atrophy of the olfactory nerves in the adjacent submucosal tissues. This nerve atrophy was considered to be secondary to the loss of sensory neurons in the olfactory epithelium and was not given a separate diagnosis in this study. In respiratory metaplasia of the olfactory epithelium, the normal olfactory epithelium was replaced by ciliated columnar epithelium similar in appearance to normal respiratory epithelium and was observed in the dorsal meatus of Level II and along the ethmoid turbinates of Level III. The incidence of minimal to mild olfactory epithelium degeneration in 300 mg/kg males was also significantly increased. Degeneration of the olfactory epithelium was characterized by disorganization of the layers of sensory neurons and vacuolization of the epithelium.

Liver: Incidences of basophilic focus were significantly decreased in all exposed groups of male rats (Tables 10 and A4), while those of eosinophilic focus decreased significantly in 75 and 150 mg/kg males (Tables 10 and A4). In 300 mg/kg males, incidences of clear cell focus and bile duct hyperplasia also decreased significantly (Tables 10 and A4). These decreases were not present in female rats and have unknown significance. The incidences of mixed cell foci in male groups exposed to isoeugenol were not significantly different from the vehicle control group. Eosinophilic, mixed, basophilic, and clear cell foci consisted of localized areas of hepatocytes with characteristic altered tinctorial properties. Otherwise, the hepatocytes were generally similar in appearance. An eosinophilic focus was composed of cells with abundant eosinophilic cytoplasm. A mixed cell focus was composed of a mixture of cells with different staining properties, generally a mixture of eosinophilic cells and clear cells. A basophilic focus consisted of hepatocytes with basophilic cytoplasm, occasionally with basophilic linear (tigroid) intracytoplasmic aggregates. A clear cell focus was composed of cells having clear cytoplasm. Other than tinctorial differences, hepatocytes in foci were generally somewhat larger than hepatocytes in adjacent parenchyma and were arranged in relatively normal lobular patterns. The hepatic cords at the periphery of these foci generally merged imperceptibly with the surrounding normal liver, resulting in an indistinct border and little or no compression of the adjacent liver parenchyma.

Bile duct hyperplasia consisted of multifocal proliferations of small bile ducts within portal areas. These bile ducts were similar in appearance to those seen in portal triads of normal liver lobules. The only distinctive feature was increased numbers of ducts. They consisted of uniform basophilic, flattened to cuboidal epithelium surrounded by scant to moderate amounts of collagenous stroma.

Other Findings: Pancreas acinus atrophy occurred with a negative trend (P=0.010) with a significantly decreased incidence in 300 mg/kg males (vehicle control, 22/50; 75 mg/kg, 21/50; 150 mg/kg, 17/50; 300 mg/kg, 10/49; Table A4). Testicular interstitial cell hyperplasia occurred with a negative trend (P=0.045) with a significantly decreased incidence in 300 mg/kg males (7/50, 2/50, 4/50, 1/50; Table A4). The cause of these decreased incidences of pancreas acinus atrophy and testicular interstitial cell hyperplasia was not evident.

TABLE 10
Incidences of Nonneoplastic Lesions of the Liver in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Number Examined Microscopically	50	50	50	48
Basophilic Focus[a]	43	34*	26**	18**
Clear Cell Focus	27	20	19	14*
Eosinophilic Focus	8	2*	0**	2
Bile Duct Hyperplasia	39 (1.5)[b]	39 (1.5)	32 (1.4)	24** (1.2)

* Significantly different (P≤0.05) from the vehicle control group by the Poly-3 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-Month Study in Mice

All mice survived to the end of the study (Table 11). Decreases in the mean body weight and body weight gain of 600 mg/kg males were statistically significant compared to the vehicle control group. After 85 days of exposure, mean body weights of 75 and 150 mg/kg females exceeded that of the vehicle control group by 10% and 8%, respectively, while that of 600 mg/kg females was 7% less than vehicle controls; however, the differences were not statistically significant. No clinical findings were associated with exposure to isoeugenol.

The hematology data for mice in the 3-month gavage study of isoeugenol are listed in Table F2. There were no hematological effects in mice exposed to isoeugenol.

Increases in relative liver weights of all male groups administered isoeugenol and absolute liver weights of 300 and 600 mg/kg males correlated with dose and were statistically significant (Table G2). Also, decreases in absolute kidney weights of 150, 300, and 600 mg/kg males and absolute lung weights of 600 mg/kg females were statistically significant and considered related to exposure; however, the differences in liver, kidney, and lung weights were not associated with any microscopic findings.

Incidences of mild to moderate olfactory epithelial atrophy and minimal to mild atrophy of olfactory nerve bundles increased significantly in 600 mg/kg males and females (Table 12). Olfactory epithelial atrophy was characterized by a decrease in the number of cells, resulting in thinning of the olfactory epithelium. The atrophy occurred in the most distal portion of the nasal cavity along the junction of the nasal septum with the dorsal wall of the nasal meatus in Level III. The atrophic epithelium was simple or pseudostratified, ciliated, and columnar and resembled normal respiratory epithelium. Glands within the lamina propria under the affected olfactory epithelium were often slightly dilated. Some of these glands contained secretory material with occasional inflammatory cells and cell debris. Some glands were lined by minimally hyperplastic epithelial cells.

Table 11
Survival and Body Weights of Mice in the 3-Month Gavage Study of Isoeugenol

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight[c] Relative to Controls (%)
		Initial	Terminal	Change	
Male					
0	10/10	23.3 ± 0.3	37.7 ± 0.9	14.4 ± 0.9	
37.5	10/10	23.3 ± 0.3	36.0 ± 1.0	12.7 ± 1.0	95
75	10/10	22.9 ± 0.3	36.1 ± 1.6	13.2 ± 1.5	96
150	10/10	23.4 ± 0.4	35.7 ± 1.1	12.3 ± 1.0	95
300	10/10	23.4 ± 0.3	37.1 ± 1.2	13.7 ± 0.9	98
600	10/10	23.2 ± 0.4	33.1 ± 1.0*	9.9 ± 0.7*	88
Female					
0	10/10	17.7 ± 0.2	26.3 ± 0.6	8.6 ± 0.5	
37.5	10/10	18.0 ± 0.2	27.7 ± 1.1	9.7 ± 0.9	105
75	10/10	18.1 ± 0.2	28.8 ± 0.7	10.7 ± 0.8	110
150	10/10	18.6 ± 0.3*	28.3 ± 0.9	9.7 ± 0.8	108
300	10/10	18.1 ± 0.2	26.9 ± 0.8	8.9 ± 0.7	102
600	10/10	18.1 ± 0.2	24.6 ± 0.3	6.5 ± 0.4	93

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunnett's test
[a] Number of animals surviving at 3 months/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Day 85

Table 12
Incidences of Nonneoplastic Lesions in the Nose of Mice in the 3-Month Gavage Study of Isoeugenol

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
Number Examined Microscopically	10	10	10	10	10	10
Olfactory Epithelium, Atrophy[a]	0	0	0	0	0	10** (2.0)[b]
Nerve, Atrophy	0	0	0	0	0	5* (1.0)
Female						
Number Examined Microscopically	10	10	10	10	10	10
Olfactory Epithelium, Atrophy	0	0	0	0	0	10** (2.3)
Nerve, Atrophy	0	0	0	0	0	8* (1.6)

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

Atrophy of olfactory nerves consisted of reductions in the number and size of nerve bundles within the lamina propria beneath areas of atrophic epithelium. The nerve atrophy was considered to be secondary to the loss of sensory neurons in the overlying atrophic olfactory epithelium.

Dose Selection Rationale: Mice exposed to isoeugenol in corn oil by gavage for 3 months showed significant decreases in body weights of males and increases in liver weights of the 600 mg/kg group. Although increased incidences of olfactory lesions in 600 mg/kg males and females were moderate, they were statistically significant. These nasal lesions were considered to have no effect on survival in longer exposures; however, the importance of olfaction in rodent feeding behavior increased concern that they might affect weight gain, so 300 mg/kg was selected as the highest dose for the 2-year study. The dosing regimen for the 2-year study of isoeugenol in mice was 0, 75, 150, and 300 mg/kg.

2-Year Study in Mice

Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 13 and in the Kaplan-Meier survival curves (Figure 4). The survival rate of 300 mg/kg males was significantly decreased compared to that of the vehicle controls. The survival rates of all other exposed groups were similar to those of the vehicle controls.

Twenty-three of the 50 male mice exposed to 300 mg/kg isoeugenol died before terminal sacrifice; 16 were natural deaths and seven were sacrificed in moribund condition. Liver neoplasia was the likely cause of death for 16 of these mice, as follows: 13 had hepatocellular carcinoma, one had hepatoblastoma, one had hepatocholangiocarcinoma, and one had hepatocellular adenoma. In the 300 mg/kg group of female mice, half of the 16 early deaths occurred between days 553 and 555. Examination of gross observations made at necropsy and histopathologic diagnoses recorded for these eight animals indicated that the likely cause of death for five of them was moderate or marked liver necrosis.

Body Weights and Clinical Findings

Mean body weights are shown in Tables 14 and 15 and Figure 5. The mean body weights of 300 mg/kg male and female mice were less than 95% of the vehicle controls after 60 and 28 weeks, respectively, of exposure. No clinical findings related to isoeugenol exposure were observed.

Table 13
Survival of Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Male				
Animals initially in study	50	50	50	50
Moribund	7	10	5	7
Natural deaths	4	2	9	16
Animals surviving to study termination	39	38	36	27
Percent probability of survival at end of study[a]	78	76	72	54
Mean survival (days)[b]	688	698	703	647
Survival analysis[c]	P = 0.004	P = 1.000	P = 0.757	P = 0.019
Female				
Animals initially in study	50	50	50	50
Accidental deaths[d]	2	0	0	1
Missing[d]	1	0	0	0
Moribund	5	7	5	5
Natural deaths	8	4	7	11
Animals surviving to study termination	34	39	38	33
Percent probability of survival at end of study	72	78	76	67
Mean survival (days)	679	707	705	663
Survival analysis	P = 0.317	P = 0.666N	P = 0.794N	P = 0.547

[a] Kaplan-Meier determinations
[b] Mean of all deaths (uncensored, censored, and terminal sacrifice)
[c] The result of the life table trend test (Tarone, 1975) is in the vehicle control column, and the result of the life table pairwise comparison (Cox, 1972) with the vehicle controls is in the dosed group column. A lower mortality in a dosed group is indicated by **N**.
[d] Censored from survival analysis

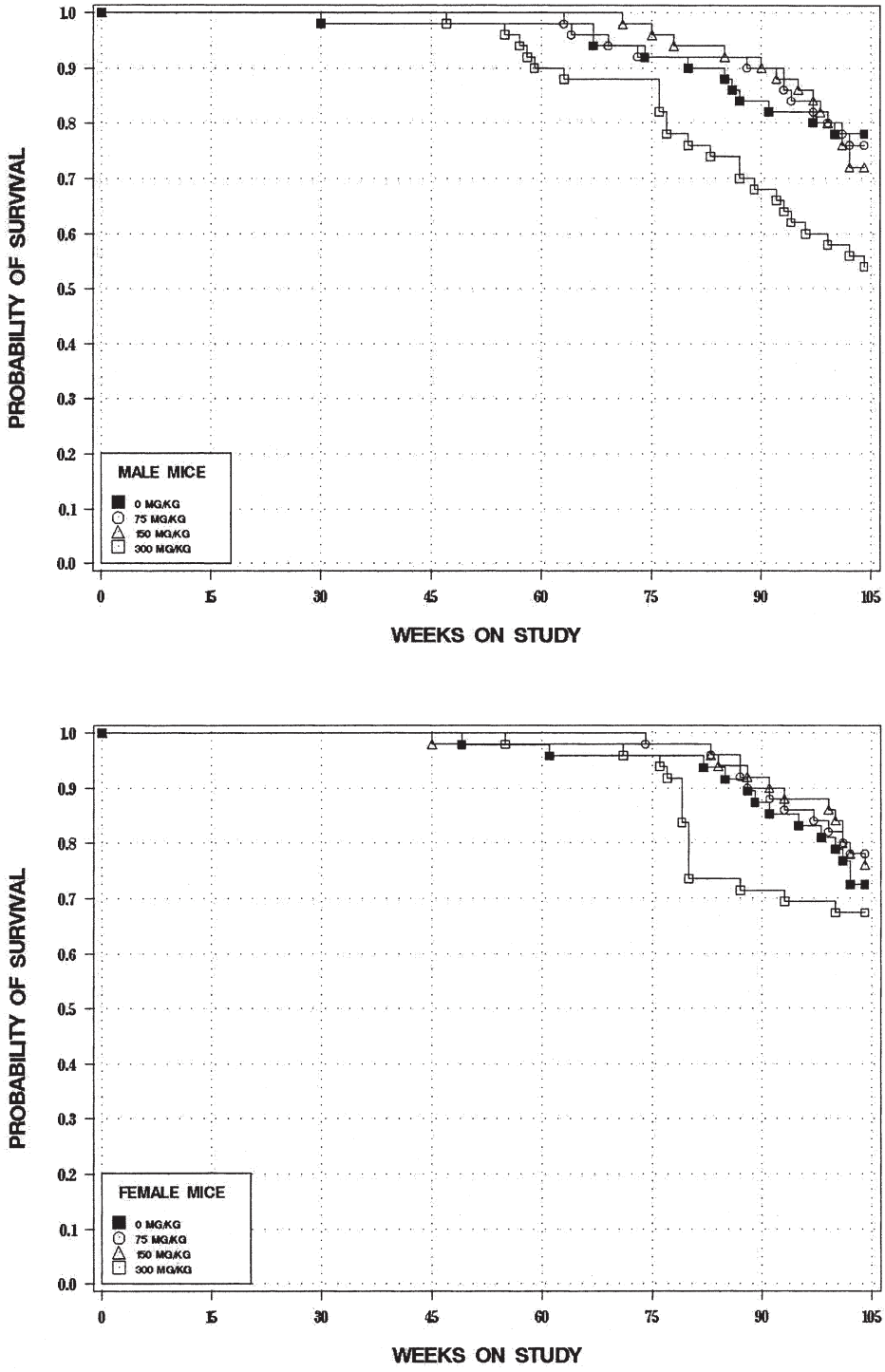


FIGURE 4
Kaplan-Meier Survival Curves for Male and Female Mice Administered Isoeugenol by Gavage for 2 Years

Table 14
Mean Body Weights and Survival of Male Mice in the 2-Year Gavage Study of Isoeugenol

Days on Study	Vehicle Control		75 mg/kg			150 mg/kg			300 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	23.2	50	23.2	100	50	23.0	100	50	23.1	100	50
8	24.3	50	24.6	101	50	24.4	100	50	24.4	100	50
15	25.5	50	25.7	101	50	25.3	99	50	25.5	100	50
22	26.5	50	26.8	101	50	26.4	100	50	26.5	100	50
29	28.2	50	28.6	102	50	28.2	100	50	28.2	100	50
36	29.8	50	30.0	100	50	29.6	99	50	29.3	98	50
43	31.3	50	31.8	102	50	31.1	99	50	30.7	98	50
50	32.8	50	33.3	102	50	32.6	100	50	32.0	98	50
57	32.6	50	33.7	103	50	32.7	101	50	32.1	99	50
64	35.5	50	36.0	101	50	34.9	99	50	34.0	96	50
71	35.5	50	35.9	101	50	35.1	99	50	34.6	97	50
78	37.1	50	38.1	103	50	37.0	100	50	36.5	98	50
85	37.2	50	38.0	102	50	37.5	101	50	36.6	98	50
113	41.1	50	41.6	101	50	40.4	98	50	39.8	97	50
141	43.9	50	44.6	102	50	43.3	99	50	42.1	96	50
169	46.6	50	46.9	101	50	46.2	99	50	44.6	96	50
197	47.9	50	48.3	101	50	47.9	100	50	45.9	96	50
225	49.8	49	50.1	101	50	49.6	100	50	48.0	96	50
253	50.2	49	50.4	100	50	50.3	100	50	48.9	97	50
281	51.9	49	51.7	100	50	51.8	100	50	50.0	96	50
309	52.3	49	51.9	99	50	52.2	100	50	50.8	97	50
337	53.8	49	53.5	99	50	53.6	100	50	51.8	96	49
365	53.5	49	53.2	100	50	53.9	101	50	51.8	97	49
393	54.5	49	54.2	100	50	55.0	101	50	52.3	96	48
421	54.6	49	54.5	100	50	54.6	100	50	52.7	97	45
449	55.5	49	54.8	99	48	55.1	99	50	52.6	95	44
477	54.8	47	53.6	98	48	54.4	99	50	51.8	95	44
505	56.7	47	55.5	98	47	55.8	98	49	53.0	94	44
533	55.5	46	54.1	98	46	54.1	97	48	51.1	92	40
561	55.9	45	54.4	97	46	54.7	98	47	50.9	91	38
589	55.5	44	53.7	97	46	54.5	98	47	50.3	91	37
617	55.0	42	53.8	98	45	54.2	99	46	49.4	90	35
645	53.9	41	52.6	98	45	52.5	97	44	48.2	90	33
673	54.2	41	53.7	99	42	51.7	95	43	48.2	89	30
701	53.9	39	53.8	100	40	50.7	94	39	48.3	90	29
Mean for weeks											
1-13	30.7		31.2	102		30.6	100		30.3	99	
14-52	48.6		48.8	100		48.4	99		46.9	96	
53-101	54.9		54.0	98		53.9	98		50.8	93	

TABLE 15
Mean Body Weights and Survival of Female Mice in the 2-Year Gavage Study of Isoeugenol

Days on Study	Vehicle Control		75 mg/kg			150 mg/kg			300 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	18.6	50	18.7	100	50	18.5	99	50	18.5	99	50
8	19.0	50	19.3	101	50	19.1	100	50	19.3	102	50
15	20.1	50	20.5	102	50	20.1	100	50	20.2	101	50
22	20.9	50	21.6	103	50	21.4	102	50	21.3	102	50
29	22.2	50	22.6	102	50	22.4	101	50	22.5	101	50
36	23.5	50	23.8	101	50	23.7	101	50	23.6	100	50
43	24.8	50	25.2	102	50	24.8	100	50	24.4	98	50
50	25.8	50	26.6	103	50	26.3	102	50	25.5	99	50
57	26.8	50	26.7	100	50	26.3	98	50	26.0	97	50
64	27.6	50	27.5	100	50	27.4	99	50	27.0	98	50
71	28.4	50	28.8	102	50	28.2	99	50	27.7	98	50
78	29.0	50	29.4	101	50	28.7	99	50	28.3	98	50
85	30.2	50	30.3	100	50	29.5	98	50	29.2	97	50
113	33.0	50	33.6	102	50	33.4	101	50	32.8	99	50
141	37.3	50	38.1	102	50	37.6	101	50	37.0	99	50
169	40.5	50	41.1	101	50	39.9	99	50	39.3	97	50
197	40.8	50	42.8	105	50	40.9	100	50	39.4	97	50
225	45.2	50	46.7	103	50	45.0	100	50	42.1	93	50
253	47.0	50	48.7	104	50	47.3	101	50	44.3	94	50
281	50.7	50	52.5	104	50	49.2	97	50	46.6	92	49
309	53.1	48	54.6	103	50	52.0	98	50	48.3	91	49
337	56.3	48	57.9	103	50	54.1	96	49	49.9	89	49
365	57.4	47	58.4	102	50	55.3	96	49	50.6	88	49
393	59.7	47	61.0	102	50	58.5	98	49	52.1	87	48
421	60.0	47	62.6	104	50	59.4	99	49	51.8	86	48
449	62.5	46	63.9	102	50	60.4	97	49	52.9	85	48
477	62.6	45	64.1	102	50	60.7	97	49	53.0	85	48
505	64.8	45	65.8	102	50	63.6	98	49	54.5	84	47
533	65.0	45	65.7	101	49	63.5	98	49	53.9	83	46
561	64.1	45	65.7	102	49	63.7	99	49	54.1	84	36
589	62.5	44	63.6	102	48	60.9	97	47	54.3	87	36
617	63.4	41	62.5	98	45	60.4	95	46	53.9	85	35
645	63.1	40	61.5	97	44	58.8	93	45	53.7	85	35
673	63.0	39	62.0	99	43	60.3	96	44	54.6	87	34
701	61.7	36	60.0	97	40	57.8	94	42	52.8	86	33
Mean for weeks											
1-13	24.4		24.7	101		24.3	100		24.1	99	
14-52	44.9		46.2	103		44.4	99		42.2	95	
53-101	62.3		62.8	101		60.3	97		53.2	86	

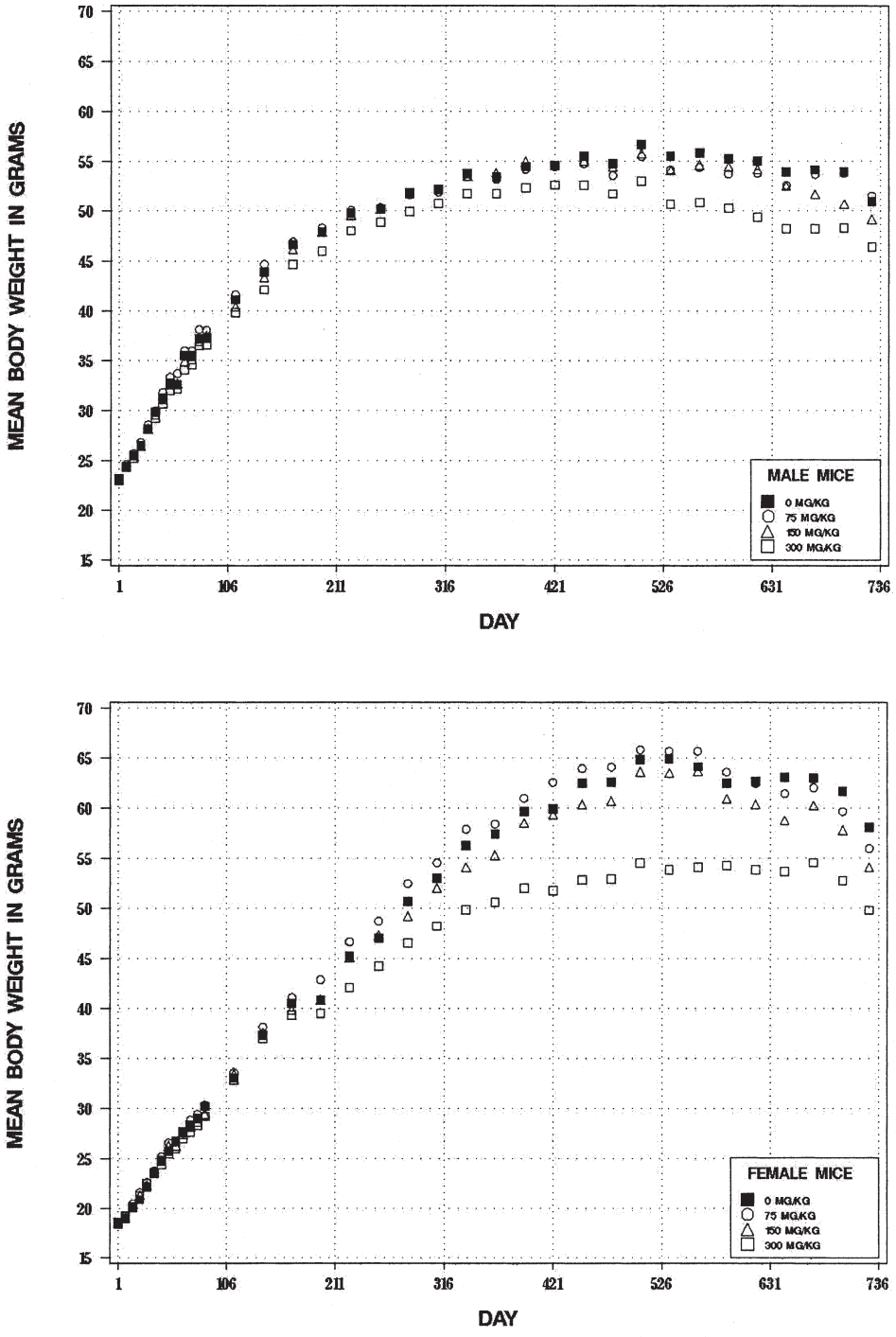


FIGURE 5
Growth Curves for Male and Female Mice Administered Isoeugenol by Gavage for 2 Years

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of histiocytic sarcoma and neoplasms and/or nonneoplastic lesions of the liver, nose, kidney, stomach, and spleen. Summaries of the incidences of neoplasms and nonneoplastic lesions, statistical analysis of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix C for male mice and Appendix D for female mice.

Liver: In all exposed male groups, incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined) were significantly greater than those in the vehicle control group; the incidences of multiple hepatocellular adenoma were also significantly increased (Tables 16, C1, and C2). The incidences of these neoplasms exceeded the historical control range for corn oil vehicle control groups, and the incidences of hepatocellular adenoma or carcinoma (combined) exceeded the range for controls by all routes (Tables 16 and C3). Hepatocellular adenomas were usually discrete masses having solid growth patterns that caused compression of the surrounding normal hepatic parenchyma. They consisted of hepatocytes having clear, eosinophilic, or basophilic cytoplasm and were sometimes difficult to distinguish from hepatocellular foci. However, the lack of normal lobular architecture and the presence of plates of neoplastic hepatocytes that intersected the surrounding normal liver plates at sharp angles, rather than merging with them as seen in foci, were characteristics used to differentiate adenomas from foci. Hepatocellular carcinomas were large, poorly demarcated masses that generally had irregular borders due to growth into the surrounding normal parenchyma.

Table 16
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Male				
Number Examined Microscopically	50	50	50	50
Basophilic Focus[a]	5	5	3	7
Clear Cell Focus	16	26*	29**	13
Eosinophilic Focus	8	13	11	5
Hepatocellular Adenoma, Multiple	10	26**	28**	20*
Hepatocellular Adenoma (includes multiple)[b]				
Overall rate[c]	24/50 (48%)	35/50 (70%)	37/50 (74%)	33/50 (66%)
Adjusted rate[d]	53.0%	75.7%	76.9%	77.7%
Terminal rate[e]	22/39 (56%)	31/38 (82%)	29/36 (81%)	23/27 (85%)
First incidence (days)	469	646	491	408
Poly-3 test[f]	P = 0.012	P = 0.015	P = 0.010	P = 0.009
Hepatocellular Carcinoma, Multiple	2	6	6	4
Hepatocellular Carcinoma (includes multiple)[g]				
Overall rate	8/50 (16%)	18/50 (36%)	19/50 (38%)	18/50 (36%)
Adjusted rate	17.4%	37.9%	38.7%	40.4%
Terminal rate	5/39 (13%)	10/38 (26%)	9/36 (25%)	5/27 (19%)
First incidence (days)	469	481	491	385
Poly-3 test	P = 0.027	P = 0.022	P = 0.017	P = 0.012
Hepatocellular Adenoma or Carcinoma[h]				
Overall rate	28/50 (56%)	43/50 (86%)	43/50 (86%)	43/50 (86%)
Adjusted rate	60.5%	90.0%	86.3%	90.3%
Terminal rate	24/39 (62%)	34/38 (90%)	30/36 (83%)	24/27 (89%)
First incidence (days)	469	481	491	385
Poly-3 test	P < 0.001	P < 0.001	P = 0.003	P < 0.001

TABLE 16
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Female				
Number Examined Microscopically	49	50	49	50
Necrosis	3 (3.7)[i]	0	1 (3.0)	8 (3.4)
Hepatocellular Adenoma, multiple	0	3	1	0
Hepatocellular Adenoma (includes multiple)[j]				
Overall rate	11/49 (22%)	10/50 (20%)	9/49 (18%)	4/50 (8%)
Adjusted rate	25.7%	20.8%	19.3%	9.9%
Terminal rate	8/34 (24%)	5/39 (13%)	8/38 (21%)	3/33 (9%)
First incidence (days)	699	577	693	698
Poly-3 test	P = 0.048N	P = 0.380N	P = 0.321N	P = 0.053N
Hepatocellular Carcinoma, multiple	1	0	2	0
Hepatocellular Carcinoma (includes multiple)[k]	3	8	9	6
Hepatocellular Adenoma or Carcinoma[l]				
Overall rate	13/49 (27%)	16/50 (32%)	15/49 (31%)	9/50 (18%)
Adjusted rate	29.6%	33.2%	31.9%	22.2%
Terminal rate	8/34 (24%)	11/39 (28%)	11/38 (29%)	8/33 (24%)
First incidence (days)	423	577	647	698
Poly-3 test	P = 0.229N	P = 0.442	P = 0.495	P = 0.297N

* Significantly different (P≤0.05) from the vehicle control group by the Poly-3 test
** P≤0.01
[a] Number of animals with lesion
[b] Historical incidence for 2-year gavage studies with corn oil vehicle control groups (mean ± standard deviation): 50/100 (50.0% ± 2.8%), range, 48%-52%; all routes: 544/1,146 (47.5% ± 14.9%), range 14%-72%
[c] Number of animals with neoplasm per number of animals with liver examined microscopically
[d] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[e] Observed incidence at terminal kill
[f] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence is the P value corresponding to pairwise comparison between the vehicle controls and that dosed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A negative trend or lower incidence in a dosed group is indicated by **N**.
[g] Historical incidence for 2-year corn oil gavage studies: 22/100 (22.0% ± 8.5%), range, 16%-28%; all routes: 317/1,146 (27.7% ± 9.2%), range 8%-48%
[h] Historical incidence for 2-year corn oil gavage studies: 61/100 (61.0% ± 7.1%), range, 56%-66%; all routes: 729/1,146 (63.6% ± 15.6%), range 20%-84%
[i] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked
[j] Historical incidence for 2-year corn oil gavage studies: 17/99 (17.2% ± 7.4%), range, 12%-22%; all routes: 345/1,245 (27.8% ± 17.0%), range 2%-62%
[k] Historical incidence for 2-year corn oil gavage studies: 4/99 (4.1% ± 2.9%), range, 2%-6%; all routes: 131/1,245 (10.5% ± 7.7%), range 0%-28%
[l] Historical incidence for 2-year corn oil gavage studies: 20/99 (20.3% ± 8.9%), range, 14%-27%; all routes: 419/1,245 (33.7% ± 19.1%), range 8%-64%

The neoplastic hepatocytes often were somewhat atypical in appearance, but the major distinguishing feature of carcinomas was the presence of abnormal patterns of growth. The most common abnormal growth pattern was formation of trabeculae by neoplastic hepatocytes that were three or more cell layers thick, while less commonly the neoplastic cells formed glandular structures or solid masses. Several growth patterns were often seen within a single neoplasm. Areas of hemorrhage or necrosis were sometimes present. Metastases of carcinomas to the lungs occurred in all dosed groups of male mice and in 150 and 300 mg/kg females and were often multiple.

In contrast to the effect in males, incidences of hepatocellular adenoma in females occurred with a negative trend, increases in the incidences of hepatocellular carcinoma were not significant, and incidences of these neoplasms in exposed females individually or combined, were within their respective historical control (all routes) ranges (Tables 16, D2, and D3a). The magnitude of the overall decrease in combined incidences in females is similar to that expected for the observed decreases in body weight (Haseman *et al*., 1997).

Incidences of clear cell focus were significantly increased in 75 and 150 mg/kg males (Tables 16 and C4). The incidences of eosinophilic foci were also increased in these two groups, but the differences from vehicle controls were not significant. Clear cell foci were irregularly shaped, poorly circumscribed, and had a variable amount of cytoplasmic clear space that is thought to be accumulations of glycogen. The hepatocytes were normal or slightly enlarged in size with centrally located and often condensed nuclei. The affected hepatocytes lacked the discrete cytoplasmic vacuoles of fatty change and formed normal hepatic cords that merged with the surrounding normal hepatocytes. Incidences of basophilic foci were similar for all male groups. Basophilic foci were composed of well-demarcated collections of hepatocytes with abundant, characteristic darkly basophilic-staining cytoplasm. These hepatocytes were arranged in normal hepatic cords that merged with surrounding normal hepatocytes. As with the other types of foci, there was little or no compression of the surrounding normal hepatocytes. Eosinophilic foci consisted of well-demarcated collections of enlarged hepatocytes with abundant dark, homogeneous eosinophilic cytoplasm. These hepatocytes were arranged in normal hepatic cords that merged with the surrounding normal hepatocytes. There was usually little or no compression of the surrounding normal hepatocytes. Foci of altered hepatocytes, hepatocellular adenomas, and hepatocellular carcinomas are thought to represent a continuum.

A positive trend in liver necrosis in females was significant, but the incidence in the 300 mg/kg group was not significant compared to the vehicle control group (Tables 16 and D4). Moderate to marked liver necrosis was the likely cause of death for five of eight female mice in the 300 mg/kg group that died between days 553 and 555. The proximate cause of this liver necrosis was undetermined.

Histiocytic Sarcoma: The positive trend in the incidences of histiocytic sarcoma in females was statistically significant. Histiocytic sarcoma has not been observed in vehicle controls in corn oil gavage studies, and the incidence in the 300 mg/kg group was at the upper end of the historical range for controls by all routes (Tables 17, D2, and D3b). These histiocytic sarcomas were invasive with a variety of distributions in multiple organs, including liver, ovary, uterus, spleen, lung, lymph nodes, kidney, thymus, and bone marrow. Histologically, cells that are characteristic of neoplastic histiocytes were large with relatively abundant, pale eosinophilic cytoplasm. Their nuclei were dark basophilic with round to oval shapes and inconspicuous nucleoli. Variation in the size and shape of some neoplastic cells and high cytoplasmic-to-nuclear ratios were observed. Occasional multinucleated giant cells were present. Fibrosis was usually scant.

Nose: Incidences of olfactory epithelial respiratory metaplasia in all exposed groups, atrophy and hyaline droplet accumulation in all exposed groups of males and in 150 and 300 mg/kg females, and degeneration in 150 and 300 mg/kg males were significantly increased (Tables 18, C4, and D4). Small increases of atrophy and hyaline droplet accumulation were also observed in 75 mg/kg females. The severity of respiratory metaplasia generally increased with increasing dose; severity of the other olfactory lesions were minimal to mild and similar to those of the vehicle controls. Respiratory metaplasia of the olfactory epithelium was characterized by replacement of the olfactory epithelium by ciliated, columnar epithelium that was similar in appearance to normal respiratory epithelium. Metaplastic respiratory epithelium was low columnar rather than tall columnar

Table 17
Incidences of Histiocytic Sarcoma in Female Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Histiocytic Sarcoma[a]				
Overall rate[b]	0/49 (0%)	1/50 (2%)	1/50 (2%)	4/50 (8%)
Adjusted rate[c]	0.0%	2.1%	2.1%	9.7%
Terminal rate[d]	0/34 (0%)	0/39 (0%)	0/38 (0%)	2/33 (6%)
First incidence (days)	—[f]	605	616	538
Poly-3 test[e]	P = 0.015	P = 0.519	P = 0.519	P = 0.056

[a] Historical incidence for 2-year gavage studies with corn oil vehicle control groups (mean ± standard deviation): 0/99; all routes: 31/1,249 (2.5% ± 2.5%), range 0%-8%
[b] Number of animals with neoplasm per number of animals necropsied
[c] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[d] Observed incidence at terminal kill
[e] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence is the P value corresponding to pairwise comparison between the vehicle controls and that dosed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.
[f] Not applicable; no neoplasms in animal group

and typically lacked or had fewer goblet cells than the normal respiratory epithelium. Also, in some areas, the metaplastic epithelium was mildly hyperplastic and had mucosal invaginations resulting in the formation of "pseudoglands." Degeneration of the olfactory epithelium was characterized by vacuolation and disorganization of the epithelium with apoptosis, necrosis, and loss of epithelial cells. Olfactory epithelial atrophy consisted of small focal areas, primarily in the epithelium lining of the dorsal meatus in Level III nasal sections and occasionally in ethmoid turbinates. Atrophy of the olfactory epithelium was frequently associated with atrophy of the olfactory nerves in adjacent submucosal tissues. This nerve atrophy was considered to be secondary to loss of sensory neurons in the overlying olfactory epithelium and was not given a separate diagnosis in this study. The atrophic olfactory epithelium was often accompanied by metaplasia to ciliated columnar epithelium. Hyaline droplet accumulation consisted of intracellular, homogenous, eosinophilic globules that distended affected epithelial cells.

The incidences of minimal to moderate hyperplasia of the Bowman's gland were significantly increased in all exposed groups; severities increased with increasing dose (Tables 18, C4, and D4). Hyperplasia of Bowman's glands occurred in the same areas of olfactory epithelium having atrophy and metaplastic changes and consisted of increased numbers of glandular epithelial cells in the lamina adjacent to affected olfactory epithelium. This hyperplasia was accompanied by dilation of affected glands.

Kidney: Low incidences of minimal to moderate necrosis of the renal papilla and mild to moderate renal tubule necrosis in 300 mg/kg females were significantly greater than those in the vehicle controls (Tables 18 and D4). Necrosis was observed in all or portions of the renal papilla. These necrotic areas were clearly delineated from the adjacent viable tissue and frequently had infiltration by inflammatory cells and tubular interstitial mineralization. In renal tubule necrosis, portions or all of the renal tubules in the kidney were necrotic with intratubular cell protein debris that sometimes formed tubular casts and had varying degrees of tubular epithelial cell regeneration.

Stomach: Low incidences of forestomach squamous hyperplasia, inflammation, and ulceration in male mice increased with increasing dose and were significantly increased in the 300 mg/kg group. Similarly, low incidences of squamous hyperplasia and inflammation in the forestomach of female mice were increased in the 75 mg/kg and 150 mg/kg groups and were significantly increased in the 300 mg/kg group (Tables 18, C4, and

TABLE 18
Incidences of Selected Nonneoplastic Lesions Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control		75 mg/kg		150 mg/kg		300 mg/kg	
Male								
Nose[a]	50		50		50		50	
Olfactory Epithelium, Respiratory Metaplasia[b]	4	(1.0)[c]	31**	(1.3)	47**	(2.5)	49**	(2.1)
Olfactory Epithelium, Degeneration	1	(1.0)	1	(1.0)	7*	(1.1)	6*	(1.0)
Olfactory Epithelium, Atrophy	5	(1.2)	13*	(1.1)	36**	(1.1)	41**	(1.3)
Olfactory Epithelium, Accumulation, Hyaline Droplet	0		6*	(1.2)	26**	(1.4)	19**	(1.3)
Glands, Hyperplasia	3	(1.3)	34**	(1.5)	49**	(2.7)	48**	(2.7)
Spleen	50		50		50		49	
Cellular Depletion	0		0		0		2	(2.0)
Stomach, Forestomach	50		49		50		49	
Hyperplasia, Squamous	7	(1.1)	8	(2.8)	8	(2.6)	14*	(2.2)
Inflammation	5	(1.4)	8	(2.5)	9	(2.3)	14*	(2.4)
Ulcer	1	(3.0)	4	(2.5)	4	(2.8)	9**	(2.7)
Stomach, Glandular	50		49		49		44	
Ulcer	0		1	(2.0)	4	(1.5)	5*	(2.6)
Inflammation	0		1	(2.0)	3	(1.0)	3	(2.0)
Female								
Kidney	47		50		49		49	
Papilla, Necrosis	0		0		1	(2.0)	14**	(1.4)
Papilla, Necrosis, Bilateral	0		1	(3.0)	0		4	(2.8)
Renal Tubule, Necrosis	0		1	(2.0)	0		6*	(2.7)
Nose	48		50		50		50	
Olfactory Epithelium, Respiratory Metaplasia	6	(1.0)	37**	(1.2)	49**	(2.0)	50**	(2.6)
Olfactory Epithelium, Atrophy	3	(1.3)	8	(1.0)	36**	(1.2)	43**	(1.3)
Olfactory Epithelium, Accumulation, Hyaline Droplet	0		4	(1.0)	18**	(1.1)	12**	(1.3)
Glands, Hyperplasia	6	(1.0)	38**	(1.3)	49**	(2.4)	49**	(3.3)
Spleen	48		50		49		50	
Cellular Depletion	0		0		0		9**	(2.4)
Stomach, Forestomach	48		50		49		50	
Hyperplasia, Squamous	2	(1.5)	8	(2.5)	5	(1.4)	8*	(2.0)
Inflammation	2	(1.5)	8	(2.0)	5	(1.6)	8*	(1.4)
Stomach, Glandular	46		48		47		48	
Ulcer	0		1	(1.0)	1	(2.0)	7**	(2.1)
Inflammation	0		1	(1.0)	1	(1.0)	6*	(1.7)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the the Poly-3 test
** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

D4). In males, the three forestomach lesions often were present in the same mouse. Squamous hyperplasia was the result of thickening of the squamous epithelium caused by increased numbers of cell layers, primarily of the prickle cell layer. The affected epithelium was five to six cell layers thick as compared to the three to four cell layers for normal squamous epithelium. Ulcers of the forestomach were observed in male mice only and resulted when minimal to moderate damage of the mucosal surface caused a complete loss of the squamous epithelium and extended through the basement membrane. The margins of the ulcers often had epithelial hyperplasia. Ulcers were usually accompanied by inflammation. Inflammation of the forestomach had variable numbers of mixed inflammatory cells, primarily neutrophils and macrophages with some lymphocytes, and, rarely, eosinophils in the lamina propria; congestion; and various degrees of increased fibrous connective tissue. Inflammation usually occurred concurrently with squamous epithelial hyperplasia.

Low incidences of ulcers of the glandular stomach were statistically significant in 300 mg/kg males and females (Tables 18, C4, and D4). Ulcers of the glandular stomach resulted when minimal to moderate damage to the mucosal surface caused a complete loss of the mucosal epithelium and extended through the basement membrane. Ulcers were usually accompanied by minimal to moderate inflammation, and the incidence of inflammation in 300 mg/kg females was significantly increased.

Spleen: In 300 mg/kg female mice, the incidence of splenic cellular depletion was significantly increased (Tables 18 and D4). The lesion consisted of minimal to marked reductions in lymphocytes accompanied by variable decreases in hematopoietic cells. These findings were considered secondary to stress because they occurred in mice that were found dead or sacrificed moribund and seven of the nine female mice with cellular depletion also had liver and/or kidney necrosis.

Toxicokinetics

Single administration toxicokinetic studies of isoeugenol were conducted via intravenous and oral gavage routes in male and female F344/N rats and B6C3F1 mice (Appendix L). Plasma concentrations of isoeugenol were measured using a validated gas chromatography-mass spectrometric method. Secondary peaks, consistent with enterohepatic recirculation, were observed in the terminal phase of the plasma concentration versus time plots for both routes, species, and sexes tested. Their presence precluded precise calculations, so datapoint values were estimated.

Plasma concentration versus time curves from intravenously exposed animals (17 mg/kg for rats and 35 mg/kg for mice in Cremophor® EL:ethanol:water 1:1:8) were modeled by applying a biexponential model to the data using a nonlinear least squares fitting program. The data for rats indicated no differences in toxicokinetic parameters estimated for males and females. Data for mice showed differences between sexes, in that Cl_{tot} and AUC_{∞} did not have overlapping 95% confidence intervals. Absorption of isoeugenol following exposure by gavage in corn oil was rapid in both sexes of both species with t_{max} occurring within 2 to 20 minutes for all dose groups; however, large secondary peaks observed during the terminal phase of elimination precluded more precise determination of half lives and rate constants. With each dose group and species, Cl_{tot} was greater and $AUC_{ti\text{-}tf}$ was smaller for males than females. Bioavailability ranged from 11% (males) to 17% (females) in rats and 34% (males) to 36% (females) in mice. Isoeugenol was absorbed rapidly by both species following exposure by gavage in corn oil. However, it was also eliminated from systemic circulation rapidly and extensively. The collective toxicokinetic evidence indicates that the low bioavailability is the result of extensive first-pass metabolism. There was no evidence of saturation in either male or female rats or mice.

Genetic Toxicology

Isoeugenol (3.3 to 2,000 µg/plate) was not active in either of two independent assays for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* strain WP2 *uvrA* conducted with and without exogenous metabolic activation (S9 liver enzymes) (Table E1). Isoeugenol (concentrations up to 200 µg/mL in medium) did not induce chromosomal aberrations in cultured Chinese hamster ovary cells, with or without S9 activation (Table E2). *In vivo*, no increases in the frequencies of micronucleated erythrocytes were seen in peripheral blood samples of male B6C3F1 mice treated with 37.5 to 600 mg/kg isoeugenol by gavage for 3 months; in contrast, results of this test in female mice were judged to be positive, based on a 3.2-fold increase of micronucleated erythrocytes in the 600 mg/kg group and a significant trend (Table E3). No significant changes in the percentage of polychromatic erythrocytes were observed over the dose range tested in either males or females, indicating an absence of exposure-related toxicity to bone marrow.

DISCUSSION AND CONCLUSIONS

Isoeugenol is used as a flavor additive to foods and as a fragrance in cosmetics and household cleaning products. In food products, isoeugenol concentrations range from 0.57 ppm in nonalcoholic beverages to 3.27 ppm in frozen dairy products; it may also be added to baked goods, soft candy, gelatins, and puddings. Isoeugenol, as the active ingredient in AQUI-S®, is used as a "zero-withdrawal anesthetic" to manage the aquaculture and harvest of finfish and shellfish in Australia, Chile, the Faeroe Islands, Korea, and New Zealand (Schnick, 1999, 2006). The Food and Drug Administration (2006) has added four designations for isoeugenol-AQUI-S® to its Animal Drugs for Minor Uses and Minor Species list.

Isoeugenol was selected for toxicity and carcinogenicity evaluations based on high potential for human exposure, general lack of toxicity information, and structural similarity to carcinogenic phenylpropenoid analogs such as methyleugenol and safrole. The National Toxicology Program (NTP) performed 3-month and 2-year studies to characterize the potential toxicity and carcinogenicity of isoeugenol in male and female rats and mice. The highest dose selected for the 2-year studies was 300 mg/kg, based on results from the 3-month study that indicated this would approximate the minimally toxic dose.

Exposure of male and female rats and mice to isoeugenol for 3 months at doses of 37.5, 75, 150, 300, or 600 mg/kg by gavage in corn oil induced slight toxic responses. Survival was unaffected. Body weights of male rats and mice in the 600 mg/kg groups were decreased relative to vehicle controls. No clinical signs were observed, and the only exposure-related organ weight change was an increase in liver weights of 300 and 600 mg/kg male mice and female rats. Minimal to mild atrophy of nasal olfactory epithelium and olfactory nerve bundles was observed in male and female rats and mice. The incidences and severities of these nonneoplastic lesions were slightly greater in rats than in mice, where they occurred only in 600 mg/kg animals. In rats, mild atrophy of olfactory epithelium was observed in about half of the males and females in the 300 and 600 mg/kg groups and males in the 150 mg/kg group. Secondary atrophy of olfactory nerve bundles was minimal to mild in severity in 600 mg/kg female rats. Minimal to mild periportal hepatocellular cytoplasmic alteration occurred in all female rats exposed to 300 or 600 mg/kg.

Because allylbenzene analogs induce cytochrome P450 enzyme systems (Ioannides *et al*., 1981; Gardner *et al*., 1997; Jeurissen *et al*., 2004, 2006, 2007), P450 enzyme activities were assayed for rats exposed to isoeugenol for 31 days. P450 (CYP) isoenzyme activities for rats exposed to isoeugenol were unchanged in female rats and slightly decreased in males. Decreases of 25% to 30% in CYP1A1 (EROD) and CYP2B1/2 (PROD) activities were statistically significant at the highest dose and exhibited an inverse dose-response trend, indicating that these enzymes were mildly suppressed by isoeugenol. The small magnitude of the changes and the absence of any effect in female rats make it unlikely that the observed decreases in P450 activities have any physiological significance. However, these *in vivo* results are similar to those obtained *in vitro*, where isoeugenol and eugenol inhibited EROD and PROD activities in hepatic microsomes by about 12% and 30%, respectively (Zhao and O'Brien, 1996). The methylenedioxybenzene (MDB) compounds safrole, isosafrole, and dihydrosafrole exerted complete inhibition of CYP2B1 (PROD), while isosafrole and dihydrosafrole were better inhibitors of CYP1A1 than safrole. In contrast, induction of rat hepatic microsomes *in vitro* results in activation of methyleugenol to the putative proximate carcinogen 1-hydroxy-methyleugenol. Hydroxylation is catalyzed by CYP2E1 and probably CYP2C6 in rats (Gardner *et al*., 1997). When the hepatic microsomes were isolated from rats that were pretreated with methyleugenol, CYP2B (PROD) and 1A2 (A4H) were found to be induced, along with lesser induced CYPs (Gardner *et al*., 1997). Like methyleugenol, estragole induced an approximately fivefold increase in CYP2B-associated PROD activity in both male and female rats (NTP, 2010).

Exposure of male and female rats to isoeugenol for 2 years had no effect on survival. Body weights of

exposed groups were approximately equal to or greater than those of vehicle controls; those of 300 mg/kg males and females were 10% and 15% greater, respectively, than those of the vehicle control groups after 2 years. Nonneoplastic lesions in the nose were of low incidence and severity. Two thymic thymomas and two mammary gland carcinomas were observed in 300 mg/kg males; trends in the occurrence of these rare neoplasms were statistically significant, and the thymoma incidence exceeded the historical control range for all routes of exposure. While the low incidence of mammary gland carcinoma had a significant positive trend and exceeded or equaled historical control rates for studies by gavage in corn oil vehicle or by all routes of exposure, its significance was not supported by biological evidence; specifically, it was not accompanied by hyperplasia or adenoma. While these bioassays provide equivocal evidence for carcinogenic activity of isoeugenol in male rats, they provide no indication of carcinogenic activity in female rats. However, the general absence of chronic toxicity indicates that both male and female rats may have tolerated higher doses of isoeugenol.

When male mice were administered isoeugenol for 2 years at doses of 75, 150, and 300 mg/kg, survival in the 300 mg/kg group compared to vehicle controls and the decreasing trend in survival across all groups were statistically significant; liver neoplasms were the likely cause of death for many of the early-death animals. Exposure to isoeugenol had no effect on survival of female mice. Mean body weights of the 300 mg/kg groups were 10% to 15% less than those of the vehicle controls, and those for lower exposed groups were similar to those of vehicle controls. Male mice exhibited significantly increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined). The dose-response curves for these neoplasms were flat; for example, hepatocellular adenoma or carcinoma (combined) was diagnosed in 86% of the animals in each dosed group and in 56% of the vehicle controls. By comparison, methyleugenol not only caused hepatocellular adenomas and hepatocellular carcinomas in male and female rats and mice, it also caused rare hepatoblastomas in mice and hepatocholangiomas or hepatocholangiocarcinomas in rats (NTP, 2000).

The differences in liver response noted for methyleugenol, an allylbenzene, and isoeugenol, a propenylbenzene (isoallylbenzene), may be governed by competition between two or perhaps more metabolic pathways. Miller *et al.* (1983) have shown that carcinogenic activity of allylbenzenes such as safrole, methyleugenol, and estragole may be related to phase I hydroxylation of the 1-methylene carbon of the allyl side chain, followed by phase II formation of electrophilic sulfate esters. Unlike the allylbenzenes, propenylbenzene analogs undergo 3-hydroxylation rather 1-hydroxylation; specifically, the 3-hydroxy metabolite of isoeugenol occurs naturally as coniferyl alcohol, a key intermediate in the biosynthesis of phenylpropenoid and lignan compounds (Koeduka *et al.*, 2006, 2008; Dexter *et al.*, 2007). Isomerization of the 3-hydroxy metabolite to the 1-isomer is thermodynamically unfavorable because it breaks the conjugated system into isolated, higher energy phenyl and olefin groups. Although allylic hydroxylation catalyzed by cytochrome P450 may occur with rearrangement (Groves and Subramanian, 1984), the resistance to isomerization presented by propenylbenzenes may prevent formation of the 1-sulfate ester conjugate, the putative ultimate carcinogen formed by metabolism of allylbenzenes. Recent studies show that epoxides of anethole and asarone, both propenylbenzene analogs of isoeugenol, induce hepatomas in B6C3F1 mice (Kim *et al*., 1999). These results and observations across studies suggest that a different metabolic pathway, perhaps involving epoxidation or formation of a quinone-methide, is responsible for the liver tumors induced by isoeugenol in male mice.

Histiocytic sarcomas were observed in six female mice exposed to 75, 150, or 300 mg/kg isoeugenol for 2 years. Although the incidence in the 300 mg/kg group compared with the vehicle control group was not statistically significant (P=0.056), the positive trend across all groups was significant (P=0.015). Histiocytic sarcomas are highly invasive and were observed at 23 different sites including liver, ovary, uterus, and spleen. Histiocytic sarcoma was not observed in male mice but was diagnosed in one male and one female rat in each of the 75 and 150 mg/kg groups exposed to isoeugenol. In two previous gavage studies using corn oil as the vehicle, the incidences of histiocytic sarcoma were 0/99 in female mice and 1/100 in male rats. In the present study the lesion was not observed in female rats or male mice. The incidence (8%) of histiocytic sarcoma in female mice exposed to 300 mg/kg isoeugenol exceeded the overall historical range for vehicle controls in 2-year gavage studies with corn oil as the vehicle; it was threefold greater than the mean (2.50) but equal to the maximum

of the historical range for controls by all routes. The incidence for historical controls from 17 NTP studies where female mice were housed five per cage was 6/1,008. Histiocytic sarcomas are classified as hematopoietic neoplasms of the mononuclear phagocyte system, based upon the morphology of the neoplastic cells and the presence of lysozyme, Mac-2, and mononuclear phagocyte antigens. The specific origin of the neoplastic histiocytic cells is undetermined, but in female mice, the liver, uterus, and vagina often appear to be the primary site (Frith *et al*., 1993). One or more cell subtypes may give rise to this neoplasm. Kupffer's cells in the liver, bone marrow cells, tissue histiocytes, and circulating macrophages have been proposed. Although the spontaneous incidence of this neoplasm is low in both B6C3F1 mice and F344 rats, it varies among different strains; from 0% in male BALB/c mice to about 5.9% in female C57BL/6 mice and from 0% in female F344 rats to 1.3% in male Sprague-Dawley rats (Chandra and Frith, 1992; Frith *et al*., 1993).

Increased incidences of dose-related histiocytic sarcoma have been observed in a few other NTP studies; it served as the sole basis for assigning a level of evidence only for the study of benzophenone in female mice. The incidences of histiocytic sarcoma in female mice exposed to isoeugenol increased with increasing dose and exceeded the historical control range for 2-year corn oil gavage studies but were not statistically significant. Because the incidence of histiocytic sarcoma in the high dose group was not statistically significant relative to the concurrent vehicle controls and was within the historical control range for all routes of exposure, the increased incidence was considered to be equivocal evidence of carcinogenicity.

In the 2-year study in mice, olfactory epithelial atrophy, olfactory epithelial respiratory metaplasia, hyaline droplet accumulation in the olfactory epithelium, and hyperplasia of nasal glands were observed with minimal to mild severity; the incidences of these nonneoplastic lesions increased with increasing dose, indicating they were exposure related. Atrophy of olfactory nerves occurred frequently in submucosal tissues adjacent to atrophic areas of olfactory epithelium. This nerve atrophy was observed in both the 3-month and 2-year studies. It was considered to be secondary to loss of sensory neurons in the overlying olfactory epithelium. Because primary nasal lesions were predominant, olfactory nerve atrophy was not given a separate diagnosis in the 2-year study.

Significant renal lesions occurred in the medulla and cortex of female, but not male, mice exposed to isoeugenol for 2 years. Papillary necrosis of the medulla affected 18 of 49 females in the 300 mg/kg group, including four with bilateral involvement. This incidence of papillary necrosis in mice at 2 years is unusual for NTP studies and is regarded as the result of chronic exposure to isoeugenol. Low incidences of papillary necrosis have been observed to occur spontaneously in both control and exposed groups of mice and rats during many NTP studies; however, only two other NTP chronic toxicity studies have displayed increases like those in mice exposed to isoeugenol. Papillary necrosis was increased significantly in both sexes of high-dose mice, as well as in high-dose female rats exposed by gavage to C.I. Acid Orange 3 for 2 years (NTP, 1988). Papillary necrosis was also increased significantly in male mice exposed to 2,2-bis(bromomethyl)-1,3-propanediol for 3 months by gavage or in feed (Elwell *et al.*, 1989; NTP, 1996). Papillary necrosis in humans has been frequently associated with exposure to analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) and is reported in animals exposed to these drugs (Bach and Nguyen, 1998; Brix, 2002). For example, papillary necrosis was significantly increased in male and female rats, but not mice, exposed to the NSAID phenylbutazone for 3 months or 2 years (NTP, 1990). Papillary necrosis is also observed in mice, rats, and other animal species exposed to chemicals that are structurally unrelated to analgesics and NSAIDs (Montgomery and Seely, 1990; Bach and Nguyen, 1998). Early sequential changes in the development of papillary necrosis have been investigated in rats and mice (Montgomery and Seely, 1990; Wolf *et al.*, 1992). Medullary ischemia, possibly the result of endothelial cell damage or decreased blood flow, as well as direct toxicity to the medullary interstitial cells have been suggested as possible mechanisms (Bach and Nguyen, 1998; Seely, 1999; Brix, 2002). Less frequent lesions in female mice were necrosis of the tubular epithelium in the renal cortex of the 300 mg/kg group. Renal tubule degeneration and necrosis have been observed concurrent with papillary necrosis after exposure to other chemicals (Wolf *et al.*, 1992; Elwell *et al.*, 1989).

Low incidences of forestomach squamous hyperplasia and inflammation in male and female mice and of forestomach ulcer in male mice were only significantly increased in animals exposed to 300 mg/kg isoeugenol for 2 years. Incidences of glandular stomach ulcer in males and females and inflammation in females were low but significantly increased in 300 mg/kg mice.

Nonneoplastic glandular stomach lesions observed in rats and mice exposed to methyleugenol were accompanied by glandular stomach neoplasms (NTP, 2000). Glandular stomach neoplasms induced by methyleugenol are thought to result from the cytotoxic loss of parietal cells, resulting in mucosal atrophy, decreased gastric secretion, increased intragastric pH, and increased serum gastrin. Increased gastric pH and gastrin secretion may stimulate hyperplasia of neuroendocrine cells, ultimately leading to tumor formation. In the NTP studies of methyleugenol and estragole, gastric pH and serum gastrin levels were significantly increased in F344/N rats after 30 days of exposure (NTP, 2000, 2010). Similar to methyleugenol, estragole administration resulted in significantly increased incidences of glandular stomach atrophy (NTP, 2010). In contrast, isoeugenol administration to F344/N rats for 30 days decreased intragastric pH in female rats and had no effect on serum gastrin levels. There was no indication of glandular neuroendocrine hyperplasia or neoplasia in stomachs of animals exposed to isoeugenol.

Conclusions

Under the conditions of these 2-year gavage studies, there was *equivocal evidence of carcinogenic activity** of isoeugenol in male F344/N rats based on increased incidences of rarely occurring thymoma and mammary gland carcinoma. There was *no evidence of carcinogenic activity* of isoeugenol in female F344/N rats administered 75, 150, or 300 mg/kg. There was *clear evidence of carcinogenic activity* of isoeugenol in male B6C3F1 mice based on increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined). There was *equivocal evidence of carcinogenic activity* of iso-eugenol in female B6C3F1 mice based on increased incidences of histiocytic sarcoma.

Exposure to isoeugenol resulted in nonneoplastic lesions of the nose in male and female rats; of the nose, forestomach, and glandular stomach in male and female mice; and of the kidney in female mice.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 12. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 14.

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APPENDIX A
SUMMARY OF LESIONS IN MALE RATS IN THE 2-YEAR GAVAGE STUDY OF ISOEUGENOL

Table A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of Isoeugenol[a]

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths		1		2
Moribund	14	9	14	11
Natural deaths	1	6	3	7
Survivors				
Terminal sacrifice	35	34	33	30
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Intestine large, cecum	(49)	(46)	(50)	(45)
Intestine large, colon	(50)	(46)	(50)	(47)
Adenoma				1 (2%)
Carcinoma			1 (2%)	
Intestine large, rectum	(50)	(46)	(50)	(48)
Intestine small, duodenum	(50)	(46)	(50)	(45)
Intestine small, ileum	(50)	(46)	(50)	(45)
Intestine small, jejunum	(50)	(46)	(50)	(45)
Leiomyoma	1 (2%)			
Liver	(50)	(50)	(50)	(48)
Hemangiosarcoma	1 (2%)			
Hepatocellular adenoma	1 (2%)			2 (4%)
Mesentery	(12)	(18)	(7)	(11)
Rhabdomyosarcoma, metastatic, skeletal muscle		1 (6%)		
Oral mucosa	(1)	(0)	(1)	(3)
Gingival, squamous cell carcinoma	1 (100%)		1 (100%)	1 (33%)
Pharyngeal, squamous cell carcinoma				1 (33%)
Pancreas	(50)	(50)	(50)	(49)
Rhabdomyosarcoma, metastatic, skeletal muscle		1 (2%)		
Acinus, adenoma	7 (14%)	1 (2%)	1 (2%)	4 (8%)
Acinus, adenoma, multiple				1 (2%)
Acinus, carcinoma	1 (2%)			
Salivary glands	(50)	(50)	(49)	(50)
Schwannoma malignant				1 (2%)
Stomach, forestomach	(50)	(49)	(50)	(49)
Squamous cell papilloma				1 (2%)
Stomach, glandular	(50)	(46)	(50)	(45)
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (2%)

Table A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(49)
Adenoma				1 (2%)
Adrenal medulla	(50)	(50)	(50)	(49)
Pheochromocytoma benign	4 (8%)	9 (18%)	9 (18%)	7 (14%)
Pheochromocytoma complex		1 (2%)		
Pheochromocytoma malignant	3 (6%)		1 (2%)	1 (2%)
Bilateral, pheochromocytoma benign	1 (2%)	1 (2%)		
Islets, pancreatic	(50)	(49)	(50)	(49)
Adenoma	4 (8%)	1 (2%)	1 (2%)	3 (6%)
Pituitary gland	(50)	(48)	(47)	(47)
Pars distalis, adenoma	19 (38%)	13 (27%)	19 (40%)	15 (32%)
Pars intermedia, adenoma	1 (2%)			
Thyroid gland	(50)	(49)	(50)	(47)
Bilateral, C-cell, adenoma				1 (2%)
C-cell, adenoma	9 (18%)	12 (24%)	8 (16%)	5 (11%)
C-cell, adenoma, multiple			1 (2%)	
C-cell, carcinoma	6 (12%)	3 (6%)	3 (6%)	2 (4%)
Follicular cell, adenoma	1 (2%)			
Follicular cell, carcinoma	1 (2%)	2 (4%)	2 (4%)	1 (2%)
General Body System				
None				
Genital System				
Epididymis	(50)	(50)	(50)	(50)
Preputial gland	(50)	(49)	(50)	(50)
Adenoma	2 (4%)	3 (6%)	4 (8%)	5 (10%)
Carcinoma	2 (4%)	3 (6%)	1 (2%)	4 (8%)
Prostate gland	(50)	(49)	(50)	(50)
Adenoma		2 (4%)	2 (4%)	1 (2%)
Adenoma, multiple			1 (2%)	
Chemodectoma benign		1 (2%)		
Seminal vesicle	(50)	(49)	(50)	(49)
Adenoma			1 (2%)	
Testes	(50)	(50)	(50)	(50)
Bilateral, interstitial cell, adenoma	40 (80%)	40 (80%)	37 (74%)	41 (82%)
Interstitial cell, adenoma	6 (12%)	3 (6%)	7 (14%)	3 (6%)
Hematopoietic System				
Bone marrow	(50)	(49)	(50)	(50)
Lymph node	(3)	(4)	(4)	(4)
Deep cervical, carcinoma, metastatic, thyroid gland	2 (67%)			
Mediastinal, schwannoma malignant, metastatic, salivary glands				1 (25%)
Lymph node, mesenteric	(50)	(48)	(50)	(47)
Spleen	(50)	(46)	(50)	(47)
Hemangiosarcoma	1 (2%)			
Leiomyoma				1 (2%)
Thymus	(47)	(43)	(49)	(48)
Thymoma benign				1 (2%)
Thymoma malignant				1 (2%)

Table A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Integumentary System				
Mammary gland	(50)	(49)	(50)	(50)
Carcinoma				2 (4%)
Fibroadenoma	4 (8%)	3 (6%)	1 (2%)	1 (2%)
Skin	(50)	(50)	(50)	(50)
Basal cell adenoma				1 (2%)
Basal cell carcinoma	1 (2%)		2 (4%)	
Keratoacanthoma	7 (14%)	5 (10%)	1 (2%)	1 (2%)
Squamous cell papilloma				1 (2%)
Sebaceous gland, adenoma	1 (2%)			
Subcutaneous tissue, fibroma	5 (10%)	1 (2%)	6 (12%)	2 (4%)
Subcutaneous tissue, fibrosarcoma	1 (2%)	1 (2%)		
Subcutaneous tissue, hemangiosarcoma			2 (4%)	
Subcutaneous tissue, lipoma	2 (4%)			
Subcutaneous tissue, sarcoma	1 (2%)	1 (2%)		
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Hemangiosarcoma			2 (4%)	
Osteoma		1 (2%)		
Osteosarcoma	2 (4%)	1 (2%)		
Vertebra, chordoma				1 (2%)
Skeletal muscle	(0)	(2)	(0)	(1)
Rhabdomyosarcoma		1 (50%)		
Sarcoma		1 (50%)		
Nervous System				
Brain	(50)	(50)	(50)	(50)
Glioma malignant		1 (2%)	1 (2%)	
Oligodendroglioma malignant	1 (2%)			
Peripheral nerve	(0)	(1)	(1)	(0)
Schwannoma malignant			1 (100%)	
Spinal cord	(0)	(1)	(1)	(0)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	3 (6%)	3 (6%)	2 (4%)	2 (4%)
Alveolar/bronchiolar carcinoma	1 (2%)	2 (4%)	1 (2%)	2 (4%)
Carcinoma, metastatic, preputial gland		1 (2%)		
Carcinoma, metastatic, thyroid gland	2 (4%)			
Carcinoma, metastatic, Zymbal's gland	1 (2%)			
Hemangiosarcoma	1 (2%)		1 (2%)	
Osteosarcoma, metastatic, bone	2 (4%)			
Pheochromocytoma malignant, metastatic, adrenal medulla			1 (2%)	
Sarcoma, metastatic, skin		1 (2%)		
Schwannoma malignant, metastatic, salivary glands				1 (2%)
Nose	(50)	(48)	(49)	(49)
Pleura	(0)	(0)	(0)	(2)
Trachea	(50)	(49)	(50)	(48)

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Special Senses System				
Eye	(50)	(46)	(50)	(45)
Harderian gland	(50)	(47)	(50)	(45)
Zymbal's gland	(2)	(1)	(0)	(0)
Carcinoma	2 (100%)	1 (100%)		
Urinary System				
Kidney	(50)	(48)	(50)	(49)
Hemangiosarcoma	1 (2%)			
Transitional epithelium, papilloma		1 (2%)		
Urinary bladder	(50)	(48)	(50)	(47)
Transitional epithelium, papilloma				1 (2%)
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Histiocytic sarcoma		1 (2%)		1 (2%)
Leukemia mononuclear	10 (20%)	11 (22%)	14 (28%)	7 (14%)
Mesothelioma malignant	3 (6%)	1 (2%)	2 (4%)	4 (8%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	50	47	46	50
Total primary neoplasms	158	131	136	131
Total animals with benign neoplasms	49	46	46	49
Total benign neoplasms	118	100	101	102
Total animals with malignant neoplasms	29	25	27	25
Total malignant neoplasms	40	31	35	29
Total animals with metastatic neoplasms	5	3	1	2
Total metastatic neoplasms	7	4	1	3

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate[a]	5/50 (10%)	10/50 (20%)	9/50 (18%)	7/49 (14%)
Adjusted rate[b]	10.9%	22.6%	21.4%	17.2%
Terminal rate[c]	4/35 (11%)	9/34 (27%)	6/33 (18%)	6/30 (20%)
First incidence (days)	674	726	607	719
Poly-3 test[d]	P = 0.342	P = 0.113	P = 0.148	P = 0.301
Adrenal Medulla: Malignant Pheochromocytoma				
Overall rate	3/50 (6%)	0/50 (0%)	1/50 (2%)	1/49 (2%)
Adjusted rate	6.6%	0.0%	2.4%	2.5%
Terminal rate	3/35 (9%)	0/34 (0%)	1/33 (3%)	1/30 (3%)
First incidence (days)	729 (T)	—[e]	729 (T)	729 (T)
Poly-3 test	P = 0.313N	P = 0.124N	P = 0.341N	P = 0.346N
Adrenal Medulla: Benign, Complex, or Malignant Pheochromocytoma				
Overall rate	7/50 (14%)	10/50 (20%)	9/50 (18%)	8/49 (16%)
Adjusted rate	15.3%	22.6%	21.4%	19.6%
Terminal rate	6/35 (17%)	9/34 (27%)	6/33 (18%)	7/30 (23%)
First incidence (days)	674	726	607	719
Poly-3 test	P = 0.406	P = 0.270	P = 0.324	P = 0.405
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	3/50 (6%)	3/50 (6%)	2/50 (4%)	2/50 (4%)
Adjusted rate	6.6%	6.7%	4.8%	4.8%
Terminal rate	2/35 (6%)	2/34 (6%)	1/33 (3%)	2/30 (7%)
First incidence (days)	715	533	624	729 (T)
Poly-3 test	P = 0.412N	P = 0.655	P = 0.541N	P = 0.540N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	4/50 (8%)	5/50 (10%)	3/50 (6%)	4/50 (8%)
Adjusted rate	8.8%	11.1%	7.2%	9.4%
Terminal rate	3/35 (9%)	3/34 (9%)	2/33 (6%)	2/30 (7%)
First incidence (days)	715	533	624	443
Poly-3 test	P = 0.545N	P = 0.491	P = 0.549N	P = 0.605
Mammary Gland: Fibroadenoma				
Overall rate	4/50 (8%)	3/50 (6%)	1/50 (2%)	1/50 (2%)
Adjusted rate	8.8%	6.8%	2.4%	2.4%
Terminal rate	2/35 (6%)	3/34 (9%)	1/33 (3%)	0/30 (0%)
First incidence (days)	688	729 (T)	729 (T)	692
Poly-3 test	P = 0.107N	P = 0.518N	P = 0.211N	P = 0.207N
Mammary Gland: Carcinoma				
Overall rate	0/50 (0%)	0/50 (0%)	0/50 (0%)	2/50 (4%)
Adjusted rate	0.0%	0.0%	0.0%	4.8%
Terminal rate	0/35	0/34	0/33	2/30 (7%)
First incidence (days)	—	—	—	729 (T)
Poly-3 test	P = 0.042	—[f]	—	P = 0.218

Table A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Mammary Gland: Fibroadenoma or Carcinoma				
Overall rate	4/50 (8%)	3/50 (6%)	1/50 (2%)	3/50 (6%)
Adjusted rate	8.8%	6.8%	2.4%	7.2%
Terminal rate	2/35 (6%)	3/34 (9%)	1/33 (3%)	2/30 (7%)
First incidence (days)	688	729 (T)	729 (T)	692
Poly-3 test	P = 0.431N	P = 0.518N	P = 0.211N	P = 0.548N
Pancreas: Adenoma				
Overall rate	7/50 (14%)	1/50 (2%)	1/50 (2%)	5/49 (10%)
Adjusted rate	15.4%	2.3%	2.4%	12.1%
Terminal rate	7/35 (20%)	1/34 (3%)	1/33 (3%)	5/30 (17%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P = 0.497N	P = 0.033N	P = 0.041N	P = 0.450N
Pancreas: Adenoma or Carcinoma				
Overall rate	8/50 (16%)	1/50 (2%)	1/50 (2%)	5/49 (10%)
Adjusted rate	17.6%	2.3%	2.4%	12.1%
Terminal rate	8/35 (23%)	1/34 (3%)	1/33 (3%)	5/30 (17%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P = 0.370N	P = 0.017N	P = 0.023N	P = 0.342N
Pancreatic Islets: Adenoma				
Overall rate	4/50 (8%)	1/49 (2%)	1/50 (2%)	3/49 (6%)
Adjusted rate	8.8%	2.3%	2.4%	7.3%
Terminal rate	4/35 (11%)	1/34 (3%)	1/33 (3%)	3/30 (10%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P = 0.550N	P = 0.194N	P = 0.210N	P = 0.554N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	19/50 (38%)	13/48 (27%)	19/47 (40%)	15/47 (32%)
Adjusted rate	40.7%	29.2%	48.2%	36.7%
Terminal rate	13/35 (37%)	7/34 (21%)	15/30 (50%)	12/30 (40%)
First incidence (days)	577	477	549	630
Poly-3 test	P = 0.513	P = 0.175N	P = 0.313	P = 0.437N
Preputial Gland: Adenoma				
Overall rate	2/50 (4%)	3/49 (6%)	4/50 (8%)	5/50 (10%)
Adjusted rate	4.4%	6.9%	9.6%	12.0%
Terminal rate	2/35 (6%)	3/33 (9%)	3/33 (9%)	5/30 (17%)
First incidence (days)	729 (T)	729 (T)	624	729 (T)
Poly-3 test	P = 0.123	P = 0.477	P = 0.297	P = 0.182
Preputial Gland: Carcinoma				
Overall rate	2/50 (4%)	3/49 (6%)	1/50 (2%)	4/50 (8%)
Adjusted rate	4.4%	6.9%	2.4%	9.3%
Terminal rate	1/35 (3%)	2/33 (6%)	0/33 (0%)	1/30 (3%)
First incidence (days)	577	568	605	485
Poly-3 test	P = 0.267	P = 0.477	P = 0.534N	P = 0.307

Table A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Preputial Gland: Adenoma or Carcinoma				
Overall rate	4/50 (8%)	5/49 (10%)	5/50 (10%)	9/50 (18%)
Adjusted rate	8.7%	11.4%	11.9%	20.9%
Terminal rate	3/35 (9%)	4/33 (12%)	3/33 (9%)	6/30 (20%)
First incidence (days)	577	568	605	485
Poly-3 test	P = 0.056	P = 0.468	P = 0.444	P = 0.090
Prostate Gland: Adenoma				
Overall rate	0/50 (0%)	2/49 (4%)	3/50 (6%)	1/50 (2%)
Adjusted rate	0.0%	4.6%	7.3%	2.4%
Terminal rate	0/35 (0%)	1/34 (3%)	3/33 (9%)	1/30 (3%)
First incidence (days)	—	710	729 (T)	729 (T)
Poly-3 test	P = 0.394	P = 0.227	P = 0.102	P = 0.483
Skin: Keratoacanthoma				
Overall rate	7/50 (14%)	5/50 (10%)	1/50 (2%)	1/50 (2%)
Adjusted rate	15.3%	11.3%	2.4%	2.4%
Terminal rate	5/35 (14%)	4/34 (12%)	1/33 (3%)	1/30 (3%)
First incidence (days)	681	701	729 (T)	729 (T)
Poly-3 test	P = 0.012N	P = 0.402N	P = 0.042N	P = 0.041N
Skin: Squamous Cell Papilloma or Keratoacanthoma				
Overall rate	7/50 (14%)	5/50 (10%)	1/50 (2%)	2/50 (4%)
Adjusted rate	15.3%	11.3%	2.4%	4.8%
Terminal rate	5/35 (14%)	4/34 (12%)	1/33 (3%)	2/30 (7%)
First incidence (days)	681	701	729 (T)	729 (T)
Poly-3 test	P = 0.038N	P = 0.402N	P = 0.042N	P = 0.102N
Skin: Squamous Cell Papilloma, Keratoacanthoma, Basal Cell Adenoma, or Basal Cell Carcinoma				
Overall rate	8/50 (16%)	5/50 (10%)	3/50 (6%)	3/50 (6%)
Adjusted rate	17.3%	11.3%	7.3%	7.2%
Terminal rate	5/35 (14%)	4/34 (12%)	3/33 (9%)	3/30 (10%)
First incidence (days)	617	701	729 (T)	729 (T)
Poly-3 test	P = 0.084N	P = 0.302N	P = 0.137N	P = 0.133N
Skin: Squamous Cell Papilloma, Keratoacanthoma, Basal Cell Adenoma, Basal Cell Carcinoma, or Sebaceous Gland Adenoma				
Overall rate	9/50 (18%)	5/50 (10%)	3/50 (6%)	3/50 (6%)
Adjusted rate	19.5%	11.3%	7.3%	7.2%
Terminal rate	6/35 (17%)	4/34 (12%)	3/33 (9%)	3/30 (10%)
First incidence (days)	617	701	729 (T)	729 (T)
Poly-3 test	P = 0.051N	P = 0.215N	P = 0.088N	P = 0.084N
Skin (Subcutaneous Tissue): Fibroma				
Overall rate	5/50 (10%)	1/50 (2%)	6/50 (12%)	2/50 (4%)
Adjusted rate	10.9%	2.3%	14.2%	4.8%
Terminal rate	4/35 (11%)	1/34 (3%)	2/33 (6%)	1/30 (3%)
First incidence (days)	635	729 (T)	624	719
Poly-3 test	P = 0.365N	P = 0.110N	P = 0.441	P = 0.255N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Skin (Subcutaneous Tissue): Fibroma, Fibrosarcoma, or Sarcoma				
Overall rate	7/50 (14%)	3/50 (6%)	6/50 (12%)	2/50 (4%)
Adjusted rate	15.1%	6.7%	14.2%	4.8%
Terminal rate	5/35 (14%)	1/34 (3%)	2/33 (6%)	1/30 (3%)
First incidence (days)	607	485	624	719
Poly-3 test	P = 0.136N	P = 0.169N	P = 0.572N	P = 0.105N
Testes: Adenoma				
Overall rate	46/50 (92%)	43/50 (86%)	44/50 (88%)	44/50 (88%)
Adjusted rate	95.4%	92.0%	96.2%	90.1%
Terminal rate	35/35 (100%)	33/34 (97%)	33/33 (100%)	27/30 (90%)
First incidence (days)	556	533	411	415
Poly-3 test	P = 0.222N	P = 0.388N	P = 0.644	P = 0.257N
Thyroid Gland (C-Cell): Adenoma				
Overall rate	9/50 (18%)	12/49 (24%)	9/50 (18%)	6/47 (13%)
Adjusted rate	19.7%	27.5%	21.6%	14.6%
Terminal rate	7/35 (20%)	9/34 (27%)	7/33 (21%)	2/30 (7%)
First incidence (days)	674	648	624	628
Poly-3 test	P = 0.228N	P = 0.267	P = 0.517	P = 0.367N
Thyroid Gland (C-Cell): Carcinoma				
Overall rate	6/50 (12%)	3/49 (6%)	3/50 (6%)	2/47 (4%)
Adjusted rate	13.1%	6.9%	7.2%	5.0%
Terminal rate	5/35 (14%)	3/34 (9%)	2/33 (6%)	2/30 (7%)
First incidence (days)	688	729 (T)	603	729 (T)
Poly-3 test	P = 0.142N	P = 0.269N	P = 0.288N	P = 0.178N
Thyroid Gland (C-Cell): Adenoma or Carcinoma				
Overall rate	15/50 (30%)	15/49 (31%)	11/50 (22%)	8/47 (17%)
Adjusted rate	32.7%	34.3%	26.1%	19.5%
Terminal rate	12/35 (34%)	12/34 (35%)	8/33 (24%)	4/30 (13%)
First incidence (days)	674	648	603	628
Poly-3 test	P = 0.068N	P = 0.522	P = 0.330N	P = 0.123N
All Organs: Hemangiosarcoma				
Overall rate	1/50 (2%)	0/50 (0%)	4/50 (8%)	0/50 (0%)
Adjusted rate	2.2%	0.0%	9.5%	0.0%
Terminal rate	0/35 (0%)	0/34 (0%)	3/33 (9%)	0/30 (0%)
First incidence (days)	607	—	485	—
Poly-3 test	P = 0.574N	P = 0.508N	P = 0.152	P = 0.519N
All Organs: Mononuclear Leukemia				
Overall rate	10/50 (20%)	11/50 (22%)	14/50 (28%)	7/50 (14%)
Adjusted rate	21.5%	24.5%	32.7%	16.6%
Terminal rate	5/35 (14%)	7/34 (21%)	8/33 (24%)	4/30 (13%)
First incidence (days)	624	648	549	676
Poly-3 test	P = 0.363N	P = 0.461	P = 0.169	P = 0.378N

Table A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
All Organs: Malignant Mesothelioma				
Overall rate	3/50 (6%)	1/50 (2%)	2/50 (4%)	4/50 (8%)
Adjusted rate	6.5%	2.3%	4.9%	9.5%
Terminal rate	2/35 (6%)	1/34 (3%)	2/33 (6%)	3/30 (10%)
First incidence (days)	556	729 (T)	729 (T)	674
Poly-3 test	P = 0.261	P = 0.320N	P = 0.550N	P = 0.449
All Organs: Benign Neoplasms				
Overall rate	49/50 (98%)	46/50 (92%)	46/50 (92%)	49/50 (98%)
Adjusted rate	99.7%	95.7%	99.5%	99.5%
Terminal rate	35/35 (100%)	33/34 (97%)	33/33 (100%)	30/30 (100%)
First incidence (days)	556	477	411	415
Poly-3 test	P = 0.447	P = 0.234N	P = 1.000N	P = 1.000N
All Organs: Malignant Neoplasms				
Overall rate	29/50 (58%)	25/50 (50%)	27/50 (54%)	25/50 (50%)
Adjusted rate	58.3%	53.4%	59.2%	54.7%
Terminal rate	16/35 (46%)	16/34 (47%)	16/33 (49%)	13/30 (43%)
First incidence (days)	401	485	411	415
Poly-3 test	P = 0.456N	P = 0.389N	P = 0.549	P = 0.441N
All Organs: Benign or Malignant Neoplasms				
Overall rate	50/50 (100%)	47/50 (94%)	46/50 (92%)	50/50 (100%)
Adjusted rate	100.0%	96.3%	99.5%	100.0%
Terminal rate	35/35 (100%)	33/34 (97%)	33/33 (100%)	30/30 (100%)
First incidence (days)	401	477	411	415
Poly-3 test	P = 0.446	P = 0.259N	P = 1.000N	—

(T) Terminal sacrifice
[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, lung, pancreas, pancreatic islets, pituitary gland, preputial gland, prostate gland, testes, and thyroid gland; for other tissues, denominator is number of animals necropsied.
[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[c] Observed incidence at terminal kill
[d] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence is the P value corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in a dosed group is indicated by **N**.
[e] Not applicable; no neoplasms in animal group
[f] Value of statistic cannot be computed.

TABLE A3a
Historical Incidence of Thymoma in Control Male F344/N Rats[a]

Study	Incidence in Controls		
	Benign	Malignant	Benign or Malignant
Historical Incidence: Corn Oil Gavage Studies			
Beta-myrcene	0/47	0/47	0/47
Isoeugenol	0/47	0/47	0/47
Overall Historical Incidence: Corn Oil Gavage Studies			
Total (%)	0/94	0/94	0/94
Overall Historical Incidence: All Routes			
Total (%)	2/1,146 (0.2%)	1/1,146 (0.1%)	3/1,146 (0.3%)
Mean ± standard deviation	0.2% ± 0.6%	0.1% ± 0.4%	0.3% ± 0.7%
Range	0%-2%	0%-2%	0%-2%

[a] Data as of October 4, 2007

TABLE A3b
Historical Incidence of Mammary Gland Carcinoma in Control Male F344/N Rats[a]

Study	Incidence in Controls
Historical Incidence: Corn Oil Gavage Studies	
Beta-myrcene	0/50
Isoeugenol	0/50
Overall Historical Incidence: Corn Oil Gavage Studies	
Total (%)	0/100
Overall Historical Incidence: All Routes	
Total (%)	8/1,199 (0.7%)
Mean ± standard deviation	0.7% ± 1.3%
Range	0%-4%

[a] Data as of October 4, 2007

TABLE A3c
Historical Incidence of Keratoacanthoma of the Skin in Control Male F344/N Rats[a]

Study	Incidence in Controls
Historical Incidence: Corn Oil Gavage Studies	
Beta-myrcene	2/50
Isoeugenol	7/50
Overall Historical Incidence: Corn Oil Gavage Studies	
Total (%)	9/100 (9.0%)
Mean ± standard deviation	9.0% ± 7.1%
Range	4%-14%
Overall Historical Incidence: All Routes	
Total (%)	71/1,199 (5.9%)
Mean ± standard deviation	5.9% ± 5.2%
Range	0%-20%

[a] Data as of October 4, 2007

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Isoeugenol[a]

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths		1		2
Moribund	14	9	14	11
Natural deaths	1	6	3	7
Survivors				
Terminal sacrifice	35	34	33	30
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Perforation				2 (4%)
Intestine large, cecum	(49)	(46)	(50)	(45)
Inflammation, acute		1 (2%)		
Intestine large, colon	(50)	(46)	(50)	(47)
Parasite metazoan	1 (2%)	1 (2%)	2 (4%)	
Intestine large, rectum	(50)	(46)	(50)	(48)
Parasite metazoan	4 (8%)	1 (2%)		
Intestine small, duodenum	(50)	(46)	(50)	(45)
Inflammation, acute				1 (2%)
Intestine small, ileum	(50)	(46)	(50)	(45)
Intestine small, jejunum	(50)	(46)	(50)	(45)
Inflammation, chronic			1 (2%)	
Liver	(50)	(50)	(50)	(48)
Angiectasis	1 (2%)			2 (4%)
Basophilic focus	43 (86%)	34 (68%)	26 (52%)	18 (38%)
Clear cell focus	27 (54%)	20 (40%)	19 (38%)	14 (29%)
Degeneration, cystic	4 (8%)	1 (2%)	2 (4%)	4 (8%)
Eosinophilic focus	8 (16%)	2 (4%)		2 (4%)
Fatty change	2 (4%)	1 (2%)	2 (4%)	2 (4%)
Hematopoietic cell proliferation		1 (2%)		
Hepatodiaphragmatic nodule	5 (10%)	3 (6%)	3 (6%)	3 (6%)
Infarct				1 (2%)
Malformation		1 (2%)		
Mixed cell focus	6 (12%)	4 (8%)	6 (12%)	2 (4%)
Necrosis	1 (2%)			
Bile duct, hyperplasia	39 (78%)	39 (78%)	32 (64%)	24 (50%)
Centrilobular, necrosis	2 (4%)	1 (2%)		1 (2%)
Hepatocyte, vacuolization cytoplasmic				1 (2%)
Mesentery	(12)	(18)	(7)	(11)
Fat, hemorrhage	1 (8%)			
Fat, necrosis	10 (83%)	17 (94%)	7 (100%)	9 (82%)
Oral mucosa	(1)	(0)	(1)	(3)
Pancreas	(50)	(50)	(50)	(49)
Basophilic focus	1 (2%)	1 (2%)		
Metaplasia, hepatocyte	1 (2%)			
Acinus, atrophy	22 (44%)	21 (42%)	17 (34%)	10 (20%)
Acinus, hyperplasia	21 (42%)	12 (24%)	13 (26%)	19 (39%)
Salivary glands	(50)	(50)	(49)	(50)
Hyperplasia	1 (2%)			

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

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Alimentary System *(continued)*				
Stomach, forestomach	(50)	(49)	(50)	(49)
Hyperplasia, squamous	2 (4%)	1 (2%)	3 (6%)	5 (10%)
Inflammation	1 (2%)			
Ulcer	3 (6%)	1 (2%)	4 (8%)	2 (4%)
Stomach, glandular	(50)	(46)	(50)	(45)
Atrophy	2 (4%)	3 (7%)	2 (4%)	3 (7%)
Inflammation, chronic active			1 (2%)	
Mineralization				1 (2%)
Necrosis		1 (2%)	1 (2%)	
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	48 (96%)	44 (88%)	47 (94%)	46 (92%)
Thrombosis	1 (2%)	2 (4%)		
Pericardium, inflammation, acute				1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(49)
Degeneration, cystic	2 (4%)			
Hyperplasia	31 (62%)	22 (44%)	21 (42%)	31 (63%)
Hypertrophy	5 (10%)	1 (2%)	4 (8%)	1 (2%)
Vacuolization cytoplasmic		2 (4%)		
Adrenal medulla	(50)	(50)	(50)	(49)
Hyperplasia	24 (48%)	18 (36%)	16 (32%)	15 (31%)
Islets, pancreatic	(50)	(49)	(50)	(49)
Hyperplasia	1 (2%)		1 (2%)	
Pituitary gland	(50)	(48)	(47)	(47)
Hemorrhage				1 (2%)
Pars distalis, angiectasis	6 (12%)		3 (6%)	2 (4%)
Pars distalis, hyperplasia	18 (36%)	20 (42%)	15 (32%)	16 (34%)
Thyroid gland	(50)	(49)	(50)	(47)
C-cell, hyperplasia	9 (18%)	5 (10%)	7 (14%)	9 (19%)
Follicular cell, hyperplasia	3 (6%)	3 (6%)		5 (11%)
General Body System				
None				
Genital System				
Epididymis	(50)	(50)	(50)	(50)
Granuloma sperm		1 (2%)		
Preputial gland	(50)	(49)	(50)	(50)
Cyst		1 (2%)		
Hyperplasia	1 (2%)	2 (4%)	1 (2%)	4 (8%)
Inflammation, chronic active		1 (2%)		
Prostate gland	(50)	(49)	(50)	(50)
Hyperplasia	10 (20%)	9 (18%)	15 (30%)	11 (22%)
Inflammation, chronic active	3 (6%)	1 (2%)		3 (6%)
Seminal vesicle	(50)	(49)	(50)	(49)
Hyperplasia				1 (2%)
Testes	(50)	(50)	(50)	(50)
Atrophy	1 (2%)	4 (8%)	3 (6%)	
Interstitial cell, hyperplasia	7 (14%)	2 (4%)	4 (8%)	1 (2%)

Table A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Hematopoietic System				
Bone marrow	(50)	(49)	(50)	(50)
Hyperplasia, reticulum cell			1 (2%)	
Lymph node	(3)	(4)	(4)	(4)
Deep cervical, hyperplasia, lymphoid			1 (25%)	
Mediastinal, ectasia	1 (33%)			
Pancreatic, inflammation, chronic active			1 (25%)	
Lymph node, mesenteric	(50)	(48)	(50)	(47)
Spleen	(50)	(46)	(50)	(47)
Fibrosis	2 (4%)		2 (4%)	
Hematopoietic cell proliferation	3 (6%)	3 (7%)	1 (2%)	2 (4%)
Hemorrhage	1 (2%)			
Hyperplasia, lymphoid		1 (2%)		
Inflammation, acute		1 (2%)		
Necrosis	2 (4%)	1 (2%)	1 (2%)	
Thymus	(47)	(43)	(49)	(48)
Integumentary System				
Mammary gland	(50)	(49)	(50)	(50)
Skin	(50)	(50)	(50)	(50)
Cyst epithelial inclusion		2 (4%)		2 (4%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Skeletal muscle	(0)	(2)	(0)	(1)
Nervous System				
Brain	(50)	(50)	(50)	(50)
Hydrocephalus	2 (4%)			
Thrombosis		1 (2%)		
Meninges, hemorrhage			1 (2%)	
Peripheral nerve	(0)	(1)	(1)	(0)
Spinal cord	(0)	(1)	(1)	(0)
Hemorrhage			1 (100%)	
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Foreign body			3 (6%)	1 (2%)
Inflammation, chronic active		1 (2%)	1 (2%)	
Metaplasia, squamous	1 (2%)	2 (4%)		
Thrombosis		1 (2%)		
Alveolar epithelium, hyperplasia	27 (54%)	20 (40%)	16 (32%)	25 (50%)
Alveolus, infiltration cellular, histiocyte				1 (2%)
Bronchiole, hyperplasia		1 (2%)	1 (2%)	1 (2%)
Perivascular, inflammation, chronic active		1 (2%)		

Table A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Respiratory System *(continued)*				
Nose	(50)	(48)	(49)	(49)
Foreign body	3 (6%)		3 (6%)	4 (8%)
Inflammation, suppurative	12 (24%)	11 (23%)	13 (27%)	12 (24%)
Inflammation, chronic	2 (4%)	1 (2%)	2 (4%)	2 (4%)
Thrombosis			1 (2%)	
Glands, dilatation		2 (4%)	1 (2%)	2 (4%)
Nasolacrimal duct, cyst			1 (2%)	
Nasolacrimal duct, inflammation				1 (2%)
Olfactory epithelium, accumulation, hyaline droplet	50 (100%)	37 (77%)	42 (86%)	42 (86%)
Olfactory epithelium, atrophy	1 (2%)	5 (10%)	9 (18%)	13 (27%)
Olfactory epithelium, degeneration	1 (2%)		2 (4%)	6 (12%)
Olfactory epithelium, hyperplasia				1 (2%)
Olfactory epithelium, hyperplasia, basal cell			1 (2%)	
Olfactory epithelium, metaplasia, respiratory	4 (8%)	6 (13%)	10 (20%)	15 (31%)
Olfactory epithelium, metaplasia, squamous	1 (2%)	1 (2%)		
Respiratory epithelium, accumulation, hyaline droplet	3 (6%)	5 (10%)	1 (2%)	2 (4%)
Respiratory epithelium, hyperplasia	12 (24%)	3 (6%)	14 (29%)	8 (16%)
Respiratory epithelium, metaplasia, squamous	1 (2%)	4 (8%)	3 (6%)	5 (10%)
Pleura				(2)
Inflammation, acute				2 (100%)
Trachea	(50)	(49)	(50)	(48)
Epithelium, necrosis		1 (2%)		
Special Senses System				
Eye	(50)	(46)	(50)	(45)
Cataract		1 (2%)		2 (4%)
Retina, atrophy		1 (2%)	1 (2%)	2 (4%)
Harderian gland	(50)	(47)	(50)	(45)
Hyperplasia	1 (2%)	5 (11%)	5 (10%)	1 (2%)
Inflammation, chronic active	1 (2%)			
Zymbal's gland	(2)	(1)	(0)	(0)
Urinary System				
Kidney	(50)	(48)	(50)	(49)
Cyst				1 (2%)
Degeneration, mucoid, focal				1 (2%)
Hyperplasia, oncocytic				1 (2%)
Inflammation, suppurative				1 (2%)
Nephropathy	49 (98%)	46 (96%)	47 (94%)	47 (96%)
Renal tubule, degeneration, hyaline				1 (2%)
Transitional epithelium, hyperplasia		1 (2%)		2 (4%)
Urinary bladder	(50)	(48)	(50)	(47)

APPENDIX B
SUMMARY OF LESIONS IN FEMALE RATS IN THE 2-YEAR GAVAGE STUDY OF ISOEUGENOL

Table B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Gavage Study of Isoeugenol[a]

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death		1		
Moribund	15	8	9	9
Natural deaths	2	6	7	10
Survivors				
Terminal sacrifice	33	35	34	31
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Intestine large, colon	(50)	(48)	(47)	(48)
Intestine large, rectum	(49)	(48)	(47)	(48)
Intestine small, ileum	(49)	(47)	(45)	(45)
Liver	(50)	(50)	(50)	(50)
Mesentery	(11)	(11)	(11)	(14)
Schwannoma malignant				1 (7%)
Oral mucosa	(0)	(1)	(0)	(1)
Pharyngeal, squamous cell papilloma		1 (100%)		
Pancreas	(50)	(49)	(48)	(50)
Salivary glands	(49)	(50)	(50)	(50)
Stomach, forestomach	(50)	(50)	(49)	(50)
Stomach, glandular	(50)	(47)	(46)	(47)
Tongue	(0)	(0)	(1)	(0)
Squamous cell carcinoma			1 (100%)	
Tooth	(0)	(1)	(0)	(0)
Odontoma		1 (100%)		
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Schwannoma malignant, metastatic, mesentery				1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(47)	(49)
Adenoma		1 (2%)		
Adrenal medulla	(50)	(50)	(46)	(49)
Pheochromocytoma benign	2 (4%)	3 (6%)		2 (4%)
Bilateral, pheochromocytoma benign		1 (2%)		
Islets, pancreatic	(50)	(49)	(47)	(49)
Adenoma			1 (2%)	
Parathyroid gland	(47)	(44)	(48)	(44)
Pituitary gland	(50)	(48)	(49)	(49)
Pars distalis, adenoma	25 (50%)	20 (42%)	20 (41%)	20 (41%)
Pars intermedia, adenoma			1 (2%)	
Thyroid gland	(50)	(46)	(46)	(48)
Bilateral, C-cell, adenoma		1 (2%)		1 (2%)
Bilateral, C-cell, carcinoma	1 (2%)			
C-cell, adenoma	7 (14%)	7 (15%)	7 (15%)	5 (10%)
C-cell, carcinoma	2 (4%)	2 (4%)	2 (4%)	2 (4%)
Follicular cell, adenoma		1 (2%)		
Follicular cell, carcinoma	1 (2%)		1 (2%)	

Table B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
General Body System				
None				
Genital System				
Clitoral gland	(48)	(50)	(49)	(50)
Adenoma	3 (6%)	3 (6%)	4 (8%)	5 (10%)
Carcinoma	1 (2%)	2 (4%)		2 (4%)
Bilateral, carcinoma	1 (2%)			
Ovary	(50)	(50)	(49)	(50)
Cystadenoma		1 (2%)		
Granulosa-theca tumor malignant				1 (2%)
Schwannoma malignant, metastatic, mesentery				1 (2%)
Uterus	(50)	(50)	(49)	(50)
Deciduoma benign	1 (2%)			
Polyp stromal	8 (16%)	15 (30%)	6 (12%)	5 (10%)
Polyp stromal, multiple		1 (2%)		
Sarcoma stromal			1 (2%)	
Vagina	(1)	(0)	(1)	(0)
Polyp	1 (100%)			
Hematopoietic System				
Bone marrow	(50)	(49)	(49)	(49)
Lymph node	(0)	(3)	(3)	(3)
Lymph node, mesenteric	(50)	(49)	(48)	(49)
Schwannoma malignant, metastatic, mesentery				1 (2%)
Spleen	(50)	(49)	(49)	(49)
Thymus	(47)	(47)	(46)	(49)
Integumentary System				
Mammary gland	(49)	(50)	(50)	(49)
Carcinoma	4 (8%)	1 (2%)	2 (4%)	4 (8%)
Fibroadenoma	17 (35%)	19 (38%)	12 (24%)	13 (27%)
Fibroadenoma, multiple	9 (18%)	9 (18%)	7 (14%)	5 (10%)
Skin	(50)	(50)	(50)	(50)
Squamous cell carcinoma				1 (2%)
Squamous cell papilloma			1 (2%)	
Subcutaneous tissue, fibroma		1 (2%)		
Subcutaneous tissue, lipoma		1 (2%)	1 (2%)	
Subcutaneous tissue, neural crest tumor			1 (2%)	
Subcutaneous tissue, sarcoma			1 (2%)	
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Osteosarcoma		1 (2%)		
Nervous System				
Brain	(50)	(50)	(50)	(50)
Astrocytoma malignant			1 (2%)	
Granular cell tumor malignant				1 (2%)
Oligodendroglioma malignant	1 (2%)			

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	1 (2%)	2 (4%)	1 (2%)	4 (8%)
Granulosa-theca tumor malignant, metastatic, ovary				1 (2%)
Schwannoma malignant, metastatic, mesentery				1 (2%)
Nose	(50)	(49)	(49)	(49)
Respiratory epithelium, adenoma		1 (2%)		
Pleura	(0)	(0)	(0)	(1)
Trachea	(50)	(49)	(48)	(49)
Special Senses System				
Eye	(49)	(46)	(47)	(48)
Zymbal's gland	(0)	(1)	(0)	(1)
Carcinoma		1 (100%)		1 (100%)
Urinary System				
Kidney	(50)	(49)	(47)	(50)
Transitional epithelium, carcinoma			1 (2%)	
Urinary bladder	(50)	(50)	(48)	(49)
Leiomyoma				1 (2%)
Sarcoma stromal, metastatic, uterus			1 (2%)	
Transitional epithelium, papilloma			1 (2%)	
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Histiocytic sarcoma		1 (2%)		1 (2%)
Leukemia mononuclear	8 (16%)	5 (10%)	12 (24%)	8 (16%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	46	46	46	43
Total primary neoplasms	93	102	85	83
Total animals with benign neoplasms	41	45	36	40
Total benign neoplasms	74	89	62	61
Total animals with malignant neoplasms	18	12	21	20
Total malignant neoplasms	19	13	22	22
Total animals with metastatic neoplasms			1	2
Total metastatic neoplasms			1	5
Total animals with uncertain neoplasm – benign or malignant			1	
Total uncertain neoplasms			1	

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

Table B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate[a]	2/50 (4%)	4/50 (8%)	0/46 (0%)	2/49 (4%)
Adjusted rate[b]	4.4%	9.3%	0.0%	5.1%
Terminal rate[c]	1/33 (3%)	3/35 (9%)	0/33 (0%)	1/31 (3%)
First incidence (days)	559	708	—[e]	623
Poly-3 test[d]	P = 0.450N	P = 0.313	P = 0.261N	P = 0.641
Clitoral Gland: Adenoma				
Overall rate	3/48 (6%)	3/50 (6%)	4/49 (8%)	5/50 (10%)
Adjusted rate	7.0%	7.0%	9.3%	12.7%
Terminal rate	3/31 (10%)	3/35 (9%)	4/34 (12%)	5/31 (16%)
First incidence (days)	730 (T)	730 (T)	730 (T)	730 (T)
Poly-3 test	P = 0.209	P = 0.662N	P = 0.506	P = 0.312
Clitoral Gland: Adenoma or Carcinoma				
Overall rate	5/48 (10%)	5/50 (10%)	4/49 (8%)	7/50 (14%)
Adjusted rate	11.7%	11.7%	9.3%	17.7%
Terminal rate	4/31 (13%)	5/35 (14%)	4/34 (12%)	7/31 (23%)
First incidence (days)	713	730 (T)	730 (T)	730 (T)
Poly-3 test	P = 0.262	P = 0.630N	P = 0.495N	P = 0.320
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	1/50 (2%)	2/50 (4%)	1/50 (2%)	4/50 (8%)
Adjusted rate	2.2%	4.7%	2.3%	10.1%
Terminal rate	1/33 (3%)	2/35 (6%)	1/34 (3%)	4/31 (13%)
First incidence (days)	730 (T)	730 (T)	730 (T)	730 (T)
Poly-3 test	P = 0.091	P = 0.485	P = 0.756	P = 0.142
Mammary Gland: Fibroadenoma				
Overall rate	26/50 (52%)	28/50 (56%)	19/50 (38%)	18/50 (36%)
Adjusted rate	56.3%	64.1%	42.4%	43.6%
Terminal rate	20/33 (61%)	24/35 (69%)	15/34 (44%)	13/31 (42%)
First incidence (days)	617	660	529	606
Poly-3 test	P = 0.053N	P = 0.292	P = 0.127N	P = 0.162N
Mammary Gland: Carcinoma				
Overall rate	4/50 (8%)	1/50 (2%)	2/50 (4%)	4/50 (8%)
Adjusted rate	8.9%	2.3%	4.6%	10.0%
Terminal rate	3/33 (9%)	1/35 (3%)	2/34 (6%)	3/31 (10%)
First incidence (days)	627	730 (T)	730 (T)	623
Poly-3 test	P = 0.400	P = 0.194N	P = 0.351N	P = 0.573
Mammary Gland: Fibroadenoma or Carcinoma				
Overall rate	28/50 (56%)	29/50 (58%)	21/50 (42%)	22/50 (44%)
Adjusted rate	60.2%	66.3%	46.9%	52.9%
Terminal rate	21/33 (64%)	25/35 (71%)	17/34 (50%)	16/31 (52%)
First incidence (days)	617	660	529	606
Poly-3 test	P = 0.148N	P = 0.345	P = 0.138N	P = 0.314N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	25/50 (50%)	20/48 (42%)	20/49 (41%)	20/49 (41%)
Adjusted rate	52.6%	46.2%	43.9%	47.0%
Terminal rate	16/33 (49%)	15/35 (43%)	14/34 (41%)	12/31 (39%)
First incidence (days)	559	389	529	496
Poly-3 test	P = 0.343N	P = 0.345N	P = 0.264N	P = 0.374N

Table B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Thyroid Gland (C-Cell): Adenoma				
Overall rate	7/50 (14%)	8/46 (17%)	7/46 (15%)	6/48 (13%)
Adjusted rate	15.5%	19.6%	17.0%	15.2%
Terminal rate	6/33 (18%)	8/35 (23%)	6/34 (18%)	4/31 (13%)
First incidence (days)	647	730 (T)	660	547
Poly-3 test	P = 0.487N	P = 0.417	P = 0.544	P = 0.601N
Thyroid Gland (C-Cell): Carcinoma				
Overall rate	3/50 (6%)	2/46 (4%)	2/46 (4%)	2/48 (4%)
Adjusted rate	6.7%	4.9%	4.9%	5.2%
Terminal rate	3/33 (9%)	1/35 (3%)	2/34 (6%)	2/31 (7%)
First incidence (days)	730 (T)	660	730 (T)	730 (T)
Poly-3 test	P = 0.480N	P = 0.539N	P = 0.540N	P = 0.565N
Thyroid Gland (C-Cell): Adenoma or Carcinoma				
Overall rate	9/50 (18%)	10/46 (22%)	9/46 (20%)	8/48 (17%)
Adjusted rate	20.0%	24.3%	21.8%	20.2%
Terminal rate	8/33 (24%)	9/35 (26%)	8/34 (24%)	6/31 (19%)
First incidence (days)	647	660	660	547
Poly-3 test	P = 0.515N	P = 0.410	P = 0.521	P = 0.594
Uterus: Stromal Polyp				
Overall rate	8/50 (16%)	16/50 (32%)	6/50 (12%)	5/50 (10%)
Adjusted rate	17.7%	34.8%	13.5%	12.6%
Terminal rate	5/33 (15%)	10/35 (29%)	5/34 (15%)	4/31 (13%)
First incidence (days)	673	389	573	632
Poly-3 test	P = 0.104N	P = 0.050	P = 0.402N	P = 0.363N
Uterus: Stromal Polyp or Stromal Sarcoma				
Overall rate	8/50 (16%)	16/50 (32%)	7/50 (14%)	5/50 (10%)
Adjusted rate	17.7%	34.8%	15.6%	12.6%
Terminal rate	5/33 (15%)	10/35 (29%)	5/34 (15%)	4/31 (13%)
First incidence (days)	673	389	529	632
Poly-3 test	P = 0.115N	P = 0.050	P = 0.506N	P = 0.363N
All Organs: Mononuclear Leukemia				
Overall rate	8/50 (16%)	5/50 (10%)	12/50 (24%)	8/50 (16%)
Adjusted rate	17.4%	11.6%	25.8%	20.1%
Terminal rate	3/33 (9%)	4/35 (11%)	6/34 (18%)	7/31 (23%)
First incidence (days)	617	671	499	632
Poly-3 test	P = 0.268	P = 0.318N	P = 0.232	P = 0.483
All Organs: Benign Neoplasms				
Overall rate	41/50 (82%)	45/50 (90%)	36/50 (72%)	40/50 (80%)
Adjusted rate	83.9%	94.6%	76.5%	91.2%
Terminal rate	28/33 (85%)	33/35 (94%)	27/34 (79%)	29/31 (94%)
First incidence (days)	400	389	529	496
Poly-3 test	P = 0.397	P = 0.075	P = 0.248N	P = 0.213

Table B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
All Organs: Malignant Neoplasms				
Overall rate	18/50 (36%)	12/50 (24%)	21/50 (42%)	20/50 (40%)
Adjusted rate	38.5%	27.1%	44.1%	47.0%
Terminal rate	10/33 (30%)	8/35 (23%)	12/34 (35%)	13/31 (42%)
First incidence (days)	617	469	499	541
Poly-3 test	P = 0.115	P = 0.173N	P = 0.365	P = 0.274
All Organs: Benign or Malignant Neoplasms				
Overall rate	46/50 (92%)	46/50 (92%)	46/50 (92%)	43/50 (86%)
Adjusted rate	92.0%	96.7%	92.0%	95.7%
Terminal rate	29/33 (88%)	34/35 (97%)	30/34 (88%)	30/31 (97%)
First incidence (days)	400	389	499	496
Poly-3 test	P = 0.388	P = 0.285	P = 0.642	P = 0.370

(T) Terminal sacrifice
[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, clitoral gland, lung, pituitary gland, and thyroid gland; for other tissues, denominator is number of animals necropsied.
[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[c] Observed incidence at terminal kill
[d] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence is the P value corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in a dosed group is indicated by **N**.
[e] Not applicable; no neoplasms in animal group

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Isoeugenol[a]

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death		1		
Moribund	15	8	9	9
Natural deaths	2	6	7	10
Survivors				
Terminal sacrifice	33	35	34	31
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Perforation		1 (2%)		
Intestine large, colon	(50)	(48)	(47)	(48)
Parasite metazoan	2 (4%)	1 (2%)	1 (2%)	
Intestine large, rectum	(49)	(48)	(47)	(48)
Parasite metazoan	1 (2%)	1 (2%)	1 (2%)	2 (4%)
Intestine small, ileum	(49)	(47)	(45)	(45)
Parasite metazoan			1 (2%)	
Liver	(50)	(50)	(50)	(50)
Angiectasis			2 (4%)	3 (6%)
Basophilic focus	50 (100%)	49 (98%)	48 (96%)	46 (92%)
Clear cell focus	12 (24%)	9 (18%)	9 (18%)	10 (20%)
Degeneration, cystic	1 (2%)			
Eosinophilic focus	2 (4%)	2 (4%)	5 (10%)	5 (10%)
Fatty change	2 (4%)		1 (2%)	
Hepatodiaphragmatic nodule	8 (16%)	11 (22%)	7 (14%)	4 (8%)
Mixed cell focus	5 (10%)	7 (14%)	3 (6%)	3 (6%)
Necrosis		1 (2%)		
Bile duct, hyperplasia	3 (6%)	4 (8%)	4 (8%)	4 (8%)
Hepatocyte, mitotic alteration				1 (2%)
Mesentery	(11)	(11)	(11)	(14)
Fat, hemorrhage	1 (9%)			
Fat, necrosis	11 (100%)	10 (91%)	11 (100%)	13 (93%)
Oral mucosa	(0)	(1)	(0)	(1)
Pancreas	(50)	(49)	(48)	(50)
Basophilic focus	1 (2%)			
Acinus, atrophy	9 (18%)	4 (8%)	2 (4%)	4 (8%)
Acinus, hyperplasia	5 (10%)	2 (4%)		5 (10%)
Salivary glands	(49)	(50)	(50)	(50)
Stomach, forestomach	(50)	(50)	(49)	(50)
Hyperplasia, squamous	2 (4%)		1 (2%)	2 (4%)
Inflammation	1 (2%)			1 (2%)
Ulcer	1 (2%)		2 (4%)	2 (4%)
Stomach, glandular	(50)	(47)	(46)	(47)
Atrophy	1 (2%)	3 (6%)		3 (6%)
Mineralization	3 (6%)	1 (2%)	2 (4%)	1 (2%)
Tongue	(0)	(0)	(1)	(0)
Tooth	(0)	(1)	(0)	(0)

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

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	41 (82%)	42 (84%)	38 (76%)	39 (78%)
Pericardium, inflammation, granulomatous				1 (2%)
Pericardium, inflammation, acute		1 (2%)		1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(47)	(49)
Degeneration, cystic		2 (4%)	4 (9%)	3 (6%)
Hyperplasia	25 (50%)	17 (34%)	18 (38%)	18 (37%)
Hypertrophy	4 (8%)	3 (6%)	2 (4%)	2 (4%)
Necrosis	1 (2%)	2 (4%)	1 (2%)	
Vacuolization cytoplasmic				1 (2%)
Adrenal medulla	(50)	(50)	(46)	(49)
Hyperplasia	5 (10%)	6 (12%)	5 (11%)	5 (10%)
Islets, pancreatic	(50)	(49)	(47)	(49)
Parathyroid gland	(47)	(44)	(48)	(44)
Hyperplasia	1 (2%)			
Pituitary gland	(50)	(48)	(49)	(49)
Cyst	1 (2%)			
Pars distalis, angiectasis	3 (6%)	2 (4%)	2 (4%)	3 (6%)
Pars distalis, hyperplasia	17 (34%)	21 (44%)	21 (43%)	20 (41%)
Thyroid gland	(50)	(46)	(46)	(48)
C-cell, hyperplasia	9 (18%)	4 (9%)	7 (15%)	6 (13%)
Follicular cell, hyperplasia	2 (4%)	2 (4%)		
General Body System				
None				
Genital System				
Clitoral gland	(48)	(50)	(49)	(50)
Hyperplasia	7 (15%)	5 (10%)	5 (10%)	4 (8%)
Inflammation, chronic active		1 (2%)		1 (2%)
Ovary	(50)	(50)	(49)	(50)
Cyst	1 (2%)	6 (12%)	1 (2%)	4 (8%)
Uterus	(50)	(50)	(49)	(50)
Inflammation, acute			1 (2%)	
Necrosis	1 (2%)			
Endometrium, hyperplasia, cystic	2 (4%)		1 (2%)	
Vagina	(1)	(0)	(1)	(0)
Inflammation, suppurative			1 (100%)	
Hematopoietic System				
Bone marrow	(50)	(49)	(49)	(49)
Hyperplasia, reticulum cell	1 (2%)	1 (2%)	1 (2%)	
Lymph node	(0)	(3)	(3)	(3)
Deep cervical, ectasia		1 (33%)		1 (33%)
Deep cervical, hemorrhage				1 (33%)
Lymph node, mesenteric	(50)	(49)	(48)	(49)
Spleen	(50)	(49)	(49)	(49)
Hematopoietic cell proliferation		1 (2%)	2 (4%)	3 (6%)
Inflammation, granulomatous			1 (2%)	1 (2%)
Necrosis				1 (2%)
Thymus	(47)	(47)	(46)	(49)
Inflammation, acute				1 (2%)

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Integumentary System				
Mammary gland	(49)	(50)	(50)	(49)
Galactocele				1 (2%)
Hyperplasia	1 (2%)			
Skin	(50)	(50)	(50)	(50)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Hyperostosis			1 (2%)	
Nervous System				
Brain	(50)	(50)	(50)	(50)
Hemorrhage			1 (2%)	
Hydrocephalus	1 (2%)			
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Cyst				1 (2%)
Inflammation, acute	1 (2%)			3 (6%)
Inflammation, chronic active	2 (4%)	4 (8%)	3 (6%)	2 (4%)
Alveolar epithelium, hyperplasia	17 (34%)	11 (22%)	16 (32%)	14 (28%)
Alveolus, infiltration cellular, histiocyte				1 (2%)
Bronchiole, hyperplasia	1 (2%)		1 (2%)	1 (2%)
Nose	(50)	(49)	(49)	(49)
Foreign body	1 (2%)			
Inflammation, suppurative	3 (6%)	5 (10%)	2 (4%)	3 (6%)
Inflammation, chronic	5 (10%)	4 (8%)		2 (4%)
Thrombosis	1 (2%)	1 (2%)	1 (2%)	
Glands, dilatation	2 (4%)			2 (4%)
Glands, hyperplasia		1 (2%)	1 (2%)	1 (2%)
Olfactory epithelium, accumulation, hyaline droplet	48 (96%)	36 (73%)	37 (76%)	33 (67%)
Olfactory epithelium, atrophy				4 (8%)
Olfactory epithelium, degeneration			1 (2%)	
Olfactory epithelium, metaplasia, respiratory	5 (10%)	5 (10%)	9 (18%)	12 (24%)
Olfactory epithelium, metaplasia, squamous	3 (6%)			1 (2%)
Respiratory epithelium, accumulation, hyaline droplet	4 (8%)	3 (6%)	2 (4%)	
Respiratory epithelium, hyperplasia	6 (12%)	9 (18%)	4 (8%)	6 (12%)
Respiratory epithelium, metaplasia, squamous	2 (4%)	2 (4%)		2 (4%)
Respiratory epithelium, necrosis		1 (2%)		
Pleura	(0)	(0)	(0)	(1)
Inflammation, suppurative				1 (100%)
Trachea	(50)	(49)	(48)	(49)
Inflammation, suppurative				1 (2%)

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Special Senses System				
Eye	(49)	(46)	(47)	(48)
Cataract			3 (6%)	
Cornea, inflammation, acute		1 (2%)		
Cornea, mineralization	1 (2%)			
Retina, atrophy	1 (2%)		3 (6%)	1 (2%)
Zymbal's gland	(0)	(1)	(0)	(1)
Urinary System				
Kidney	(50)	(49)	(47)	(50)
Infarct		1 (2%)		1 (2%)
Inflammation, suppurative				2 (4%)
Nephropathy	41 (82%)	39 (80%)	40 (85%)	40 (80%)
Renal tubule, necrosis	1 (2%)			
Transitional epithelium, hyperplasia	1 (2%)		1 (2%)	2 (4%)
Urinary bladder	(50)	(50)	(48)	(49)
Inflammation, chronic active				1 (2%)

APPENDIX C
SUMMARY OF LESIONS IN MALE MICE IN THE 2-YEAR GAVAGE STUDY OF ISOEUGENOL

Table C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of Isoeugenol[a]

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	7	10	5	7
Natural deaths	4	2	9	16
Survivors				
Terminal sacrifice	39	38	36	27
Animals examined microscopically	50	50	50	50
Alimentary System				
Gallbladder	(44)	(47)	(46)	(38)
Intestine large, colon	(48)	(49)	(46)	(46)
Carcinoma		1 (2%)		
Intestine small, jejunum	(47)	(48)	(46)	(42)
Adenoma			1 (2%)	
Carcinoma			1 (2%)	
Hemangiosarcoma	1 (2%)			
Liver	(50)	(50)	(50)	(50)
Hemangiosarcoma	1 (2%)	2 (4%)	1 (2%)	
Hepatoblastoma	3 (6%)			1 (2%)
Hepatoblastoma, multiple		1 (2%)		1 (2%)
Hepatocellular adenoma	14 (28%)	9 (18%)	9 (18%)	13 (26%)
Hepatocellular adenoma, multiple	10 (20%)	26 (52%)	28 (56%)	20 (40%)
Hepatocellular carcinoma	6 (12%)	12 (24%)	13 (26%)	14 (28%)
Hepatocellular carcinoma, multiple	2 (4%)	6 (12%)	6 (12%)	4 (8%)
Hepatocholangiocarcinoma	2 (4%)	1 (2%)		1 (2%)
Mesentery	(5)	(4)	(4)	(1)
Hepatoblastoma, metastatic, liver		1 (25%)		1 (100%)
Pancreas	(50)	(50)	(50)	(49)
Hepatocholangiocarcinoma, metastatic, liver	1 (2%)			
Salivary glands	(50)	(50)	(50)	(50)
Stomach, forestomach	(50)	(49)	(50)	(49)
Squamous cell papilloma		2 (4%)	2 (4%)	
Stomach, glandular	(50)	(49)	(49)	(44)
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Hemangiosarcoma		1 (2%)		
Hepatocholangiocarcinoma, metastatic, liver	2 (4%)			1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(49)
Subcapsular, adenoma	3 (6%)		3 (6%)	
Adrenal medulla	(50)	(50)	(50)	(49)
Pheochromocytoma benign				1 (2%)
Islets, pancreatic	(50)	(50)	(50)	(49)
Pituitary gland	(50)	(48)	(50)	(48)
Pars intermedia, adenoma				1 (2%)
Thyroid gland	(50)	(49)	(50)	(49)
Follicular cell, adenoma		1 (2%)		
Follicular cell, carcinoma	1 (2%)			

Table C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
General Body System				
None				
Genital System				
Coagulating gland	(0)	(1)	(0)	(0)
Adenoma		1 (100%)		
Epididymis	(50)	(50)	(50)	(50)
Preputial gland	(50)	(50)	(50)	(50)
Prostate gland	(50)	(50)	(50)	(50)
Seminal vesicle	(49)	(50)	(50)	(48)
Testes	(50)	(50)	(50)	(50)
Interstitial cell, adenoma	2 (4%)	1 (2%)	1 (2%)	
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hemangiosarcoma		1 (2%)		
Lymph node	(1)	(0)	(2)	(2)
Bronchial, hepatocholangiocarcinoma, metastatic, liver	1 (100%)			
Pancreatic, hepatoblastoma, metastatic, liver				1 (50%)
Lymph node, mandibular	(49)	(49)	(50)	(47)
Lymph node, mesenteric	(48)	(50)	(50)	(48)
Spleen	(50)	(50)	(50)	(49)
Hemangiosarcoma		1 (2%)	1 (2%)	
Thymus	(48)	(48)	(48)	(42)
Alveolar/bronchiolar carcinoma, metastatic, lung		1 (2%)		
Hepatocholangiocarcinoma, metastatic, liver	2 (4%)			1 (2%)
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Subcutaneous tissue, fibrosarcoma		1 (2%)		
Subcutaneous tissue, hemangiosarcoma	1 (2%)			
Subcutaneous tissue, lipoma		1 (2%)		
Subcutaneous tissue, sarcoma			1 (2%)	
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Hemangiosarcoma				1 (2%)
Skeletal muscle	(1)	(0)	(0)	(0)
Hepatocholangiocarcinoma, metastatic, liver	1 (100%)			
Nervous System				
Brain	(50)	(50)	(50)	(50)

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Respiratory System				
Lung	(50)	(49)	(50)	(50)
Alveolar/bronchiolar adenoma	5 (10%)	5 (10%)	2 (4%)	8 (16%)
Alveolar/bronchiolar adenoma, multiple	1 (2%)			
Alveolar/bronchiolar carcinoma	2 (4%)	7 (14%)	5 (10%)	2 (4%)
Alveolar/bronchiolar carcinoma, multiple			2 (4%)	
Carcinoma, metastatic, Harderian gland		1 (2%)	1 (2%)	
Hepatoblastoma, metastatic, liver	1 (2%)			1 (2%)
Hepatocellular carcinoma, metastatic, liver		10 (20%)	13 (26%)	12 (24%)
Hepatocholangiocarcinoma, metastatic, liver	2 (4%)	1 (2%)		1 (2%)
Nose	(50)	(50)	(50)	(50)
Pleura	(0)	(1)	(0)	(0)
Alveolar/bronchiolar carcinoma, metastatic, lung		1 (100%)		
Special Senses System				
Eye	(48)	(48)	(46)	(43)
Harderian gland	(49)	(50)	(50)	(49)
Adenoma	7 (14%)	4 (8%)	2 (4%)	3 (6%)
Carcinoma	4 (8%)	3 (6%)	2 (4%)	1 (2%)
Bilateral, adenoma	1 (2%)		1 (2%)	
Urinary System				
Kidney	(50)	(50)	(50)	(49)
Hepatocholangiocarcinoma, metastatic, liver	1 (2%)			
Renal tubule, adenoma	1 (2%)			
Renal tubule, carcinoma		1 (2%)		1 (2%)
Urinary bladder	(49)	(50)	(50)	(45)
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Lymphoma malignant			3 (6%)	2 (4%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	43	48	46	46
Total primary neoplasms	67	88	84	74
Total animals with benign neoplasms	33	37	37	36
Total benign neoplasms	44	50	49	46
Total animals with malignant neoplasms	20	27	29	28
Total malignant neoplasms	23	38	35	28
Total animals with metastatic neoplasms	3	14	14	14
Total metastatic neoplasms	11	15	14	18

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Adrenal Cortex: Adenoma				
Overall rate[a]	3/50 (6%)	0/50 (0%)	3/50 (6%)	0/49 (0%)
Adjusted rate[b]	6.8%	0.0%	6.5%	0.0%
Terminal rate[c]	3/39 (8%)	0/38 (0%)	3/36 (8%)	0/27 (0%)
First incidence (days)	728 (T)	—[e]	728 (T)	—
Poly-3 test[d]	P = 0.202N	P = 0.115N	P = 0.647N	P = 0.148N
Harderian Gland: Adenoma				
Overall rate	8/50 (16%)	4/50 (8%)	3/50 (6%)	3/50 (6%)
Adjusted rate	18.0%	8.8%	6.5%	7.8%
Terminal rate	7/39 (18%)	4/38 (11%)	3/36 (8%)	3/27 (11%)
First incidence (days)	700	728 (T)	728 (T)	728 (T)
Poly-3 test	P = 0.102N	P = 0.167N	P = 0.089N	P = 0.152N
Harderian Gland: Carcinoma				
Overall rate	4/50 (8%)	3/50 (6%)	2/50 (4%)	1/50 (2%)
Adjusted rate	8.9%	6.5%	4.4%	2.6%
Terminal rate	3/39 (8%)	1/38 (3%)	2/36 (6%)	1/27 (4%)
First incidence (days)	468	441	728 (T)	728 (T)
Poly-3 test	P = 0.148N	P = 0.484N	P = 0.330N	P = 0.233N
Harderian Gland: Adenoma or Carcinoma				
Overall rate	12/50 (24%)	7/50 (14%)	5/50 (10%)	4/50 (8%)
Adjusted rate	26.5%	15.1%	10.9%	10.5%
Terminal rate	10/39 (26%)	5/38 (13%)	5/36 (14%)	4/27 (15%)
First incidence (days)	468	441	728 (T)	728 (T)
Poly-3 test	P = 0.034N	P = 0.135N	P = 0.048N	P = 0.055N
Liver: Hepatocellular Adenoma				
Overall rate	24/50 (48%)	35/50 (70%)	37/50 (74%)	33/50 (66%)
Adjusted rate	53.0%	75.7%	76.9%	77.7%
Terminal rate	22/39 (56%)	31/38 (82%)	29/36 (81%)	23/27 (85%)
First incidence (days)	469	646	491	408
Poly-3 test	P = 0.012	P = 0.015	P = 0.010	P = 0.009
Liver: Hepatocellular Carcinoma				
Overall rate	8/50 (16%)	18/50 (36%)	19/50 (38%)	18/50 (36%)
Adjusted rate	17.4%	37.9%	38.7%	40.4%
Terminal rate	5/39 (13%)	10/38 (26%)	9/36 (25%)	5/27 (19%)
First incidence (days)	469	481	491	385
Poly-3 test	P = 0.027	P = 0.022	P = 0.017	P = 0.012
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	28/50 (56%)	43/50 (86%)	43/50 (86%)	43/50 (86%)
Adjusted rate	60.5%	90.0%	86.3%	90.3%
Terminal rate	24/39 (62%)	34/38 (90%)	30/36 (83%)	24/27 (89%)
First incidence (days)	469	481	491	385
Poly-3 test	P<0.001	P<0.001	P = 0.003	P<0.001
Liver: Hepatoblastoma				
Overall rate	3/50 (6%)	1/50 (2%)	0/50 (0%)	2/50 (4%)
Adjusted rate	6.7%	2.2%	0.0%	5.2%
Terminal rate	2/39 (5%)	1/38 (3%)	0/36 (0%)	1/27 (4%)
First incidence (days)	599	728 (T)	—	640
Poly-3 test	P = 0.449N	P = 0.301N	P = 0.115N	P = 0.568N

Table C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Liver: Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	11/50 (22%)	19/50 (38%)	19/50 (38%)	20/50 (40%)
Adjusted rate	23.7%	40.0%	38.7%	44.6%
Terminal rate	7/39 (18%)	11/38 (29%)	9/36 (25%)	6/27 (22%)
First incidence (days)	469	481	491	385
Poly-3 test	P = 0.042	P = 0.068	P = 0.085	P = 0.027
Liver: Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma				
Overall rate	30/50 (60%)	43/50 (86%)	43/50 (86%)	43/50 (86%)
Adjusted rate	64.2%	90.0%	86.3%	90.3%
Terminal rate	25/39 (64%)	34/38 (90%)	30/36 (83%)	24/27 (89%)
First incidence (days)	469	481	491	385
Poly-3 test	P = 0.003	P = 0.002	P = 0.008	P < 0.001
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	6/50 (12%)	5/49 (10%)	2/50 (4%)	8/50 (16%)
Adjusted rate	13.4%	11.2%	4.4%	20.4%
Terminal rate	5/39 (13%)	4/37 (11%)	2/36 (6%)	5/27 (19%)
First incidence (days)	589	658	728 (T)	607
Poly-3 test	P = 0.254	P = 0.505N	P = 0.126N	P = 0.284
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	2/50 (4%)	7/49 (14%)	7/50 (14%)	2/50 (4%)
Adjusted rate	4.5%	15.5%	15.2%	5.2%
Terminal rate	2/39 (5%)	4/37 (11%)	6/36 (17%)	2/27 (7%)
First incidence (days)	728 (T)	510	710	728 (T)
Poly-3 test	P = 0.530N	P = 0.083	P = 0.087	P = 0.640
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	7/50 (14%)	12/49 (24%)	9/50 (18%)	10/50 (20%)
Adjusted rate	15.6%	26.3%	19.6%	25.6%
Terminal rate	6/39 (15%)	8/37 (22%)	8/36 (22%)	7/27 (26%)
First incidence (days)	589	510	710	607
Poly-3 test	P = 0.253	P = 0.160	P = 0.412	P = 0.195
All Organs: Hemangiosarcoma				
Overall rate	3/50 (6%)	4/50 (8%)	2/50 (4%)	1/50 (2%)
Adjusted rate	6.8%	8.7%	4.4%	2.6%
Terminal rate	3/39 (8%)	2/38 (5%)	1/36 (3%)	0/27 (0%)
First incidence (days)	728 (T)	510	682	408
Poly-3 test	P = 0.198N	P = 0.522	P = 0.483N	P = 0.352N
All Organs: Malignant Lymphoma				
Overall rate	0/50 (0%)	0/50 (0%)	3/50 (6%)	2/50 (4%)
Adjusted rate	0.0%	0.0%	6.5%	5.2%
Terminal rate	0/39 (0%)	0/38 (0%)	1/36 (3%)	2/27 (7%)
First incidence (days)	—	—	629	728 (T)
Poly-3 test	P = 0.063	—[f]	P = 0.127	P = 0.206

Table C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
All Organs: Benign Neoplasms				
Overall rate	33/50 (66%)	37/50 (74%)	37/50 (74%)	36/50 (72%)
Adjusted rate	70.9%	80.1%	76.9%	84.0%
Terminal rate	28/39 (72%)	33/38 (87%)	29/36 (81%)	25/27 (93%)
First incidence (days)	469	646	491	408
Poly-3 test	P = 0.107	P = 0.208	P = 0.329	P = 0.095
All Organs: Malignant Neoplasms				
Overall rate	20/50 (40%)	27/50 (54%)	29/50 (58%)	28/50 (56%)
Adjusted rate	41.5%	54.9%	58.2%	60.9%
Terminal rate	13/39 (33%)	16/38 (42%)	16/36 (44%)	12/27 (44%)
First incidence (days)	468	441	491	385
Poly-3 test	P = 0.046	P = 0.131	P = 0.071	P = 0.043
All Organs: Benign or Malignant Neoplasms				
Overall rate	43/50 (86%)	48/50 (96%)	46/50 (92%)	46/50 (92%)
Adjusted rate	87.7%	97.5%	92.0%	96.6%
Terminal rate	33/39 (85%)	37/38 (97%)	32/36 (89%)	27/27 (100%)
First incidence (days)	468	441	491	385
Poly-3 test	P = 0.128	P = 0.067	P = 0.355	P = 0.097

(T) Terminal sacrifice
[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, liver, and lung; for other tissues, denominator is number of animals necropsied.
[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[c] Observed incidence at terminal kill
[d] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence is the P value corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in a dosed group is indicated by **N**.
[e] Not applicable; no neoplasms in animal group
[f] Value of statistic cannot be computed.

TABLE C3
Historical Incidence of Hepatocelluar Neoplasms in Control Male B6C3F1 Mice[a]

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Historical Incidence: Corn Oil Gavage Studies			
Beta-myrcene	26/50	14/50	33/50
Isoeugenol	24/50	8/50	28/50
Overall Historical Incidence: Corn Oil Gavage Studies			
Total (%)	50/100 (50.0%)	22/100 (22.0%)	61/100 (61.0%)
Mean ± standard deviation	50.0% ± 2.8%	22.0% ± 8.5%	61.0% ± 7.1%
Range	48%-52%	16%-28%	56%-66%
Overall Historical Incidence: All Routes			
Total (%)	544/1,146 (47.5%)	317/1,146 (27.7%)	729/1,146 (63.6%)
Mean ± standard deviation	47.5% ± 14.9%	27.7% ± 9.2%	63.6% ± 15.6%
Range	14%-72%	8%-48%	20%-84%

[a] Data as of October 4, 2007

Table C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of Isoeugenol[a]

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	7	10	5	7
Natural deaths	4	2	9	16
Survivors				
Terminal sacrifice	39	38	36	27
Animals examined microscopically	50	50	50	50
Alimentary System				
Gallbladder	(44)	(47)	(46)	(38)
Hyperplasia				1 (3%)
Intestine large, colon	(48)	(49)	(46)	(46)
Intestine small, jejunum	(47)	(48)	(46)	(42)
Necrosis		1 (2%)		
Ulcer				1 (2%)
Liver	(50)	(50)	(50)	(50)
Angiectasis			1 (2%)	
Basophilic focus	5 (10%)	5 (10%)	3 (6%)	7 (14%)
Clear cell focus	16 (32%)	26 (52%)	29 (58%)	13 (26%)
Eosinophilic focus	8 (16%)	13 (26%)	11 (22%)	5 (10%)
Hepatodiaphragmatic nodule			2 (4%)	
Infarct	2 (4%)	1 (2%)		
Necrosis	1 (2%)		2 (4%)	5 (10%)
Mesentery	(5)	(4)	(4)	(1)
Fat, necrosis	5 (100%)	3 (75%)	4 (100%)	
Pancreas	(50)	(50)	(50)	(49)
Atrophy	2 (4%)	1 (2%)	2 (4%)	
Hemorrhage		1 (2%)		
Hyperplasia				1 (2%)
Necrosis, fatty			1 (2%)	
Duct, cyst			1 (2%)	
Salivary glands	(50)	(50)	(50)	(50)
Stomach, forestomach	(50)	(49)	(50)	(49)
Hyperplasia, squamous	7 (14%)	8 (16%)	8 (16%)	14 (29%)
Inflammation	5 (10%)	8 (16%)	9 (18%)	14 (29%)
Metaplasia, hepatocyte		1 (2%)		
Ulcer	1 (2%)	4 (8%)	4 (8%)	9 (18%)
Stomach, glandular	(50)	(49)	(49)	(44)
Atrophy				1 (2%)
Hyperplasia			1 (2%)	
Inflammation		1 (2%)	3 (6%)	3 (7%)
Inflammation, acute			1 (2%)	2 (5%)
Metaplasia, hepatocyte		1 (2%)		
Mineralization	1 (2%)			
Ulcer		1 (2%)	4 (8%)	5 (11%)

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

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	9 (18%)	11 (22%)	14 (28%)	12 (24%)
Inflammation, suppurative	1 (2%)		1 (2%)	
Mineralization	1 (2%)			2 (4%)
Thrombosis	1 (2%)	1 (2%)	1 (2%)	
Artery, inflammation, chronic active	1 (2%)	2 (4%)		1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(49)
Hyperplasia	13 (26%)	16 (32%)	12 (24%)	7 (14%)
Hypertrophy	13 (26%)	13 (26%)	14 (28%)	6 (12%)
Adrenal medulla	(50)	(50)	(50)	(49)
Hyperplasia		1 (2%)	2 (4%)	
Islets, pancreatic	(50)	(50)	(50)	(49)
Hyperplasia	3 (6%)		1 (2%)	
Pituitary gland	(50)	(48)	(50)	(48)
Pars distalis, hyperplasia	1 (2%)	3 (6%)	3 (6%)	
Thyroid gland	(50)	(49)	(50)	(49)
Follicular cell, hyperplasia		2 (4%)		1 (2%)
General Body System				
None				
Genital System				
Coagulating gland	(0)	(1)	(0)	(0)
Epididymis	(50)	(50)	(50)	(50)
Granuloma sperm		1 (2%)		1 (2%)
Inflammation, acute		1 (2%)	1 (2%)	
Necrosis			1 (2%)	
Preputial gland	(50)	(50)	(50)	(50)
Ectasia	5 (10%)	4 (8%)	5 (10%)	6 (12%)
Inflammation, granulomatous		1 (2%)		
Prostate gland	(50)	(50)	(50)	(50)
Inflammation, chronic active		1 (2%)		1 (2%)
Seminal vesicle	(49)	(50)	(50)	(48)
Dilatation		1 (2%)		
Testes	(50)	(50)	(50)	(50)
Atrophy	1 (2%)	1 (2%)		4 (8%)
Inflammation, suppurative		1 (2%)		
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Atrophy			1 (2%)	
Hyperplasia, reticulum cell				1 (2%)
Necrosis		1 (2%)		
Lymph node	(1)	(0)	(2)	(2)
Lymph node, mandibular	(49)	(49)	(50)	(47)
Lymph node, mesenteric	(48)	(50)	(50)	(48)
Spleen	(50)	(50)	(50)	(49)
Depletion cellular				2 (4%)
Hematopoietic cell proliferation			1 (2%)	1 (2%)
Thymus	(48)	(48)	(48)	(42)

Table C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Inflammation, acute	1 (2%)			
Inflammation, chronic active		3 (6%)	1 (2%)	2 (4%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Arthrosis	1 (2%)			
Skeletal muscle	(1)	(0)	(0)	(0)
Nervous System				
Brain	(50)	(50)	(50)	(50)
Inflammation, granulomatous				1 (2%)
Necrosis		2 (4%)		
Respiratory System				
Lung	(50)	(49)	(50)	(50)
Thrombosis	1 (2%)		1 (2%)	
Alveolar epithelium, hyperplasia	3 (6%)	4 (8%)	4 (8%)	2 (4%)
Alveolus, infiltration cellular, histiocyte		1 (2%)		
Bronchiole, hyperplasia	1 (2%)		1 (2%)	
Nose	(50)	(50)	(50)	(50)
Polyp, inflammatory		1 (2%)		
Glands, hyperplasia	3 (6%)	34 (68%)	49 (98%)	48 (96%)
Olfactory epithelium, accumulation, hyaline droplet		6 (12%)	26 (52%)	19 (38%)
Olfactory epithelium, atrophy	5 (10%)	13 (26%)	36 (72%)	41 (82%)
Olfactory epithelium, degeneration	1 (2%)	1 (2%)	7 (14%)	6 (12%)
Olfactory epithelium, inflammation, granulomatous				1 (2%)
Olfactory epithelium, metaplasia, squamous			2 (4%)	1 (2%)
Olfactory epithelium, respiratory metaplasia	4 (8%)	31 (62%)	47 (94%)	49 (98%)
Pleura	(0)	(1)	(0)	(0)
Special Senses System				
Eye	(48)	(48)	(46)	(43)
Inflammation, acute		1 (2%)		
Cornea, inflammation, chronic active		1 (2%)	1 (2%)	
Harderian gland	(49)	(50)	(50)	(49)
Hyperplasia	3 (6%)	4 (8%)	4 (8%)	4 (8%)
Urinary System				
Kidney	(50)	(50)	(50)	(49)
Infarct	1 (2%)	1 (2%)	2 (4%)	1 (2%)
Inflammation, suppurative		2 (4%)		2 (4%)
Metaplasia, osseous			1 (2%)	
Nephropathy	47 (94%)	48 (96%)	47 (94%)	47 (96%)
Renal tubule, hyperplasia	2 (4%)	2 (4%)	1 (2%)	2 (4%)
Urinary bladder	(49)	(50)	(50)	(45)
Inflammation, chronic active		1 (2%)		

APPENDIX D
SUMMARY OF LESIONS IN FEMALE MICE IN THE 2-YEAR GAVAGE STUDY OF ISOEUGENOL

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of Isoeugenol[a]

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths	2			1
Moribund	5	7	5	5
Natural deaths	8	4	7	11
Survivors				
Terminal sacrifice	34	39	38	33
Missing	1			
Animals examined microscopically	49	50	50	50
Alimentary System				
Gallbladder	(42)	(47)	(47)	(40)
Intestine large, cecum	(46)	(47)	(45)	(42)
Intestine large, colon	(47)	(47)	(47)	(47)
Intestine large, rectum	(47)	(48)	(45)	(46)
Intestine small, duodenum	(42)	(47)	(45)	(40)
Intestine small, ileum	(45)	(47)	(46)	(44)
Intestine small, jejunum	(44)	(48)	(45)	(45)
Liver	(49)	(50)	(49)	(50)
Carcinoma, metastatic, uncertain primary site		1 (2%)		
Hemangiosarcoma		2 (4%)		
Hepatocellular adenoma	11 (22%)	7 (14%)	8 (16%)	4 (8%)
Hepatocellular adenoma, multiple		3 (6%)	1 (2%)	
Hepatocellular carcinoma	2 (4%)	8 (16%)	7 (14%)	6 (12%)
Hepatocellular carcinoma, multiple	1 (2%)		2 (4%)	
Mesentery	(15)	(14)	(14)	(6)
Pancreas	(47)	(50)	(47)	(49)
Carcinoma, metastatic, uncertain primary site		1 (2%)		
Salivary glands	(49)	(50)	(50)	(50)
Stomach, forestomach	(48)	(50)	(49)	(50)
Carcinoma, metastatic, uncertain primary site		1 (2%)		
Squamous cell papilloma	1 (2%)	1 (2%)	5 (10%)	2 (4%)
Squamous cell papilloma, multiple				1 (2%)
Stomach, glandular	(46)	(48)	(47)	(48)
Adenoma	1 (2%)			
Carcinoma, metastatic, uncertain primary site		1 (2%)		
Cardiovascular System				
Blood vessel	(2)	(1)	(1)	(2)
Heart	(49)	(50)	(50)	(50)
Endocrine System				
Adrenal cortex	(48)	(50)	(49)	(50)
Capsule, adenoma			1 (2%)	
Adrenal medulla	(48)	(50)	(49)	(50)
Pheochromocytoma benign		1 (2%)		
Islets, pancreatic	(47)	(50)	(48)	(49)
Pituitary gland	(47)	(48)	(50)	(50)
Pars distalis, adenoma	4 (9%)		4 (8%)	1 (2%)
Thyroid gland	(48)	(50)	(49)	(50)
Follicular cell, adenoma			1 (2%)	

Table D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
General Body System				
None				
Genital System				
Clitoral gland	(47)	(49)	(49)	(48)
Fibrosarcoma				1 (2%)
Ovary	(48)	(50)	(50)	(50)
Cystadenocarcinoma			1 (2%)	
Cystadenoma	2 (4%)		1 (2%)	1 (2%)
Granulosa-theca tumor malignant		1 (2%)		
Tubulostromal adenoma		1 (2%)		
Bilateral, cystadenoma	1 (2%)			
Uterus	(48)	(50)	(50)	(50)
Carcinoma		1 (2%)		
Hemangiosarcoma			1 (2%)	1 (2%)
Polyp stromal			2 (4%)	1 (2%)
Sarcoma stromal	1 (2%)			
Hematopoietic System				
Bone marrow	(48)	(49)	(50)	(50)
Hemangiosarcoma				1 (2%)
Lymph node	(4)	(5)	(3)	(2)
Lymph node, mandibular	(49)	(50)	(50)	(50)
Lymph node, mesenteric	(47)	(49)	(47)	(50)
Sarcoma		1 (2%)		
Spleen	(48)	(50)	(49)	(50)
Hemangiosarcoma	1 (2%)			
Thymus	(47)	(50)	(49)	(47)
Integumentary System				
Mammary gland	(48)	(50)	(50)	(50)
Carcinoma		1 (2%)		
Skin	(49)	(50)	(50)	(50)
Subcutaneous tissue, hemangiosarcoma	1 (2%)			
Subcutaneous tissue, sarcoma	2 (4%)		1 (2%)	
Subcutaneous tissue, sarcoma, multiple	1 (2%)			
Musculoskeletal System				
Bone	(49)	(50)	(50)	(50)
Osteosarcoma	1 (2%)		1 (2%)	1 (2%)
Skeletal muscle	(0)	(1)	(0)	(0)
Rhabdomyosarcoma		1 (100%)		
Nervous System				
Brain	(49)	(50)	(50)	(50)
Peripheral nerve	(2)	(2)	(1)	(0)

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Respiratory System				
Lung	(48)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	4 (8%)	2 (4%)	1 (2%)	1 (2%)
Alveolar/bronchiolar carcinoma		1 (2%)	3 (6%)	1 (2%)
Alveolar/bronchiolar carcinoma, multiple		1 (2%)		
Carcinoma, metastatic, Harderian gland	1 (2%)	1 (2%)		
Carcinoma, metastatic, uterus		1 (2%)		
Hepatocellular carcinoma, metastatic, liver			6 (12%)	3 (6%)
Osteosarcoma, metastatic, bone	1 (2%)		1 (2%)	
Sarcoma, metastatic, Harderian gland			1 (2%)	
Nose	(48)	(50)	(50)	(50)
Carcinoma, metastatic, Harderian gland				1 (2%)
Mast cell tumor malignant, metastatic, uncertain primary site				1 (2%)
Sarcoma, metastatic, Harderian gland			1 (2%)	
Special Senses System				
Eye	(44)	(47)	(46)	(42)
Sarcoma, metastatic, Harderian gland			1 (2%)	
Harderian gland	(46)	(50)	(49)	(48)
Adenoma	3 (7%)	3 (6%)	2 (4%)	5 (10%)
Carcinoma	1 (2%)	2 (4%)	2 (4%)	1 (2%)
Sarcoma			1 (2%)	
Urinary System				
Kidney	(47)	(50)	(49)	(49)
Urinary bladder	(47)	(49)	(47)	(46)
Systemic Lesions				
Multiple organs[b]	(49)	(50)	(50)	(50)
Histiocytic sarcoma		1 (2%)	1 (2%)	4 (8%)
Lymphoma malignant	11 (22%)	10 (20%)	8 (16%)	7 (14%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	34	33	37	26
Total primary neoplasms	49	48	54	39
Total animals with benign neoplasms	20	18	22	13
Total benign neoplasms	27	18	26	16
Total animals with malignant neoplasms	21	27	25	19
Total malignant neoplasms	22	30	28	23
Total animals with metastatic neoplasms	2	3	8	5
Total metastatic neoplasms	2	6	10	5
Total animals with malignant neoplasms of uncertain primary site		1		1

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

Table D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Harderian Gland: Adenoma				
Overall rate[a]	3/49 (6%)	3/50 (6%)	2/50 (4%)	5/50 (10%)
Adjusted rate[b]	7.0%	6.5%	4.3%	12.1%
Terminal rate[c]	3/34 (9%)	3/39 (8%)	2/38 (5%)	3/33 (9%)
First incidence (days)	727 (T)	727 (T)	727 (T)	553
Poly-3 test[d]	P = 0.248	P = 0.622N	P = 0.460N	P = 0.341
Harderian Gland: Adenoma or Carcinoma				
Overall rate	4/49 (8%)	5/50 (10%)	4/50 (8%)	5/50 (10%)
Adjusted rate	9.4%	10.7%	8.6%	12.1%
Terminal rate	4/34 (12%)	4/39 (10%)	3/38 (8%)	3/33 (9%)
First incidence (days)	727 (T)	577	695	553
Poly-3 test	P = 0.436	P = 0.561	P = 0.592N	P = 0.483
Liver: Hepatocellular Adenoma				
Overall rate	11/49 (22%)	10/50 (20%)	9/49 (18%)	4/50 (8%)
Adjusted rate	25.7%	20.8%	19.3%	9.9%
Terminal rate	8/34 (24%)	5/39 (13%)	8/38 (21%)	3/33 (9%)
First incidence (days)	699	577	693	698
Poly-3 test	P = 0.048N	P = 0.380N	P = 0.321N	P = 0.053N
Liver: Hepatocellular Carcinoma				
Overall rate	3/49 (6%)	8/50 (16%)	9/49 (18%)	6/50 (12%)
Adjusted rate	6.9%	17.2%	19.2%	14.8%
Terminal rate	1/34 (3%)	8/39 (21%)	6/38 (16%)	6/33 (18%)
First incidence (days)	423	727 (T)	647	727 (T)
Poly-3 test	P = 0.231	P = 0.119	P = 0.077	P = 0.205
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	13/49 (27%)	16/50 (32%)	15/49 (31%)	9/50 (18%)
Adjusted rate	29.6%	33.2%	31.9%	22.2%
Terminal rate	8/34 (24%)	11/39 (28%)	11/38 (29%)	8/33 (24%)
First incidence (days)	423	577	647	698
Poly-3 test	P = 0.229N	P = 0.442	P = 0.495	P = 0.297N
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	4/48 (8%)	2/50 (4%)	1/50 (2%)	1/50 (2%)
Adjusted rate	9.6%	4.3%	2.2%	2.5%
Terminal rate	4/34 (12%)	1/39 (3%)	1/38 (3%)	1/33 (3%)
First incidence (days)	727 (T)	632	727 (T)	727 (T)
Poly-3 test	P = 0.115N	P = 0.287N	P = 0.148N	P = 0.188N
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	0/48 (0%)	2/50 (4%)	3/50 (6%)	1/50 (2%)
Adjusted rate	0.0%	4.3%	6.4%	2.5%
Terminal rate	0/34 (0%)	1/39 (3%)	2/38 (5%)	1/33 (3%)
First incidence (days)	—[e]	647	577	727 (T)
Poly-3 test	P = 0.401	P = 0.263	P = 0.141	P = 0.493
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	4/48 (8%)	4/50 (8%)	4/50 (8%)	2/50 (4%)
Adjusted rate	9.6%	8.5%	8.5%	4.9%
Terminal rate	4/34 (12%)	2/39 (5%)	3/38 (8%)	2/33 (6%)
First incidence (days)	727 (T)	632	577	727 (T)
Poly-3 test	P = 0.283N	P = 0.577N	P = 0.577N	P = 0.352N

Table D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Ovary: Cystadenoma				
Overall rate	3/48 (6%)	0/50 (0%)	1/50 (2%)	1/50 (2%)
Adjusted rate	7.2%	0.0%	2.2%	2.5%
Terminal rate	3/34 (9%)	0/39 (0%)	1/38 (3%)	1/33 (3%)
First incidence (days)	727 (T)	—	727 (T)	727 (T)
Poly-3 test	P = 0.303N	P = 0.101N	P = 0.268N	P = 0.317N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	4/47 (9%)	0/48 (0%)	4/50 (8%)	1/50 (2%)
Adjusted rate	9.7%	0.0%	8.6%	2.5%
Terminal rate	3/34 (9%)	0/38 (0%)	4/38 (11%)	1/33 (3%)
First incidence (days)	699	—	727 (T)	727 (T)
Poly-3 test	P = 0.284N	P = 0.050N	P = 0.576N	P = 0.184N
Skin: Sarcoma				
Overall rate	3/49 (6%)	0/50 (0%)	1/50 (2%)	0/50 (0%)
Adjusted rate	6.9%	0.0%	2.1%	0.0%
Terminal rate	0/34 (0%)	0/39 (0%)	0/38 (0%)	0/33 (0%)
First incidence (days)	342	—	587	—
Poly-3 test	P = 0.089N	P = 0.108N	P = 0.280N	P = 0.133N
Stomach (Forestomach): Squamous Cell Papilloma				
Overall rate	1/49 (2%)	1/50 (2%)	5/50 (10%)	3/50 (6%)
Adjusted rate	2.4%	2.2%	10.7%	7.4%
Terminal rate	1/34 (3%)	1/39 (3%)	3/38 (8%)	3/33 (9%)
First incidence (days)	727 (T)	727 (T)	693	727 (T)
Poly-3 test	P = 0.126	P = 0.741N	P = 0.124	P = 0.287
All Organs: Histiocytic Sarcoma				
Overall rate	0/49 (0%)	1/50 (2%)	1/50 (2%)	4/50 (8%)
Adjusted rate	0.0%	2.1%	2.1%	9.7%
Terminal rate	0/34 (0%)	0/39 (0%)	0/38 (0%)	2/33 (6%)
First incidence (days)	—	605	616	538
Poly-3 test	P = 0.015	P = 0.519	P = 0.519	P = 0.056
All Organs: Malignant Lymphoma				
Overall rate	11/49 (22%)	10/50 (20%)	8/50 (16%)	7/50 (14%)
Adjusted rate	25.0%	21.0%	17.2%	17.1%
Terminal rate	7/34 (21%)	7/39 (18%)	8/38 (21%)	6/33 (18%)
First incidence (days)	569	518	727 (T)	605
Poly-3 test	P = 0.211N	P = 0.419N	P = 0.257N	P = 0.267N
All Organs: Benign Neoplasms				
Overall rate	20/49 (41%)	18/50 (36%)	22/50 (44%)	13/50 (26%)
Adjusted rate	46.7%	37.1%	46.2%	31.3%
Terminal rate	17/34 (50%)	12/39 (31%)	19/38 (50%)	10/33 (30%)
First incidence (days)	699	577	313	553
Poly-3 test	P = 0.150N	P = 0.237N	P = 0.567N	P = 0.105N

Table D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
All Organs: Malignant Neoplasms				
Overall rate	21/49 (43%)	27/50 (54%)	25/50 (50%)	20/50 (40%)
Adjusted rate	44.7%	54.8%	51.2%	47.7%
Terminal rate	10/34 (29%)	19/39 (49%)	16/38 (42%)	16/33 (49%)
First incidence (days)	342	518	577	538
Poly-3 test	P = 0.505	P = 0.216	P = 0.336	P = 0.472
All Organs: Benign or Malignant Neoplasms				
Overall rate	34/49 (69%)	33/50 (66%)	37/50 (74%)	26/50 (52%)
Adjusted rate	72.3%	66.5%	74.1%	61.2%
Terminal rate	22/34 (65%)	24/39 (62%)	26/38 (68%)	21/33 (64%)
First incidence (days)	342	518	313	538
Poly-3 test	P = 0.218N	P = 0.347N	P = 0.508	P = 0.183N

(T) Terminal sacrifice
[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for liver, lung, ovary, and pituitary gland; for other tissues, denominator is number of animals necropsied.
[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[c] Observed incidence at terminal kill
[d] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence is the P value corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in a dosed group is indicated by **N**.
[e] Not applicable; no neoplasms in animal group

TABLE D3a
Historical Incidence of Hepatocellular Neoplasms in Control Female B6C3F1 Mice[a]

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Historical Incidence: Corn Oil Gavage Studies			
Beta-myrcene	6/50	1/50	7/50
Isoeugenol	11/49	3/49	13/49
Overall Historical Incidence: Corn Oil Gavage Studies			
Total (%)	17/99 (17.2%)	4/99 (4.0%)	20/99 (20.2%)
Mean ± standard deviation	17.2% ± 7.4%	4.1% ± 2.9%	20.3% ± 8.9%
Range	12%-22%	2%-6%	14%-27%
Overall Historical Incidence: All Routes			
Total (%)	345/1,245 (27.7%)	131/1,245 (10.5%)	419/1,245 (33.7%)
Mean ± standard deviation	27.8% ± 17.0%	10.5% ± 7.7%	33.7% ± 19.1%
Range	2%-62%	0%-28%	8%-64%

[a] Data as of October 4, 2007

TABLE D3b
Historical Incidence of Histiocytic Sarcoma in Control Female B6C3F1 Mice[a]

Study	Incidence in Controls
Historical Incidence: Corn Oil Gavage Studies	
Beta-myrcene	0/50
Isoeugenol	0/49
Overall Historical Incidence: Corn Oil Gavage Studies	
Total	0/99
Overall Historical Incidence: All Routes	
Total (%)	31/1,249 (2.5%)
Mean ± standard deviation	2.5% ± 2.5%
Range	0%-8%

[a] Data as of October 4, 2007

Table D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of Isoeugenol[a]

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths	2			1
Moribund	5	7	5	5
Natural deaths	8	4	7	11
Survivors				
Terminal sacrifice	34	39	38	33
Missing	1			
Animals examined microscopically	49	50	50	50
Alimentary System				
Gallbladder	(42)	(47)	(47)	(40)
Degeneration, hyaline			1 (2%)	
Intestine large, cecum	(46)	(47)	(45)	(42)
Inflammation, acute				1 (2%)
Necrosis				1 (2%)
Intestine large, colon	(47)	(47)	(47)	(47)
Intestine large, rectum	(47)	(48)	(45)	(46)
Necrosis				1 (2%)
Intestine small, duodenum	(42)	(47)	(45)	(40)
Intestine small, ileum	(45)	(47)	(46)	(44)
Intestine small, jejunum	(44)	(48)	(45)	(45)
Liver	(49)	(50)	(49)	(50)
Amyloid deposition			1 (2%)	
Angiectasis	2 (4%)	2 (4%)	2 (4%)	
Basophilic focus	5 (10%)	4 (8%)	8 (16%)	4 (8%)
Clear cell focus	1 (2%)	1 (2%)	2 (4%)	
Eosinophilic focus	5 (10%)	5 (10%)	4 (8%)	1 (2%)
Fatty change	3 (6%)	1 (2%)	1 (2%)	
Hematopoietic cell proliferation	1 (2%)	1 (2%)		
Inflammation, chronic				1 (2%)
Mineralization				2 (4%)
Mixed cell focus	1 (2%)			
Necrosis	3 (6%)		1 (2%)	8 (16%)
Hepatocyte, mitotic alteration			1 (2%)	
Mesentery	(15)	(14)	(14)	(6)
Fat, hemorrhage			1 (7%)	
Fat, necrosis	14 (93%)	14 (100%)	13 (93%)	6 (100%)
Pancreas	(47)	(50)	(47)	(49)
Amyloid deposition		2 (4%)		
Atrophy		1 (2%)	1 (2%)	
Basophilic focus	1 (2%)	2 (4%)	1 (2%)	1 (2%)
Hyperplasia				1 (2%)
Duct, cyst	1 (2%)	1 (2%)		
Salivary glands	(49)	(50)	(50)	(50)
Necrosis		1 (2%)		
Stomach, forestomach	(48)	(50)	(49)	(50)
Hyperplasia	2 (4%)	8 (16%)	5 (10%)	8 (16%)
Inflammation	2 (4%)	8 (16%)	5 (10%)	8 (16%)
Ulcer	2 (4%)	4 (8%)	3 (6%)	4 (8%)

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

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Alimentary System *(continued)*				
Stomach, glandular	(46)	(48)	(47)	(48)
Amyloid deposition		1 (2%)		
Inflammation		1 (2%)	1 (2%)	6 (13%)
Mineralization	1 (2%)			
Ulcer		1 (2%)	1 (2%)	7 (15%)
Cardiovascular System				
Blood vessel	(2)	(1)	(1)	(2)
Mineralization	1 (50%)			1 (50%)
Heart	(49)	(50)	(50)	(50)
Cardiomyopathy	2 (4%)	2 (4%)		3 (6%)
Inflammation, suppurative		1 (2%)		2 (4%)
Mineralization	3 (6%)	2 (4%)	2 (4%)	5 (10%)
Thrombosis		1 (2%)		2 (4%)
Endocrine System				
Adrenal cortex	(48)	(50)	(49)	(50)
Amyloid deposition		1 (2%)	1 (2%)	
Hyperplasia	4 (8%)	2 (4%)	4 (8%)	2 (4%)
Hypertrophy		1 (2%)	3 (6%)	1 (2%)
Necrosis				1 (2%)
Vacuolization cytoplasmic	1 (2%)			
Adrenal medulla	(48)	(50)	(49)	(50)
Hyperplasia	1 (2%)		1 (2%)	2 (4%)
Islets, pancreatic	(47)	(50)	(48)	(49)
Hyperplasia	1 (2%)	1 (2%)	1 (2%)	2 (4%)
Pituitary gland	(47)	(48)	(50)	(50)
Pars distalis, hyperplasia	9 (19%)	17 (35%)	6 (12%)	9 (18%)
Thyroid gland	(48)	(50)	(49)	(50)
Follicular cell, hyperplasia	1 (2%)			1 (2%)
General Body System				
None				
Genital System				
Clitoral gland	(47)	(49)	(49)	(48)
Ovary	(48)	(50)	(50)	(50)
Angiectasis	2 (4%)			1 (2%)
Cyst	5 (10%)	12 (24%)	5 (10%)	4 (8%)
Thrombosis		1 (2%)		
Uterus	(48)	(50)	(50)	(50)
Angiectasis	2 (4%)	3 (6%)	2 (4%)	
Inflammation, suppurative		1 (2%)		
Thrombosis			1 (2%)	
Endometrium, hyperplasia, cystic	24 (50%)	32 (64%)	19 (38%)	21 (42%)

Table D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Hematopoietic System				
Bone marrow	(48)	(49)	(50)	(50)
Necrosis		1 (2%)		
Lymph node	(4)	(5)	(3)	(2)
Lymph node, mandibular	(49)	(50)	(50)	(50)
Lymph node, mesenteric	(47)	(49)	(47)	(50)
Hyperplasia, lymphoid		1 (2%)		
Inflammation, acute				1 (2%)
Spleen	(48)	(50)	(49)	(50)
Amyloid deposition			1 (2%)	
Depletion cellular				9 (18%)
Hematopoietic cell proliferation	1 (2%)	5 (10%)	2 (4%)	
Hyperplasia, lymphoid		1 (2%)		
Necrosis	1 (2%)			
Thymus	(47)	(50)	(49)	(47)
Integumentary System				
Mammary gland	(48)	(50)	(50)	(50)
Hyperplasia	1 (2%)			
Skin	(49)	(50)	(50)	(50)
Hemorrhage	1 (2%)			
Inflammation, chronic active			1 (2%)	
Necrosis	1 (2%)	1 (2%)		
Musculoskeletal System				
Bone	(49)	(50)	(50)	(50)
Skeletal muscle	(0)	(1)	(0)	(0)
Nervous System				
Brain	(49)	(50)	(50)	(50)
Demyelination		2 (4%)		
Gliosis	1 (2%)			
Necrosis		1 (2%)		1 (2%)
Peripheral nerve	(2)	(2)	(1)	(0)
Radicular neuropathy	1 (50%)	1 (50%)	1 (100%)	
Respiratory System				
Lung	(48)	(50)	(50)	(50)
Hemorrhage		1 (2%)		
Inflammation, suppurative		1 (2%)		
Mineralization	1 (2%)			
Thrombosis				1 (2%)
Alveolar epithelium, hyperplasia	2 (4%)	1 (2%)	4 (8%)	
Bronchiole, hyperplasia				1 (2%)
Bronchiole, necrosis				1 (2%)
Nose	(48)	(50)	(50)	(50)
Thrombosis				1 (2%)
Glands, hyperplasia	6 (13%)	38 (76%)	49 (98%)	49 (98%)
Olfactory epithelium, accumulation, hyaline droplet		4 (8%)	18 (36%)	12 (24%)
Olfactory epithelium, atrophy	3 (6%)	8 (16%)	36 (72%)	43 (86%)
Olfactory epithelium, degeneration			1 (2%)	2 (4%)
Olfactory epithelium, respiratory metaplasia	6 (13%)	37 (74%)	49 (98%)	50 (100%)
Respiratory epithelium, necrosis				1 (2%)

Table D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of Isoeugenol

	Vehicle Control	75 mg/kg	150 mg/kg	300 mg/kg
Special Senses System				
Eye	(44)	(47)	(46)	(42)
Cataract			1 (2%)	1 (2%)
Degeneration			1 (2%)	1 (2%)
Harderian gland	(46)	(50)	(49)	(48)
Hyperplasia	5 (11%)	5 (10%)	6 (12%)	2 (4%)
Urinary System				
Kidney	(47)	(50)	(49)	(49)
Amyloid deposition	1 (2%)	1 (2%)	1 (2%)	
Inflammation, suppurative				1 (2%)
Nephropathy	23 (49%)	30 (60%)	22 (45%)	33 (67%)
Bilateral, papilla, necrosis		1 (2%)		4 (8%)
Papilla, mineralization				1 (2%)
Papilla, necrosis			1 (2%)	14 (29%)
Renal tubule, necrosis		1 (2%)		6 (12%)
Urinary bladder	(47)	(49)	(47)	(46)

APPENDIX E
GENETIC TOXICOLOGY

GENETIC TOXICOLOGY

Salmonella typhimurium Mutagenicity Test Protocol

Two independent assays for bacterial mutagenicity were conducted with isoeugenol. The first study was performed as reported by Mortelmans *et al.* (1986). Isoeugenol was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537, 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. The second assay, conducted with the same lot of isoeugenol tested in the 2-year study, used a slightly modified protocol (activation only with rat liver S9) and also employed *Escherichia coli* strain WP2 *uvrA*/pKM101 as a bacterial tester strain in addition to *S. typhimurium* strains TA98 and TA100. Isoeugenol was sent to the testing laboratory as a coded aliquot. It was incubated with the bacterial tester strains either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague Dawley rat livers) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added to the cultures, 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 incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of isoeugenol. The high dose was limited by toxicity. All trials were repeated.

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.

Chinese Hamster Ovary Cell Cytogenetics Protocols

Testing was performed as reported by Galloway *et al.* (1987). Isoeugenol was sent to the testing laboratory as a coded aliquot. It was tested in cultured Chinese hamster ovary (CHO) cells for induction of chromosomal aberrations (Abs), both in the presence and absence of Aroclor 1254-induced male Sprague Dawley rat liver S9 and cofactor mix. Cultures were handled under gold lights to prevent photolysis of bromodeoxyuridine-substituted DNA. Each test consisted of concurrent solvent and positive controls and of at least three doses of isoeugenol; the high dose was limited by toxicity. A single flask per dose was used.

In the Abs test without S9, cells were incubated in McCoy's 5A medium with isoeugenol for 10 hours; Colcemid was added, and incubation continued for 2 hours. The cells were then harvested by mitotic shake-off, fixed, and stained with Giemsa. For the Abs test with S9, cells were treated with isoeugenol and S9 for 2 hours, after which the treatment medium was removed and the cells were incubated for 10 hours in fresh medium, with Colcemid present for the final 2 hours. Cells were harvested in the same manner as for the treatment without S9.

Cells were selected for scoring on the basis of good morphology and completeness of karyotype (21 ± 2 chromosomes). All slides were scored blind, and those from a single test were read by the same person. Two hundred first-division metaphase cells were scored at each dose level. Classes of aberrations included simple (breaks and terminal deletions), complex (rearrangements and translocations), and other (pulverized cells, despiralized chromosomes, and cells containing 10 or more aberrations).

Chromosomal aberration data are presented as percentage of cells with aberrations. To arrive at a statistical call for a trial, analyses were conducted on both the dose response curve and individual dose points. For a single trial, a statistically significant ($P \leq 0.05$) difference for one dose point and a significant trend ($P \leq 0.015$) were considered weak evidence for a positive response; significant differences for two or more doses indicated the trial was positive. A positive trend test in the absence of a statistically significant increase at any one dose resulted in an equivocal call (Galloway *et al.,* 1987). Ultimately, the trial calls were based on a consideration of the statistical analyses as well as the biological information available to the reviewers.

Mouse Peripheral Blood Micronucleus Test Protocol

A detailed discussion of this assay is presented by MacGregor *et al.* (1990). At the end of the 3-month toxicity study, peripheral blood samples were obtained from male and female B6C3F1 mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes (NCEs) in each animal per treatment group. In addition, the percentage of polychromatic erythrocytes (PCEs) in a population of 1,000 erythrocytes was determined as a measure of bone marrow 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 NCEs 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. 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). Results of the 3-month studies were accepted without repeat tests, because additional test data could not be obtained. Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects.

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 table in the Abstract of this Technical Report presents a result that represents a scientific judgement of the overall evidence for activity of the chemical in an assay.

Results

Isoeugenol (3.3 to 2,000 µg/plate) was not active in either of two independent assays for mutagenicity in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 *uvrA* conducted with and without exogenous metabolic activation (S9 liver enzymes) (Table E1). Isoeugenol (in medium concentrations up to 200 µg/mL) did not induce chromosomal aberrations in cultured CHO cells, with or without S9 activation (Table E2). *In vivo*, the frequencies of micronucleated erythrocytes were not increased in peripheral blood of male B6C3F1 mice exposed to 37.5 to 600 mg/kg isoeugenol by gavage for 3 months; in contrast, results of this test in female mice were judged to be positive, based on a 3.2-fold increase of micronucleated erythrocytes in the 600 mg/kg group and a significant trend (Table E3). No significant changes in the percentage of PCEs were observed over the dose range tested in either males or females, indicating an absence of treatment-related toxicity to the bone marrow.

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

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+10% hamster S9 Trial 1	+10% hamster S9 Trial 2	+10% rat S9 Trial 1	+10% rat S9 Trial 2
Study performed at EG&G Mason Research Institute[c]							
TA100	0	137 ± 9.0	165 ± 5.0	162 ± 7.0	156 ± 13.0	151 ± 1.0	134 ± 5.0
	3.3	142 ± 7.0	143 ± 2.0				
	10	146 ± 1.0	148 ± 11.0	159 ± 2.0	167 ± 11.0	151 ± 11.0	130 ± 1.0
	33	142 ± 2.0	147 ± 7.0	155 ± 2.0	150 ± 3.0	147 ± 11.0	121 ± 7.0
	100	147 ± 3.0	161 ± 3.0	137 ± 4.0	165 ± 5.0	140 ± 4.0	124 ± 5.0
	250		145 ± 9.0[d]				
	333	Toxic		131 ± 4.0	155 ± 3.0	135 ± 5.0	122 ± 8.0
	800				Toxic		Toxic
	1,000			Toxic		Toxic	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[e]		1,384 ± 44.0	1,460 ± 37.0	1,415 ± 50.0	974 ± 16.0	1,019 ± 56.0	1,308 ± 16.0
TA1535	0	22 ± 1.0	45 ± 4.0	14 ± 2.0	13 ± 1.0	10 ± 3.0	11 ± 1.0
	3.3	22 ± 4.0	35 ± 4.0				
	10	21 ± 2.0	39 ± 1.0	9 ± 1.0	13 ± 3.0	9 ± 2.0	12 ± 2.0
	33	17 ± 5.0	42 ± 1.0	13 ± 1.0	10 ± 2.0	14 ± 3.0	7 ± 1.0
	100	17 ± 3.0	35 ± 3.0	10 ± 3.0	12 ± 3.0	8 ± 1.0	9 ± 2.0
	250		22 ± 1.0[d]				
	333	Toxic		10 ± 1.0	9 ± 1.0	8 ± 1.0	11 ± 3.0
	800				Toxic		Toxic
	1,000			Toxic		Toxic	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		1,029 ± 45.0	1,347 ± 43.0	104 ± 3.0	78 ± 3.0	64 ± 2.0	107 ± 6.0
TA1537	0	6 ± 1.0	8 ± 2.0	7 ± 2.0	9 ± 2.0	9 ± 1.0	8 ± 1.0
	3.3	5 ± 1.0	6 ± 2.0				
	10	4 ± 2.0	9 ± 1.0	7 ± 1.0	10 ± 2.0	5 ± 1.0	7 ± 1.0
	33	4 ± 1.0	8 ± 3.0	9 ± 1.0	8 ± 1.0	9 ± 1.0	8 ± 2.0
	100	6 ± 1.0	5 ± 1.0	10 ± 1.0	7 ± 1.0	6 ± 0.0	8 ± 3.0
	250		4 ± 1.0[d]				
	333	Toxic		9 ± 1.0	9 ± 2.0	9 ± 1.0	6 ± 1.0
	800				Toxic		Toxic
	1,000			Toxic		Toxic	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		274 ± 80.0	559 ± 19.0	117 ± 7.0	74 ± 3.0	93 ± 8.0	105 ± 5.0
TA98	0	20 ± 4.0	18 ± 2.0	20 ± 2.0	33 ± 1.0	23 ± 4.0	35 ± 1.0
	3.3	14 ± 2.0	20 ± 2.0				
	10	14 ± 1.0	16 ± 3.0	20 ± 1.0	33 ± 3.0	25 ± 2.0	26 ± 1.0
	33	16 ± 1.0	16 ± 1.0	24 ± 1.0	28 ± 1.0	23 ± 1.0	34 ± 4.0
	100	15 ± 4.0	17 ± 1.0	22 ± 1.0	26 ± 4.0	26 ± 1.0	27 ± 4.0
	250		15 ± 3.0[d]				
	333	12 ± 3.0[d]		16 ± 2.0	33 ± 2.0	23 ± 1.0	27 ± 3.0
	800				Toxic		Toxic
	1,000			Toxic		Toxic	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		1,452 ± 41.0	1,671 ± 20.0	1,008 ± 33.0	1,079 ± 30.0	689 ± 13.0	1,220 ± 47.0

Table E1
Mutagenicity of Isoeugenol in *Salmonella typhimurium*

Strain	Dose (µg/plate)	Revertants/Plate –S9 Trial 1	–S9 Trial 2	–S9 Trial 3	+10% rat S9 Trial 1	+10% rat S9 Trial 2	+10% rat S9 Trial 3
Study performed at SITEK Research Laboratories							
TA100	0	42 ± 2.0	45 ± 3.0		83 ± 12.0	64 ± 2.0	58 ± 2.0
	10	49 ± 3.0	56 ± 8.0				
	50	48 ± 1.0	44 ± 4.0		63 ± 2.0	63 ± 5.0	49 ± 8.0
	100	29 ± 9.0	38 ± 4.0		70 ± 4.0	64 ± 9.0	64 ± 2.0
	250	29 ± 5.0	36 ± 4.0				
	500	32 ± 3.0	27 ± 1.0		47 ± 7.0	47 ± 7.0	40 ± 2.0
	1,000				20 ± 2.0	43 ± 5.0	26 ± 4.0
	1,500				10 ± 0.0	23 ± 2.0	
	2,000						Toxic
Trial summary		Negative	Negative		Negative	Negative	Negative
Positive control		574 ± 1.0	653 ± 32.0		738 ± 31.0	1,161 ± 65.0	510 ± 27.0
TA98	0	23 ± 3.0	14 ± 3.0	20 ± 3.0	21 ± 2.0	18 ± 2.0	22 ± 2.0
	10	35 ± 3.0	15 ± 3.0	19 ± 2.0			
	50	21 ± 2.0	13 ± 2.0	16 ± 3.0	17 ± 3.0	24 ± 3.0	26 ± 1.0
	100	18 ± 1.0[d]	12 ± 3.0	12 ± 3.0	17 ± 4.0	22 ± 5.0	27 ± 1.0
	250	15 ± 1.0[d]	9 ± 1.0	18 ± 2.0			
	500	9 ± 1.0[d]	8 ± 0.0	Toxic	23 ± 4.0	22 ± 4.0	27 ± 2.0
	1,000				16 ± 3.0	23 ± 2.0	27 ± 2.0
	1,500				6 ± 1.0		9 ± 7.0
	2,000					Toxic	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		468 ± 17.0	506 ± 17.0	417 ± 31.0	1,313 ± 18.0	1,008 ± 32.0	372 ± 38.0
***Escherichia coli* WP2 *uvrA*/pKM101 (Analogous to TA102)**							
	0	181 ± 46.0	106 ± 5.0	133 ± 5.0	266 ± 54.0	125 ± 6.0	176 ± 0.0
	10	115 ± 3.0	124 ± 2.0	96 ± 23.0	170 ± 7.0	157 ± 6.0	192 ± 4.0
	50	135 ± 3.0	109 ± 5.0	164 ± 6.0	178 ± 7.0	156 ± 3.0	196 ± 4.0
	100	131 ± 1.0	115 ± 5.0	138 ± 6.0	167 ± 5.0	139 ± 4.0	145 ± 15.0
	500	93 ± 18.0	116 ± 6.0	135 ± 4.0	141 ± 7.0	95 ± 6.0	133 ± 11.0
	750	36 ± 1.0	117 ± 1.0		153 ± 8.0	82 ± 6.0	
	1,000	109 ± 8.0	111 ± 11.0	Toxic	110 ± 2.0	55 ± 10.0	21 ± 3.0
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		1,183 ± 140.0	930 ± 19.0	1,529 ± 121.0	710 ± 32.0	878 ± 12.0	1,204 ± 24.0

[a] 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] The detailed protocol and these data are presented by Mortelmans *et al.* (1986).
[d] Slight toxicity
[e] The positive controls in the absence of metabolic activation were sodium azide (TA100 and 1535), 9-aminoacridine (TA1537), 4-nitro-o-phenylenediamine (TA98), and methyl methanesulfonate (pKM101). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE E2
Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Isoeugenol[a]

Compound	Dose (µg/mL)	Total Cells Scored	Number of Aberrations	Aberrations/ Cell	Cells with Aberrations (%)
–S9					
Harvest time: 12.0 hours Summary: Negative					
Dimethylsulfoxide[b]		200	2	0.01	1.0
Isoeugenol	50.0	200	1	0.01	0.5
	100.0	200	1	0.01	0.5
	200.0	200	1	0.01	0.5
	300.0[c]				
	400.0[c]				
	500.0[c]				
					P = 0.710[d]
Mitomycin-C[e]	0.4	25	22	0.88	60.0
+S9					
Harvest time: 12.0 hours Summary: Negative					
Dimethylsulfoxide		200	4	0.02	2.0
Isoeugenol	150.0	200	4	0.02	2.0
	160.0	200	5	0.03	2.5
	170.0	200	3	0.02	1.5
	180.0[c]				
	190.0[f]				
	200.0[f]				
					P = 0.588
Cyclophosphamide[e]	20.0	25	23	0.92	48.0

[a] Study was performed at SITEK Research Laboratories. The detailed protocol is presented by Galloway *et al.* (1987).
[b] Solvent control
[c] No dividing cells
[d] Significance of percent cells with aberrations tested by the linear regression trend test versus log of the dose
[e] Positive control
[f] Cells did not survive.

Table E3
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice Following Exposure to Isoeugenol by Gavage for 3 Months[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs (%)
Male					
Corn oil[d]	0	5	0.90 ± 0.37		2.3
Isoeugenol	37.5	5	1.60 ± 0.46	0.0806	2.8
	75	5	0.70 ± 0.25	0.6915	3.1
	150	5	0.90 ± 0.24	0.5000	2.8
	300	5	0.30 ± 0.12	0.9584	2.5
	600	5	0.90 ± 0.19	0.5000	2.9
			P = 0.841[e]		
Female					
Corn oil	0	8	0.50 ± 0.16		2.8
Isoeugenol	37.5	5	1.10 ± 0.19	0.0408	3.5
	75	5	0.20 ± 0.12	0.8850	3.1
	150	5	0.70 ± 0.30	0.2568	2.7
	300	5	1.00 ± 0.35	0.0680	3.4
	600	5	1.60 ± 0.40	0.0022	2.4
			P = 0.001		

[a] Study was performed at SITEK Research Laboratories. The detailed protocol is presented by MacGregor et al. (1990). NCE = normochromatic erythrocyte; PCE = polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the vehicle control; dosed group values are significant at P≤0.005
[d] Vehicle control
[e] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test; significant at P≤0.025

APPENDIX F
CLINICAL PATHOLOGY RESULTS

Table F1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Isoeugenol[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
Hematology						
n						
Day 4	10	10	10	10	10	10
Day 23	9	9	9	9	8	10
Week 14	10	9	10	9	10	9
Hematocrit (%)						
Day 4	37.6 ± 0.3	38.0 ± 0.3	38.4 ± 0.6	37.5 ± 0.3	38.5 ± 0.4	37.2 ± 0.4
Day 23	42.0 ± 0.4	41.2 ± 0.4	41.0 ± 0.3	42.3 ± 0.5	41.7 ± 0.3	41.9 ± 0.4
Week 14	45.2 ± 0.4	45.5 ± 0.3	45.5 ± 0.3	46.0 ± 0.4	45.8 ± 0.4	46.2 ± 0.6
Hemoglobin (g/dL)						
Day 4	13.2 ± 0.1	13.3 ± 0.1	13.4 ± 0.2	13.2 ± 0.1	13.5 ± 0.1	13.1 ± 0.2
Day 23	15.3 ± 0.1	14.8 ± 0.1	14.7 ± 0.2	15.2 ± 0.1	14.9 ± 0.1	14.9 ± 0.1
Week 14	14.9 ± 0.1	15.0 ± 0.1	15.0 ± 0.1	15.2 ± 0.1	15.0 ± 0.2	15.1 ± 0.2
Erythrocytes (10^6/µL)						
Day 4	6.87 ± 0.06	6.90 ± 0.07	7.01 ± 0.09	6.74 ± 0.12	6.98 ± 0.07	6.76 ± 0.07
Day 23	7.68 ± 0.09	7.49 ± 0.06	7.52 ± 0.07	7.67 ± 0.10	7.67 ± 0.07	7.73 ± 0.07
Week 14	8.75 ± 0.09	8.87 ± 0.05	8.91 ± 0.07	8.97 ± 0.08*	8.95 ± 0.07*	9.14 ± 0.10**
Reticulocytes (10^6/µL)						
Day 4	0.38 ± 0.04	0.33 ± 0.03	0.38 ± 0.02	0.45 ± 0.06	0.39 ± 0.03	0.28 ± 0.02
Day 23	0.29 ± 0.02	0.33 ± 0.03	0.27 ± 0.03	0.34 ± 0.03	0.29 ± 0.02	0.30 ± 0.02
Week 14	0.12 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
Nucleated erythrocytes/100 leukocytes						
Day 4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 23	10.56 ± 10.56	0.00 ± 0.00	7.33 ± 7.33	0.00 ± 0.00	0.00 ± 0.00	9.10 ± 9.10
Week 14	10.10 ± 10.10	0.00 ± 0.00	7.60 ± 7.60	0.00 ± 0.00	8.80 ± 8.80	0.00 ± 0.00
Mean cell volume (fL)						
Day 4	54.7 ± 0.3	55.0 ± 0.2	54.8 ± 0.2	55.7 ± 0.7	55.2 ± 0.3	55.1 ± 0.3
Day 23	54.8 ± 0.2	55.0 ± 0.2	54.5 ± 0.2	55.1 ± 0.3	54.3 ± 0.3	54.2 ± 0.2
Week 14	51.6 ± 0.1	51.3 ± 0.2	51.1 ± 0.1**	51.2 ± 0.1*	51.1 ± 0.1*	50.6 ± 0.1**
Mean cell hemoglobin (pg)						
Day 4	19.2 ± 0.1	19.3 ± 0.1	19.1 ± 0.1	19.6 ± 0.3	19.3 ± 0.1	19.4 ± 0.1
Day 23	19.9 ± 0.1	19.8 ± 0.2	19.5 ± 0.2	19.8 ± 0.2	19.4 ± 0.1	19.3 ± 0.1**
Week 14	17.0 ± 0.1	16.9 ± 0.1	16.8 ± 0.1	16.9 ± 0.0	16.8 ± 0.0**	16.5 ± 0.1**
Mean cell hemoglobin concentration (g/dL)						
Day 4	35.1 ± 0.2	35.1 ± 0.2	34.9 ± 0.1	35.2 ± 0.2	35.0 ± 0.2	35.3 ± 0.2
Day 23	36.4 ± 0.2	35.9 ± 0.3	35.9 ± 0.3	35.9 ± 0.2	35.8 ± 0.2	35.5 ± 0.2
Week 14	32.9 ± 0.2	33.0 ± 0.2	33.0 ± 0.2	33.0 ± 0.1	32.8 ± 0.1	32.7 ± 0.1
Platelets (10^3/µL)						
Day 4	812.4 ± 31.4	799.6 ± 48.0	842.7 ± 15.3	884.3 ± 21.7	908.3 ± 15.0**	902.1 ± 24.0**
Day 23	790.1 ± 13.2	753.6 ± 31.8	751.3 ± 22.6	821.9 ± 13.0	837.8 ± 18.3	831.1 ± 26.3
Week 14	729.0 ± 18.3	692.8 ± 22.2	718.9 ± 17.9	727.8 ± 11.5	756.5 ± 8.4	754.9 ± 14.8
Leukocytes (10^3/µL)						
Day 4	7.63 ± 0.37	7.28 ± 0.23	7.98 ± 0.51	8.18 ± 0.38	7.53 ± 0.26	8.21 ± 0.41
Day 23	9.84 ± 0.45	10.31 ± 0.51	9.28 ± 0.54	9.07 ± 0.34	9.76 ± 0.57	9.58 ± 0.58
Week 14	8.97 ± 0.41	10.02 ± 0.18	8.88 ± 0.47	10.12 ± 0.43	8.25 ± 0.53	9.47 ± 0.36
Segmented neutrophils (10^3/µL)						
Day 4	0.83 ± 0.08	0.89 ± 0.12	0.97 ± 0.10	0.91 ± 0.11	0.89 ± 0.09	0.97 ± 0.07
Day 23	1.10 ± 0.17	1.25 ± 0.14	0.92 ± 0.09	0.94 ± 0.13	1.13 ± 0.09	1.20 ± 0.16
Week 14	1.13 ± 0.07	1.11 ± 0.11	1.01 ± 0.08	1.21 ± 0.12	1.06 ± 0.08	1.16 ± 0.12

Table F1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Isoeugenol

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male *(continued)*						
Hematology *(continued)*						
n						
Day 4	10	10	10	10	10	10
Day 23	9	9	9	9	8	10
Week 14	10	9	10	9	10	9
Bands (10^3/μL)						
Day 4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 23	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/μL)						
Day 4	6.70 ± 0.37	6.27 ± 0.19	6.91 ± 0.43	7.17 ± 0.30	6.54 ± 0.24	7.10 ± 0.42
Day 23	8.51 ± 0.39	8.92 ± 0.51	8.11 ± 0.50	7.95 ± 0.29	8.51 ± 0.59	8.20 ± 0.46
Week 14	7.50 ± 0.38	8.61 ± 0.19	7.47 ± 0.44	8.48 ± 0.41	6.93 ± 0.47	7.92 ± 0.36
Monocytes (10^3/μL)						
Day 4	0.08 ± 0.02	0.10 ± 0.02	0.06 ± 0.02	0.05 ± 0.02	0.08 ± 0.02	0.12 ± 0.03
Day 23	0.19 ± 0.06	0.14 ± 0.05	0.22 ± 0.07	0.16 ± 0.05	0.09 ± 0.03	0.15 ± 0.03
Week 14	0.29 ± 0.07	0.29 ± 0.05	0.31 ± 0.03	0.36 ± 0.06	0.24 ± 0.03	0.33 ± 0.07
Basophils (10^3/μL)						
Day 4	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 23	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/μL)						
Day 4	0.03 ± 0.01	0.02 ± 0.01	0.05 ± 0.02	0.05 ± 0.02	0.02 ± 0.02	0.03 ± 0.01
Day 23	0.05 ± 0.02	0.00 ± 0.00	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.02	0.04 ± 0.02
Week 14	0.04 ± 0.02	0.01 ± 0.01	0.09 ± 0.04	0.08 ± 0.02	0.03 ± 0.01	0.06 ± 0.03
Clinical Chemistry						
n						
Day 4	10	10	10	10	10	10
Day 23	10	10	10	10	10	10
Week 14	10	10	10	10	10	9
Urea nitrogen (mg/dL)						
Day 4	9.1 ± 0.5	10.1 ± 0.5	9.1 ± 0.6	9.3 ± 0.5	9.7 ± 0.6	9.1 ± 0.6
Day 23	14.4 ± 0.5	14.0 ± 0.4	13.3 ± 0.5	13.6 ± 0.4	14.2 ± 0.6	13.7 ± 0.4
Week 14	10.7 ± 0.3	10.7 ± 0.5	11.1 ± 0.3	10.6 ± 0.4	11.8 ± 0.4	11.6 ± 0.6
Creatinine (mg/dL)						
Day 4	0.40 ± 0.00	0.40 ± 0.00	0.40 ± 0.00	0.43 ± 0.02*	0.41 ± 0.01	0.40 ± 0.00
Day 23	0.44 ± 0.02	0.43 ± 0.02	0.42 ± 0.02	0.42 ± 0.01	0.44 ± 0.02	0.44 ± 0.02
Week 14	0.61 ± 0.01	0.60 ± 0.00	0.58 ± 0.01	0.58 ± 0.01	0.60 ± 0.00	0.56 ± 0.02**
Total protein (g/dL)						
Day 4	5.5 ± 0.1	5.6 ± 0.1	5.5 ± 0.1	5.5 ± 0.1	5.8 ± 0.1	5.7 ± 0.1
Day 23	6.3 ± 0.1	6.3 ± 0.1	6.1 ± 0.1	6.4 ± 0.1	6.4 ± 0.1	6.3 ± 0.0
Week 14	6.8 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	6.7 ± 0.0	6.9 ± 0.1	6.9 ± 0.1
Albumin (g/dL)						
Day 4	3.9 ± 0.0	3.9 ± 0.0	3.9 ± 0.0	3.8 ± 0.0	3.9 ± 0.1	3.9 ± 0.0
Day 23	4.2 ± 0.0	4.2 ± 0.0	4.1 ± 0.0	4.3 ± 0.0	4.2 ± 0.0	4.3 ± 0.0
Week 14	4.4 ± 0.0	4.3 ± 0.0	4.3 ± 0.0	4.3 ± 0.0	4.5 ± 0.1	4.5 ± 0.0
Alanine aminotransferase (IU/L)						
Day 4	84 ± 2	83 ± 2	77 ± 2	82 ± 2	82 ± 3	86 ± 2
Day 23	44 ± 1	43 ± 1	43 ± 2	49 ± 1*	49 ± 2*	63 ± 13**
Week 14	48 ± 3	48 ± 1	49 ± 2	45 ± 1	47 ± 1	47 ± 1

Table F1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Isoeugenol

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male *(continued)*						
Clinical Chemistry *(continued)*						
n						
Day 4	10	10	10	10	10	10
Day 23	10	10	10	10	10	10
Week 14	10	10	10	10	10	9
Alkaline phosphatase (IU/L)						
Day 4	882 ± 14	843 ± 17	852 ± 16	899 ± 19	830 ± 20	865 ± 33
Day 23	635 ± 12	640 ± 14	620 ± 14	654 ± 24	641 ± 13	654 ± 20
Week 14	248 ± 6	263 ± 5	257 ± 6	246 ± 5	252 ± 8	238 ± 6
Creatine kinase (IU/L)						
Day 4	337 ± 28	337 ± 30	351 ± 41	245 ± 23	298 ± 27	346 ± 45
Day 23	500 ± 67	477 ± 47	388 ± 38	374 ± 46	529 ± 131	463 ± 87
Week 14	114 ± 14	155 ± 22	110 ± 19	129 ± 51	90 ± 15	116 ± 21
Sorbitol dehydrogenase (IU/L)						
Day 4	17 ± 1	18 ± 1	17 ± 0	17 ± 1	18 ± 1	17 ± 1
Day 23	23 ± 2[b]	24 ± 1	24 ± 2	24 ± 1	24 ± 1	38 ± 14
Week 14	20 ± 2	18 ± 1	18 ± 1	17 ± 1	14 ± 0**	14 ± 1**
Bile acids (μmol/L)						
Day 4	31.7 ± 3.0	24.2 ± 1.3	29.4 ± 2.5	32.2 ± 2.1	29.4 ± 2.4	38.1 ± 4.2
Day 23	19.5 ± 1.1	18.3 ± 1.3	21.1 ± 1.5	23.4 ± 1.6	30.4 ± 3.8*	33.0 ± 3.0**
Week 14	18.2 ± 2.5	18.7 ± 2.5	23.0 ± 3.2	26.7 ± 3.5	28.8 ± 3.5	27.1 ± 3.3
Female						
Hematology						
n						
Day 4	10	10	10	10	9	10
Day 23	10	10	9	10	10	10
Week 14	10	9	9	10	10	10
Hematocrit (%)						
Day 4	40.6 ± 0.5	41.0 ± 0.4	40.1 ± 0.4	41.4 ± 0.5	39.6 ± 0.8	39.9 ± 0.4
Day 23	42.5 ± 0.5	42.6 ± 0.4	43.2 ± 0.6	42.7 ± 0.5	43.3 ± 0.4	43.0 ± 0.3
Week 14	43.7 ± 0.3	44.8 ± 0.4	44.5 ± 0.5	44.6 ± 0.4	44.7 ± 0.3	44.6 ± 0.5
Hemoglobin (g/dL)						
Day 4	14.2 ± 0.2	14.4 ± 0.2	14.1 ± 0.1	14.6 ± 0.2	13.9 ± 0.3	14.0 ± 0.2
Day 23	15.2 ± 0.2	15.2 ± 0.1	15.4 ± 0.2	15.3 ± 0.2	15.5 ± 0.1	15.3 ± 0.1
Week 14	14.6 ± 0.1	14.9 ± 0.2	14.8 ± 0.2	14.9 ± 0.1	14.8 ± 0.1	14.6 ± 0.1
Erythrocytes (10^6/μL)						
Day 4	7.27 ± 0.09	7.43 ± 0.08	7.24 ± 0.07	7.52 ± 0.10	7.14 ± 0.14	7.29 ± 0.06
Day 23	7.76 ± 0.09	7.76 ± 0.08	7.89 ± 0.12	7.81 ± 0.09	7.91 ± 0.07	7.89 ± 0.08
Week 14	8.10 ± 0.07	8.27 ± 0.08	8.23 ± 0.10	8.27 ± 0.07	8.29 ± 0.05	8.26 ± 0.08
Reticulocytes (10^6/μL)						
Day 4	0.25 ± 0.03	0.27 ± 0.01	0.27 ± 0.02	0.26 ± 0.02	0.32 ± 0.02	0.27 ± 0.02
Day 23	0.21 ± 0.01	0.22 ± 0.03	0.18 ± 0.01	0.19 ± 0.01	0.23 ± 0.03	0.21 ± 0.01
Week 14	0.10 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.14 ± 0.01*	0.12 ± 0.01	0.13 ± 0.01
Nucleated erythrocytes/100 leukocytes						
Day 4	19.20 ± 19.20	12.20 ± 12.20	14.90 ± 9.95	27.60 ± 14.48	15.78 ± 15.78	0.00 ± 0.00
Day 23	0.00 ± 0.00	0.00 ± 0.00	11.56 ± 11.56	22.30 ± 14.93	0.00 ± 0.00	10.40 ± 10.40
Week 14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Isoeugenol

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Female *(continued)*						
Hematology *(continued)*						
n						
Day 4	10	10	10	10	9	10
Day 23	10	10	9	10	10	10
Week 14	10	9	9	10	10	10
Mean cell volume (fL)						
Day 4	55.9 ± 0.2	55.1 ± 0.2*	55.3 ± 0.2	55.1 ± 0.2*	55.5 ± 0.2	54.8 ± 0.3*
Day 23	54.8 ± 0.2	54.9 ± 0.1	54.8 ± 0.2	54.7 ± 0.3	54.7 ± 0.2	54.4 ± 0.2
Week 14	53.9 ± 0.1	54.1 ± 0.1	54.1 ± 0.1	53.9 ± 0.1	53.9 ± 0.2	54.0 ± 0.2
Mean cell hemoglobin (pg)						
Day 4	19.6 ± 0.1	19.4 ± 0.2	19.5 ± 0.1	19.4 ± 0.1	19.5 ± 0.1	19.2 ± 0.1*
Day 23	19.6 ± 0.1	19.6 ± 0.1	19.6 ± 0.1	19.6 ± 0.1	19.6 ± 0.1	19.4 ± 0.1
Week 14	18.1 ± 0.1	18.0 ± 0.1	18.0 ± 0.1	18.0 ± 0.1	17.9 ± 0.0*	17.7 ± 0.1**
Mean cell hemoglobin concentration (g/dL)						
Day 4	35.0 ± 0.2	35.2 ± 0.2	35.1 ± 0.1	35.1 ± 0.1	35.2 ± 0.2	35.1 ± 0.2
Day 23	35.8 ± 0.2	35.6 ± 0.2	35.7 ± 0.1	35.9 ± 0.1	35.8 ± 0.2	35.6 ± 0.2
Week 14	33.5 ± 0.1	33.2 ± 0.1	33.3 ± 0.1	33.4 ± 0.1	33.2 ± 0.1*	32.8 ± 0.1**
Platelets (10^3/μL)						
Day 4	800.7 ± 25.3	717.8 ± 31.0	813.9 ± 19.6[b]	755.1 ± 34.7	823.6 ± 33.0	762.6 ± 24.5
Day 23	768.7 ± 12.2	782.1 ± 25.6	762.0 ± 23.4	783.9 ± 18.8	747.6 ± 36.7	800.6 ± 19.4
Week 14	745.1 ± 9.9	738.7 ± 15.6	740.0 ± 15.9	769.7 ± 14.7	747.2 ± 23.0	715.1 ± 14.1
Leukocytes (10^3/μL)						
Day 4	9.46 ± 0.46	10.00 ± 0.57	8.56 ± 0.53	10.22 ± 0.59	8.86 ± 0.57	10.38 ± 0.64
Day 23	11.07 ± 0.51	9.48 ± 0.23	10.36 ± 0.41	10.19 ± 0.44	9.84 ± 0.50	10.46 ± 0.41
Week 14	8.79 ± 0.61	8.78 ± 0.62	8.42 ± 0.55	8.20 ± 0.80	9.29 ± 0.61	9.13 ± 0.63
Segmented neutrophils (10^3/μL)						
Day 4	0.97 ± 0.12	1.08 ± 0.18	0.88 ± 0.13	1.07 ± 0.08	1.02 ± 0.11	1.18 ± 0.14
Day 23	0.90 ± 0.13	0.90 ± 0.08	1.03 ± 0.13	1.08 ± 0.13	0.80 ± 0.08	1.08 ± 0.10
Week 14	1.22 ± 0.15	1.02 ± 0.11	0.92 ± 0.11	1.37 ± 0.19	0.94 ± 0.12	1.02 ± 0.15
Bands (10^3/μL)						
Day 4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 23	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/μL)						
Day 4	8.32 ± 0.37	8.75 ± 0.48	7.57 ± 0.47	9.02 ± 0.57	7.71 ± 0.47	8.94 ± 0.62
Day 23	9.96 ± 0.52	8.35 ± 0.24	9.20 ± 0.44	8.90 ± 0.34	8.82 ± 0.52	9.18 ± 0.41
Week 14	7.12 ± 0.61	7.33 ± 0.49	7.12 ± 0.53	6.36 ± 0.70	7.94 ± 0.54	7.71 ± 0.51
Monocytes (10^3/μL)						
Day 4	0.15 ± 0.02	0.13 ± 0.04	0.07 ± 0.03	0.13 ± 0.05	0.10 ± 0.04	0.19 ± 0.04
Day 23	0.15 ± 0.02	0.17 ± 0.04	0.09 ± 0.02	0.13 ± 0.04	0.16 ± 0.03	0.15 ± 0.03
Week 14	0.37 ± 0.06	0.36 ± 0.07	0.29 ± 0.04	0.35 ± 0.05	0.35 ± 0.03	0.35 ± 0.04
Basophils (10^3/μL)						
Day 4	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 23	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/μL)						
Day 4	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.01 ± 0.01	0.03 ± 0.02	0.07 ± 0.02
Day 23	0.05 ± 0.03	0.06 ± 0.02	0.04 ± 0.02	0.07 ± 0.03	0.07 ± 0.03	0.05 ± 0.02
Week 14	0.09 ± 0.02	0.07 ± 0.03	0.09 ± 0.02	0.13 ± 0.04	0.06 ± 0.02	0.06 ± 0.02

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Isoeugenol

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Female *(continued)*						
Clinical Chemistry						
n						
Day 4	10	10	10	10	10	10
Day 23	10	10	10	10	10	10
Week 14	10	9	9	10	10	10
Urea nitrogen (mg/dL)						
Day 4	9.3 ± 0.5	9.9 ± 0.4	9.4 ± 0.6	10.8 ± 0.7	10.2 ± 0.9	8.7 ± 0.4
Day 23	14.4 ± 0.5	14.5 ± 0.5	14.8 ± 0.6	12.8 ± 0.4	13.0 ± 0.6	12.8 ± 0.7
Week 14	11.4 ± 0.7	12.4 ± 0.4	11.3 ± 0.6	11.7 ± 0.3	11.9 ± 0.4	12.3 ± 0.5
Creatinine (mg/dL)						
Day 4	0.47 ± 0.02	0.47 ± 0.02	0.48 ± 0.01	0.48 ± 0.01	0.46 ± 0.02	0.44 ± 0.02
Day 23	0.43 ± 0.02	0.42 ± 0.01	0.43 ± 0.02	0.43 ± 0.02	0.43 ± 0.02	0.42 ± 0.01
Week 14	0.60 ± 0.02	0.61 ± 0.01	0.56 ± 0.02	0.60 ± 0.02	0.59 ± 0.01	0.57 ± 0.02
Total protein (g/dL)						
Day 4	5.8 ± 0.0	5.8 ± 0.0	5.7 ± 0.1	5.8 ± 0.1	5.8 ± 0.1	5.8 ± 0.1
Day 23	6.2 ± 0.1	6.3 ± 0.1	6.2 ± 0.1	6.1 ± 0.0	6.3 ± 0.1	6.3 ± 0.0
Week 14	6.7 ± 0.1	6.7 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	6.6 ± 0.1	6.6 ± 0.1
Albumin (g/dL)						
Day 4	4.1 ± 0.0	4.2 ± 0.0	4.1 ± 0.0	4.1 ± 0.1	4.1 ± 0.1	4.1 ± 0.0
Day 23	4.4 ± 0.0	4.4 ± 0.0	4.4 ± 0.0	4.3 ± 0.0	4.4 ± 0.1	4.4 ± 0.0
Week 14	4.7 ± 0.1	4.7 ± 0.0	4.8 ± 0.1	4.7 ± 0.0	4.7 ± 0.0	4.7 ± 0.1
Alanine aminotransferase (IU/L)						
Day 4	76 ± 2	71 ± 3	70 ± 2	68 ± 1*	73 ± 2	81 ± 5
Day 23	35 ± 1	36 ± 1	34 ± 1	34 ± 1	41 ± 2*	41 ± 2*
Week 14	47 ± 2	53 ± 2	42 ± 2	43 ± 3	45 ± 1	45 ± 1
Alkaline phosphatase (IU/L)						
Day 4	689 ± 18	663 ± 22	660 ± 21	667 ± 10	694 ± 16	699 ± 21
Day 23	446 ± 8	468 ± 12	457 ± 8	473 ± 10	498 ± 16**	501 ± 13**
Week 14	222 ± 5	233 ± 4	222 ± 9	204 ± 12	210 ± 6	202 ± 8
Creatine kinase (IU/L)						
Day 4	535 ± 53	479 ± 57	445 ± 45	462 ± 53	612 ± 93	509 ± 91
Day 23	369 ± 54	263 ± 41	508 ± 103	378 ± 24	388 ± 76	388 ± 57
Week 14	173 ± 36	315 ± 101	240 ± 80	199 ± 27	241 ± 38	220 ± 42
Sorbitol dehydrogenase (IU/L)						
Day 4	20 ± 1	21 ± 1	20 ± 1	20 ± 1	19 ± 1	20 ± 1
Day 23	16 ± 0	18 ± 1	19 ± 1	18 ± 1	18 ± 1	18 ± 1
Week 14	13 ± 1	18 ± 1*	13 ± 1	14 ± 1	14 ± 1	16 ± 1
Bile acids (µmol/L)						
Day 4	17.1 ± 1.3	17.4 ± 1.4	16.3 ± 0.7	17.9 ± 1.2	17.9 ± 1.8	26.3 ± 3.5
Day 23	24.7 ± 3.2	14.5 ± 2.5	11.4 ± 1.1	21.8 ± 3.3	25.0 ± 2.4	31.5 ± 4.9
Week 14	20.4 ± 2.3	27.8 ± 3.2	21.6 ± 2.3	20.3 ± 3.3	23.3 ± 3.1	24.2 ± 3.3

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test
** $P \leq 0.01$
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n = 9

TABLE F2
Hematology Data for Mice in the 3-Month Gavage Study of Isoeugenol[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
n	10	10	10	10	10	10
Hematocrit (%)	50.5 ± 0.6	49.0 ± 0.7	51.3 ± 1.0	51.5 ± 0.9	50.3 ± 1.2	50.4 ± 1.1
Hemoglobin (g/dL)	16.2 ± 0.2	15.7 ± 0.2	16.5 ± 0.4	16.6 ± 0.3	16.1 ± 0.4	16.2 ± 0.4
Erythrocytes (10^6/µL)	10.84 ± 0.14	10.51 ± 0.16	10.94 ± 0.22	11.05 ± 0.23	10.78 ± 0.27	10.87 ± 0.25
Reticulocytes (10^6/µL)	0.23 ± 0.03	0.19 ± 0.02	0.30 ± 0.03	0.26 ± 0.02	0.23 ± 0.02	0.24 ± 0.02
Nucleated erythrocytes/100 leukocytes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	46.5 ± 0.2	46.7 ± 0.1	46.8 ± 0.1	46.7 ± 0.2	46.7 ± 0.1	46.3 ± 0.1
Mean cell hemoglobin (pg)	15.0 ± 0.1	15.0 ± 0.1	15.0 ± 0.1	15.1 ± 0.1	15.0 ± 0.1	14.9 ± 0.0
Mean cell hemoglobin concentration (g/dL)	32.2 ± 0.1	32.1 ± 0.1	32.1 ± 0.1	32.3 ± 0.1	32.0 ± 0.1	32.2 ± 0.1
Platelets (10^3/µL)	710.5 ± 33.8	726.6 ± 41.3	670.6 ± 35.0	713.6 ± 38.8	804.9 ± 44.9	777.5 ± 47.7
Leukocytes (10^3/µL)	2.98 ± 0.36	3.02 ± 0.36	3.15 ± 0.33	2.75 ± 0.26	2.87 ± 0.32	2.81 ± 0.31
Segmented neutrophils (10^3/µL)	0.42 ± 0.08	0.37 ± 0.04	0.37 ± 0.03	0.34 ± 0.04	0.46 ± 0.05	0.41 ± 0.06
Bands (10^3/µL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/µL)	2.47 ± 0.29	2.54 ± 0.35	2.70 ± 0.31	2.34 ± 0.26	2.34 ± 0.29	2.34 ± 0.27
Monocytes (10^3/µL)	0.03 ± 0.01	0.06 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
Basophils (10^3/µL)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/µL)	0.06 ± 0.02	0.05 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	0.05 ± 0.01	0.03 ± 0.01
Female						
n	10	10	10	9	10	10
Hematocrit (%)	49.9 ± 0.7	50.3 ± 0.7	49.0 ± 0.6	50.2 ± 0.6	49.8 ± 0.5	48.9 ± 0.6
Hemoglobin (g/dL)	16.4 ± 0.2	16.4 ± 0.2	16.1 ± 0.2	16.4 ± 0.2	16.2 ± 0.2	16.0 ± 0.2
Erythrocytes (10^6/µL)	10.63 ± 0.18	10.75 ± 0.16	10.51 ± 0.12	10.69 ± 0.13	10.61 ± 0.10	10.43 ± 0.13
Reticulocytes (10^6/µL)	0.31 ± 0.02	0.29 ± 0.03	0.30 ± 0.02	0.28 ± 0.02	0.32 ± 0.02	0.29 ± 0.03
Nucleated erythrocytes/100 leukocytes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	46.9 ± 0.2	46.8 ± 0.2	46.7 ± 0.1	47.0 ± 0.1	46.9 ± 0.1	46.8 ± 0.1
Mean cell hemoglobin (pg)	15.5 ± 0.1	15.3 ± 0.1	15.4 ± 0.1	15.4 ± 0.1	15.3 ± 0.1	15.3 ± 0.1
Mean cell hemoglobin concentration (g/dL)	32.9 ± 0.1	32.6 ± 0.1	32.9 ± 0.1	32.7 ± 0.1	32.6 ± 0.1	32.7 ± 0.1
Platelets (10^3/µL)	663.1 ± 45.7	625.5 ± 34.4	642.5 ± 30.6	622.0 ± 40.2	624.0 ± 28.3	739.3 ± 37.0
Leukocytes (10^3/µL)	4.17 ± 0.28	3.74 ± 0.27	3.21 ± 0.24*	3.41 ± 0.23	3.48 ± 0.26	3.64 ± 0.15
Segmented neutrophils (10^3/µL)	0.44 ± 0.05	0.39 ± 0.05	0.29 ± 0.04	0.37 ± 0.06	0.39 ± 0.08	0.41 ± 0.04
Bands (10^3/µL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/µL)	3.62 ± 0.22	3.27 ± 0.24	2.84 ± 0.20*	3.00 ± 0.20	3.05 ± 0.20	3.15 ± 0.11
Monocytes (10^3/µL)	0.08 ± 0.04	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.04 ± 0.01
Basophils (10^3/µL)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/µL)	0.03 ± 0.01	0.02 ± 0.01	0.05 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.04 ± 0.02

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunn's test
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.

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

TABLE G1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 3-Month Gavage Study of Isoeugenol[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
n	10	10	10	10	10	9
Necropsy body wt	360 ± 8	333 ± 3*	343 ± 5*	342 ± 7*	334 ± 6**	313 ± 7**
Heart						
Absolute	1.035 ± 0.028	0.970 ± 0.022	1.000 ± 0.021	0.997 ± 0.031	1.007 ± 0.024	1.108 ± 0.166
Relative	2.874 ± 0.050	2.912 ± 0.059	2.917 ± 0.054	2.917 ± 0.054	3.020 ± 0.064	3.562 ± 0.561
R. Kidney						
Absolute	1.155 ± 0.072	1.049 ± 0.024	1.053 ± 0.023	1.113 ± 0.029	1.153 ± 0.029	1.130 ± 0.039
Relative	3.207 ± 0.194	3.151 ± 0.071	3.070 ± 0.050	3.260 ± 0.049	3.455 ± 0.068	3.607 ± 0.073**
Liver						
Absolute	13.04 ± 0.44	12.81 ± 0.19	12.84 ± 0.32	13.01 ± 0.48	13.73 ± 0.42	12.81 ± 0.43
Relative	36.140 ± 0.627	38.474 ± 0.515*	37.404 ± 0.561	38.006 ± 0.695*	41.070 ± 0.712**	40.897 ± 0.676**
Lung						
Absolute	1.874 ± 0.097	1.849 ± 0.105	1.647 ± 0.067	1.747 ± 0.063	1.813 ± 0.099	1.498 ± 0.040*
Relative	5.220 ± 0.282	5.543 ± 0.287	4.807 ± 0.196	5.132 ± 0.211	5.433 ± 0.294	4.798 ± 0.125
R. Testis						
Absolute	1.469 ± 0.046	1.370 ± 0.038	1.446 ± 0.020	1.449 ± 0.035	1.420 ± 0.028	1.415 ± 0.014
Relative	4.076 ± 0.091	4.115 ± 0.114	4.218 ± 0.033	4.243 ± 0.043	4.254 ± 0.050	4.536 ± 0.088**
Thymus						
Absolute	0.375 ± 0.023	0.323 ± 0.013*	0.329 ± 0.006	0.296 ± 0.013**	0.331 ± 0.012	0.312 ± 0.013*
Relative	1.039 ± 0.052	0.970 ± 0.038	0.959 ± 0.020	0.868 ± 0.032*	0.993 ± 0.041	0.999 ± 0.039
Female						
n	10	9	9	10	10	10
Necropsy body wt	193 ± 4	195 ± 4	197 ± 4	187 ± 3	199 ± 3	193 ± 4
Heart						
Absolute	0.647 ± 0.015	0.638 ± 0.010	0.626 ± 0.013	0.618 ± 0.015	0.657 ± 0.012	0.632 ± 0.015
Relative	3.355 ± 0.083	3.289 ± 0.080	3.185 ± 0.058	3.314 ± 0.091	3.301 ± 0.035	3.271 ± 0.049
R. Kidney						
Absolute	0.627 ± 0.018	0.664 ± 0.019	0.662 ± 0.018	0.639 ± 0.013	0.673 ± 0.007	0.696 ± 0.018**
Relative	3.251 ± 0.069	3.412 ± 0.067	3.366 ± 0.054	3.422 ± 0.061	3.387 ± 0.035	3.596 ± 0.043**
Liver						
Absolute	6.524 ± 0.168	6.657 ± 0.147	6.443 ± 0.222	6.327 ± 0.116	7.150 ± 0.186*	7.459 ± 0.257**
Relative	33.817 ± 0.714	34.263 ± 0.776	32.682 ± 0.599	33.892 ± 0.698	35.901 ± 0.543*	38.513 ± 0.797**
Lung						
Absolute	1.105 ± 0.048	1.208 ± 0.055	1.097 ± 0.069	1.051 ± 0.036	1.247 ± 0.080	1.041 ± 0.031
Relative	5.730 ± 0.239	6.228 ± 0.302	5.577 ± 0.319	5.635 ± 0.213	6.278 ± 0.420	5.388 ± 0.129
Thymus						
Absolute	0.248 ± 0.017	0.227 ± 0.010	0.263 ± 0.011	0.236 ± 0.008	0.244 ± 0.007	0.246 ± 0.008
Relative	1.283 ± 0.079	1.173 ± 0.055	1.335 ± 0.034	1.260 ± 0.029	1.224 ± 0.030	1.272 ± 0.023

* 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).

TABLE G2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 3-Month Gavage Study of Isoeugenol[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
n	10	10	10	10	10	10
Male						
Necropsy body wt	39.3 ± 0.9	37.5 ± 1.0	37.6 ± 1.6	37.0 ± 1.2	37.8 ± 1.2	33.7 ± 1.1**
Heart						
Absolute	0.164 ± 0.006	0.173 ± 0.009	0.166 ± 0.009	0.165 ± 0.010	0.170 ± 0.011	0.152 ± 0.008
Relative	4.174 ± 0.146	4.656 ± 0.272	4.406 ± 0.147	4.458 ± 0.231	4.561 ± 0.370	4.499 ± 0.176
R. Kidney						
Absolute	0.296 ± 0.006	0.287 ± 0.006	0.285 ± 0.005	0.273 ± 0.007*	0.274 ± 0.004*	0.268 ± 0.008**
Relative	7.559 ± 0.148	7.691 ± 0.208	7.662 ± 0.248	7.399 ± 0.118	7.311 ± 0.218	7.976 ± 0.164
Liver						
Absolute	1.569 ± 0.044	1.655 ± 0.047	1.707 ± 0.059	1.735 ± 0.063	1.908 ± 0.075**	1.794 ± 0.064**
Relative	40.009 ± 0.880	44.248 ± 1.115**	45.609 ± 0.998**	46.816 ± 0.727**	50.570 ± 1.217**	53.238 ± 0.851**
Lung						
Absolute	0.295 ± 0.014	0.276 ± 0.018	0.295 ± 0.021	0.295 ± 0.014	0.270 ± 0.020	0.286 ± 0.019
Relative	7.512 ± 0.334	7.394 ± 0.513	7.965 ± 0.694	7.984 ± 0.314	7.224 ± 0.634	8.588 ± 0.658
R. Testis						
Absolute	0.120 ± 0.002	0.119 ± 0.002	0.119 ± 0.003	0.117 ± 0.003	0.116 ± 0.001	0.115 ± 0.002
Relative	3.051 ± 0.061	3.198 ± 0.128	3.197 ± 0.147	3.181 ± 0.067	3.120 ± 0.128	3.423 ± 0.102
Thymus						
Absolute	0.050 ± 0.003	0.053 ± 0.003	0.052 ± 0.003	0.049 ± 0.004	0.051 ± 0.004	0.040 ± 0.002
Relative	1.272 ± 0.057	1.404 ± 0.075	1.396 ± 0.077	1.332 ± 0.103	1.339 ± 0.063	1.180 ± 0.051
Female						
Necropsy body wt	26.9 ± 0.7	28.3 ± 1.1	29.5 ± 1.1	29.1 ± 0.9	27.8 ± 0.9	24.6 ± 0.5
Heart						
Absolute	0.132 ± 0.003	0.130 ± 0.004	0.138 ± 0.006	0.133 ± 0.003	0.126 ± 0.003	0.118 ± 0.003
Relative	4.929 ± 0.102	4.646 ± 0.254	4.721 ± 0.229	4.595 ± 0.129	4.570 ± 0.124	4.794 ± 0.161
R. Kidney						
Absolute	0.172 ± 0.004	0.166 ± 0.004	0.166 ± 0.004	0.180 ± 0.004	0.166 ± 0.004	0.164 ± 0.004
Relative	6.401 ± 0.148	5.917 ± 0.163	5.695 ± 0.194*	6.240 ± 0.249	6.007 ± 0.168	6.652 ± 0.191
Liver						
Absolute	1.185 ± 0.035	1.136 ± 0.032	1.235 ± 0.042	1.278 ± 0.022	1.212 ± 0.033	1.187 ± 0.024
Relative	44.016 ± 0.618	40.389 ± 1.033	41.977 ± 0.829	44.151 ± 0.923	43.766 ± 0.702	48.254 ± 0.948**
Lung						
Absolute	0.274 ± 0.013	0.293 ± 0.009	0.270 ± 0.013	0.286 ± 0.010	0.253 ± 0.011	0.224 ± 0.012**
Relative	10.287 ± 0.619	10.538 ± 0.595	9.287 ± 0.593	9.878 ± 0.336	9.218 ± 0.528	9.117 ± 0.475
Thymus						
Absolute	0.052 ± 0.002	0.053 ± 0.003	0.052 ± 0.004	0.056 ± 0.002	0.049 ± 0.002	0.044 ± 0.003
Relative	1.916 ± 0.055	1.851 ± 0.036	1.771 ± 0.094	1.928 ± 0.055	1.776 ± 0.073	1.782 ± 0.097

* 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).

APPENDIX H
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

Procurement and Characterization

Isoeugenol

Isoeugenol was obtained from Penta International Corporation (Livingston, NJ) in one lot (60449) that was used in the 3-month and 2-year studies. Identity and purity analyses were performed by the analytical chemistry laboratory at Battelle Columbus Operations (Chemistry Support Services, Columbus, OH) and the study laboratory at Battelle Columbus Operations (Columbus, OH); Karl Fischer titration and elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN). Reports on analyses performed in support of the isoeugenol studies are on file at the National Institute of Environmental Health Sciences.

Lot 60449 of the chemical, a yellow liquid, was identified as isoeugenol by the analytical chemistry laboratory using infrared (IR) spectral analysis and by both proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy. The study laboratory confirmed the identity of the test article by IR spectroscopy. All spectra were consistent with literature spectra (*Aldrich*, 1985, 1992), frozen reference spectra, and a previously reported spectrum from the same lot of isoeugenol. The NMR spectra indicated that lot 60449 was approximately a 1:7 mixture of *Z*-:*E*-isoeugenol (Figures H1, H2, and H3).

The purity of lot 60449 was determined by the analytical chemistry laboratory using gas chromatography (GC) by system A and high performance liquid chromatography (HPLC) using a Hewlett Packard (Palo Alto, CA) instrument with a Luna® C18 column (150 mm × 4.6 mm, 5-µm particle size; Phenomenex, Torrance, CA), and a mobile phase of A: 50:50:1 acetonitrile:Milli-Q® Water:glacial acetic acid and B: 90:10:0.1 acetonitrile:Milli-Q® Water:glacial acetic acid, held at 100% A for 20 minutes, then linear to 100% B in 5 minutes, held at 100% B for 20 minutes, then linear to 100% A in 5 minutes, held at 100% A for 15 minutes; the flow rate was 0.7 mL/minute, and ultraviolet detection at 230 nm was used. The study laboratory determined purity using GC by system B.

A) Hewlett-Packard gas chromatograph, a RTX-5 (15 m × 0.53 mm ID, 1.0-µm film thickness) column (Restek, Bellefonte, PA), flame ionization detection, helium as a carrier gas at a flow rate of 5 mL/minute, and an oven temperature program of 50° C for 3 minutes, then 10° C/minute to 300° C, then held for 2 minutes

B) Hewlett-Packard gas chromatograph, a Restek Stabilwax® Crossbond® (30 m × 0.25 mm ID, 0.25-µm film thickness) column, flame ionization detection, helium as a carrier gas at a flow rate of 3 mL/minute, and an oven temperature program of 80° C for 2 minutes, then 20° C/minute to 240° C, then held for 9 minutes

For lot 60449, Karl Fischer titration indicated 0.57% water. Elemental analyses for carbon and hydrogen were in agreement with the theoretical values for isoeugenol. GC using system A indicated one major peak and four smaller peaks. The major peak, 87% of the total peak area, was determined to be *E*-isoeugenol, and the second largest peak, 12% of the total peak area, was determined to be *Z*-isoeugenol. The identity of three minor peaks with a combined area of approximately 1% of the total area was not determined. GC using system B yielded purity estimates of 101.7% and 99.2% relative to a frozen reference sample of the same lot. HPLC indicated one major peak, believed to be the coelution of *Z*- and *E*-isoeugenol, and one impurity with an area of 0.5% relative to the total peak area. The overall purity of lot 60449 was determined to be 99% or greater.

To ensure stability, the bulk chemical was stored at or less than –20° C, protected from light, in 1-L Teflon® bottles. The study laboratory monitored stability during the 3-month and 2-year studies by periodic analyses using GC by system B. No degradation of the bulk chemical was detected.

Corn Oil

Corn oil was obtained in multiple lots from Spectrum Chemicals and Laboratory Products (Gardena, CA) and was used as the vehicle during the 3-month and 2-year studies. The study laboratory analyzed peroxide levels prior to use and every 2 months during the studies using potentiometric titration; all peroxide concentrations were less than the acceptable limit of 3 mEq/kg.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared by mixing isoeugenol with corn oil to give the required concentrations (Table H1). The dose formulations were stored at room temperature in amber glass bottles with Teflon®-lined lids for up to 35 days.

Homogeneity studies of 0.2 and 120 mg/mL dose formulations and stability studies of a 0.2 mg/mL dose formulation were performed by the analytical chemistry laboratory using GC by system B on a different lot (46928) of isoeugenol obtained from Penta International Corporation. Homogeneity was confirmed, and the 120 mg/mL dose formulation was found to be suitable for gavage. Stability was confirmed for up to 35 days for dose formulations stored in amber glass bottles with Teflon®-lined lids at –20° C, 5° C, and room temperature and for 3 hours under simulated animal room conditions.

Periodic analyses of the dose formulations of isoeugenol were conducted by the study laboratory using GC by system B. During the 3-month studies, the dose formulations were analyzed three times; animal room samples were also analyzed. All 15 dose formulations for rats and mice were within 10% of the target concentrations; all 15 animal room samples analyzed for rats and 14 of 15 for mice were within 10% of the target concentrations (Table H2). During the 2-year studies, the dose formulations were analyzed approximately every 3 months; animal room samples were also analyzed (Table H3). All 27 dose formulations for rats and 27 of 28 for mice were within 10% of the target concentrations. The 7.5 mg/mL dose formulation prepared for mice on December 19, 2002, was determined to be 13% below the target concentration after being administered to animals for 4 days; use of this batch was discontinued, and an acceptable 7.5 mg/mL dose formulation was subsequently prepared and used. All nine animal room samples analyzed for rats and mice were within 10% of the target concentrations.

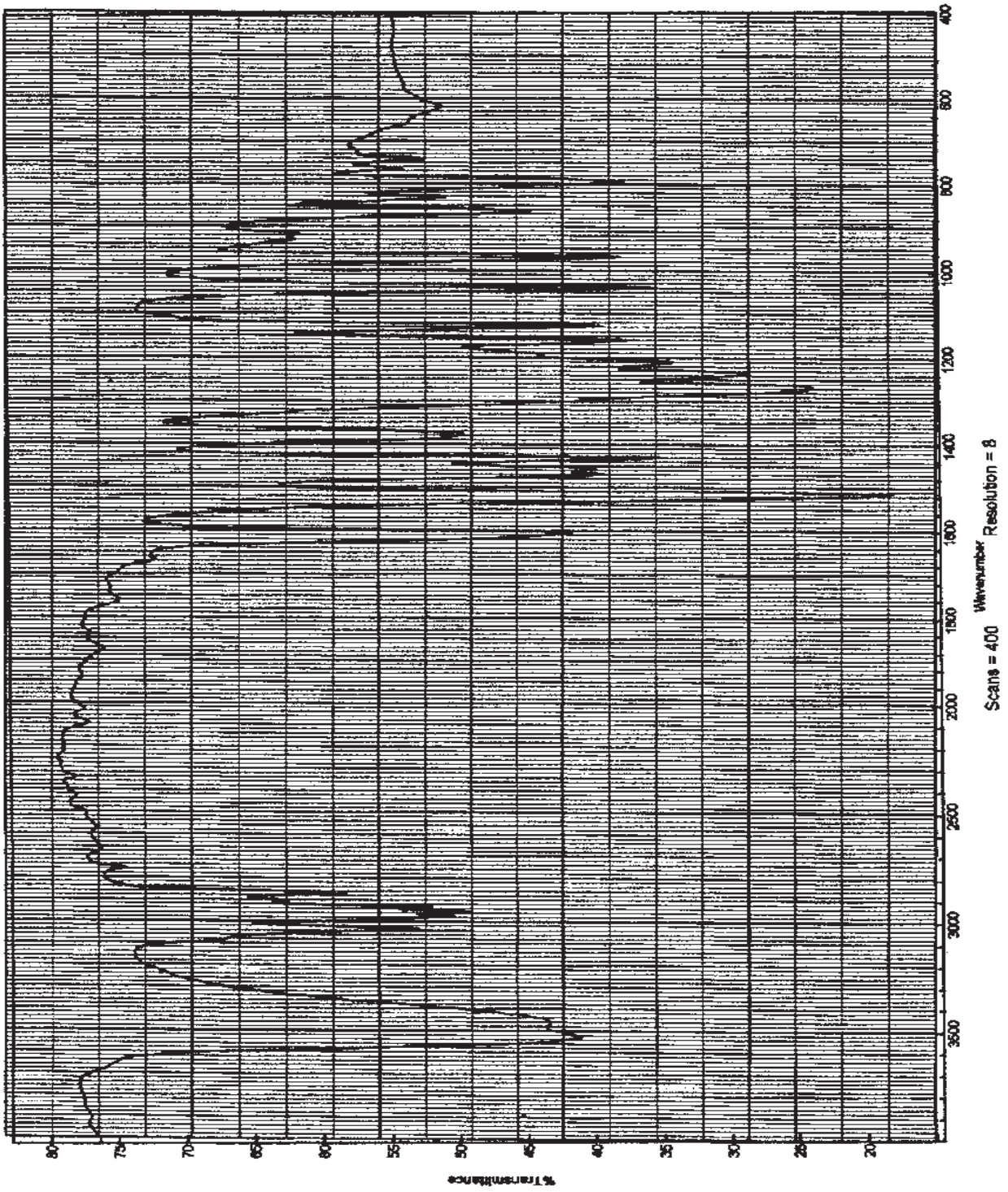

Figure H1
Infrared Absorption Spectrum of Isoeugenol

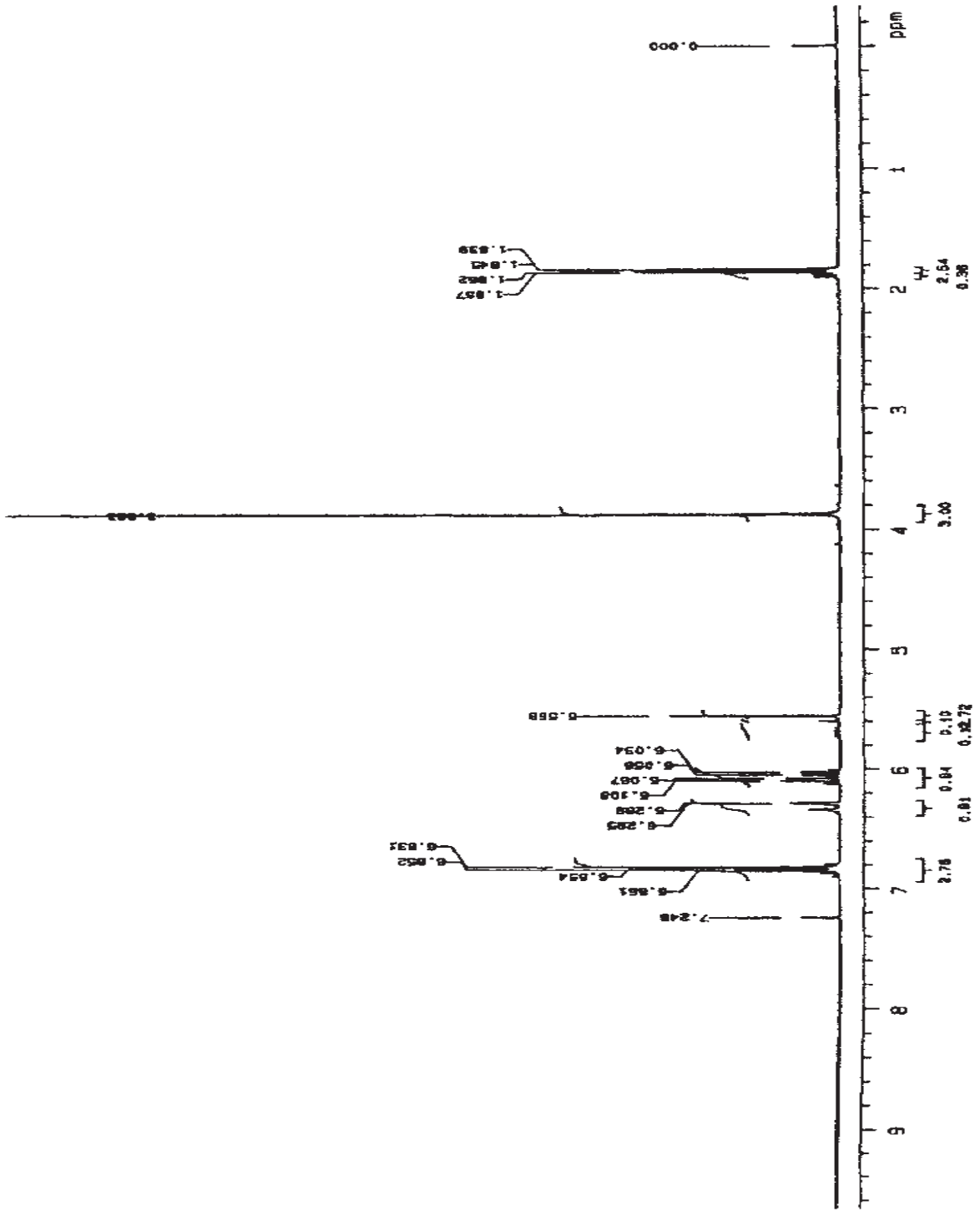

Figure H2
Proton Nuclear Magnetic Resonance Spectrum of Isoeugenol

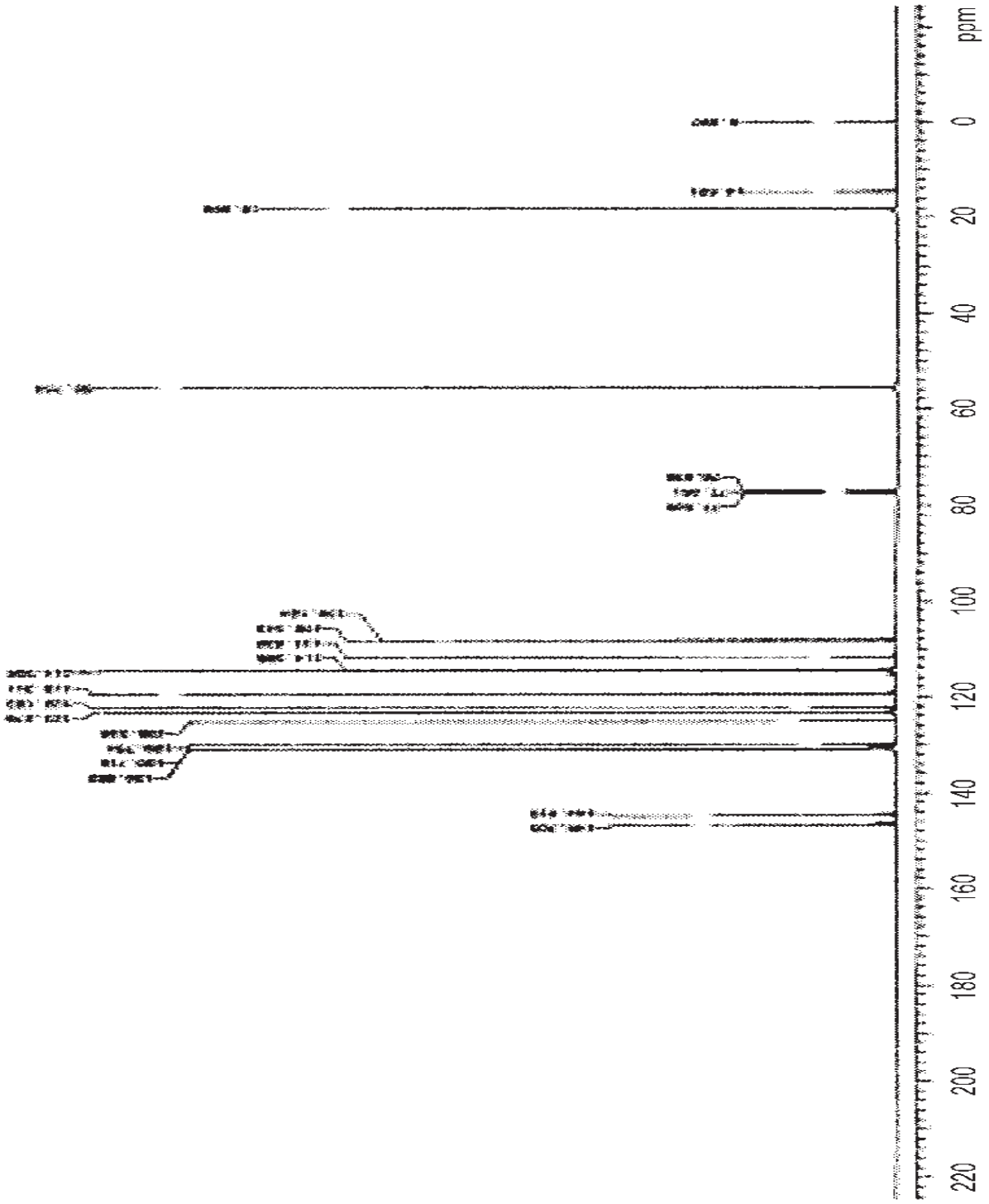

Figure H3
Carbon-13 Nuclear Magnetic Resonance Spectrum of Isoeugenol

Table H1
Preparation and Storage of Dose Formulations in the 3-Month and 2-Year Gavage Studies of Isoeugenol

Preparation
The appropriate amounts of isoeugenol and corn oil were placed in a glass mixing container, capped, and thoroughly mixed with a paint shaker for approximately 5 minutes. Dose formulations were prepared approximately monthly during the 3-month and 2-year studies.

Chemical Lot Number
60449

Maximum Storage Time
35 days

Storage Conditions
Stored in amber glass bottles with Teflon®-lined lids at room temperature

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

TABLE H2
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 3-Month Gavage Studies of Isoeugenol

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Rats				
April 3, 2001	April 5-6, 2001	7.5	7.710	+3
		15	15.07	+1
		30	29.75	–1
		60	61.54	+3
		120	125.7	+5
	May 8-9, 2001[b]	7.5	7.199	–4
		15	14.85	–1
		30	29.13	–3
		60	58.37	–3
		120	115.8	–4
April 30, 2001	May 1-2, 2001	7.5	7.346	–2
		15	14.74	–2
		30	29.91	0
		60	59.68	–1
		120	118.1	–2
	June 5-6, 2001[b]	7.5	7.293	–3
		15	14.83	–1
		30	29.54	–2
		60	59.73	–1
		120	117.7	–2
June 18, 2001	June 21-22, 2001	7.5	7.032	–6
		15	15.39	+3
		30	29.82	–1
		60	61.81	+3
		120	119.1	–1
	July 19-20, 2001[b]	7.5	6.780	–10
		15	14.81	–1
		30	29.14	–3
		60	60.67	+1
		120	118.1	–2

TABLE H2
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 3-Month Gavage Studies of Isoeugenol

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
Mice				
April 3, 2001	April 5-6, 2001	3.75	3.715	–1
		7.5	7.710	+3
		15	15.07	+1
		30	29.75	–1
		60	61.54	+3
	May 8-9, 2001[b]	3.75	3.613	–4
		7.5	7.446	–1
		15	14.83	–1
		30	29.03	–3
		60	58.04	–3
April 30, 2001	May 1-2, 2001	3.75	3.787	+1
		7.5	7.346	–2
		15	14.74	–2
		30	29.91	0
		60	59.68	–1
	June 5-6, 2001[b]	3.75	3.577	–5
		7.5	7.242	–3
		15	14.58	–3
		30	29.19	–3
		60	59.36	–1
June 18, 2001	June 21-22, 2001	3.75	3.768	+1
		7.5	7.032	–6
		15	15.39	+3
		30	29.82	–1
		60	61.81	+3
	July 19-20, 2001[b]	3.75	3.331	–11
		7.5	6.776	–10
		15	14.95	0
		30	28.94	–4
		60	60.55	+1

[a] Results of duplicate analyses. For rats, dosing volume = 5 mL/kg; 7.5 mg/mL = 37.5 mg/kg, 15 mg/mL = 75 mg/kg, 30 mg/mL = 150 mg/kg, 60 mg/mL = 300 mg/kg, 120 mg/mL = 600 mg/kg. For mice, dosing volume = 10 mL/kg; 3.75 mg/mL = 37.5 mg/kg, 7.5 mg/ml = 75 mg/kg, 15 mg/mL = 150 mg/kg, 30 mg/mL = 300 mg/kg, 60 mg/mL = 600 mg/kg.
[b] Animal room samples

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Gavage Studies of Isoeugenol

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Rats				
April 10, 2002	April 12-13, 2002	15	14.81	–1
		30	29.93	0
		60	60.32	+1
	May 15-16, 2002[b]	15	16.45	+10
		30	32.63	+9
		60	65.76	+10
July 2, 2002	July 8-10, 2002	15	15.23	2
		30	29.85	–1
		60	60.65	+1
September 26, 2002	September 27-30, 2002	15	15.75	+5
		30	31.21	+4
		60	64.03	+7
December 19, 2002	December 20-21, 2002	15	14.69	–2
		30	30.52	+2
		60	61.70	+3
	January 23-24, 2003[b]	15	14.65	–2
		30	29.85	–1
		60	60.10	0
March 13, 2003	March 18-19, 2003	15	15.59	+4
		30	31.48	+5
		60	64.33	+7
June 5, 2003	June 9-10, 2003	15	15.63	+4
		30	30.97	+3
		60	61.66	+3
August 28, 2003	August 29-30, 2003	15	15.86	+6
		30	31.58	+5
		60	63.84	+6
	October 9-10, 2003[b]	15	14.50	–3
		30	29.10	–3
		60	59.65	–1
November 20, 2003	November 26-27, 2003	15	15.13	+1
		30	30.35	+1
		60	61.48	+3
February 12, 2004	February 13-14, 2004	15	14.08	–6
		30	28.81	–4
		60	59.20	–1

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Gavage Studies of Isoeugenol

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
Mice				
April 10, 2002	April 12-13, 2002	7.5	7.157	–5
		15	14.81	–1
		30	29.93	0
	May 15-16, 2002[b]	7.5	7.728	+3
		15	15.26	+2
		30	32.26	+8
July 2, 2002	July 8-10, 2002	7.5	7.505	0
		15	15.23	+2
		30	29.85	–1
September 26, 2002	September 27-30, 2002	7.5	7.507	0
		15	15.75	+5
		30	31.21	+4
December 19, 2002	December 20-21, 2002	7.5	6.517[c]	–13
		15	14.69	–2
		30	30.52	+2
	January 23-24, 2003[b]	15	14.53	–3
		30	29.55	–2
January 7, 2003	January 7, 2003	7.5	7.239[d]	–4
	January 23-24, 2003[b]	7.5	7.134	–5
March 13, 2003	March 18-19, 2003	7.5	7.352	–2
		15	15.59	+4
		30	31.48	+5
June 5, 2003	June 9-10, 2003	7.5	7.901	+5
		15	15.63	+4
		30	30.97	+3
August 28, 2003	August 29-30, 2003	7.5	7.806	+4
		15	15.86	+6
		30	31.58	+5
	October 9-10, 2003[b]	7.5	7.203	–4
		15	14.60	–3
		30	29.58	–1

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Gavage Studies of Isoeugenol

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
Mice *(continued)*				
November 20, 2003	November 26-27, 2003	7.5	7.716	+3
		15	15.13	+1
		30	30.35	+1
February 12, 2004	February 13-14, 2004	7.5	6.778	–10
		15	14.08	–6
		30	28.81	–4

[a] Results of duplicate analyses. For rats, dosing volume = 5 mL/kg; 15 mg/mL = 75 mg/kg, 30 mg/mL = 150 mg/kg, 60 mg/mL = 300 mg/kg. For mice, dosing volume = 10 mL/kg; 7.5 mg/mL = 75 mg/kg, 15 mg/mL = 150 mg/kg, 30 mg/mL = 300 mg/kg.
[b] Animal room samples
[c] Remixed; used in study for 4 days (January 2, 3, 6, and 7, 2003)
[d] Results of remix

APPENDIX I
INGREDIENTS, NUTRIENT COMPOSITION, AND CONTAMINANT LEVELS IN NTP-2000 RAT AND MOUSE RATION

TABLE I1
Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground hard winter wheat	22.26
Ground #2 yellow shelled corn	22.18
Wheat middlings	15.0
Oat hulls	8.5
Alfalfa meal (dehydrated, 17% protein)	7.5
Purified cellulose	5.5
Soybean meal (49% protein)	5.0
Fish meal (60% protein)	4.0
Corn oil (without preservatives)	3.0
Soy oil (without preservatives)	3.0
Dried brewer's yeast	1.0
Calcium carbonate (USP)	0.9
Vitamin premix[a]	0.5
Mineral premix[b]	0.5
Calcium phosphate, dibasic (USP)	0.4
Sodium chloride	0.3
Choline chloride (70% choline)	0.26
Methionine	0.2

[a] Wheat middlings as carrier
[b] Calcium carbonate as carrier

TABLE I2
Vitamins and Minerals in NTP-2000 Rat and Mouse Ration[a]

	Amount	Source
Vitamins		
A	4,000 IU	Stabilized vitamin A palmitate or acetate
D	1,000 IU	D-activated animal sterol
K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl acetate	100 IU	
Niacin	23 mg	
Folic acid	1.1 mg	
d-Pantothenic acid	10 mg	*d*-Calcium pantothenate
Riboflavin	3.3 mg	
Thiamine	4 mg	Thiamine mononitrate
B_{12}	52 µg	
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	*d*-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a] Per kg of finished product

TABLE I3
Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	14.8 ± 0.45	13.9 – 15.7	25
Crude Fat (% by weight)	8.0 ± 0.27	7.4 – 8.6	25
Crude Fiber (% by weight)	9.0 ± 0.39	8.4 – 9.9	25
Ash (% by weight)	5.0 ± 0.25	4.4 – 5.6	25
Amino Acids (% of total diet)			
Arginine	0.750 ± 0.048	0.670 – 0.850	15
Cystine	0.225 ± 0.025	0.150 – 0.250	15
Glycine	0.701 ± 0.039	0.620 – 0.750	15
Histidine	0.365 ± 0.090	0.310 – 0.680	15
Isoleucine	0.533 ± 0.038	0.430 – 0.590	15
Leucine	1.077 ± 0.059	0.960 – 1.150	15
Lysine	0.703 ± 0.125	0.310 – 0.830	15
Methionine	0.402 ± 0.049	0.260 – 0.460	15
Phenylalanine	0.615 ± 0.035	0.540 – 0.660	15
Threonine	0.492 ± 0.040	0.430 – 0.590	15
Tryptophan	0.135 ± 0.018	0.110 – 0.160	15
Tyrosine	0.378 ± 0.048	0.280 – 0.460	15
Valine	0.658 ± 0.043	0.550 – 0.710	15
Essential Fatty Acids (% of total diet)			
Linoleic	3.90 ± 0.256	3.49 – 4.54	15
Linolenic	0.30 ± 0.035	0.21 – 0.35	15
Vitamins			
Vitamin A (IU/kg)	4,951 ± 114	3,400 – 8,900	25
Vitamin D (IU/kg)	1,000[a]		
α-Tocopherol (ppm)	84.2 ± 16.60	52.0 – 110.0	15
Thiamine (ppm)[b]	8.6 ± 3.66	5.9 – 25.2	25
Riboflavin (ppm)	6.8 ± 2.11	4.20 – 11.20	15
Niacin (ppm)	79.0 ± 10.50	66.4 – 98.2	15
Pantothenic acid (ppm)	23.9 ± 3.73	17.4 – 29.8	15
Pyridoxine (ppm)[b]	9.21 ± 2.20	6.4 – 13.7	15
Folic acid (ppm)	1.75 ± 0.54	1.20 – 3.27	15
Biotin (ppm)	0.332 ± 0.12	0.225 – 0.704	15
Vitamin B_{12} (ppb)	60.5 ± 46.5	18.3 – 174.0	15
Choline (ppm)[b]	3,064 ± 270	2,700 – 3,790	15
Minerals			
Calcium (%)	0.977 ± 0.048	0.873 – 1.150	25
Phosphorus (%)	0.593 ± 0.026	0.549 – 0.641	25
Potassium (%)	0.665 ± 0.023	0.626 – 0.694	15
Chloride (%)	0.376 ± 0.041	0.300 – 0.474	15
Sodium (%)	0.191 ± 0.017	0.160 – 0.222	15
Magnesium (%)	0.201 ± 0.009	0.185 – 0.217	15
Sulfur (%)	0.170 ± 0.029	0.116 – 0.209	15
Iron (ppm)	182 ± 46.7	135 – 311	15
Manganese (ppm)	54.1 ± 7.89	42.1 – 73.1	15
Zinc (ppm)	55.0 ± 9.55	43.3 – 78.5	15
Copper (ppm)	6.65 ± 1.790	3.21 – 10.50	15
Iodine (ppm)	0.512 ± 0.221	0.233 – 0.972	15
Chromium (ppm)	0.604 ± 0.253	0.330 – 1.380	14
Cobalt (ppm)	0.25 ± 0.074	0.20 – 0.47	14

[a] From formulation
[b] As hydrochloride (thiamine and pyridoxine) or chloride (choline)

TABLE I4
Contaminant Levels in NTP-2000 Rat and Mouse Ration[a]

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.39 ± 0.154	0.14 – 0.50	25
Cadmium (ppm)	0.06 ± 0.023	0.04 – 0.10	25
Lead (ppm)	0.08 ± 0.030	0.05 – 0.17	25
Mercury (ppm)	<0.02		25
Selenium (ppm)	0.19 ± 0.029	0.14 – 0.23	25
Aflatoxins (ppb)	<5.00		25
Nitrate nitrogen (ppm)[c]	14.6 ± 3.54	10.00 – 23.2	25
Nitrite nitrogen (ppm)[c]	<0.61		25
BHA (ppm)[d]	<1.0		25
BHT (ppm)[d]	<1.0		25
Aerobic plate count (CFU/g)	26 ± 70	10 – 360	25
Coliform (MPN/g)	3.0 ± 0.0	3.0 – 3.0	25
Escherichia coli (MPN/g)	<10		25
Salmonella (MPN/g)	Negative		25
Total nitrosoamines (ppb)[e]	3.9 ± 1.65	2.3 – 8.4	25
N-Nitrosodimethylamine (ppb)[e]	2.5 ± 1.50	1.1 – 6.9	25
N-Nitrosopyrrolidine (ppb)[e]	1.5 ± 0.54	0.9 – 3.1	25
Pesticides (ppm)			
α-BHC	<0.01		25
β-BHC	<0.02		25
γ-BHC	<0.01		25
δ-BHC	<0.01		25
Heptachlor	<0.01		25
Aldrin	<0.01		25
Heptachlor epoxide	<0.01		25
DDE	<0.01		25
DDD	<0.01		25
DDT	<0.01		25
HCB	<0.01		25
Mirex	<0.01		25
Methoxychlor	<0.05		25
Dieldrin	<0.01		25
Endrin	<0.01		25
Telodrin	<0.01		25
Chlordane	<0.05		25
Toxaphene	<0.10		25
Estimated PCBs	<0.20		25
Ronnel	<0.01		25
Ethion	<0.02		25
Trithion	<0.05		25
Diazinon	<0.10		25
Methyl chlorpyrifos	0.093 ± 0.067	0.020 – 0.259	25
Methyl parathion	<0.02		25
Ethyl parathion	<0.02		25
Malathion	0.306 ± 0.475	0.020 – 1.850	25
Endosulfan 1	<0.01		25
Endosulfan 2	<0.01		25
Endosulfane Sulfate	<0.03		25

[a] All samples were irradiated. CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride
[b] For values less than the limit of detection, the detection limit is given as the mean.
[c] Sources of contamination: alfalfa, grains, and fish meal
[d] Sources of contamination: soy oil and fish meal
[e] All values were corrected for percent recovery.

APPENDIX J
SENTINEL ANIMAL PROGRAM

SENTINEL ANIMAL PROGRAM

METHODS

Rodents used in the Carcinogenesis Program of the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of chemical compounds. Under this program, the disease state of the rodents is monitored via serology on sera from extra (sentinel) animals in the study rooms. These animals and the study animals are subject to identical environmental conditions. The sentinel animals come from the same production source and weanling groups as the animals used for the studies of chemical compounds.

Serum samples were collected from randomly selected animals, five male (three male rats were sampled at 18 months in the 2-year studies) and five female sentinel rats and mice for each study. Serum samples were collected at 1 month and at the end of the 3-month studies and at 1, 6, 12, 18, and 24 months (300 mg/kg male and female rats and mice) for the 2-year studies. Blood from each animal was collected and allowed to clot, and the serum was separated; fecal samples were collected from five male and five female mice at 18 months in the 2-year studies. Samples were processed appropriately and sent to BioReliance (Rockville, MD) for determination of antibody titers. The laboratory serology methods and viral agents for which testing was performed are tabulated below; the times at which blood was collected during the studies are also listed.

Method and Test	Time of Collection
Rats	
3-Month Study	
ELISA	
Mycoplasma arthritidis	3 months
Mycoplasma pulmonis	3 months
PVM (pneumonia virus of mice)	1 and 3 months
RCV/SDA (rat coronavirus/sialodacryoadenitis virus)	1 and 3 months
Sendai	1 and 3 months
Immunofluorescence Assay	
M. arthritidis	3 months
Parvovirus	1 and 3 months
2-Year Study	
ELISA	
M. arthritidis	24 months
M. pulmonis	24 months
PVM	1, 6, 12, 18, and 24 months
RCV/SDA	1, 6, 12, 18, and 24 months
Sendai	1, 6, 12, 18, and 24 months
Immunofluorescence Assay	
Parvovirus	1, 6, 12, 18, and 24 months

Method and Test	Time of Collection
Mice	
3-Month Study	
ELISA	
Ectromelia virus	1 and 3 months
EDIM (epizootic diarrhea of infant mice)	1 and 3 months
GDVII (mouse encephalomyelitis virus)	1 and 3 months
LCM (lymphocytic choriomeningitis virus)	1 and 3 months
Mouse adenoma virus-FL	1 and 3 months
MHV (mouse hepatitis virus)	1 and 3 months
M. arthritidis	3 months
M. pulmonis	3 months
PVM	1 and 3 months
Reovirus 3	1 and 3 months
Sendai	1 and 3 months
Immunofluorescence Assay	
LCM	1 month
MCMV (mouse cytomegalovirus)	3 months
Parvovirus	1 and 3 months
2-Year Study	
ELISA	
Ectromelia virus	1, 6, 12, 18, and 24 months
EDIM	1, 6, 12, 18, and 24 months
GDVII	1, 6, 12, 18, and 24 months
LCM	1, 6, 12, 18, and 24 months
Mouse adenoma virus-FL	1, 6, 12, 18, and 24 months
MHV	1, 6, 12, 18, and 24 months
M. arthritidis	24 months
M. pulmonis	24 months
PVM	1, 6, 12, 18, and 24 months
Reovirus 3	1, 6, 12, 18, and 24 months
Sendai	1, 6, 12, 18, and 24 months
Immunofluorescence Assay	
EDIM	18 months
GDVII	24 months
Mouse adenoma virus-FL	18 months
MCMV	24 months
MHV	18 months
PVM	18 months
Parvovirus	1, 6, 12, 18, and 24 months
Polymerase Chain Reaction	
Helicobacter species	18 months

RESULTS

All results were negative.

APPENDIX K
SPECIAL STUDY

Table K1
Serum Gastrin Concentrations and Stomach pH in Rats Administered Isoeugenol by Gavage for 31 Days[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
n	10	10	10	10	10	10
Male						
Serum gastrin (pg/mL)	213.4 ± 20.6	301.3 ± 73.4	215.5 ± 29.5	244.0 ± 24.2	286.2 ± 21.4	210.4 ± 18.1
Stomach pH	3.16 ± 0.26	2.79 ± 0.09	2.96 ± 0.15	2.80 ± 0.12	2.58 ± 0.10	2.96 ± 0.24
Female						
Serum gastrin (pg/mL)	213.4 ± 25.7	220.3 ± 26.8	205.6 ± 18.1	242.7 ± 48.2	289.2 ± 48.4	219.8 ± 29.3
Stomach pH	4.90 ± 0.30	4.38 ± 0.30	4.28 ± 0.35	3.72 ± 0.31**	3.70 ± 0.15**	3.53 ± 0.09**

** Significantly different ($P \leq 0.01$) from the vehicle control group by Shirley's test
[a] Data are presented as mean ± standard error.

Table K2
Liver Results for Rats Administered Isoeugenol by Gavage for 31 Days[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
n	10	10	10	10	10	10
Male						
Acetanilide-4-hydroxylase (A4H) (nmole/minute per mg microsomal protein)						
	0.550 ± 0.024	0.490 ± 0.028	0.519 ± 0.031	0.524 ± 0.028	0.591 ± 0.028	0.438 ± 0.009**
7-Ethoxyresorufin-*O*-deethylase (EROD) (pmole/minute per mg microsomal protein)						
	42.03 ± 2.150	36.44 ± 2.090	33.16 ± 1.600**	34.20 ± 1.060**	36.30 ± 1.570*	29.27 ± 1.130**
7-Pentoxyresorufin-*O*-deethylase (PROD) (pmole/minute per mg microsomal protein)						
	10.449 ± 0.434	9.681 ± 0.480	8.634 ± 0.332**	8.013 ± 0.262**	7.919 ± 0.358**	7.565 ± 0.279**
Female						
Acetanilide-4-hydroxylase (A4H) (nmole/minute per mg microsomal protein)						
	0.507 ± 0.017	0.519 ± 0.021	0.458 ± 0.017	0.479 ± 0.030	0.510 ± 0.024	0.551 ± 0.034
7-Ethoxyresorufin-*O*-deethylase (EROD) (pmole/minute per mg microsomal protein)						
	34.70 ± 1.930	36.71 ± 1.990	37.16 ± 2.030	32.92 ± 1.310	38.72 ± 1.840	40.65 ± 2.300
7-Pentoxyresorufin-*O*-deethylase (PROD) (pmole/minute per mg microsomal protein)						
	3.734 ± 0.213	4.494 ± 0.237	4.767 ± 0.296	3.786 ± 0.157	3.959 ± 0.182	4.393 ± 0.246

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test
** $P \leq 0.01$
[a] Data are presented as mean ± standard error.

Table K3
Incidences of Glandular Stomach Lesions in Rats Administered Isoeugenol by Gavage for 31 Days

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
Cyst, epithelium	0	0	0	0	0	0
Necrosis, epithelium	0	0	0	0	0	0
Inflammation, chronic	0	0	0	0	0	0
Female						
Cyst, epithelium	0	0	1 (1.0)[a]	0	0	0
Necrosis, epithelium	0	0	1 (1.0)	0	0	0
Inflammation, chronic	0	0	0	0	0	1 (1.0)

[a] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

APPENDIX L
SINGLE-DOSE TOXICOKINETIC STUDIES IN F344/N RATS AND B6C3F1 MICE

SINGLE-DOSE TOXICOKINETIC STUDIES IN F344/N RATS AND B6C3F1 MICE

Introduction

Single-dose toxicokinetic studies of isoeugenol were designed to estimate toxicokinetic parameters for the determination of concentration of isoeugenol in F344/N rat and B6C3F1 mouse plasma, establish basic toxicokinetic parameters, and determine the extent of bioavailability to improve the usefulness of toxicity study results in risk assessment. Male and female rats received a single intravenous injection of 17 mg isoeugenol/kg body weight or a single gavage dose of 17, 70, or 140 mg/kg. Male and female mice received a single intravenous injection of 35 mg/kg or a single gavage dose of 35, 70, or 140 mg/kg. Postdose plasma samples were analyzed for isoeugenol concentrations, and the results were used to calculate toxicokinetic parameters.

Materials and Methods

Isoeugenol (Lot #55130) was obtained from Penta Manufacturing Company (Livingston, NJ) and stored at room temperature. The material was analyzed for identity using infrared and nuclear magnetic resonance spectroscopy; analyses confirmed the material as isoeugenol. Karl-Fischer titrimetry indicated that the test article contained 0.46% water. The material was analyzed for purity using gas chromatography, high performance liquid chromatography, and elemental analysis; the test article was determined to be approximately 99% pure. The analytical systems used were similar to those described in Appendix H.

On the day of dosing, groups of 21 male and female F344/N nonfasted rats were approximately 13 weeks old and ranged in weight from 204 to 276 g and from 149 to 169 g, respectively; groups of 42 male and female B6C3F1 nonfasted mice were approximately 13 weeks old and ranged in weight from 25.9 to 32.4 g and from 20.2 to 27.1 g, respectively. Intravenous doses were administered through jugular vein catheters implanted by the animal supplier (Charles River Laboratories, Raleigh, NC) and were formulated in Cremophor® EL:ethanol:water (1:1:8; v/v/v). Gavage doses were formulated in corn oil. All dose formulations were prepared gravimetrically and analyzed pre- and postadministration to assure that they were within 10% of target concentrations. Additional details of the study design and animal maintenance are summarized in Table L1.

After dosing, animals were anesthetized with approximately 70% CO_2 (30% O_2), and blood was collected from the retroorbital sinus of rats and by cardiac puncture from mice at the times listed in Table L1. Three rats and three mice of each dose group were bled at each time point, with the exception of the last time point for male mice, where only two animals were available. Up to 2 mL of blood were collected. Rats were bled a second time with a minimum of 1 hour of recovery time. Blood samples were collected into glass tubes containing EDTA anticoagulant and mixed gently, and plasma was separated within 60 minutes of collection by centrifugation. The plasma was stored at –70° C until analyzed. Following final blood collection, animals were sacrificed under 100% CO_2.

For analysis, plasma samples were thawed to room temperature and 100 µL of sample were combined with 1.0 mL of ethyl acetate solution containing the internal standard methyleugenol at 0.50 µg/mL. Following vortexing for 15 seconds and a 2-minute settling time, samples were vortexed again for 15 seconds and then centrifuged for approximately 1 minute to facilitate separation of the organic and aqueous phases. The organic phase was transferred to another vial, and 1 µL of the organic layer was analyzed by gas chromatography with mass spectrometric detection. Chromatography was performed on a DB-WAXetr column (J&W Scientific, Folsom, CA) with an oven program of 45° C for 1.0 minute, then 30° C/minute to 200° C, then 10° C/minute to 250° C, and then held for 1 minute. The mass spectrometer was operated in selected ion monitoring mode for *m/z* 164 (100 ms dwell) for isoeugenol and *m/z* 178 (100 ms dwell) for methyleugenol. Sample concentrations of isoeugenol were

calculated using linear regression analysis of data weighted $1/x^2$ relating the peak area response ratio of instrument response to calibration standards prepared in blank F344/N rat plasma.

The analytical method for determining isoeugenol in plasma samples was validated with a range of 0.015 to 32 µg isoeugenol/mL plasma. The limit of detection for the method was 0.0009 µg/mL plasma; the limit of quantitation was 0.0031 µg/mL plasma, and the experimental limit of quantitation (ELOQ) was 0.015 µg/mL plasma. Precision, based on the standard deviation of spiked plasma samples, was less than or equal to 5.1%, and accuracy was within 10%, also based on analysis of spiked plasma samples.

TOXICOKINETICS

Toxicokinetic parameter estimates following intravenous administration were derived only from those plasma isoeugenol measurements that were above the ELOQ. Toxicokinetic parameters were determined by fitting the following equation to the data collected in the intravenous phase of the study, using a nonlinear least-squares fitting program (SAS PROC NLIN; SAS Institute, Inc., Cary, NC):

$$C(t) = A_0 e^{-\alpha t} + B_0 e^{-\beta t}$$

where $C(t)$ is the plasma concentration of isoeugenol at any postadministration time (t), α and β are the rate constants (per minute) obtained from the fit, and A_0 and B_0 are the intercepts on the ordinate (concentration) axis of the extrapolated initial and terminal phases, respectively. Estimates for these values, with the asymptotic standard errors and approximate 95% confidence intervals, were obtained directly from the model. The elimination half-lives for the initial and terminal phases of the concentration versus time profiles were calculated as ln2/α and ln2/β, respectively. The maximum plasma isoeugenol concentration (C_0) was assumed to occur at $t = 0$ and was calculated as $A_0 + B_0$.

The area under the curve (AUC_T) was estimated to the last sampling time point (T) using the trapezoidal rule:

$$AUC_T = \sum \frac{C_{n-1} + C_n}{2} \times (t_n - t_{n-1})$$

where C_{n-1} and C_n are the plasma isoeugenol concentrations measured at two consecutive time points, t_{n-1} and t_n, respectively.

The area under the curve extrapolated to infinity (AUC_∞) was estimated using C_0 and the following equation:

$$AUC_\infty = AUC_T + \frac{C_T}{\beta}$$

where C_T is the plasma isoeugenol concentration measured at T and β is the rate constant for the terminal elimination phase.

For the gavage data, the time (t_{max}) at which the maximum plasma isoeugenol concentration (C_{max}) occurred was determined empirically from plots of the data. AUC_T was calculated from the observed plasma isoeugenol concentrations using the trapezoidal rule described above. AUC_∞ was calculated using extrapolations to time $t = 0$ minutes assuming an initial concentration of 0 µg isoeugenol/mL plasma and infinite time ($t = \infty$) using β derived from intravenous dosing.

Total clearance (Cl_{tot}) determined from intravenous dosing was calculated using the following equation:

$$Cl_{tot} = Dose / AUC_\infty$$

The apparent clearance (Cl_{app}) determined from gavage dosing was calculated using the following equation:

$$Cl_{app} = Dose / AUC_{\infty}$$

The apparent volume of distribution (V_{app}) following intravenous dosing was calculated using the following equation:

$$V_{app} = Cl_{tot} / \beta$$

Absolute bioavailability was expressed as the fraction (F) of the oral dose that reached the systemic circulation and was calculated using the following equation:

$$F = \frac{Dose_{intravenous} \times AUC_{\infty(gavage)}}{Dose_{gavage} \times AUC_{\infty(intravenous)}}$$

RESULTS

Isoeugenol Toxicokinetics in F344/N Rats

Plasma isoeugenol concentration-versus-time profiles following intravenous administration to male and female rats were fit well by a biexponential model, exhibiting a rapid initial and slower terminal elimination phase that included a minor secondary peak (Figure L1). Toxicokinetic parameters estimated from these data are presented in Table L2.

C_{max} and C_0 (simultaneous for an intravenous study) occurred in the earliest samples taken (2 minutes) and were not significantly different between the sexes. No observable sex differences were observed for α, β, $t_{1/2\alpha}$, $t_{1/2\beta}$, AUC_{∞}, Cl_{tot}, or V_{app}. There was evidence for extensive distribution to extravascular tissues and/or high tissue uptake/binding following isoeugenol administration because the V_{app} values greatly exceeded the total body water volume of 0.668 L/kg (Davies and Morris, 1993).

Following gavage administration, the plasma isoeugenol concentration-versus-time profiles were characterized by a rapid absorption phase that occurred within 20 minutes postdosing (Figure L2). Since there were only one to three time points during this absorption phase, further characterization of absorption could not be made from these profiles. The terminal elimination phase generally exhibited at least one secondary peak, and these secondary peaks were observed up to approximately 360 minutes with a tendency toward later times with increasing dose. As a result of the secondary peaks, the typical exponential model was not adequate to define the gavage data and model-independent toxicokinetic parameters were estimated (Table L3).

Absorption of isoeugenol was rapid in both sexes of rats following gavage administration as revealed by t_{max} values within 20 minutes (Table L3). Based on C_{max} values, there was an apparent trend towards increasing plasma isoeugenol concentrations with increasing dose. However, due to rapid changes in isoeugenol concentrations during the early absorption/desorption phase, AUC_{∞} values provide a more appropriate measure of the amount of isoeugenol absorbed. AUC_{∞} values increased supraproportionately with dose for both sexes (Figure L2 and Table L3). Cl_{app} values decreased with dose and were greater (approximately two times) in the 17 mg/kg groups than in the 140 mg/kg groups, independent of sex. Within each dose group, Cl_{app} was significantly greater (1.5 to 1.9 times) in males than females. Absolute bioavailability (± standard error), based on intravenous and gavage doses of 17 mg isoeugenol/kg, was significantly greater in females than males, with values of 17% ± 2% and 11% ± 2%, respectively. Because calculations were based on gavage data using $AUC_{ti\text{-}tf}$ instead of AUC_{∞}, these volumes may slightly underestimate absolute bioavailability.

Isoeugenol Toxicokinetics in B6C3F1 Mice

Plasma isoeugenol concentration-versus-time profiles following intravenous administration to male and female mice were fit well by a biexponential model, exhibiting a rapid initial and slower terminal elimination phase that included a minor secondary peak (Figure L3). Toxicokinetic parameters estimated from these data are presented in Table L4.

C_{max} occurred in the earliest samples taken (2 minutes), and neither C_{max} nor C_0 values were significantly different between the sexes. No significant sex differences were discovered for α, β, $t_{1/2\alpha}$, or $t_{1/2\beta}$. AUC_∞ values were significantly greater (1.4 times) in females than males, leading to significantly greater Cl_{tot} in males. V_{app} values were not significantly different between the sexes. There was evidence for extensive distribution to extravascular tissues and/or high tissue uptake/binding following isoeugenol administration because the V_{app} values greatly exceeded the total body water volume of 0.725 L/kg (Davies and Morris, 1993).

The plasma isoeugenol concentration-versus-time profiles following gavage administration of isoeugenol to mice were characterized by a rapid absorption phase that occurred within 20 minutes postdosing (Figure L4). Similar to the rat data, further characterization of absorption could not be made from these profiles. The terminal elimination phase generally exhibited at least one secondary peak in the plasma concentration-versus-time profiles. Following the initial absorption phase, secondary peaks were observed up to approximately 240 minutes with a tendency toward later times with increasing dose. As with the rat data, only a limited set of model-independent toxicokinetic parameters were estimated (Table L5).

Absorption of isoeugenol was rapid in both sexes of mice following gavage administration as revealed by t_{max} values within 20 minutes (Table L5). Based on C_{max} values, there was an apparent trend towards increasing plasma isoeugenol concentrations with increasing dose. However, AUC_∞ values provide a more appropriate measure of the amount of isoeugenol absorbed. A notable sex difference was observed regarding the relationship between dose-normalized AUC_∞ values and isoeugenol dose. For male mice, dose-normalized AUC_∞ values decreased slightly with increasing dose, whereas for female mice, there was no significant change in the dose-normalized AUC_∞ values, indicating a proportionate increase in AUC_∞ values with dose. Cl_{app} values increased only marginally with dose for males but did not change significantly with dose for females. Within each dose group, Cl_{app} was significantly greater in males than females. Absolute bioavailability (± standard error), based on intravenous and gavage doses of 35 mg isoeugenol/kg, was not significantly different between the sexes with values of 34% ± 4% and 36% ± 3% for males and females, respectively. Because calculations were based on gavage data using $AUC_{ti\text{-}tf}$ instead of AUC_∞, these volumes may slightly underestimate absolute bioavailability.

Reference

Davies, B., and Morris, T. (1993). Physiological parameters in laboratory animals and humans. *Pharm. Res*. **10**, 1093-1095.

Table L1
Materials and Methods in the Single-Dose Toxicokinetic Studies of Isoeugenol

	Intravenous Studies	Gavage Studies
Study Laboratory	Battelle Toxicology Northwest (Richland, WA)	Battelle Toxicology Northwest (Richland, WA)
Strain and Species	F344/N rats B6C3F1 mice	F344/N rats B6C3F1 mice
Animal Source	Charles River Laboratories (Raleigh, NC)	Charles River Laboratories (Raleigh, NC)
Time Held Before Studies	1 (males) or 4 (females) days	Rats: 13 to 19 days Mice: 13 to 20 days
Average Age When Studies Began	13 to 14 weeks	13 to 14 weeks
Date of First Dose	Rats: January 7 (males) or 10 (females), 2000 Mice: January 14 (males) or 17 (females), 2000	Rats: December 1, 2, or 7, 1999 Mice: December 13, 15, or 17 (males) or 14, 16, or 20 (females), 1999
Doses	Rats: 17 mg/kg Mice: 35 mg/kg	Rats: 17, 70, or 140 mg/kg Mice: 35, 70, or 140 mg/kg
Vehicle	Cremophor® EL:ethanol:water (1:1:8; v/v/v)	Corn oil
Dosing Volume	Rats: 2 mL/kg Mice: 4 mL/kg Doses were delivered as a bolus injection within approximately 60 seconds, followed by approximately 0.5 mL (rats) or 0.2 mL (mice) of heparinized saline solution (10 units/mL).	Rats: 5 mL/kg Mice: 10 mL/kg
Type of Observation	Animals were weighed the morning of dosing for calculation of the dosing volume.	Animals were weighed the day prior to dosing for calculation of the dosing volume.
Size of Study Groups	Rats: 21 males and 21 females Mice: 42 males and 42 females	Rats: 21 males and 21 females Mice: 42 males and 42 females
Method of Distribution	Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as intravenous studies
Diet	NTP-2000 irradiated pelleted diet (Zeigler Brothers, Inc., Gardners, PA); available *ad libitum*	Same as intravenous studies
Water	Tap water (Richland, WA, municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI); available *ad libitum*	Same as intravenous studies
Cages	Polycarbonate solid-bottom with slotted feeders, (Hazleton Systems, Inc., Aberdeen, MD)	Wire-mesh units with slotted feeders, (Hazleton Systems, Inc., Aberdeen, MD)
Animal Room Environment	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: 14/hour	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: 14/hour

Table L1
Materials and Methods in the Single-Dose Toxicokinetic Studies of Isoeugenol

	Intravenous Studies	Gavage Studies
Postdosing Blood Sample Collection Times	2, 5, 10, 20, 30, and 45 minutes and 1, 1.5, 2, 2.5, 3, 4, 5, and 6 hours	17 mg/kg (rats only): 2, 5, 10, 20, 30, and 45 minutes and 1, 1.5, 2, 3, 4, 5, 6, and 8 hours 35 mg/kg (mice only): 2, 5, 10, 20, 30, and 45 minutes and 1, 1.5, 2, 2.5, 3, 4, 5, and 6 hours 70 mg/kg: 2, 5, 10, 20, 30, and 45 minutes and 1, 1.5, 2, 3, 4, 5, 6, and 8 hours 140 mg/kg: 2, 5, 10, 20, and 30 minutes and 1, 1.5, 2, 3, 4, 5, 6, 8, and 10 hours
Analyte	Plasma isoeugenol concentration	Plasma isoeugenol concentration

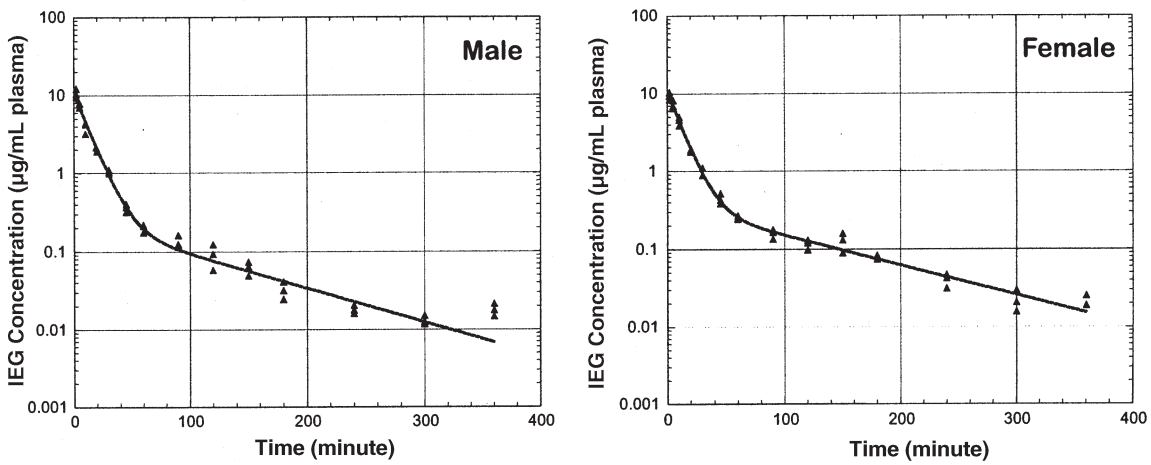

Figure L1
Plasma Concentrations of Isoeugenol (IEG) in F344/N Rats after a Single Intravenous Injection of 17 mg/kg Isoeugenol
n = up to three plasma samples per time point

Table L2
Toxicokinetic Parameter Estimates in F344/N Rats after a Single Intravenous Injection of 17 mg/kg Isoeugenol[a]

Parameter	Male	Female
C_0 (µg/mL)	10.5 ± 1.2	10.6 ± 0.9
α (minute^{-1})	0.0869 ± 0.0064	0.0920 ± 0.0052
$t_{1/2\alpha}$ (minute)	7.98 ± 0.59	7.54 ± 0.42
β (minute^{-1})	0.0100 ± 0.0009	0.00872 ± 0.00044
$t_{1/2\beta}$ (minute)	69.1 ± 6.2	79.5 ± 4.1
AUC_∞ (µg·minute/mL)	155 ± 3	162 ± 3
$AUC_\infty/Dose$ [(µg·minute/mL)/(mg/kg)]	9.09 ± 0.18	9.54 ± 0.17
Cl_{tot} (mL/minute·kg)	110 ± 2	105 ± 2
V_{app} (L/kg)	11.0 ± 1.0	12.0 ± 0.7

[a] Values are reported as the mean ± standard error. C_0 = estimated plasma concentration of isoeugenol at time zero; α and β = elimination rate constants for the initial and terminal phases of the plasma elimination curve, respectively; $t_{1/2\alpha}$ and $t_{1/2\beta}$ = elimination half-lives for the initial and terminal phases of the plasma elimination curve, respectively; AUC_∞ = area under the curve extrapolated to infinity; Cl_{tot} = total clearance; V_{app} = apparent volume of distribution.

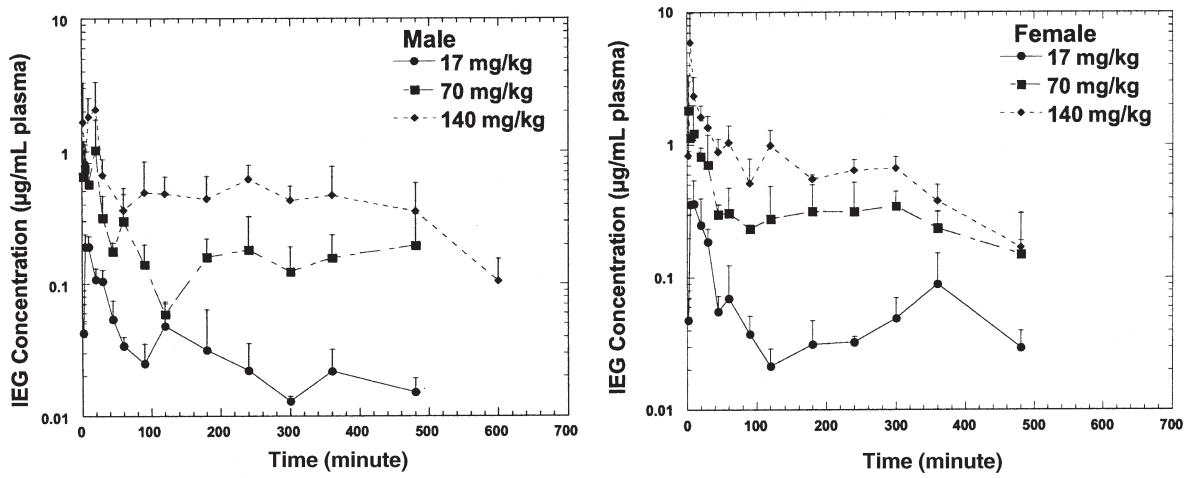

Figure L2
Plasma Concentrations of Isoeugenol (IEG) in F344/N Rats after a Single Gavage Dose of 17, 70, or 140 mg/kg Isoeugenol
n = up to three plasma samples per time point

Table L3
Toxicokinetic Parameter Estimates in F344/N Rats after a Single Gavage Dose of Isoeugenol[a]

	17 mg/kg	70 mg/kg	140 mg/kg
Male			
C_{max} (µg/mL)	0.192 ± 0.022	1.02 ± 0.41	2.06 ± 0.73
t_{max} (minute)	10	20	20
$AUC_{ti\text{-}tf}$(µg·minute/mL)	16.0 ± 1.1	92.7 ± 8.0	280 ± 19
$AUC_{ti\text{-}tf}/Dose$ [(µg·minute/mL)/(mg/kg)]	0.941 ± 0.066	1.32 ± 0.11	2.00 ± 0.14
Cl_{app} (mL/minute·kg)	1062 ± 74	755 ± 65	499 ± 35
Female			
C_{max} (µg/mL)	0.364 ± 0.103	1.82 ± 0.88	5.91 ± 2.28
t_{max} (minute)	10	2	5
$AUC_{ti\text{-}tf}$(µg·minute/mL)	30.1 ±2.8	154 ± 10	413 ± 16
$AUC_{ti\text{-}tf}/Dose$ [(µg·minute/mL)/(mg/kg)]	1.77 ± 0.17	2.20 ± 0.14	2.95 ± 0.12
Cl_{app} (mL/minute·kg)	564 ± 53	455 ± 29	339 ± 13

[a] All values except those for *tmax* are reported as the mean ± standard error. C_{max} = maximum plasma isoeugenol concentration; t_{max} = time at which *Cmax* was observed; $AUC_{ti\text{-}tf}$ = area under the curve from initial to final measured time; Cl_{app} = apparent clearance.

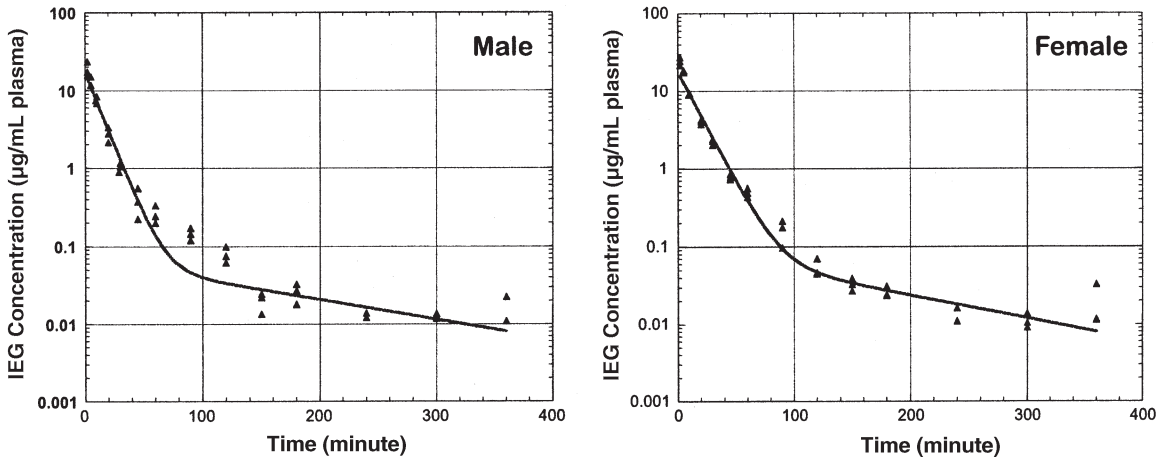

Figure L3
Plasma Concentrations of Isoeugenol (IEG) in B6C3F1 Mice after a Single Intravenous Injection of 35 mg/kg Isoeugenol
n = up to three plasma samples per time point

Table L4
Toxicokinetic Parameter Estimates in B6C3F1 Mice after a Single Intravenous Injection of 35 mg/kg Isoeugenol[a]

Parameter	Male	Female
C_0 (µg/mL)	17.1 ± 3.0	18.0 ± 2.5
α (minute^{-1})	0.0872 ± 0.0068	0.0666 ± 0.0045
$t_{1/2\alpha}$ (minute)	7.95 ± 0.62	10.4 ± 0.7
β (minute^{-1})	0.00587 ± 0.00162	0.00679 ± 0.00131
$t_{1/2\beta}$ (minute)	118 ± 33	102 ± 20
AUC_∞ (µg·minute/mL)	237 ± 7	323 ± 5
$AUC_\infty/Dose$ [(µg·minute/mL)/(mg/kg)]	6.76 ± 0.21	9.23 ± 0.13
Cl_{tot} (mL/minute·kg)	148 ± 5	108 ± 2
V_{app} (L/kg)	25.2 ± 7.0	16.0 ± 3.1

a Values are reported as the mean ± standard error. C_0 = estimated plasma concentration of isoeugenol at time zero; α and β = elimination rate constants for the initial and terminal phases of the plasma elimination curve, respectively; $t_{1/2\alpha}$ and $t_{1/2\beta}$ = elimination half-lives for the initial and terminal phases of the plasma elimination curve, respectively; AUC_∞ = area under the curve extrapolated to infinity; Cl_{tot} = total clearance; V_{app} = apparent volume of distribution.

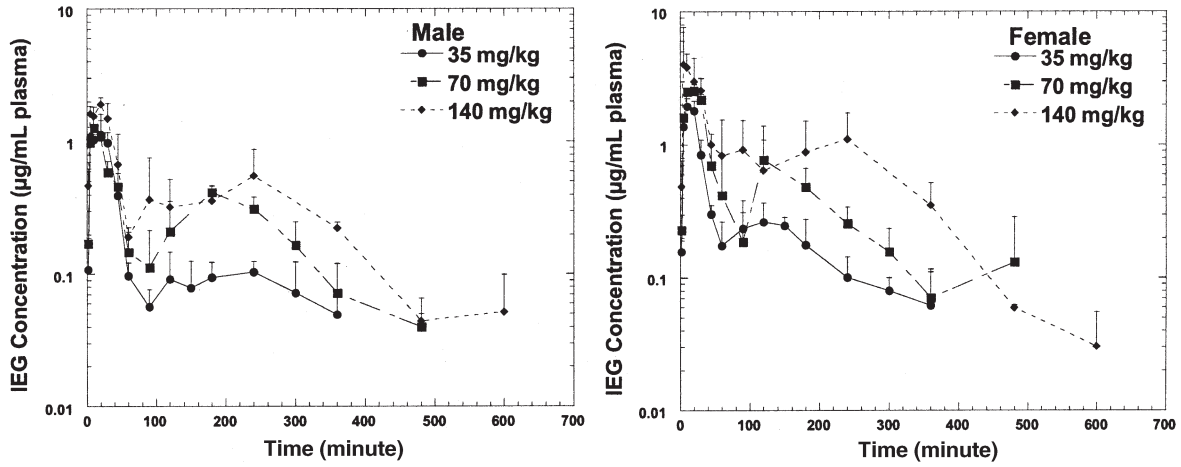

Figure L4
Plasma Concentrations of Isoeugenol (IEG) in B6C3F1 Mice after a Single Gavage Dose of 35, 70, or 140 mg/kg Isoeugenol
n = up to three plasma samples per time point

Table L5
Toxicokinetic Parameter Estimates in B6C3F1 Mice after a Single Gavage Dose of Isoeugenol[a]

	17 mg/kg	70 mg/kg	140 mg/kg
Male			
C_{max} (µg/mL)	1.13 ± 0.18	1.27 ± 0.13	1.91 ± 0.14
t_{max} (minute)	20	10	20
$AUC_{ti\text{-}tf}$ (µg·minute/mL)	67 ± 3.1	118 ± 5	203 ± 15
$AUC_{ti\text{-}tf}/Dose$ [(µg·minute/mL)/(mg/kg)]	1.91 ± 0.09	1.68 ± 0.07	1.45 ± 0.11
Cl_{app} (mL/minute·kg)	522 ± 24	595 ± 26	690 ± 50
Female			
C_{max} (µg/mL)	1.94 ± 0.17	2.54 ± 0.17	3.99 ± 2.10
t_{max} (minute)	10	20	5
$AUC_{ti\text{-}tf}$ (µg·minute/mL)	101 ± 4	207 ± 11	400 ± 36
$AUC_{ti\text{-}tf}/Dose$ [(µg·minute/mL)/(mg/kg)]	2.87 ± 0.12	2.96 ± 0.16	2.86 ± 0.26
Cl_{app} (mL/minute·kg)	348 ± 14	338 ± 18	350 ± 31

a All values except those for t_{max} are reported as the mean ± standard error. C_{max} = maximum plasma isoeugenol concentration; t_{max} = time at which C_{max} was observed; $AUC_{ti\text{-}tf}$ = area under the curve from initial to final measured time; Cl_{app} = apparent clearance.

National Toxicology Program
National Institute of Environmental Health Sciences
National Institutes of Health
P.O. Box 12233, MD K2-05
Durham, NC 27709
Tel: 984-287-3211
ntpwebrequest@niehs.nih.gov

https://ntp.niehs.nih.gov

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