NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDIES OF PULEGONE
(CAS NO. 89-82-7)

IN F344/N RATS AND B6C3F1 MICE
(GAVAGE STUDIES)

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

August 2011

NTP TR 563

NIH Publication No. 11-5905

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.

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CONTRIBUTORS

National Toxicology Program
Evaluated and interpreted results and reported findings

S.S. Auerbach, Ph.D., Study Scientist
S.A. Elmore, D.V.M., M.S., Study Pathologist
J.B. Bishop, Ph.D.
J.R. Bucher, Ph.D.
P.C. Chan, 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.
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
D.M. Sells, D.V.M., Ph.D.
A.J. Skowronek, 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
A.E. Brix, D.V.M., Ph.D.
G.C. Hard, B.V.Sc., Ph.D., D.Sc.
H.M. Kolenda-Roberts, D.V.M., Ph.D.
J.C. Peckham, D.V.M., M.S., Ph.D.

TherImmune Research Corporation
Provided SMVCE analysis

G.W. Wolfe, Ph.D., Principal Investigator
H.S. Seung, M.S.

Dynamac Corporation
Prepared quality assessment 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 2-year rats (August 30, 2007)

J.P. Morrison, D.V.M., Coordinator
Pathology Associates, A Division of Charles River Laboratories, Inc.
S.A. Elmore, D.V.M., M.S.
National Toxicology Program
G.P. Flake, M.D.
National Toxicology Program
G.C. Hard, B.V.Sc., Ph.D., D.Sc.
Experimental Pathology Laboratories
H.M. Kolenda-Roberts, D.V.M., Ph.D.
Experimental Pathology Laboratories
D.E. Malarkey, D.V.M., Ph.D.
National Toxicology Program
R.R. Maronpot, D.V.M.
Consultant
J.C. Peckham, D.V.M., M.S., Ph.D.
Experimental Pathology Laboratories

NTP Pathology Working Group (continued)

Evaluated slides and contributed to pathology report on 2-year mice (April 10, 2007)

J.P. Morrison, D.V.M., Coordinator
Pathology Associates, A Division of Charles River Laboratories, Inc.
F. Belpoggi, Ph.D.
Ramazzini Foundation, Italy
A.E. Brix, D.V.M., Ph.D.
Experimental Pathology Laboratories
S.A. Elmore, D.V.M., M.S.
National Toxicology Program
G.P. Flake, M.D.
National Toxicology Program
G.C. Hard, B.V.Sc., Ph.D., D.Sc.
Experimental Pathology Laboratories
R.A. Herbert, D.V.M., Ph.D.
National Toxicology Program
D.E. Malarkey, D.V.M., Ph.D.
National Toxicology Program
R.R. Maronpot, D.V.M.
Consultant
N. Wakamatsu, D.V.M., Ph.D.
National Toxicology Program

SRA International, 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
K.K. Coker, Ph.D.
L.M. Harper, B.S.
D.C. Serbus, Ph.D.

CONTENTS

SUMMARY

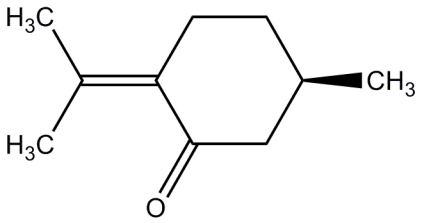
Background
Pulegone is a component of essential oils of plants including pennyroyal, mint, and peppermint. It is used primarily as a mint flavoring in foods. We studied the effects of pulegone on male and female rats and mice to identify potential toxic or cancer-related hazards.

Methods
We deposited pulegone dissolved in corn oil through a tube directly into the stomach to groups of 50 male and female rats and mice for up to two years. Male rats received 18.75, 37.5, or 75 milligrams of pulegone per kilogram of body weight five times per week; female rats and male and female mice received 37.5, 75, or 150 mg/kg five days per week. Control animals received corn oil with no chemical added by the same method. After 60, weeks many of the male rats receiving 75 mg/kg and female rats receiving 150 mg/kg had died, so the surviving animals from those groups received undosed corn oil for the duration of the study. At the end of the study, tissues from more than 40 sites were examined for every animal.

Results
A unique kidney lesion, hyaline glomerulopathy, was seen in all dosed groups of male and female mice and in the groups of rats receiving the highest doses of pulegone. Female rats receiving pulegone had increased incidences of urinary bladder tumors. Male and female mice had increased incidences of benign and malignant tumors of the liver, and female mice also had a small increase in rare bone lesions (osteoma or osteosarcoma).

Conclusions
We conclude that pulegone caused cancer of the urinary bladder in female rats and cancer of the liver in male and female mice. Neoplasms of the bone in female mice were also possibly associated with administration of pulegone. There were no increases in cancers in male rats receiving pulegone. Pulegone also caused an unusual kidney lesion, hyaline glomerulopathy, in male and female rats and mice.

ABSTRACT



PULEGONE

CAS No. 89-82-7

Chemical Formula: $C_{10}H_{16}O$ Molecular Weight: 152.2

Synonyms: Cyclohexanone, 5-methyl-2-(1-methylethylidene)-, *R*-(9CI); *d*-pulegone; *p*-menth-4(8)-en-3-one, *R*-(+)- (8CI); pulegon; (+)-pulegone; (1*R*)-(+)-*p*-menth-4(8)-en-3-one; (+)-*R*-pulegone

Several essential oils contain pulegone and are used for flavoring foods, drinks, and dental products, as fragrance agents, and in herbal medicines. Pulegone was nominated for study by the National Institute of Environmental Health Sciences based on the potential for human exposure and the absence of carcinogenicity data. Male and female F344/N rats and B6C3F1 mice received pulegone (approximately 96% pure) by gavage for 2 weeks, 3 months, or 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium, Escherichia coli*, and mouse peripheral blood erythrocytes.

2-WEEK STUDY IN RATS

Groups of five male and five female rats were administered 0, 37.5, 75, 150, 300, or 600 mg pulegone/kg body weight in corn oil by gavage, 5 days per week for 16 days. All male rats and nearly all female rats in the 300 and 600 mg/kg groups died prior to the end of the study. All moribund sacrifices and early deaths were attributed to liver toxicity. Mean body weight gains of males administered 37.5 or 150 mg/kg were significantly less than that of the vehicle controls. Clinical findings in 300 and 600 mg/kg rats included nasal/eye discharge, thinness, lethargy, and ruffled fur. Liver and kidney weights of dosed groups of females were generally significantly greater than those of the vehicle control group. The incidences of necrosis and cytoplasmic vacuolization of the liver in 300 and 600 mg/kg males and females were significantly greater than those in the vehicle control groups.

2-WEEK STUDY IN MICE

Groups of five male and five female mice were administered 0, 18.75, 37.5, 75, 150, or 300 mg pulegone/kg body weight in corn oil by gavage, 5 days per week for 16 days. Four females and one male in the 300 mg/kg groups died by study day 5**.** All early deaths were attributed to liver toxicity. Mean body weights of the dosed groups were similar to those of the vehicle controls. Clinical findings were observed only in 300 mg/kg mice and included thinness, lethargy, and ruffled fur. Liver weights of 300 mg/kg males were significantly greater than those of the vehicle controls. The incidences of cytoplasmic vacuolization and diffuse fatty change in 300 mg/kg females and necrosis in 300 mg/kg males were significantly greater than those in the vehicle controls.

3-MONTH STUDY IN RATS

Groups of 10 male and 10 female rats were administered 0, 9.375, 18.75, 37.5, 75, or 150 mg pulegone/kg

body weight in corn oil by gavage, 5 days per week for 14 weeks. All rats survived until the end of the study except for one female in the 150 mg/kg group that died on day 9. Mean body weights of 75 and 150 mg/kg males and 150 mg/kg females were significantly less than those of the vehicle controls. At the end of the study, there was a small dose-related decrease in the erythron, evidenced by decreases in the hematocrit and hemoglobin values and the erythrocyte counts. An apparent erythroid response to the decreased erythron was evidenced by increased reticulocyte counts. Reduced and oxidized glutathione levels were generally increased in 75 and 150 mg/kg males and in 37.5 mg/kg or greater females. Absolute and relative liver weights of 75 and 150 mg/kg females and relative liver weights of males administered 18.75 mg/kg or greater were significantly greater than those of the vehicle controls. The absolute kidney weight of 150 mg/kg females and the relative kidney weights of all dosed groups, except 9.375 mg/kg males, were significantly greater than those of the vehicle controls. Absolute and relative thymus weights of 150 mg/kg males and females and the absolute thymus weight of 75 mg/kg males were significantly less than those of the vehicle controls.

In the kidney, there was hyaline glomerulopathy in 75 mg/kg males and 150 mg/kg males and females. The incidence of renal tubule protein casts was significantly increased in the 150 mg/kg females. In the liver, incidences of bile duct hyperplasia and hepatocyte hypertrophy in 75 and 150 mg/kg males and 150 mg/kg females, hepatocyte focal necrosis in 150 mg/kg males, and oval cell hyperplasia and periportal fibrosis in 150 mg/kg males and females were increased. Incidences of bone marrow hyperplasia in 37.5 mg/kg males and 75 and 150 mg/kg males and females, heart mineralization in 150 mg/kg males, glandular stomach mineralization in 75 and 150 mg/kg females, and cellular histiocytic infiltration in the lung and ovarian cyst in 150 mg/kg females were significantly increased.

3-Month Study in Mice

Groups of 10 male and 10 female mice were administered 0, 9.375, 18.75, 37.5, 75, or 150 mg pulegone/kg body weight in corn oil by gavage, 5 days per week for 14 weeks. All mice survived to the end of the study. Mean body weights of dosed mice were similar to those of the vehicle controls. Reduced and oxidized glutathione levels were generally greater than vehicle control levels in 150 mg/kg males and in 75 and 150 mg/kg females. Liver weights of 150 mg/kg males and 75 and 150 mg/kg females were significantly greater than those of the vehicle controls. No histopathologic lesions were observed that could be attributed to the administration of pulegone.

2-Year Study in Rats

Groups of 50 male and 50 female rats were administered 0, 18.75 (males only), 37.5, 75, or 150 (females only) mg pulegone/kg body weight in corn oil by gavage, 5 days per week for up to 104 weeks. Due to excessive morbidity and mortality, 75 mg/kg males and 150 mg/kg females were not administered pulegone after week 60 (stop-exposure); these groups were administered the corn oil vehicle until the end of the study. Survival of 37.5 mg/kg males was significantly less than that of the vehicle controls; only two 75 mg/kg stop-exposure males survived, and no 150 mg/kg stop-exposure females survived to the end of the study. Compared to those of the vehicle controls, mean body weights were less in 75 mg/kg stop-exposure males after week 13 and in 75 mg/kg and 150 mg/kg stop-exposure females after weeks 21 and 9, respectively. Clinical findings included thinness, lethargy, and ruffled fur in the 75 mg/kg stop-exposure males and 150 mg/kg stop-exposure females.

The incidences of urinary bladder papilloma and of papilloma or carcinoma (combined) were significantly increased in 150 mg/kg stop-exposure females.

In the kidney, incidences of hyaline glomerulopathy were significantly increased in 37.5 mg/kg and 75 mg/kg stop-exposure males and in all dosed groups of females. The severity of chronic progressive nephropathy was increased in 37.5 mg/kg and 75 mg/kg stop-exposure males and in 75 mg/kg and 150 mg/kg stop-exposure females; the incidences of nephropathy were significantly increased in 75 mg/kg and 150 mg/kg stop-exposure females. The incidence of renal cyst was significantly increased in 75 mg/kg stop-exposure males.

In the liver, incidences of diffuse hepatocyte cellular alteration were significantly increased in 37.5 mg/kg and 75 mg/kg stop-exposure males and 75 mg/kg and 150 mg/kg stop-exposure females. There were significant increases in the incidences of other liver lesions including fatty change, bile duct cyst, hepatocyte necrosis, oval cell hyperplasia, bile duct hyperplasia, and portal fibrosis.

In the nose, 37.5 mg/kg and 75 mg/kg stop-exposure males and all dosed groups of females had significantly

increased incidences of olfactory epithelium degeneration. All dosed groups of females had significantly increased incidences of respiratory metaplasia of the olfactory epithelium and nasal inflammation.

In the forestomach, incidences of inflammation and ulcer were significantly increased in 37.5 mg/kg and 75 mg/kg stop-exposure males, and incidences of epithelial hyperplasia and perforation were increased in 75 mg/kg stop-exposure males. In the glandular stomach, the incidence of inflammation was significantly increased in 75 mg/kg stop-exposure males.

2-YEAR STUDY IN MICE

Groups of 50 male and 50 female mice were administered 0, 37.5, 75, or 150 mg pulegone/kg body weight in corn oil by gavage, 5 days per week for 105 weeks. Survival of all dosed groups was similar to that of the vehicle controls. Mean body weights of 150 mg/kg males and females were less than those of the vehicle controls after weeks 25 and 33, respectively.

The incidences of multiple hepatocellular adenoma were significantly increased in all dosed groups of males, and the incidences of hepatocellular adenoma (includes multiple) and hepatoblastoma (includes multiple) were significantly increased in the 75 mg/kg males. The combined incidences of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma occurred with positive trends and were significantly increased in 75 mg/kg males and 150 mg/kg females. The incidence of hepatocellular adenoma was significantly increased in 150 mg/kg females. The incidences of several nonneoplastic liver lesions were significantly increased, primarily in the 75 and 150 mg/kg groups. These nonneoplastic lesions included clear cell, eosinophilic, and mixed cell foci; focal fatty change; centrilobular hepatocyte hypertrophy; intravascular hepatocyte; necrosis; pigmentation; bile duct cyst and hyperplasia; and oval cell hyperplasia.

In the kidney, incidences of hyaline glomerulopathy were significantly increased in all dosed groups of males and 75 and 150 mg/kg females. The incidence of mineralization was significantly increased in 150 mg/kg females, and the incidence of nephropathy in 150 mg/kg females and severity of nephropathy in 150 mg/kg males were increased. Incidences of congestion of the glomerulus were increased in 150 mg/kg males and females.

The incidence of osteoma or osteosarcoma (combined) in all organs of 75 mg/kg females exceeded the historical control ranges. One 150 mg/kg male and one 75 mg/kg female had nasal osteoma; no nasal osteomas have been observed in historical control mice.

The incidences of olfactory epithelial degeneration of the nose were significantly increased in all dosed groups of females and in 75 and 150 mg/kg males. Incidences of inflammation, nerve atrophy, and olfactory epithelium metaplasia of the nose were significantly greater in 150 mg/kg males and females than in the vehicle control groups.

In the forestomach, incidences of squamous hyperplasia and inflammation were significantly increased in 75 mg/kg males and 150 mg/kg males and females, and the incidences of ulcer were significantly increased in 75 and 150 mg/kg males.

GENETIC TOXICOLOGY

Pulegone was tested in three independent bacterial mutagenicity assays. Results from two of the assays were negative, with and without exogenous metabolic activation enzymes (S9). One of these assays used the same lot of pulegone that was tested in the 2-year rodent bioassay. Bacterial strains tested in these two assays included *S. typhimurium* strains TA97, TA98, TA100, and TA1535 as well as *E. coli* strain WP2 *uvrA*/pKM101. Results of the third test, also conducted with the same lot of pulegone as the 2-year bioassay, were clearly positive in *Salmonella typhimurium* strain TA98 and in *Escherichia coli* strain WP2 *uvrA*/pKM101 in the presence of rat liver S9. *In vivo*, no significant increases in the frequencies of micronucleated erythrocytes were seen in peripheral blood of male or female mice in the 3-month study.

CONCLUSIONS

Under the conditions of these 2-year gavage studies, there was *no evidence of carcinogenic activity** of pulegone in male F344/N rats administered 18.75, 37.5, or 75 (stop-exposure) mg/kg. There was *clear evidence of carcinogenic activity* of pulegone in female F344/N rats based on increased incidences of urinary bladder neoplasms. There was *clear evidence of carcinogenic activity* of pulegone in male and female B6C3F1 mice based on increased incidences of hepatocellular neoplasms (adenomas in both sexes and

hepatoblastomas in males). Osteomas and osteosarcomas in female B6C3F1 mice may have been related to pulegone administration.

A unique renal lesion, hyaline glomerulopathy, was observed in all dosed groups of male and female mice and female rats and in 37.5 mg/kg and 75 mg/kg stop-exposure male rats. In rats, renal failure secondary to hyaline glomerulopathy and nephropathy contributed to the decreased survival in the 75 mg/kg stop-exposure males and 150 mg/kg stop-exposure females.

Pulegone administration was also associated with the occurrence of nonneoplastic lesions in the liver and nose of rats and mice and in the forestomach of male and female mice and male rats.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 13. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 15.

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

	Male F344/N Rats	Female F344/N Rats	Male B6C3F1 Mice	Female B6C3F1 Mice
Doses in corn oil by gavage	0, 18.75, 37.5, or 75 (stop-exposure) mg/kg	0, 37.5, 75, or 150 (stop-exposure) mg/kg	0, 37.5, 75, or 150 mg/kg	0, 37.5, 75, or 150 mg/kg
Body weights	75 mg/kg stop-exposure group 10% less than the vehicle control group after week 13	75 mg/kg group 10% less than the vehicle control group after week 21 and 150 mg/kg stop-exposure group 12% less than the vehicle control group after week 9	150 mg/kg group 10% less than the vehicle control group after week 25	150 mg/kg group 10% less than the vehicle control group after week 33
Survival rates	39/50, 32/50, 28/50, 2/50	31/50, 37/50, 38/50, 0/50	38/50, 36/50, 42/50, 41/50	35/49, 41/50, 38/50, 37/50
Nonneoplastic effects	Kidney: hyaline glomerulopathy (0/50, 0/50, 9/50, 24/50); severity of nephropathy (1.9, 1.9, 2.9, 4.0); cyst (0/50, 0/50, 2/50, 7/50) Liver: hepatocyte, cellular alteration, diffuse (0/50, 2/50, 21/50, 46/50); fatty change (1/50, 10/50, 27/50, 2/50); bile duct cyst (0/50, 0/50, 11/50, 9/50); hepatocyte, necrosis (0/50, 1/50, 6/50, 16/50); oval cell, hyperplasia (0/50, 0/50, 8/50, 44/50); bile duct, hyperplasia (29/50, 15/50, 37/50, 50/50); portal fibrosis (8/50, 6/50, 13/50, 43/50) Nose: olfactory epithelium, degeneration (1/50, 5/50, 33/46, 19/50) Forestomach: inflammation (2/50, 4/50, 8/50, 23/50); perforation (0/50, 0/50, 0/50, 5/50); ulcer (0/50, 2/50, 7/50, 16/50); epithelium, hyperplasia (16/50, 21/50, 20/50, 23/50)	Kidney: hyaline glomerulopathy (0/50, 17/50, 49/50, 48/49); nephropathy (42/50, 44/50, 49/50, 48/49); severity of nephropathy (1.2, 1.3, 2.9, 3.4) Liver: hepatocyte, cellular alteration, diffuse (0/50, 4/50, 45/50, 43/47); fatty change (7/50, 25/50, 35/50, 11/47); bile duct, cyst (1/50, 6/50, 38/50, 13/47); hepatocyte, necrosis (4/50, 2/50, 20/50, 15/47); oval cell, hyperplasia (0/50, 0/50, 45/50, 43/47); bile duct, hyperplasia (5/50, 4/50, 49/50, 43/47); portal fibrosis (0/50, 3/50, 28/50, 35/47) Nose: olfactory epithelium, degeneration (2/50, 40/50, 48/50, 37/41); olfactory epithelium, metaplasia, respiratory (1/50, 8/50, 46/50, 36/41); inflammation (12/50, 22/50, 39/50, 26/41)	Kidney: glomerulopathy, hyaline (1/50, 19/50, 30/50, 44/50); glomerulus, congestion (9/50, 14/50, 17/50, 44/50); severity of nephropathy (1.2, 1.3, 1.4, 1.9) Liver: clear cell focus (15/50, 27/50, 28/50, 34/50); eosinophilic focus (7/50, 12/50, 20/50, 36/50); mixed cell focus (18/50, 20/50, 19/50, 34/50); fatty change, focal (3/50, 8/50, 20/50, 23/50); centrilobular, hepatocyte, hypertrophy (0/50, 11/50, 23/50, 46/50); vein, intravascular hepatocyte (3/50, 1/50, 15/50, 47/50); necrosis (1/50, 8/50, 5/50, 26/50); bile duct, cyst (0/50, 0/50, 3/50, 14/50); bile duct, hyperplasia (0/50, 0/50, 1/50, 35/50); oval cell hyperplasia (1/50, 0/50, 1/50, 36/50) Nose: olfactory epithelium, degeneration (3/50, 3/50, 11/50, 46/50); inflammation (2/50, 3/50, 2/50, 22/50); nerve, atrophy (1/50, 3/50, 3/50, 45/50); olfactory epithelium, metaplasia (1/50, 5/50, 3/50, 44/50) Forestomach: hyperplasia, squamous (7/50, 10/50, 27/50, 41/50); inflammation (3/50, 9/50, 24/50, 39/50); ulcer (0/50, 3/50, 9/50, 22/50)	Kidney: glomerulopathy, hyaline (0/49, 3/50, 15/50, 41/50); mineralization (1/49, 0/50, 3/50, 20/50); nephropathy (13/49, 19/50, 12/50, 25/50); glomerulus, congestion (5/49, 2/50, 12/50, 37/50) Liver: clear cell focus (0/49, 6/50, 23/50, 32/50); eosinophilic focus (3/49, 7/50, 10/50, 31/50); mixed cell focus (4/49, 8/50, 16/50, 20/50); fatty change, focal (1/49, 2/50, 20/50, 12/50); centrilobular, hepatocyte, hypertrophy (0/49, 4/50, 12/50, 29/50); vein, intravascular hepatocyte (0/49, 2/50, 20/50, 46/50); necrosis (5/49, 2/50, 4/50, 27/50); pigmentation (0/49, 0/50, 0/50, 46/50); bile duct, cyst (0/49, 0//50, 4/50, 38/50); bile duct, hyperplasia (0/49, 0/50, 2/50, 47/50); oval cell, hyperplasia (0/49, 0/50, 3/50, 46/50) Nose: olfactory epithelium, degeneration (0/49, 5/50, 22/50, 48/50); inflammation (2/49, 1/50, 4/50, 27/50); nerve, atrophy (0/49, 1/50, 2/50, 49/50); olfactory epithelium, metaplasia (1/49, 2/50, 4/50, 49/50) Forestomach: hyperplasia, squamous (13/49, 1/50, 10/50, 26/50); inflammation (10/49, 0/50, 7/50, 20/50)

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

	Male F344/N Rats	Female F344/N Rats	Male B6C3F1 Mice	Female B6C3F1 Mice
Neoplastic effects	None	Urinary Bladder: papilloma (0/50, 0/49, 1/50, 3/47); papilloma or carcinoma (0/50, 0/49, 1/50, 5/47)	Liver: hepatocellular adenoma (22/50, 31/50, 35/50, 28/50); hepatoblastoma (1/50, 3/50, 7/50, 2/50)	Liver: hepatocellular adenoma (13/49, 15/50, 13/50, 27/50)
Equivocal findings	None	None	None	All Organs: osteoma or osteosarcoma (0/49, 0/50, 3/50, 1/50)
Level of evidence of carcinogenic activity	No evidence	Clear evidence	Clear evidence	Clear evidence
Genetic toxicology				
Bacterial gene mutations				
Study one:		Negative in *Salmonella typhimurium* strains TA97, TA98, TA100, and TA1535, with and without 10% or 30% hamster or rat liver S9		
Study two:		Negative in *S. typhimurium* strains TA98 and TA100 and in *Escherichia coli* strain WP2 *uvrA*/pKM101, with and without 10% rat liver S9		
Study three:		Positive in *S. typhimurium* strains TA98 and in *E. coli* strain WP2 *uvrA*/pKM101 with 10% rat liver S9; negative in TA98 and WP2 *uvrA*/pKM101 without S9; negative in TA100 with and without 10% rat S9		
Micronucleated erythrocytes				
Mouse peripheral blood *in vivo:*		Negative		

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 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 on 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 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 pulegone on November 19, 2009, 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.

Raymond F. Novak, Ph.D., Chairperson
Children's Hospital of Michigan
Wayne State University School of Medicine
Detroit, MI

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

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

David A. Eastmond, Ph.D.
Department of Cell Biology and Neuroscience
University of California
Riverside, CA

Stephen W. Looney, Ph.D.
Department of Biostatistics
Medical College of Georgia
Augusta, GA

Mitzi Nagarkatti, Ph.D.
Department of Pathology, Microbiology, and Immunology
University of South Carolina School of Medicine
Columbia, SC

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

Kenneth M. Portier, Ph.D.
American Cancer Society
Atlanta, GA

Jim E. Riviere, D.V.M., Ph.D.
College of Veterinary Medicine
North Carolina State University
Raleigh, NC

James L. Sherley, M.D., Ph.D.
Programs in Regenerative Biology and Cancer
Boston Biomedical Research Institute
Watertown, MA

Justin G. Teeguarden, Ph.D., Principal Reviewer
Pacific Northwest National Laboratory
Richland, WA

SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On November 19, 2009, the draft Technical Report on the toxicology and carcinogenesis studies of pulegone 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. S.S. Auerbach, NIEHS, introduced the toxicology and carcinogenesis studies of pulegone by describing the properties and uses of the essential oils constituting the herbal product, the genetic toxicity, absorption and metabolism studies of the chemical, and the design and results of the short- and long-term studies in rodents. Dr. S.A. Elmore, NIEHS, followed with a presentation characterizing the hyaline glomerulopathy observed in rats and mice in these studies. The proposed conclusions were *no evidence* of carcinogenic activity of pulegone in male F344/N rats, *some evidence* of carcinogenic activity of pulegone in female F344/N rats, and *clear evidence* of carcinogenic activity of pulegone in male and female B6C3F1 mice.

Dr. Cattley, the first principal reviewer, asked whether the observed kidney nephropathy was associated with the glomerulopathy described and if circulating immunoglobulins were assayed. He requested that the tabular analyses of the liver neoplasms be expanded to include each tumor type and asked for more detail on the methodology of the analyses of the effects of intercurrent factors (survival and body weight) on expected tumor incidence.

Regarding the proposed conclusions, Dr. Cattley recommended specifying the tumor type rather than just the tissue of occurrence. He also asked for a discussion of the level of evidence assigned for the bladder neoplasms in rats.

Dr. Auerbach replied that it was unknown if there was any association between the nephropathy and the glomerulopathy, and no assays for immunoglobulins were performed. Dr. Elmore added that further immunohistochemical studies were being performed on the short-term studies to further characterize the lesions and the findings would be published separately. Dr. Auerbach explained that the proposed conclusion for mice was framed to cover a variety of liver lesions, including adenomas, hepatoblastomas, and several preneoplastic lesions, rather than any one specific neoplasm type. Dr. Cattley suggested that at least specifying the neoplasms were hepatocellular might add clarity. Concerning the bladder tumors, Dr. Auerbach listed the various factors including rarity of the tumors, progression, early onset, and concomitant mortality due to toxicity in the urinary system that went into consideration of the proposed level of evidence.

Dr. Pino, the second principal reviewer, thought the proposed conclusion for the female rat bladder tumors should be *clear evidence*. He questioned why certain decreases in tumor incidence were not included in the conclusion statement. He also questioned whether biliary tumors were included with liver tumors, as distinguished from hepatocellular lesions. Dr. Auerbach replied that the proposed conclusion regarding bladder tumors in female rats was the subject of considerable debate among the NTP staff as well. Regarding the seeming decrease in the incidence of pituitary adenomas and thyroid gland C-cell adenomas, he noted that the mortality in the high dose group and marginal statistical significance reduced confidence that it could truly be deemed a chemical-related effect.

Dr. Teeguarden, the third principal reviewer, agreed with the proposed conclusions and felt the report was complete. In light of the toxicity and extensive mortality in the high dose group, and the presence of toxicity in the short term studies, he inquired about the dose-setting process in study design. He also suggested that language be included in the report clarifying that NTP Technical Reports are not risk assessment documents.

Dr. Auerbach replied that the dose selection for this study included consideration of a possible adaptive response to glutathione depletion, and the hyaline glomerulopathy was not fully diagnosed until a retrospective analysis of the short-term studies was conducted after the 2-year studies were completed. Dr. J.R. Bucher, NIEHS, noted that the Foreword to the report indicates that risk assessment is beyond the purview of these studies.

Dr. Eastmond suggested that, because of the highly variable, spontaneous, background rate of liver neoplasms in male mice, a conclusion of *some evidence* rather than *clear evidence* might be more appropriate. Dr. Cattley questioned whether using a response in

female mice as support for an effect in male mice is legitimate.

Dr. Auerbach agreed that male mouse liver adenomas are variable and the proposed *clear evidence* conclusion for male liver neoplasms was based on a variety of factors, including a definite dose-response, preneoplastic lesions, the presence of hepatoblastomas, and an increased tumor incidence in the presence of a marked body weight reduction. He noted that while the studies are evaluated independently, one factor considered when determining the level of evidence is a corroborating response in the companion sex/species group.

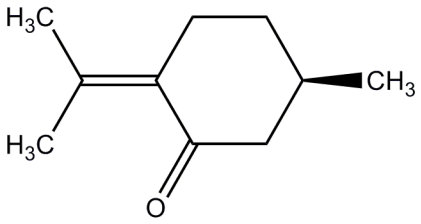
Dr. Portier moved and Dr. Sherley seconded that the proposed conclusions be accepted as written. The motion failed, with the consensus being that a modification of the descriptive language supporting the levels of evidence was needed.

Dr. Cattley recommended that the conclusion for mice be changed to *clear evidence* based on increased incidences of hepatocellular neoplasms (adenomas in both sexes and hepatoblastomas in males). A consensus vote of the panel indicated this change was satisfactory.

Dr. Pino suggested that the conclusion in female rats be changed from *some evidence* to *clear evidence* based on the presence of the rare urinary bladder tumors. Dr. D.E. Malarkey, NIEHS, said the competing considerations in drafting the conclusion were the presence of rare tumors but in the presence of toxicity at doses that exceeded the maximum tolerated dose.

Dr. Riviere moved and Dr. Bunton seconded that the conclusions be accepted with the two changes proposed. The motion was approved with six yes votes and four no votes. Drs. Sherley, Portier, Eastmond, and Teeguarden voted no. Dr. Sherley voted no because he agreed with the conclusion for mice as written, "*clear evidence*… based on increased incidences of liver neoplasms." He also did not agree with changing the conclusion for female rats to *clear evidence*. Dr. Portier agreed with conclusion for mice as *clear evidence* based on increased incidences of hepatocellular neoplasms (adenomas in both sexes and hepatoblastomas in males), but did not agree with the conclusion of *clear evidence* for female rats. Dr. Eastmond thought the conclusion should be *some evidence* rather than *clear evidence* in male mice. Dr. Teeguarden disagreed with *clear evidence* for female rats.

INTRODUCTION



PULEGONE

CAS No. 89-82-7

Chemical Formula: $C_{10}H_{16}O$ Molecular Weight: 152.2

Synonyms: Cyclohexanone, 5-methyl-2-(1-methylethylidene)-, *R*-(9CI); *d*-pulegone; *p*-menth-4(8)-en-3-one, *R*-(+)- (8CI); pulegon; (+)-pulegone; (1*R*)-(+)-*p*-menth-4(8)-en-3-one; (+)-*R*-pulegone

CHEMICAL AND PHYSICAL PROPERTIES

Pulegone [*R*-(+)-pulegone unless otherwise indicated] is a colorless, clear, oily liquid at room temperature and which has an odor between peppermint and camphor (*Merck*, 1996). It has a molecular mass of 153.23 g/mol and a density of 0.9346 g/cm^3. Pulegone has a vapor pressure of 138 mm Hg at 25° C. It is insoluble in water but is miscible with alcohol, chloroform, or ether. Pulegone has a specific gravity of 0.937 at 25° C and a partition coefficient (octanol:water) of 3.08 (Good Scents, 2009). Its boiling point is 224° C, and it freezes at less than 25° C (ChemIDplus, 2009). It is a flammable liquid (flash point of 82° C) that will ignite if moderately heated.

PRODUCTION, USE, AND HUMAN EXPOSURE

Several essential oils containing pulegone [e.g., mint oil (<2% pulegone), peppermint oil (<4% pulegone), pennyroyal oil (60%-90% pulegone)] are used for flavoring foods, drinks, and dental products (Lawrence, 2006). Pennyroyal oil has also been used as a fragrance agent and as an herbal medicine proposed to induce menstruation and abortion (Nelson *et al.*, 1992).

Pulegone can be produced synthetically (Grundschober, 1979). A survey conducted in the 1980s indicated that production of synthetic pulegone was 15 and 13 kg per year in Europe and the United States, respectively (Stofberg and Grundschober, 1987). Despite synthetic production, pulegone exposure comes predominantly from its natural occurrence in food products. Pulegone can also be produced by shoot cultures of *Mentha piperita* grown in fermenters (Hilton *et al.*, 1995). Essential oils containing pulegone are derived from many plant species (Lawrence, 2006), including *Hedeoma pulegioides* (American pennyroyal), species of the genus *Bystropogon* (evergreen shrubs), and species of the genus *Mentha* [e.g., European pennyroyal (*M. pulegium*), cornmint, Biblical mint]. Essential (i.e., volatile) plant oils are produced in the plant tissues by the reaction of certain constituents when tissues contact water; most oils are obtained from plants by steam distillation (Wright, 1999).

Human exposure to pulegone is primarily through ingestion of food products and of beverages flavored with oils or synthetic pulegone (Grundschober, 1979). Average levels of pulegone for various whole food product categories in the United States were reported as 9.07 ppm for nonalcoholic beverages, 10.5 ppm for

alcoholic beverages, 28.0 ppm for frozen dairy dessert, 27.4 ppm for candy, 35.4 ppm for baked goods, and 27.3 ppm for gelatins and puddings.

A survey of food flavorings in the United Kingdom reported pulegone contents of 18.2% in essential oil of buchu leaves, 6.2% in essential oil of mint blend, 73.3% in essential oil of pennyroyal, and 6.6% in essential oil of peppermint/cornmint (UKMAFF, 1994). Mint products and herbal teas available in the United Kingdom were reported to contain peppermint oil with pulegone content ranging from 0.2% to 2.9% (weight/weight) (2,000 to 29,000 ppm) (UKMAFF, 1996a). Other pulegone containing products available in the United Kingdom include mint-flavored sugar, chocolate, and gum-based confectionery products (0 to 119 ppm pulegone), peppermint and peppermint-containing teas (0 to 27 ppm pulegone), and mint liqueur (5 ppm pulegone) (UKMAFF, 1996b). Pulegone was not detected in meat products, processed fruit, confectioner frosting, or mint jams or jellies.

The recent estimate of the mean intake of pulegone in France was 43.9 mg per day (0.6 mg/kg per day); the 97.5th percentile of intake was 72.7 mg per day (1.0 mg/kg per day) (EFSA, 2005). A similar analysis in the United Kingdom estimated the mean and 97.5th percentile intakes of pulegone to be 0.8 mg per day (0.02 mg/kg per day) and 3.1 mg per day (0.04 mg/kg per day), respectively (EFSA, 2005). Consumption of certain products can dramatically increase daily intake of pulegone. For example, it is estimated that a 30 kg child consuming 500 mL of a mint flavored beverage or 100 g of mint confectionary would receive a 4.2 or 1.2 mg/kg dose of pulegone, respectively. According to the Committee on Herbal Medicinal Products of the European Medicines Agency, doses of up to 2.3 mg/kg body weight per day are commonly encountered in herbal medicine products in Europe (EMEA/HMPC, 2005).

REGULATORY STATUS

Pulegone has Flavor and Extracts Manufacturers Association Generally Recognized as Safe (GRAS) status and is authorized in the United States as a synthetic flavoring substance (21 CFR § 172.515; Grundschober, 1979), and American and European pennyroyal oils are authorized as natural flavorings (21 CFR § 172.510). Some European countries have regulated the use of pulegone; levels of pulegone in natural (unspecified) flavorings are limited to 20 ppm in Spain, Switzerland, the Netherlands, and the United Kingdom (Grundschober, 1979). In the United Kingdom, a provisional limit of synthetic pulegone not to be exceeded in food is 125 ppm (Grundschober, 1979). In Germany, the use of pennyroyal oil is prohibited, but the use of pulegone is not limited (Grundschober, 1979). The Committee of Experts on Flavouring Substances of the Council of Europe set a Tolerable Daily Intake of 0.1 mg/kg body weight per day based on the no-observed-effect level (NOEL) of 20 mg/kg body weight per day in a 28-day oral toxicity study in rats (Thorup, 1983a) with a safety factor of 200 (EMEA/HMPC, 2005).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

Except where noted, the following data describe the *in vivo* fate of (*R*)-(+)-pulegone, the enatiomer commonly found in essential oils and used as the test article in the present studies. Pulegone was rapidly and extensively absorbed from the gastrointestinal tract following oral administration of single ^{14}C-labeled doses (0.8 to 80 mg/kg) to male and female F344/N rats and B6C3F1 mice (Chen *et al*., 2003). The ^{14}C was primarily excreted in the urine (44% to 93% total dose) within 24 hours, although a significant amount (6% to 24%) was excreted in the feces and a trace was recovered in expired air. Some dose-, species-, and sex-dependent differences in elimination of pulegone-derived ^{14}C were observed. For instance, in rats, the proportion of ^{14}C excreted in the urine decreased with increased dose, and male rats excreted less ^{14}C at the two higher doses than did female rats. Overall, mice excreted more ^{14}C in urine than rats, and the amount of ^{14}C excretion was similar (males) or higher (females) with increased dose. Patterns of excretion were similar following intravenous or oral administration of 0.8 mg/kg to rats and mice. Studies conducted by intraperitoneal injection (150 mg/kg) in male Sprague-Dawley rats indicated a plasma half-life for pulegone of approximately 2 hours (Thomassen *et al*., 1988).

Pulegone-derived radioactivity was present in the liver, kidney, blood, and lung 24 hours following oral administration to male and female F344/N rats and B6C3F1 mice (Chen *et al*., 2003). Generally, mouse tissues contained lower amounts of ^{14}C than rat tissues and male rat tissues contained higher amounts of ^{14}C than female rat tissues. The highest concentration of ^{14}C in tissues of these animals, with the exception of the male rat, was in the liver, a known target for pulegone-mediated toxicity (Gordon *et al*., 1987; Chen *et al*., 2003). The kidney generally contained the highest

concentration of ^{14}C in the male rat, an amount up to 10-fold higher than in female rats at the low dose (Chen *et al.*, 2003). An additional study demonstrated binding of pulegone and its metabolites menthofuran and menthone (Figure 1) to α2u-globulin in the cytosol of male F344/N rat kidney following oral administration of these ^{14}C-labeled chemicals (Ferguson *et al*., 2007). However, binding of the ^{14}C to α2u-globulin was reversible and did not result in accumulation of the protein in the kidney. Multidose studies in female F344/N rats indicated a potential for bioaccumulation of pulegone-derived radioactivity in the liver (Chen *et al*., 2003).

A number of studies have been published describing the metabolism of pulegone in rodents (Moorthy *et al*., 1989a; Thomassen *et al*., 1991; Madyastha and Raj, 1993; Chen *et al*., 2001, 2003). The results indicate that metabolism of pulegone takes place through multiple pathways, including hydroxylation, reduction, and conjugation (Figure 1). The initial studies, conducted at hepatotoxic doses (250 or 400 mg/kg) in male rats, resulted in identification of 14 phase I metabolites, most arising from a common 9-hydroxypulegone intermediate (Moorthy *et al*., 1989a; Madyastha and Raj, 1993). Thomassen *et al*. (1991) identified 10 phase II metabolites consisting of glucuronide, glutathione, and glutathionyl glucuronide conjugates in the bile of Sprague-Dawley rats receiving 250 mg/kg by intraperitoneal injection. Studies in F344/N rats, conducted at putative nontoxic doses≤(80 mg/kg), identified 14 metabolites in rat urine, mostly glucuronide conjugates (Chen *et al.*, 2001). Some of the phase I metabolites in the studies conducted by Moorthy *et al*. (1989a) and Madyastha and Raj (1993) had no structural similarity to the precursors of the phase II metabolites detected by Chen *et al*. (2001). This was suggested to be attributable to dose and/or differences in isolation procedures (Chen *et al*., 2001). A follow-up study by Chen *et al*. (2003) demonstrated both qualitative and quantitative differences in metabolism of pulegone in rats and mice. A metabolic scheme for pulegone-derived metabolites identified in rat urine by Chen *et al*. (2003) is shown in Figure 1. The majority of these metabolites were detected in mice; notable exceptions were some metabolites arising through 5-hydroxypulegone. Rat urine also contained several late-eluting, radiolabeled peaks not evident in the representative high-performance liquid chromatography chromatogram for mouse urine. In contrast to rats, a larger ratio of the ^{14}C in female mouse urine consisted of some specific glucuronide conjugates of the menthones and mouse urine appeared to contain less of the glucuronide conjugate of 7α-hydroxymintlactone, derived from menthofuran. The authors did not report sex differences in pulegone metabolism, except to indicate that the initial radiolabeled peak in male rat urine was larger than in female rat urine.

Pulegone biotransformation has undergone a significant amount of study due to its critical role in pulegone toxicity. The cytochrome P450 (CYP)-dependent metabolism of pulegone to menthofuran and subsequently to a reactive γ-ketonal apparently accounted for a significant portion of the hepatotoxicity observed following pulegone exposure to mice (Gordon *et al*., 1987; McClanahan *et al*., 1989). Further, there is potential for oxidation of menthofuran to a reactive epoxide (Madyastha and Moorthy, 1989; Nelson *et al*., 1992); and Madyastha and Raj (1993) detected potentially toxic *p*-cresol, a metabolite of menthofuran (Madyastha and Raj, 1992), in the urine of pulegone-treated rats. The formation of reactive electrophiles in pulegone-treated rodents is indicated by the presence of glutathione conjugates in bile and mercapturic acids in urine, depletion of glutathione in the liver, and the enhancement of hepatotoxicity following pretreatment with other glutathione-depleting agents (Gordon *et al*., 1982; Thomassen *et al*., 1990, 1991; Chen *et al*., 2003). Metabolic activation of (*R*)-(+)-pulegone is further supported by the observation of decreased formation of metabolites along reactive pathways in rats treated with the less toxic enatiomer, (*S*)-(−)-pulegone (Gordon *et al*., 1982; Madyastha and Gaikwad, 1998). Several studies support the role of CYP-dependent metabolism in the toxicity of pulegone in rodents, including those showing decreased *in vitro* or *in vivo* covalent binding of pulegone-derived metabolites to macromolecules or decreased toxicity in animals following CYP inhibition (Gordon *et al*., 1987; Mizutani *et al*., 1987; McClanahan *et al*., 1989; Madyastha and Moorthy, 1989; Moorthy *et al*., 1989b). In contrast, results from these studies demonstrated increased pulegone-mediated hepatotoxicity and/or covalent binding to macromolecules in association with CYP induction by phenobarbital.

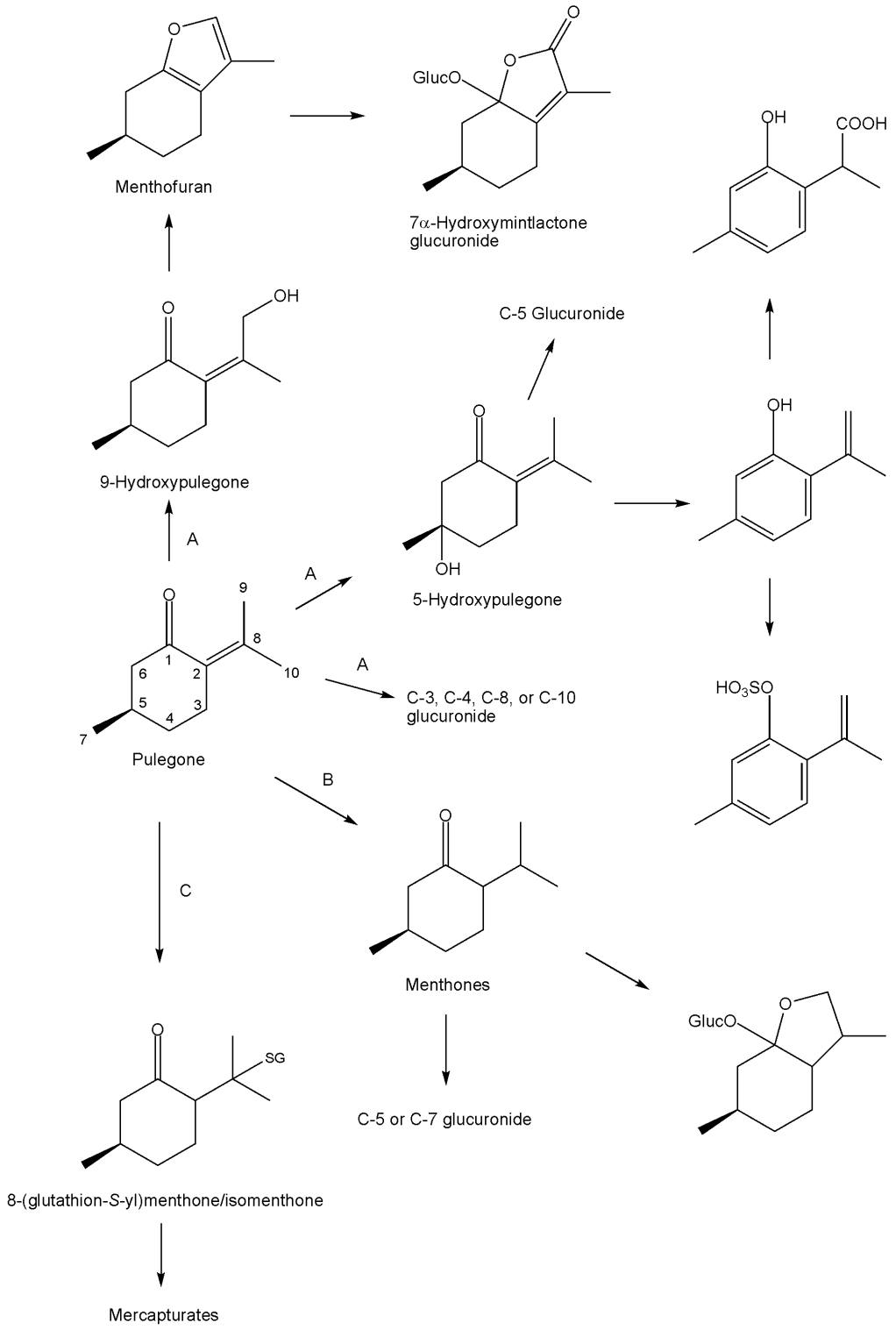


FIGURE 1
Metabolism of Pulegone by Rats

Pathways leading to formation of pulegone-derived metabolites excreted in urine of F344/N rats as identified by Chen *et al*. (2003).
A = hydroxylation followed by conjugation or further metabolism. B = reduction followed by hydroxylation/glucuronidation.
C = glutathione conjugation and metabolism to mercapturic acids.

Humans

Engel (2003) investigated the metabolism of 0.5 mg (*R*)-(+)-pulegone or 1 mg (*S*)-(−)-pulegone administered orally to six human volunteers. Six metabolites (four major) common to both enatiomers were identified in the urine following enzymatic digestion, purification, and analysis by gas chromatography-mass spectrometry. A major metabolite of (*R*)-(+)-pulegone was 10-hydroxypulegone. Although 10-hydroxypulegone was shown to convert to menthofuran following *in vitro* hydrolysis, little menthofuran or its metabolites were found in the urine of the volunteers. The other major metabolites in urine consisted of two menthone derivatives and an apparent conjugation product of pulegone. The author concluded that menthofuran was not a relevant metabolite in humans for either enatiomer at these doses. However, menthofuran was present in the serum of two individuals hours after ingesting a large amount of pennyroyal oil (Anderson *et al*., 1996). Pulegone was found to be metabolized to menthofuran by expressed human liver CYPs, primarily by CYP2E1, and to a lesser extent by CYP1A2 and CYP2C19 (Khojasteh-Bakht *et al*., 1999). Menthofuran was metabolized to the greatest extent by CYP2E1 and to a lesser extent by CYP1A2, CYP2C19, and CYP2A6. Menthofuran, but not pulegone, was shown to inactivate human liver CYP2A6 (Khojasteh-Bakht *et al*., 1998, 1999).

TOXICITY

Experimental Animals

The subcutaneous LD_{50} of pulegone has been estimated as 1,709 mg/kg in mice; the intraperitoneal LD_{50} as 150 mg/kg in rats; and the intravenous LD_{50} as 330 mg/kg in dogs (Grundschober, 1979). A gavage LD_{50} was not available.

Toxicity studies with intraperitoneal doses ranging from 150 to 400 mg/kg per day for up to 7 days in rodents have consistently revealed hepatotoxicity manifesting as hepatic necrosis and increases in circulating liver enzymes alanine amino transferase (ALT) and serum glutamate pyruvate transaminase (SGPT) (Gordon *et al.*, 1982, 1987; Mizutani *et al.*, 1987; Thomassen *et al.*, 1988, 1990, 1992; Moorthy *et al.*, 1989a). An early single dose study of pennyroyal oil toxicity performed in Swiss Webster mice (400 mg/kg intraperitoneally) found bronchiolar epithelium necrosis in addition to hepatic necrosis (Gordon *et al.*, 1982). Pennyroyal oil (60 mL) topically applied to a dog resulted in seizures and death within 30 hours; pulegone was identified in the pennyroyal oil and in the liver (Sudekum *et al.*, 1992). The necropsy showed histopathologic damage that included massive hepatocellular necrosis, lung congestion, hemorrhage, and edema.

In a 28-day study, male and female SPF rats treated by gavage with pulegone at 80 or 160 mg/kg per day had atonia, decreased blood creatinine content, lower terminal body weights, and histopathologic changes in the liver and the white matter of the cerebellum (Thorup *et al.*, 1983a). The lowest dose tested in the same study, 20 mg/kg per day, did not produce these effects. In a related study, dose-related cyst-like spaces were observed in the white matter of the cerebellum of rats treated orally with pulegone at 80 or 160 mg/kg per day for 28 days, while the lowest dose, 20 mg/kg per day, did not produce this effect (Olsen and Thorup, 1984). The same encephalopathy was observed in rats administered 40 or 100 mg peppermint oil/kg body weight per day, but not 10 mg/kg per day, for 28 days (Thorup *et al.*, 1983b). However, no corresponding clinical signs of neurotoxicity were observed. The concentration of pulegone or menthofuran in the peppermint oil was not given. In a third 28-day study, 0 or 160 mg/kg pulegone was given orally by gavage to groups of 28 female Wistar rats (Mølck *et al.*, 1998). Treatment-related increases in plasma glucose, alkaline phosphatase, and ALT were observed along with a decrease in plasma creatinine; however, there were no significant histopathologic changes in the liver nor the brain, with or without perfusion fixation. The authors of this study surmised that the lesions in the cerebellum that were previously observed were likely a result of inadequate tissue fixation procedures.

The toxicity of pulegone is largely attributed to its metabolism to reactive species, in particular menthofuran, which can undergo rearrangement to electrophilic γ-ketoenal species, 8-pulegone aldehyde (Thomassen *et al.*, 1988; McClanahan *et al.*, 1989). The reactive metabolites adduct cellular macromolecules and deplete glutathione levels leading to toxicity. Gene expression changes in the liver of male Sprague-Dawley rats 24 hours after a 400 mg/kg dose of pulegone indicated there was activation of Nrf2 (McMillian *et al.*, 2004). Activation of Nrf2 is a common response to oxidative stress and GSH depletion, an effect observed with hepatotoxic doses of pulegone (Gordon *et al.*, 1982; Thomassen *et al.*, 1990).

Humans

Ingestion of pulegone (in pennyroyal oil) has been associated with toxic effects (Anderson *et al.*, 1996). A review of published reports of pennyroyal poisoning (1869 to 1996) identified 18 cases in which there was moderate to severe toxicity from ingestion of at least 10 mL (about 2 tsp.) of pennyroyal oil. The toxic symptoms included coma, seizures, and hepatic and renal effects. Ingestion of less than 10 mL was generally associated with gastritis and mild central nervous system toxicity. However, the severity of the toxic effects was not always related strictly to dose and depended on the use of emetics or other treatments. In two cases reported before 1905, coma and seizures were associated with ingestion of 5 mL pennyroyal oil; two other cases reportedly survived after ingestion of 30 mL. Variability in the toxic effects of pennyroyal oil may be due to differing concentrations of pulegone contained in preparations from European and American pennyroyal (Lawrence, 2006).

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

No data on the reproductive and developmental toxicity of pulegone in experimental animals or humans were found in the literature.

CARCINOGENICITY

No data on the carcinogenicity of pulegone in experimental animals or epidemiology studies in humans were found in the literature.

GENETIC TOXICITY

Pulegone was not mutagenic in *Salmonella typhimurium* strains TA97, TA98, TA100, TA1535, or TA1537 at 6.4, 32, 160, or 800 mg per plate, with or without S9 metabolic activation (Andersen and Jensen, 1984).

Pulegone was reported to be weakly genotoxic in *Drosophila melanogaster* at a dose of 0.2 mL, based on the presence of small single spots in the wing spot test (Franzios *et al.*, 1997). However, pennyroyal oil reported to contain 75.7% pulegone was not mutagenic in the same assay at a dose of 2.1 mL.

STUDY RATIONALE

Pulegone is present in food and dental care products. In addition, essential oils and herbal products containing pulegone are proposed for the treatment of a variety of maladies. Toxicity and even death have been associated with pulegone exposure in humans. Pulegone was nominated for chronic toxicity and carcinogenicity testing by the National Institute of Environmental Health Sciences based on the potential for human exposure and the absence of carcinogenicity data. For these reasons, the National Toxicology Program performed a series of toxicity and carcinogenicity studies in rodents with special emphasis on hepatic effects. Humans are exposed to pulegone primarily through food, therefore an oral route of exposure was chosen. Pulegone was not palatable in feed; therefore, it was administered by gavage.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION

Pulegone

Pulegone was obtained from TCI America (Portland, OR) in one lot (OGI01) that was used in the 2-week, 3-month, and 2-year studies. Identity analyses were conducted by the analytical chemistry laboratory at Midwest Research Institute (Kansas City, MO) and by the study laboratory at Battelle Columbus Operations (Columbus, OH) (Appendix J). Purity and stability analyses were conducted by the analytical chemistry laboratory. Reports on analyses performed in support of the pulegone studies are on file at the National Institute of Environmental Health Sciences.

Lot OGI01 of the chemical, a pale yellow liquid, was identified as pulegone using infrared, ultraviolet/visible, and proton nuclear magnetic resonance spectroscopy and determinations of optical rotation, boiling point, density, and vapor pressure of the test article. Karl Fischer titration indicated 0.07% ± 0.01% water. Thin-layer chromatography indicated one major spot and no impurities. Gas chromatography indicated one major peak and 10 impurities with a combined area of 3.9% of the total peak area; two of the impurities had relative areas exceeding 1%. Of these, the first impurity was identified by gas chromatography coupled with mass spectrometry as isopulegone; the second could not be unequivocally identified due to interference from the large pulegone peak. The overall purity of lot OGI01 was determined to be approximately 96%.

Stability studies of the bulk chemical were performed using gas chromatography. These studies indicated that pulegone was stable as a bulk chemical for at least 2 weeks when stored protected from light under a nitrogen headspace at temperatures up to approximately 25° C. To ensure stability, the bulk chemical was stored at room temperature in sealed amber glass bottles. Periodic reanalyses of the bulk chemical were performed during the 2-week, 3-month, and 2-year studies using gas chromatography, and no degradation of the bulk chemical was detected.

Corn Oil

USP-grade corn oil was obtained in multiple lots from Spectrum Chemicals and Laboratory Products (Gardena, CA) and was used as the vehicle in the 2-week, 3-month, and 2-year studies. Periodic analyses of the corn oil vehicle by potentiometric titration demonstrated peroxide concentrations less than 3 mEq/kg.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared by mixing pulegone with corn oil to give the required concentrations (Table J2). The dose formulations were stored at room temperature in amber glass bottles sealed with Teflon®-lined lids for up to 28 days (2-week rat studies) or 35 days (all other studies). Because all dose formulations in the studies were determined to be solutions, no homogeneity studies were required. Stability studies of a 1.4 mg/mL formulation of a lot not used in the animal studies were performed using gas chromatography. Stability was confirmed for at least 35 days for dose formulations stored in sealed glass bottles and protected from light at room temperature and for at least 3 hours under simulated animal room conditions. Additional stability studies of a 0.9375 mg/mL formulation indicated that dose formulations were stable for 39 days when stored in sealed amber glass bottles at room temperature.

Periodic analyses of the dose formulations of pulegone were conducted by the study laboratory using gas chromatography. During the 2-week studies, the dose formulations were analyzed once; all seven dose formulations for rats and mice were within 10% of the target concentrations (Table J3). Animal room samples of these dose formulations were also analyzed; all five for rats and all five for mice were within 10% of the target concentrations. During the 3-month studies, the dose formulations were analyzed three times; animal room samples of these dose formulations were also analyzed (Table J4). Of the dose formulations analyzed and used during the studies, all 18 for rats and mice

were within 10% of the target concentrations; all 15 animal room samples for rats and all 15 for mice were within 10% of the target concentrations. During the 2-year studies, the dose formulations were analyzed approximately every 2 to 3 months; animal room samples were also analyzed (Table J5). Of the dose formulations analyzed and used during the studies, all 33 for rats and mice were within 10% of the target concentrations; 10 of 11 animal room samples for rats and all nine for mice were within 10% of the target concentrations.

2-WEEK STUDIES

Previous studies showed that no mortality was observed when rats were administered pulegone by gavage at 400 mg/kg per day for 5 days (Moorthy *et al.*, 1989a) or 160 mg/kg for 28 days (Mølck *et al.*, 1998). Moorthy and colleagues observed hepatic toxicity (necrosis) in male albino rats following pulegone administration. Mølck and colleagues reported that in the dosed female SPF Wistar rats (1) no liver histopathology or brain lesions were found; (2) plasma concentrations of alkaline phosphatase and ALT and absolute and relative liver weights were increased, indicating an adverse effect on the liver; and (3) body weight gains were reduced. Based on the observations from these previous studies in combination with the uncertainties associated with strain and sex differences, doses of 37.5, 75, 150, 300, and 600 mg pulegone/kg body weight were selected for the 2-week study in rats.

A single intraperitoneal dose of 400 mg/kg administered to Swiss-Webster mice caused centrilobular necrosis in the liver and necrosis of bronchial epithelial cells of the lung (Gordon *et al.*, 1982). A similar study done at 300 mg/kg in BALB/c and dYY mice found similar effects in the liver and increased mortality in the dYY mice (Gordon *et al.*, 1987; Mizutani *et al.*, 1987). Based on these data, it was estimated that a 300 mg/kg gavage dose of pulegone would approximate the maximum tolerated dose in a 2-week study; therefore, the doses selected for the 2-week study in mice were 18.75, 37.5, 75, 150, and 300 mg pulegone/kg body weight.

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 (rats) or 18 days (mice) and were 5 to 6 (rats) or 6 to 7 (mice) weeks old on the first day of the studies. Before the studies began, five male and five female rats and mice were examined for parasite evaluation and gross observation for evidence of disease.

Groups of five male and five female rats and mice were administered pulegone in corn oil by gavage at doses of 18.75 (mice only), 37.5, 75, 150, 300, or 600 (rats only) mg/kg body weight 5 days per week for 16 days. Groups of five male and five female control rats and mice received the corn oil vehicle alone. Feed and water were available *ad libitum*. All rats and female mice were housed five per cage; male mice were housed individually. Clinical findings were recorded daily for rats and mice. The animals were weighed initially, on day 8, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

At the end of the 2-week studies, necropsies were performed on all animals. The heart, right kidney, liver, lung, right testis, and thymus were weighed. Histopathologic examinations were performed on all organs that showed evidence of dose-related gross lesions plus corresponding organs from control animals. The brain, kidney, liver, and lung with mainstem bronchi were examined in all vehicle control and high dose animals (600 mg/kg rats and 300 mg/kg mice) and to a no-effect level in lower-dose animals. Table 1 lists the tissues and organs examined.

3-MONTH STUDIES

The 3-month studies were conducted to evaluate the cumulative toxic effects of repeated dosing with pulegone and to determine the appropriate doses to be used in 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 were 4 to 5 weeks old; the mice, 3 to 4 weeks old. Rats were quarantined for 11 (males) or 12 (females) days, and mice were quarantined for 19 (males) or 18 (females) days. Animals were 5 to 6 (male rats), 6 (female rats), or 6 to 7 (mice) 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. Serum samples were collected from five male and five female sentinel rats and mice at 4 weeks and at the end of the studies; serologic analyses were performed using the protocols of the NTP Sentinel Animal Program (Appendix L).

Groups of 10 male and 10 female core study rats and mice were administered pulegone in corn oil by gavage at doses of 9.375, 18.75, 37.5, 75, or 150 mg/kg 5 days per week for 14 weeks. Groups of 10 male and 10 female control rats and mice received the corn oil vehicle alone. Groups of 10 male and 10 female special study rats and mice were administered the same doses for 4 days, and additional groups of 10 male and 10 female special study rats were administered the same doses for 18 days. Feed and water were available *ad libitum*. Animals were housed five per cage (rats and female mice) or individually (male mice). Clinical findings were recorded weekly for rats and mice. The animals were weighed initially, weekly, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

Blood was collected from the retroorbital sinus of 10 male and 10 female special study animals on days 4 and 18 (rats only) and of all core study rats and mice at the end of the studies for hematology (rats only) and clinical chemistry analyses. Animals were anesthetized with a mixture of CO_2/O_2. Blood samples were collected into microcollection serum separator tubes, and serum was obtained by centrifugation for clinical chemistry. Blood was also collected into microcollection tubes containing potassium EDTA for hematology (rats). Using reagents obtained from the instrument manufacturer, clinical chemistry parameters were measured using a Hitachi 911 chemistry analyzer (Roche Diagnostics Corp., Indianapolis, IN), and hematology parameters were measured using a Cell Dyn 3500 automated cell counter (Abbott Laboratories, Abbott Park, IL). The parameters measured are listed in Table 1.

Groups of 10 male and 10 female special study rats and mice on day 4, 10 male and 10 female special study rats on day 18, and all core study rats and mice at the end of the studies were tested for reduced and oxidized liver glutathione. Following blood collection, animals were euthanized, and liver samples were collected, weighed, and frozen until analysis. For each frozen liver sample, a 1.0 M perchloric acid-2 mM EDTA tissue homogenate was prepared; the homogenate was then centrifuged. The resulting supernatant was placed into one tube from which two aliquots were removed, one portion for total glutathione determination and the other portion for the reduced glutathione determination. For measurement of total glutathione, the supernatant was diluted with 0.2 M phosphate-6.3 mM EDTA buffer and pipetted into a microtiter plate well. Two mM β-nicotinamide adenine dinucleotide phosphate was added. After a 5-minute wait, oxidized glutathione reductase in 6 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent) was added and mixed; the resulting reaction was monitored kinetically for 10 minutes by measuring the absorbance at 410 nm. The rate of color production was proportional to the amount of total glutathione in the sample. The concentration of total glutathione was calculated by interpolation from a standard curve conducted in the same assay plate. Reduced glutathione was determined in the same manner, except oxidized glutathione reductase was not included in the reduction mixture. Reduced glutathione concentration was subtracted from total glutathione concentration to determine oxidized glutathione concentration.

At the end of the 3-month studies, samples were collected for sperm motility and vaginal cytology evaluations on core study rats in the vehicle control, 18.75, 37.5, and 75 mg/kg groups and mice in the vehicle control, 37.5, 75, and 150 mg/kg groups. The parameters evaluated are listed in Table 1. For 12 consecutive days prior to scheduled terminal sacrifice, the vaginal vaults of the females were moistened with saline, if necessary, and samples of vaginal fluid and cells were stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus). Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides, and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65° C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

Necropsies were performed on all core study animals. The heart, right kidney, liver, lung, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (eyes were first fixed in Davidson's solution),

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 150 mg/kg groups. In rats, the bone marrow, brain, heart, kidney, liver, lung, ovary, thymus, glandular stomach, and uterus were examined to a no-effect level in the lower dose groups. In mice, the brain, kidney, liver, lung, and glandular stomach were examined to a no-effect level in the lower dose groups. Table 1 lists the tissues and organs routinely examined.

After a review of the laboratory reports and selected histopathology slides by a quality assessment pathologist, the findings and reviewed slides were submitted to a 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 by the NTP pathology review process. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, NTP pathologist, reviewing pathologist(s), if any, 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 administered pulegone in corn oil by gavage at doses of 18.75 (male rats only), 37.5, 75, or 150 mg/kg (mice and female rats) 5 days per week for up to 104 (rats) or 105 (mice) weeks. Groups of 50 male and 50 female control rats and mice received the corn oil vehicle alone. Due to excessive morbidity and mortality, the surviving high dose rats (75 mg/kg males and 150 mg/kg females) were administered only the corn oil vehicle from week 60 until the end of the study.

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. Rats were quarantined for 13 (males) or 14 (females) days and mice for 11 (females) or 12 (males) 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 were approximately 6 to 7 weeks old, and mice were 5 to 6 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 L).

Animal Maintenance

Male rats were housed up to three per cage; female rats, five per cage; male mice, individually; and female mice, three to five per cage. Feed and water were available *ad libitum*. Cages and racks were rotated every 2 weeks. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is provided in Appendix K.

Clinical Examinations and Pathology

All animals were observed twice daily. Clinical findings were recorded monthly beginning at week 5. Body weights were recorded initially, weekly for the first 13 weeks, monthly through week 61 and then every 2 weeks (rats) or monthly (mice) thereafter, and at the end of the studies.

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 first fixed in Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin for microscopic examination. In order to help differentiate renal histopathologic changes, Masson's Trichrome Method for collagen, Alkaline Congo Red Method for amyloid, and Jones' Method and Periodic Acid Schiff for basement membranes were used to stain the kidneys of five vehicle control and 15 dosed male and female rats and mice. Gormori's Method (Prussian Blue) for iron and Stein's Method for bile pigment/hemosiderin were used to stain the livers of up to five mice per sex. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The report, slides, paraffin blocks, residual wet tissues, and pathology data were sent to the NTP Archives for inventory, slide/block match, wet tissue audit, and storage. 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 kidney, liver, and nose of rats and mice; the lung of rats and female mice; the forestomach of mice and female rats; the adrenal gland (medulla), blood vessel (aorta), bone, cecum, heart, parathyroid gland, stomach (glandular), and thyroid gland of rats; the eye and ovary of female rats and female mice; the bone marrow, lymph node (mesenteric), and urinary bladder of female rats; and the spleen of female mice.

The quality assessment report and the reviewed slides were submitted to the NTP 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. 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 1
Experimental Design and Materials and Methods in the Gavage Studies of Pulegone

2-Week Studies	3-Month Studies	2-Year Studies
Study Laboratory		
Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)
Strain and Species		
F344/N rats B6C3F1 mice	F344/N rats B6C3F1 mice	F344/N rats B6C3F1 mice
Animal Source		
Taconic Farms, Inc. (Germantown, NY)	Taconic Farms, Inc. (Germantown, NY)	Taconic Farms, Inc. (Germantown, NY)
Time Held Before Studies		
Rats: 11 days Mice: 18 days	Rats: 11 (males) or 12 (females) days Mice: 18 (females) or 19 (males) days	Rats: 13 (males) or 14 (females) days Mice: 11 (females) or 12 (males) days
Average Age When Studies Began		
Rats: 5 to 6 weeks Mice: 6 to 7 weeks	Rats: 5 to 6 (males) or 6 (females) weeks Mice: 6 to 7 weeks	Rats: 6 to 7 weeks Mice: 5 to 6 weeks
Date of First Dose		
Rats: August 13, 2001 Mice: August 20, 2001	Rats: February 18 (males) or 19 (females), 2002 Mice: February 25 (females) or 26 (males), 2002	Rats: April 9 (males) or 10 (females), 2003 Mice: April 14 (females) or 15 (males), 2003
Duration of Dosing		
5 days/week for 16 days	5 days/week for 14 weeks	Rats: 5 days/week for 60 (stop-exposure groups) or 104 weeks; stop-exposure groups then received the vehicle until the end of the study Mice: 5 days/week for 105 weeks
Date of Last Dose		
Rats: August 28, 2001 Mice: September 4, 2001	Rats: May 21 (males) or 22 (females), 2002 Mice: May 29 (females) or 30 (males), 2002	Rats: April 4 (males) or 6 (females), 2005; except 75 mg/kg males and 150 mg/kg females switched to corn oil vehicle alone on May 28, 2004 Mice: April 12 (females) or 14 (males), 2005
Necropsy Dates		
Rats: August 29, 2001 Mice: September 5, 2001	Rats: May 22 (males) or 23 (females), 2002 Mice: May 30 (females) or 31 (males), 2002	Rats: April 4-5 (males) or 6-7 (females), 2005 Mice: April 11-13 (females) or 13-15 (males), 2005
Average Age at Necropsy		
Rats: 8 to 9 weeks Mice: 9 to 10 weeks	19 to 20 weeks	109 to 111 weeks
Size of Study Groups		
5 males and 5 females	10 males and 10 females (core study); 20 male and 20 female rats or 10 male and 10 female mice (special studies)	50 males and 50 females
Method of Distribution		
Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 2-week studies	Same as 2-week studies

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Pulegone

2-Week Studies	3-Month Studies	2-Year Studies
Animals per Cage		
Rats: 5 Mice: 1 (males) or 5 (females)	Rats: 5 Mice: 1 (males) or 5 (females)	Rats: up to 3 (males) or 5 (females) Mice: 1 (males) or 3 to 5 (females)
Method of Animal Identification		
Tail tattoo	Tail tattoo	Tail tattoo
Diet		
Irradiated NTP-2000 meal feed (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed twice weekly (rats and female mice) or weekly (male mice)	Irradiated NTP-2000 wafer feed (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed weekly	Same as 3-month studies
Water		
Tap water (Columbus municipal supply) via glass bottles equipped with stainless steel sipper tubes, available *ad libitum*, changed twice weekly (rats and female mice) or once weekly (male mice)	Tap water (Columbus municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*	Same as 3-month studies
Cages		
Polycarbonate cages (Lab Products, Inc., Seaford, DE), changed twice weekly (rats, female mice) or once weekly (male mice)	Same as 2-week studies	Same as 2-week studies
Bedding		
Irradiated Sani-Chips® (P.J. Murphy Forest Products Corp., Montville, NJ), changed twice weekly (rats, female mice) or once weekly (male mice)	Same as 2-week studies	Same as 2-week studies
Rack Filters		
Spun-bonded polyester (Snow Filtration Co., Cincinnati, OH), changed every 2 weeks	Same as 2-week studies	Same as 2-week studies
Racks		
Stainless Steel (Lab Products, Inc., Seaford, DE), changed every 2 weeks	Same as 2-week studies	Same as 2-week 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	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: 10/hour
Doses		
Rats: 0 (vehicle control), 37.5, 75, 150, 300, or 600 mg/kg in corn oil, dosing volume 5 mL/kg Mice: 0 (vehicle control), 18.75, 37.5, 75, 150, or 300 mg/kg in corn oil, dosing volume 10 mL/kg	0 (vehicle control), 9.375, 18.75, 37.5, 75, or 150 mg/kg; dosing volume 5 mL/kg (rats) or 10 mL/kg (mice)	Rats: 0 (vehicle control), 18.75 (males only), 37.5, 75, or 150 mg/kg (females only); dosing volume 5 mL/kg Mice: 0 (vehicle control), 37.5, 75, or 150 mg/kg; dosing volume 10 mL/kg

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Pulegone

2-Week Studies	3-Month Studies	2-Year Studies
Type and Frequency of Observation		
Observed twice daily; animals were weighed initially, on day 8, and at the end of the studies; clinical findings were recorded daily.	Observed twice daily; animals were weighed initially, weekly, and at the end of the studies; clinical findings were recorded weekly.	Observed twice daily; animals were weighed initially, weekly for the first 13 weeks, monthly through week 61 (rats) and then biweekly (rats) or monthly (mice) thereafter, and at the end of the studies. Clinical findings were recorded monthly beginning at week 5.
Method of Sacrifice		
Carbon dioxide asphyxiation	Same as 2-week studies	Same as 2-week 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 core study animals. Organs weighed were heart, right kidney, liver, lung, right testis, and thymus	Necropsies were performed on all animals.
Clinical Pathology		
None	Blood was collected from the retroorbital sinus of special study animals on days 4 or 18 (rats only) and from core study animals at the end of the studies for hematology (rats only) and clinical chemistry. ***Hematology:*** hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials ***Clinical chemistry:*** urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, γ-glutamyltransferase, 5′-nucleotidase, and bile acids	None

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Pulegone

2-Week Studies	3-Month Studies	2-Year Studies
Histopathology		
Histopathology was performed on vehicle control rats and mice, 600 mg/kg rats, and 300 mg/kg mice. In addition to gross lesions and tissue masses, the brain, kidney, liver, and lung with mainstem bronchi were examined to a no-effect level.	Complete histopathology was performed on all core study animals in the vehicle control and 150 mg/kg groups. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eye, gallbladder (mice), Harderian gland, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung and mainstem bronchii, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, stomach (forestomach and glandular), right testis with epididymis, thymus, thyroid gland, trachea, urinary bladder, and uterus. In rats, the bone marrow, brain, heart, kidney, liver, lung, ovary, glandular stomach, and uterus were examined to a no-effect level. In mice, the brain, kidney, liver, lung, and glandular stomach were examined 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, eye, gallbladder (mice), Harderian gland, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), right testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, and uterus.
Sperm Motility and Vaginal Cytology		
None	At the end of the studies, sperm samples were collected from core study male animals in the 0 (vehicle control), 18.75 (rats only), 37.5, 75, or 150 mg/kg (mice only) groups for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, spermatid counts, and epididymal spermatozoal motility and concentration. The left cauda, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from core study females dosed with 0 (vehicle control), 18.75 (rats only), 37.5, 75, or 150 mg/kg (mice only) for vaginal cytology evaluations. The percentage of time spent in the various estrous cycle stages and estrous cycle length were evaluated.	None
Hepatic Glutathione Determinations		
None	Liver samples were collected from special study animals on day 4 or 18 (rats only) and from core study animals at the end of the studies for glutathione determinations. Total glutathione, reduced glutathione, and oxidized glutathione were measured.	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; 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 neoplasms or nonneoplastic lesions are presented in Tables A1, A3, B1, B4, 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., mesentery, pleura, peripheral nerve, skeletal muscle, tongue, tooth, and Zymbal's gland) 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 Neoplasm 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, liver glutathione determinations, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) (as modified by Williams, 1986) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of 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. Because vaginal cytology data are proportions (the proportion of the observation period that an animal was in a given estrous stage), an arcsine transformation was used to bring the data into closer conformance with a normality assumption. Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for simultaneous equality of measurements across doses. Proportions of regular cycling females in each dosed group were compared to the vehicle control group using the Fisher exact test (Gart *et al.*, 1979). Tests for extended periods of estrus and diestrus were constructed based on a Markov chain model proposed by Girard and Sager (1987). For each dose group, a transition probability matrix was estimated for transitions among the proestrus, estrus, metestrus, and diestrus stages, with provision for extended stays within estrus and diestrus. Equality of transition matrices among dose groups and between the vehicle control group and each dosed group was tested using chi-square statistics.

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 treatment-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 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, these studies were audited retrospectively by an independent quality assurance contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

The genetic toxicity of pulegone was assessed by testing the ability of the chemical to induce mutations in various strains of *Salmonella typhimurium* and *Escherichia coli* 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 weak response in one sex only or negative results in both sexes 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

RATS

2-WEEK STUDY

All male rats and nearly all female rats in the 300 and 600 mg/kg groups died, or were euthanized when found in a moribund state, prior to the termination of the study (Table 2). All moribund sacrifices and early deaths were attributed to liver toxicity. Mean body weight gains of males administered 37.5 or 150 mg/kg were significantly less than that of the vehicle controls. Clinical findings in 300 and 600 mg/kg rats included nasal/eye discharge, thinness, lethargy, and ruffled fur.

Absolute liver and kidney weights of 37.5 and 150 mg/kg females and relative liver and kidney weights of all dosed groups of females were significantly greater than those of the vehicle control group (Table H1). Other organ weight differences in males and females were considered to be related to reduced body weights.

Macroscopic observations in the liver consisted of pale discoloration in four 300 mg/kg rats that corresponded microscopically to centrilobular to midzonal hepatocellular necrosis and hepatocellular vacuolization. The liver of one rat had a mottled red focus that corresponded microscopically to hepatocellular necrosis and hemorrhage, outlined by regions of hepatocellular vacuolization.

TABLE 2
Survival and Body Weights of Rats in the 2-Week Gavage Study of Pulegone

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	80 ± 4	157 ± 5	77 ± 3	
37.5	5/5	79 ± 3	141 ± 4	62 ± 3**	90
75	5/5	79 ± 4	152 ± 7	73 ± 4	97
150	5/5	80 ± 3	137 ± 5	57 ± 2**	88
300	0/5[c]	79 ± 3	—	—	—
600	0/5[c]	81 ± 4	—	—	—
Female					
0	5/5	72 ± 3	112 ± 2	40 ± 1	
37.5	5/5	73 ± 3	119 ± 3	47 ± 2	107
75	5/5	71 ± 1	112 ± 2	41 ± 2	100
150	5/5	73 ± 3	115 ± 2	42 ± 4	103
300	1/5[c]	73 ± 2	79	14	71
600	0/5[c]	73 ± 3	—	—	—

** Significantly different ($P \leq 0.01$) from the vehicle control group by Dunnett's test

[a] Number of animals surviving at 2 weeks/number initially in group

[b] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.

[c] All early deaths occurred on or before study day 5.

The incidences of necrosis and cytoplasmic vacuolization of the liver in 300 and 600 mg/kg males and females were significantly greater than those in the vehicle control groups (Table 3). Hepatocellular necrosis was characterized microscopically as centrilobular in distribution, yet it sometimes extended into zone 2 to bridge lobules. Centrilobular and midzonal necrosis were sometimes accompanied by hemorrhage. Swollen hepatocytes with fine vacuolated cytoplasm characterized hepatocellular vacuolization, often noted at the peripheral margin of regions of necrosis. One 300 mg/kg female survived until the end of the study and exhibited hepatocellular degeneration, typified by hepatocellular dissociation and generalized hepatocyte glycogen loss.

Dose Selection Rationale: Based on mortality and liver lesions observed in the 2-week study at doses of 300 and 600 mg pulegone/kg body weight, doses selected for the 3-month gavage study in rats were 9.375, 18.75, 37.5, 75, and 150 mg/kg.

TABLE 3
Incidences of Nonneoplastic Lesions of the Liver in Rats in the 2-Week Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
Number Examined Microscopically	5	0	5	5	5	5
Necrosis[a]	0		0	1 (1.0)[b]	5** (2.2)	4* (1.8)
Cytoplasmic Vacuolization	0		0	1 (1.0)	5** (2.6)	5** (2.0)
Female						
Number Examined Microscopically	5	0	5	5	5	5
Necrosis	0		0	0	4* (2.0)	5** (1.4)
Cytoplasmic Vacuolization	0		0	0	4* (2.5)	5** (2.0)
Degeneration	0		0	0	1 (1.0)	0

* 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

3-MONTH STUDY

All rats survived until the end of the study except for one female in the 150 mg/kg group that died on day 9 (Table 4). The final mean body weights and body weight gains of 75 and 150 mg/kg males and 150 mg/kg females were significantly less than those of the vehicle controls (Table 4 and Figure 2). No chemical-related clinical findings were noted.

TABLE 4
Survival and Body Weights of Rats in the 3-Month Gavage Study of Pulegone

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	104 ± 4	358 ± 5	254 ± 4	
9.375	10/10	103 ± 3	358 ± 5	255 ± 5	100
18.75	10/10	104 ± 4	348 ± 3	243 ± 4	97
37.5	10/10	104 ± 3	353 ± 5	249 ± 7	99
75	10/10	104 ± 4	324 ± 9**	220 ± 7**	91
150	10/10	104 ± 4	248 ± 3**	144 ± 5**	69
Female					
0	10/10	99 ± 2	202 ± 2	103 ± 3	
9.375	10/10	100 ± 2	204 ± 3	104 ± 4	101
18.75	10/10	100 ± 3	195 ± 3	95 ± 2	97
37.5	10/10	99 ± 3	195 ± 2	96 ± 3	96
75	10/10	99 ± 3	195 ± 3	96 ± 3	97
150	9/10[c]	100 ± 2	180 ± 3**	80 ± 4**	89

** Significantly different ($P \leq 0.01$) from the vehicle control group by Williams' test

[a] Number of animals surviving at 3 months/number initially in group

[b] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.

[c] Week of death: 2

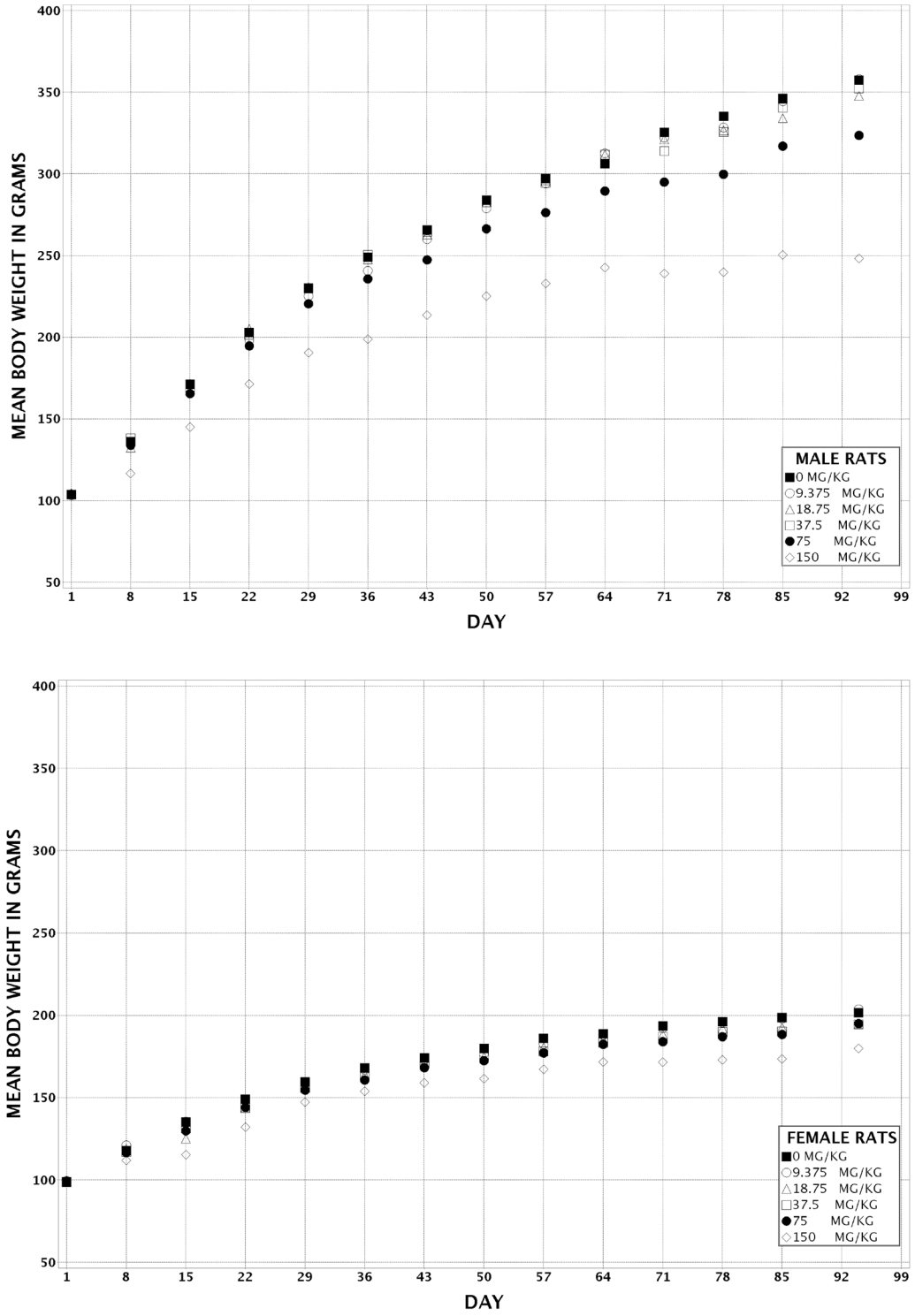


FIGURE 2
Growth Curves for Rats Administered Pulegone by Gavage for 3 Months

The hematology and clinical chemistry data for rats are listed in Tables 5 and F1. At the end of the study, there was a small (≤8%) dose-related decrease in the erythron, evidenced by decreases in the hematocrit and hemoglobin values and the erythrocyte counts. The erythron decrease was evident in the 37.5, 75, and 150 mg/kg groups. An apparent erythroid response to the decreased erythron was evidenced by increased reticulocyte counts. Platelet counts were also elevated at the end of the study in the 75 mg/kg male and 150 mg/kg male and female groups, and the elevations may be part of a generalized hematopoietic response. At week 14, the neutrophil and white blood cell numbers were slightly elevated in the 150 mg/kg groups and could suggest the occurrence of an inflammatory response.

At all time points, alanine aminotransferase (ALT) activity was increased, in a dose-related fashion, in the higher dose males and females (Tables 5 and F1). At day 4, the increases occurred in the 37.5, 75, and 150 mg/kg male and female groups. With time, however, the ALT effect ameliorated and involved only the 150 mg/kg rats by the end of the study. Sorbitol dehydrogenase activity appeared to be unaffected, suggesting the ALT effect was related to a transient enzyme induction rather than hepatocellular injury. Alkaline phosphatase and γ-glutamyl transferase activities and bile acid concentrations were increased in a dose-related fashion at all time points in the higher dose (particularly the 150 mg/kg) male and female animals and would be consistent with a cholestatic event in the liver.

Compared to the vehicle controls on day 4, reduced and total glutathione levels were significantly increased only in 75 and 150 mg/kg males (22% and 42%, respectively); oxidized glutathione levels were not significantly increased in any dosed group (Table G1). On day 18, significant increases in reduced glutathione occurred in 150 mg/kg males (79%) and 75 (24%) and 150 (120%) mg/kg females, significant increases in oxidized glutathione occurred in 37.5 mg/kg or greater males (27% to 100%) and females (24% to 78%), and total glutathione levels were significantly increased in 75 (21%) and 150 (86%) mg/kg males and in 37.5 mg/kg or greater females (22% to 107%). At week 14, reduced glutathione was significantly increased in 75 (23%) and 150 (83%) mg/kg males and in 18.75 mg/kg or greater females (17% to 95%). Oxidized glutathione levels were not significantly increased in dosed males at week 14 but were significantly increased in females administered 18.75 mg/kg or greater (64% to 144%). Total glutathione levels were significantly increased in 75 (23%) and 150 (64%) mg/kg males and in all dosed groups of females (23% to 108%). The ratios of reduced glutathione to oxidized glutathione in dosed groups of males and females were not significantly different from the vehicle controls at any time point; however, the ratios in the 150 mg/kg males and females at week 14 were increased 52% and 81%, respectively, over those of the vehicle controls.

Absolute and relative liver weights of 75 and 150 mg/kg females and relative liver weights of males administered 18.75 mg/kg or greater were significantly greater than those of the vehicle controls (Table H2). The absolute kidney weight of 150 mg/kg females and the relative kidney weights of all dosed groups, except 9.375 mg/kg males, were significantly greater than those of the vehicle controls. Absolute and relative thymus weights of 150 mg/kg males and females and the absolute thymus weight of 75 mg/kg males were significantly less than those of the vehicle controls. Other organ weight changes were related to body weight changes and were not considered chemical related.

No significant differences were observed in the sperm parameters of male rats or in the estrous cyclicity of female rats administered 18.75, 37.5, or 75 mg/kg when compared to the vehicle controls (Tables I1 and I2).

TABLE 5
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Male						
n	10	10	10	10	10	10
Hematology						
Hematocrit (%)						
Day 4	39.7 ± 0.4	38.5 ± 0.3	38.8 ± 0.5	39.3 ± 0.5	39.5 ± 0.5	42.0 ± 0.5*
Day 18	40.1 ± 0.5	41.9 ± 0.5	42.1 ± 0.3**	41.0 ± 0.3	41.6 ± 0.4	41.0 ± 0.4
Week 14	43.0 ± 0.3	43.7 ± 0.4	42.3 ± 0.4	41.6 ± 0.4*	40.8 ± 0.3**	39.4 ± 0.6**
Hemoglobin (g/dL)						
Day 4	13.0 ± 0.1	12.7 ± 0.1	12.7 ± 0.2	12.9 ± 0.1	13.0 ± 0.2	13.9 ± 0.2*
Day 18	13.4 ± 0.1	13.7 ± 0.1	13.9 ± 0.1	13.6 ± 0.1	13.6 ± 0.1	13.6 ± 0.1
Week 14	15.2 ± 0.1	15.4 ± 0.1	14.9 ± 0.1	14.9 ± 0.1	14.6 ± 0.1**	14.1 ± 0.2**
Erythrocytes (10^6/μL)						
Day 4	7.11 ± 0.07	6.84 ± 0.06	6.93 ± 0.11	7.01 ± 0.09	7.05 ± 0.09	7.71 ± 0.09**
Day 18	7.29 ± 0.09	7.62 ± 0.10	7.62 ± 0.08*	7.38 ± 0.07	7.41 ± 0.07	7.39 ± 0.09
Week 14	8.55 ± 0.07	8.58 ± 0.08	8.28 ± 0.06*	8.05 ± 0.08**	7.88 ± 0.06**	8.11 ± 0.13**
Reticulocytes (10^6/μL)						
Day 4	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0**
Day 18	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0*	0.5 ± 0.0**
Week 14	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0**	0.3 ± 0.0**
Platelets (10^3/μL)						
Day 4	912.5 ± 14.3	921.6 ± 15.5	890.1 ± 17.1	866.3 ± 21.1	854.4 ± 15.1*	737.7 ± 12.3**
Day 18	883.1 ± 16.0	821.3 ± 35.1	886.7 ± 24.1	879.2 ± 17.7	918.4 ± 8.5	1,028.4 ± 29.5**
Week 14	687.2 ± 36.9	674.8 ± 49.8	696.3 ± 29.6	773.3 ± 14.2	930.7 ± 17.0**	1,073.2 ± 43.1**
Segmented neutrophils (10^3/μL)						
Day 4	0.83 ± 0.03	0.98 ± 0.08	0.83 ± 0.06	0.96 ± 0.07	0.95 ± 0.08	1.73 ± 0.16**
Day 18	0.90 ± 0.07	0.82 ± 0.13	0.94 ± 0.08	0.90 ± 0.09	0.92 ± 0.09	1.05 ± 0.09
Week 14	1.29 ± 0.16	1.05 ± 0.10	1.36 ± 0.09	1.15 ± 0.09	1.48 ± 0.09	2.25 ± 0.17**
Clinical Chemistry						
Alanine aminotransferase (IU/L)						
Day 4	58.3 ± 2.2	62.1 ± 2.2	63.7 ± 1.9	65.8 ± 1.5*	73.9 ± 2.6**	78.5 ± 1.9**
Day 18	50.7 ± 1.5	54.1 ± 1.4	54.2 ± 1.6	54.1 ± 1.4	59.4 ± 1.5**	79.2 ± 2.4**
Week 14	45.1 ± 1.6	45.1 ± 1.9	38.0 ± 1.1	39.6 ± 1.4	43.3 ± 1.0	63.4 ± 2.9*
Sorbitol dehydrogenase (IU/L)						
Day 4	10.9 ± 0.5	10.1 ± 0.4	10.8 ± 0.5	9.8 ± 0.4	11.5 ± 0.8	16.1 ± 1.3**
Day 18	12.5 ± 0.7	14.1 ± 0.7	12.1 ± 0.5	12.0 ± 0.7	13.6 ± 0.9	14.4 ± 0.5
Week 14	18.2 ± 1.0	16.0 ± 0.8	13.4 ± 0.8**	12.8 ± 1.0**	10.9 ± 0.9**	13.9 ± 1.6**
Alkaline phosphatase (IU/L)						
Day 4	762.9 ± 24.7	801.3 ± 25.9	810.0 ± 23.7	803.1 ± 14.6	909.3 ± 22.9**	1,144.4 ± 42.2**
Day 18	619.8 ± 11.2	619.5 ± 12.0	660.0 ± 15.8	674.6 ± 14.2*	703.6 ± 7.8**	810.1 ± 17.3**
Week 14	249.9 ± 6.3	259.8 ± 5.1	263.2 ± 6.0	294.7 ± 6.9**	350.3 ± 5.8**	542.7 ± 14.7**
γ-Glutamyltransferase (IU/L)						
Day 4	0.50 ± 0.17	0.20 ± 0.13	0.40 ± 0.16	0.70 ± 0.15	0.50 ± 0.17	5.70 ± 1.04**
Day 18	2.80 ± 0.53	2.80 ± 0.51	3.60 ± 0.34	3.00 ± 0.45	3.10 ± 0.38	5.90 ± 0.91*
Week 14	0.00 ± 0.00	0.10 ± 0.10	0.20 ± 0.20	0.20 ± 0.20	0.10 ± 0.10	10.30 ± 1.10**
Bile acids (μmol/L)						
Day 4	32.3 ± 4.5	30.8 ± 3.6	33.1 ± 3.7	43.2 ± 5.3	36.1 ± 4.3	257.4 ± 44.6**
Day 18	37.3 ± 2.8	33.1 ± 3.0	44.0 ± 3.4	43.2 ± 3.2	43.1 ± 5.8	76.7 ± 11.7**
Week 14	26.9 ± 2.1	29.1 ± 2.6	27.0 ± 2.6	36.7 ± 3.2	53.6 ± 4.5**	167.4 ± 19.5**

TABLE 5
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Pulegone

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Female						
n						
Day 4	10	10	10	10	10	10
Day 18	10	10	10	10	10	10
Week 14	10	10	10	10	10	9
Hematology						
Hematocrit (%)						
Day 4	42.7 ± 0.4	42.6 ± 0.3	42.1 ± 0.5	41.8 ± 0.4	42.0 ± 0.4	41.9 ± 0.4
Day 18	44.4 ± 0.4	44.5 ± 0.5	44.6 ± 0.5	44.2 ± 0.5	43.9 ± 0.6	45.2 ± 0.6
Week 14	40.6 ± 0.4	41.1 ± 0.3	39.7 ± 0.3	39.5 ± 0.4	38.9 ± 0.3**	37.5 ± 0.3**
Hemoglobin (g/dL)						
Day 4	13.8 ± 0.2	13.8 ± 0.1	13.5 ± 0.2	13.5 ± 0.1	13.6 ± 0.1	13.6 ± 0.1
Day 18	14.6 ± 0.2	14.6 ± 0.2	14.5 ± 0.2	14.5 ± 0.2	14.4 ± 0.2	14.8 ± 0.2
Week 14	14.7 ± 0.1	14.7 ± 0.1	14.5 ± 0.1	14.3 ± 0.1*	14.3 ± 0.1**	13.5 ± 0.1**
Erythrocytes (10^6/µL)						
Day 4	7.41 ± 0.08	7.40 ± 0.06	7.24 ± 0.10	7.21 ± 0.07	7.27 ± 0.08	7.28 ± 0.07
Day 18	7.89 ± 0.08	7.86 ± 0.11	7.84 ± 0.10	7.79 ± 0.10	7.67 ± 0.10	7.96 ± 0.12
Week 14	7.55 ± 0.06	7.64 ± 0.06	7.38 ± 0.06	7.26 ± 0.08*	6.98 ± 0.05**	7.03 ± 0.06**
Reticulocytes (10^6/µL)						
Day 4	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Day 18	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0**	0.2 ± 0.0**
Week 14	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0*	0.2 ± 0.0*	0.2 ± 0.0**	0.3 ± 0.0**
Platelets (10^3/µL)						
Day 4	903.0 ± 17.7	877.8 ± 17.9	898.1 ± 29.0	898.0 ± 22.4	887.4 ± 31.5	813.2 ± 12.8**
Day 18	877.9 ± 35.4	920.2 ± 20.8	941.4 ± 16.6	868.7 ± 26.4	869.0 ± 10.6	810.2 ± 26.7
Week 14	718.8 ± 17.3	713.3 ± 10.0	758.2 ± 14.6	744.1 ± 10.9	751.6 ± 12.5	811.7 ± 17.5**
Segmented neutrophils (10^3/µL)						
Day 4	0.85 ± 0.06	0.78 ± 0.08	0.94 ± 0.08	0.79 ± 0.04	0.80 ± 0.07	0.80 ± 0.06
Day 18	0.96 ± 0.11	0.89 ± 0.08	0.94 ± 0.06	0.94 ± 0.07	0.97 ± 0.10	0.99 ± 0.10
Week 14	1.03 ± 0.09	1.01 ± 0.06	1.18 ± 0.15	1.05 ± 0.10	0.94 ± 0.10	1.89 ± 0.24*
Clinical Chemistry						
Alanine aminotransferase (IU/L)						
Day 4	45.8 ± 1.0	50.6 ± 1.5	46.2 ± 1.2	53.2 ± 1.8**	57.7 ± 1.9**	73.3 ± 2.9**
Day 18	40.7 ± 1.9	41.1 ± 1.1	41.8 ± 1.3	43.5 ± 1.6	44.6 ± 1.2*	71.3 ± 4.2**
Week 14	37.8 ± 2.7	50.6 ± 6.8	35.2 ± 1.2	32.8 ± 0.9	39.7 ± 1.2	57.8 ± 1.2**
Sorbitol dehydrogenase (IU/L)						
Day 4	10.4 ± 0.5	10.7 ± 0.7	10.6 ± 0.4	9.7 ± 0.4	11.1 ± 0.4	13.8 ± 0.4**
Day 18	10.9 ± 0.2	12.1 ± 1.0	11.0 ± 0.3	11.2 ± 0.3	10.8 ± 0.5	12.7 ± 0.4*
Week 14	11.5 ± 1.0	16.3 ± 2.1	10.2 ± 0.8	8.7 ± 0.9	11.2 ± 1.3	10.2 ± 0.6

TABLE 5
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Pulegone

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Female (continued)						
n						
Day 4	10	10	10	10	10	10
Day 18	10	10	10	10	10	10
Week 14	10	10	10	10	10	9
Clinical Chemistry (continued)						
Alkaline phosphatase (IU/L)						
Day 4	573.7 ± 11.2	579.8 ± 12.3	596.8 ± 19.3	595.3 ± 18.2	645.3 ± 12.5**	848.7 ± 32.2**
Day 18	420.4 ± 7.1	437.0 ± 11.6	432.5 ± 11.3	441.3 ± 9.2	462.9 ± 8.6**	644.6 ± 28.0**
Week 14	212.9 ± 7.7	219.8 ± 5.4	214.6 ± 8.4	234.2 ± 6.1*	284.7 ± 9.0**	535.6 ± 14.8**
γ-Glutamyltransferase (IU/L)						
Day 4	0.20 ± 0.13	0.70 ± 0.15	0.30 ± 0.15	0.80 ± 0.25	0.60 ± 0.16	1.30 ± 0.15**
Day 18	0.40 ± 0.16	0.00 ± 0.00	0.40 ± 0.16	0.30 ± 0.15	0.30 ± 0.15	2.20 ± 0.33**
Week 14	0.50 ± 0.17	0.20 ± 0.13	0.30 ± 0.15	0.20 ± 0.13	0.10 ± 0.10	4.89 ± 0.48**
Bile acids (μmol/L)						
Day 4	21.7 ± 1.9	25.2 ± 2.9	28.2 ± 3.3	33.1 ± 3.4**	29.0 ± 3.4*	38.3 ± 4.3**
Day 18	32.3 ± 2.4	29.1 ± 3.5	35.5 ± 3.1	32.1 ± 3.4	41.3 ± 6.9	75.9 ± 9.8**
Week 14	19.7 ± 1.6	27.2 ± 1.7**	30.6 ± 2.1**	27.3 ± 2.3**	45.0 ± 5.1**	87.6 ± 7.0**

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test
** $P \leq 0.01$
[a] Mean ± standard error. Statistical tests were performed on unrounded data.

Kidney hyaline glomerulopathy was present in 75 mg/kg males and in 150 mg/kg males and females (Table 6). The incidences of renal tubule protein casts were increased in 150 mg/kg males and females, and the increase in females was significant. The term 'glomerulopathy' was diagnosed when numerous, small, round, eosinophilic globules were present within the glomerular mesangium (Plate 1). The mesangium was typically expanded in the areas containing the globules. Rare aggregates of pyknotic debris were also present in small numbers of glomeruli but were not noted separately. The globules were often clustered tightly together and were located adjacent to capillary loops. The nature of the material was not clear, but protein globules were suspected based on their morphology. The globules were only apparent in a subset of glomeruli; a grade of minimal was assigned when the globules were present in less than 25% of glomeruli and a grade of mild was assigned when the globules were present in 25% to 50% of glomeruli. Mild was the highest grade assigned in this study. The globules stained weakly with Periodic Acid Schiff (PAS) and stained strongly red with Masson's Trichrome stain (Plate 2). This lesion was most common in animals that also had tubular protein casts in the medulla.

In the liver, incidences of bile duct hyperplasia and hepatocyte hypertrophy in 75 and 150 mg/kg males and 150 mg/kg females were significantly increased compared to those in the vehicle controls (Table 6). The incidence of hepatocyte focal necrosis in 150 mg/kg males was also significantly increased. In 150 mg/kg rats of both sexes, incidences of oval cell hyperplasia and periportal fibrosis were significantly increased.

Incidences of bone marrow hyperplasia in 37.5 mg/kg males and 75 and 150 mg/kg males and females were significantly increased (Table 6). Incidences of heart mineralization in 150 mg/kg males, glandular stomach mineralization in 75 and 150 mg/kg females, and cellular histiocytic infiltration in the lung and ovarian cyst in 150 mg/kg females were significantly increased. The incidence of glandular stomach mineralization was increased, but not significantly, in 150 mg/kg males.

TABLE 6
Incidences of Selected Nonneoplastic Lesions in Rats in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Male						
Kidney[a]	10	10	10	10	10	10
Hyaline Glomerulopathy[b]	0	0	0	0	2 (1.0)[c]	10** (1.0)
Renal Tubule Protein Casts	7 (1.0)	4 (1.5)	6 (1.0)	6 (1.0)	4 (1.0)	10 (2.7)
Liver	10	10	10	10	10	10
Bile Duct Hyperplasia	0	0	0	0	9** (1.0)	10** (2.0)
Hepatocyte Hypertrophy	0	0	0	0	10** (1.0)	10** (1.9)
Hepatocyte Focal Necrosis	0	0	0	0	0	6** (1.0)
Oval Cell Hyperplasia	0	0	0	0	0	10** (1.0)
Periportal Fibrosis	0	0	0	0	0	10** (1.0)
Bone Marrow	10	10	10	10	10	10
Hyperplasia	3 (1.0)	1 (1.0)	5 (1.0)	9** (1.2)	10** (1.9)	10** (2.7)
Heart	10	10	10	10	10	10
Mineralization	1 (1.0)	0	0	1 (1.0)	0	6* (1.0)
Glandular Stomach	10	10	10	10	10	10
Mineralization	5 (1.0)	3 (1.0)	5 (1.8)	2 (1.0)	3 (1.0)	9 (1.1)
Female						
Kidney	10	10	10	10	10	10
Hyaline Glomerulopathy	0	0	0	0	0	8** (1.0)
Renal Tubule Protein Casts	1 (1.0)	0	0	0	2 (1.0)	6* (1.0)
Liver	10	10	10	10	10	10
Bile Duct Hyperplasia	0	0	0	0	1 (1.0)	10** (1.7)
Hepatocyte Hypertrophy	0	0	0	0	0	10** (1.9)
Oval Cell Hyperplasia	0	0	0	0	0	9** (1.0)
Periportal Fibrosis	0	0	0	0	0	9** (1.0)
Bone Marrow	10	10	10	10	10	10
Hyperplasia	1 (1.0)	1 (1.0)	1 (1.0)	1 (1.0)	10** (1.3)	9** (1.9)
Glandular Stomach	10	10	10	10	10	10
Mineralization	0	3 (1.0)	2 (1.0)	2 (1.0)	6** (1.0)	7** (1.0)
Lung	10	10	10	10	10	10
Cellular Histiocytic Infiltration	1 (1.0)	0	0	2 (1.0)	2 (1.0)	6* (1.0)
Ovary	10	10	10	10	10	10
Cyst	1	2	2	2	3	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 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

Dose Selection Rationale: Body weight gains were the major factor in the dose selection for the 2-year rat study, as the 150 mg/kg males in the 3-month study weighed about 30% less than the vehicle controls and the 75 mg/kg males and 150 mg/kg females weighed about 10% less than their respective vehicle controls at terminal sacrifice. The dose concentrations that caused 10% body weight differences were considered close to the maximum tolerated dose and were acceptable as the dose concentrations for the 2-year study. The effects of pulegone on clinical chemistry (alkaline phosphatase) and hematology (hematocrit and bone marrow hyperplasia) in the 75 mg/kg males and 150 mg/kg females in the 3-month study were considered not serious enough to influence the decision of selecting 75 and 150 mg/kg as high doses for males and females, respectively, for the 2-year study. The liver (hepatocyte hypertrophy and bile duct hyperplasia) was considered the target organ for males and females. The hepatic reduced glutathione data were considered inconclusive, as it was not certain whether they represent a rebound after depletion. Based on these data, it was decided that the highest dose concentrations for the 2-year study of pulegone would be 75 mg/kg for male rats and 150 mg/kg for female rats. Lower doses were set by halving the high doses.

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male and female rats are shown in Table 7 and in the Kaplan-Meier survival curves (Figure 3). Because of the large number of early deaths in 75 mg/kg males and reduced body weights in 150 mg/kg females, dosing with pulegone was stopped in these groups during week 60; these groups were administered the gavage vehicle until the end of the study. These dose groups are referred to as 75 mg/kg stop-exposure and 150 mg/kg stop-exposure. Survival of the 75 mg/kg stop-exposure males was significantly decreased, with only two rats in this group surviving to the end of the study; survival of 37.5 mg/kg males was also significantly less than that of the vehicle controls. Survival of the 150 mg/kg stop-exposure females was significantly less than that of the vehicle controls, with no survivors at the end of the study. The majority of early deaths in stop-exposure male and female rats were attributed to end-stage renal disease.

TABLE 7
Survival of Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Male				
Animals initially in study	50	50	50	50
Accidental death[a]	0	1	0	0
Moribund	8	10	12	35
Natural deaths	3	7	10	13
Animals surviving to study termination	39	32	28	2
Percent probability of survival at end of study[b]	78	65	56	4
Mean survival (days)[c]	685	680	687	514
Survival analysis[d]	P<0.001	P=0.216	P=0.034	P<0.001
	Vehicle Control	**37.5 mg/kg**	**75 mg/kg**	**150 mg/kg (Stop-Exposure)**
Female				
Animals initially in study	50	50	50	50
Accidental deaths[a]	0	1	0	1
Moribund	13	8	7	29
Natural deaths	6	4	5	20
Animals surviving to study termination	31	37	38	0
Percent probability of survival at end of study	62	76	76	0
Mean survival (days)	682	697	712	536
Survival analysis	P<0.001	P=0.168N	P=0.104N	P<0.001

[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 results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. Lower mortality in a dose group is indicated by **N**.

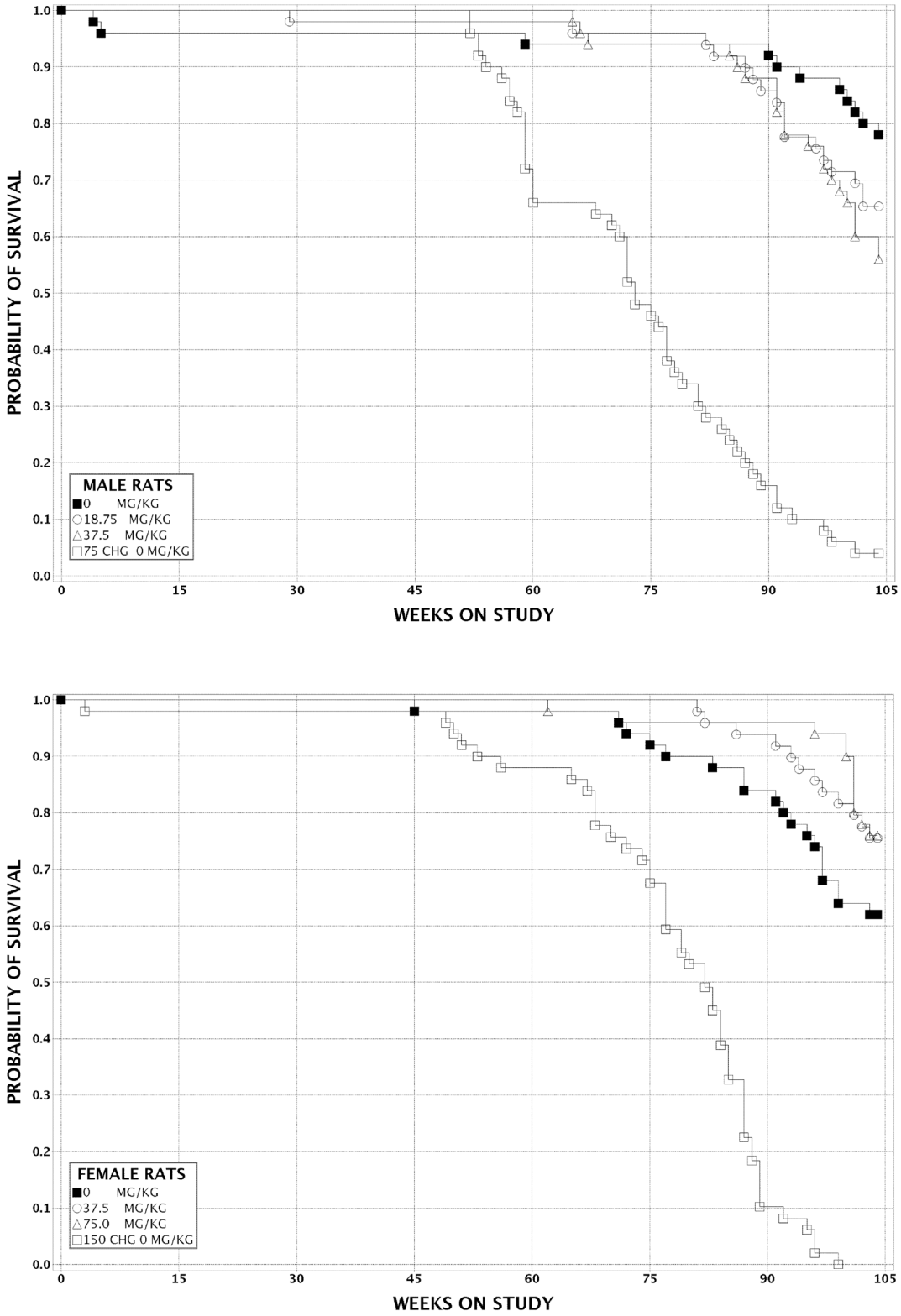


FIGURE 3
Kaplan-Meier Survival Curves for Rats Administered Pulegone by Gavage for 2 Years

Body Weights and Clinical Findings

Compared to those of the vehicle controls, mean body weights were less in 75 mg/kg stop-exposure males after week 13 and in 75 mg/kg and 150 mg/kg stop-exposure females after weeks 21 and 9, respectively (Tables 8 and 9; Figure 4). Clinical findings included thinness, lethargy, and ruffled fur in the 75 mg/kg stop-exposure males and 150 mg/kg stop-exposure females.

TABLE 8
Mean Body Weights and Survival of Male Rats in the 2-Year Gavage Study of Pulegone

Days on Study	Vehicle Control		18.75 mg/kg			37.5 mg/kg			75 mg/kg (Stop-Exposure)		
	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	121	50	122	101	50	122	100	50	122	100	50
8	152	50	154	101	50	153	100	50	150	98	50
15	188	50	191	101	50	188	100	50	182	97	50
22	220	50	225	103	50	220	100	50	211	96	50
29	241	48	246	102	50	239	99	50	227	95	50
36	263	48	268	102	50	259	98	50	248	94	50
43	279	48	282	101	50	273	98	50	261	94	50
50	292	48	296	102	50	285	98	50	272	93	50
57	305	48	312	102	50	300	98	50	287	94	50
64	318	48	325	102	50	312	98	50	295	93	50
71	329	48	335	102	50	322	98	50	303	92	50
78	339	48	347	102	50	330	97	50	311	92	50
85	347	48	357	103	50	341	98	50	319	92	50
113	381	48	389	102	50	370	97	50	342	90	50
141	404	48	413	102	50	392	97	50	361	90	50
169	424	48	434	102	50	412	97	50	377	89	50
197	437	48	445	102	50	421	97	50	380	87	50
225	450	48	457	101	49	435	97	50	390	87	50
253	462	48	469	101	49	445	96	50	399	86	50
281	471	48	476	101	49	453	96	50	407	86	50
309	472	48	482	102	49	457	97	50	407	86	50
337	486	48	492	101	49	468	96	50	408	84	50
365	490	48	497	102	49	470	96	50	398	81	48
393	499	48	505	101	48	478	96	50	401	80	43
421	507	47	513	101	48	483	95	50	399	79	33
435	504	47	513	102	48	484	96	50	409	81	33
449	506	47	518	103	48	487	96	50	413	82	33
463	508	47	518	102	47	486	96	48	409	81	33
477	512	47	522	102	47	494	96	47	408	80	32
491	515	47	525	102	47	496	96	47	405	79	31
505	515	47	526	102	47	497	97	47	397	77	26
519	509	47	523	103	47	493	97	47	399	78	24
533	507	47	516	102	47	493	97	47	385	76	21
547	510	47	515	101	47	490	96	47	397	78	17
561	512	47	517	101	47	491	96	47	393	77	15
575	512	47	518	101	46	493	96	47	388	76	14
589	508	47	514	101	45	486	96	47	377	74	13
603	506	47	512	101	45	479	95	45	369	73	11
617	503	47	507	101	43	481	96	44	372	74	9
631	500	46	504	101	42	476	95	44	363	73	8
645	500	45	513	103	38	476	95	39	359	72	6
659	499	44	512	103	38	470	94	38	369	74	5
673	499	44	509	102	36	469	94	37	352	71	4
687	495	44	507	103	35	465	94	35	349	71	3
701	499	41	504	101	34	467	94	31	336	67	3
715	492	40	500	102	32	455	92	30	331	67	2
Mean for weeks											
1-13	261		266	102		257	99		245	94	
14-52	443		451	102		428	97		386	87	
53-103	504		513	102		482	96		382	76	

TABLE 9
Mean Body Weights and Survival of Female Rats in the 2-Year Gavage Study of Pulegone

Days on Study	Vehicle Control		37.5 mg/kg			75 mg/kg			150 mg/kg (Stop-Exposure)		
	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	105	50	105	100	50	105	100	50	105	100	50
8	120	50	119	100	50	118	98	50	114	95	50
15	136	50	135	99	50	133	97	50	129	94	50
22	148	50	148	99	50	145	97	50	141	95	49
29	159	50	156	98	50	154	97	50	149	94	49
36	165	50	162	98	50	159	96	50	152	92	49
43	174	50	170	98	50	167	96	50	163	94	49
50	178	50	174	98	50	171	96	50	162	91	49
57	184	50	180	98	50	175	95	50	168	92	49
64	188	50	182	97	50	177	94	50	166	88	49
71	191	50	186	97	50	182	95	50	173	90	49
78	194	50	188	97	50	184	95	50	175	90	49
85	197	50	189	96	49	184	93	50	169	86	49
113	209	50	199	95	49	193	92	50	180	86	49
141	217	50	208	96	49	199	92	50	182	84	49
169	229	50	217	95	49	206	90	50	181	79	49
197	232	50	221	95	49	207	89	50	181	78	49
225	241	50	227	94	49	210	87	50	179	74	49
253	247	50	233	94	49	215	87	50	185	75	49
281	255	50	242	95	49	220	86	50	188	74	49
309	261	49	247	95	49	223	86	50	169	65	49
337	272	49	255	94	49	227	84	50	186	68	49
365	281	49	265	94	49	231	82	50	170	61	46
393	290	49	270	93	49	234	81	50	170	59	43
421	298	49	277	93	49	237	79	50	193	65	43
435	302	49	281	93	49	240	80	49	210	70	43
449	308	49	286	93	49	243	79	49	211	69	43
463	310	49	289	93	49	244	79	49	211	68	42
477	314	49	294	94	49	244	78	49	211	67	38
491	315	49	294	93	49	245	78	49	213	68	37
505	319	47	297	93	49	248	78	48	217	68	36
519	322	46	302	94	49	250	78	48	219	68	35
533	323	46	302	94	49	251	78	48	220	68	32
547	327	45	305	93	49	252	77	48	215	66	29
561	328	45	307	94	48	252	77	48	216	66	26
575	330	45	307	93	47	256	78	48	213	65	24
589	331	44	309	93	47	253	76	48	207	62	19
603	333	44	312	94	46	256	77	48	195	59	16
617	332	42	310	93	46	252	76	48	216	65	6
631	333	42	311	93	46	256	77	48	217	65	5
645	335	40	314	94	45	253	76	48	214	64	4
659	339	38	317	94	43	251	74	48	210	62	4
673	341	37	319	93	42	249	73	47	217	64	1
687	341	34	318	93	41	245	72	47	214	63	1
701	340	32	321	94	39	237	70	45			
715	340	32	318	94	38	244	72	39			
Mean for weeks											
1-13	165		161	98		158	96		151	92	
14-52	240		228	95		211	88		181	75	
53-103	322		301	93		247	77		208	65	

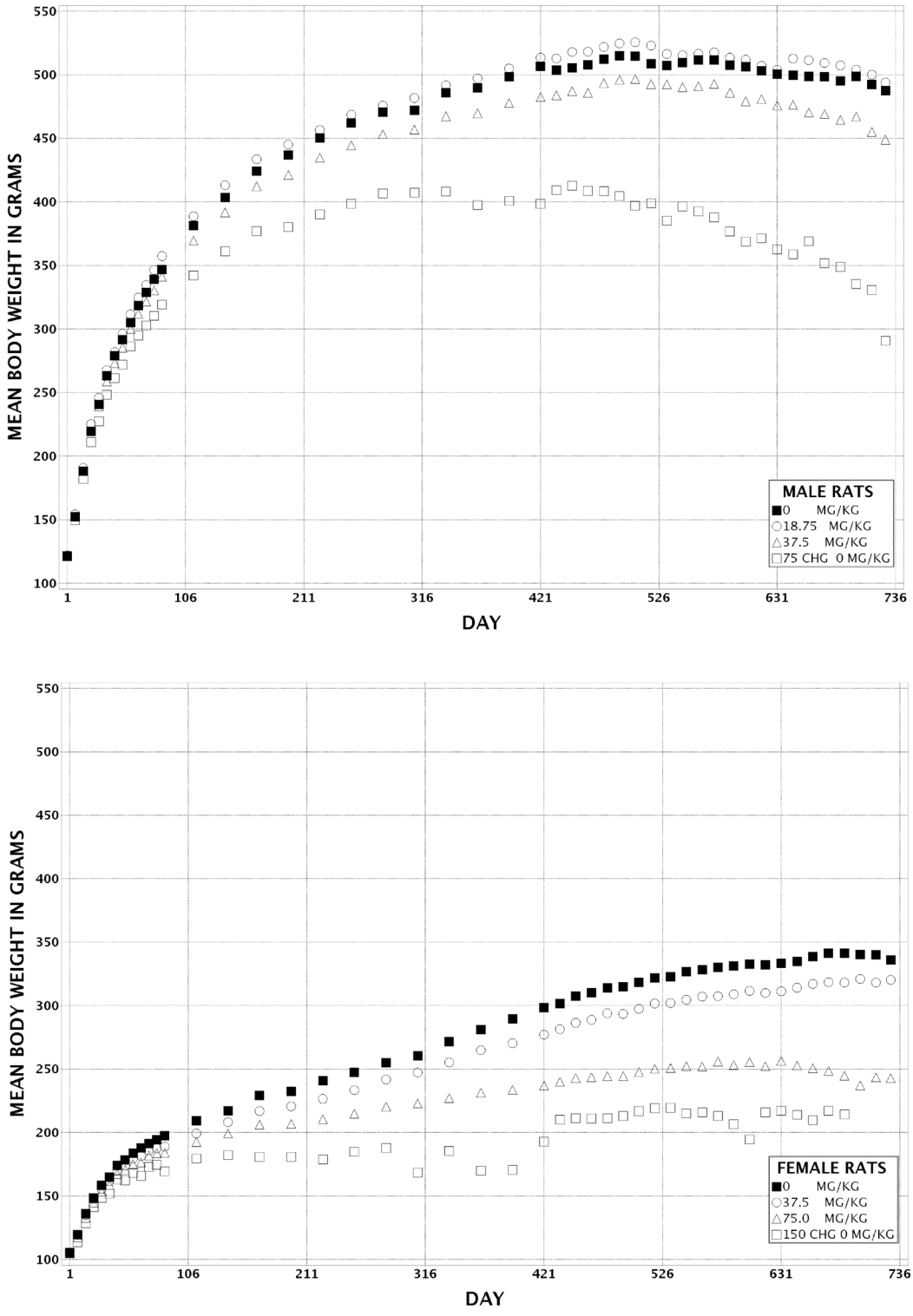


FIGURE 4
Growth Curves for Rats Administered Pulegone by Gavage for 2 Years

Pathology and Statistical Analyses

This section describes the statistically significant and biologically noteworthy changes in the study, specifically the incidences of urinary bladder papilloma and carcinoma and renal hyaline glomerulopathy and chronic progressive nephropathy (nephropathy). Various other treatment-related increases or decreases in the incidences of neoplasms and nonneoplastic lesions are also described. 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.

Urinary Bladder: The incidences of urinary bladder papilloma and of papilloma or carcinoma (combined) were significantly increased in 150 mg/kg stop-exposure females (Tables 10, B1, and B2). These neoplasms have not been observed in historical control rats from any study route (Tables 10 and B3). These neoplasms were characterized by neoplastic proliferations of transitional epithelial cells. In the papillomas, the general growth form was exophytic with a central core of fibrovascular tissue. The neoplastic cells displayed minimal atypia and rare mitotic figures. The transitional cell carcinomas had a more solid growth pattern that largely filled the bladder lumen. The cords of neoplastic cells occasionally invaded through the basement membrane zones into underlying connective tissue. Cellular atypia and the numbers of mitotic figures were increased.

TABLE 10
Incidences of Neoplasms of the Urinary Bladder in Female Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Number Examined Microscopically	50	49	50	47
Papilloma[a,b]	0	0	1	3*
Carcinoma[b]	0	0	0	2
Papilloma or Carcinoma[b]				
Overall rate[c]	0/50 (0%)	0/49 (0%)	1/50 (2%)	5/47 (11%)
Adjusted rate[d]	0.0%	0.0%	2.1%	20.8%
Terminal rate[e]	0/31 (0%)	0/36 (0%)	1/38 (3%)	0/0
First incidence (days)	—[g]	—	728 (T)	390
Poly-3 test[f]	P=0.289	—[h]	P=0.520	P=0.005

* Significantly different (P≤0.05) from the vehicle control group by the Poly-3 test
(T) Terminal sacrifice
[a] Number of animals with neoplasm
[b] Historical incidence for 2-year gavage studies with corn oil vehicle control groups: 0/200; all routes: 0/1,347
[c] Number of animals with neoplasm per number of animals with urinary bladder 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; the stop-exposure group is excluded from the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.
[g] Not applicable; no neoplasms in animal group
[h] Value of statistic cannot be computed.

Kidney: The incidences of hyaline glomerulopathy were significantly increased in 37.5 mg/kg and 75 mg/kg stop-exposure males and all female dosed groups (Tables 11, A3, and B4). The severity of nephropathy was increased in 37.5 mg/kg and 75 mg/kg stop-exposure males and 75 mg/kg and 150 mg/kg stop-exposure females; the incidences of nephropathy were significantly increased in 75 mg/kg and 150 mg/kg stop-exposure females. Both hyaline glomerulopathy and nephropathy likely contributed to the end-stage renal disease that caused the early deaths of the majority of high dose rats. The incidence of renal cyst was significantly increased in 75 mg/kg stop-exposure males.

Hyaline glomerulopathy was characterized histologically by a thickening of the glomerular mesangium and, to a lesser degree, the capillary loops by amorphous eosinophilic material, resulting in significant glomerular enlargement in the more markedly affected animals (Plate 3). While both males and females displayed an increase in incidence and severity of hyaline glomerulopathy, the lesion was particularly marked in the females. The severity of nephropathy occasionally confounded the diagnosis, particularly in male animals. Small thrombi occasionally obstructed the capillary lumens within the glomeruli.

TABLE 11
Incidences of Selected Nonneoplastic Lesions in Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Male				
Kidney[a]	50	50	50	50
Cyst[b]	0	0	2 (2.5)[c]	7** (2.1)
Glomerulopathy, Hyaline	0	0	9** (1.1)	24** (1.6)
Nephropathy	45 (1.9)	45 (1.9)	50 (2.9)	50 (4.0)
Liver	50	50	50	50
Hepatocyte, Cellular Alteration, Diffuse	0	2 (2.0)	21** (1.4)	46** (1.8)
Fatty Change	1 (2.0)	10** (1.4)	27** (1.4)	2 (1.0)
Bile Duct, Cyst	0	0	11** (2.2)	9** (2.1)
Bile Duct, Hyperplasia	29 (1.1)	15** (1.1)	37* (1.5)	50** (3.0)
Hepatocyte, Necrosis	0	1 (3.0)	6* (1.2)	16** (1.8)
Oval Cell, Hyperplasia	0	0	8** (1.3)	44** (1.6)
Portal, Fibrosis	8 (1.0)	6 (1.0)	13 (1.2)	43** (1.5)
Basophilic Focus	36	5**	2**	2**
Clear Cell Focus	22	22	20	1**
Eosinophilic Focus	1	2	12**	3
Nose	50	50	46	50
Olfactory Epithelium, Degeneration	1 (1.0)	5 (1.0)	33** (1.6)	19** (1.6)
Pancreas	50	50	50	50
Acinus, Atrophy	13 (1.6)	24* (1.4)	20 (1.4)	18** (1.2)
Acinus, Hyperplasia	11 (2.2)	8 (1.8)	7 (1.9)	1 (1.0)
Forestomach	50	50	50	50
Inflammation	2 (1.5)	4 (1.8)	8* (1.9)	23** (2.1)
Mineralization	0	1 (2.0)	0	4* (2.0)
Perforation	0	0	0	5**
Ulcer	0	2 (2.0)	7** (3.1)	16** (3.1)
Epithelium, Hyperplasia	16 (1.3)	21 (1.3)	20 (2.0)	23** (2.3)
Glandular Stomach	50	50	50	50
Inflammation	1 (1.0)	3 (1.0)	1 (1.0)	6** (1.2)

TABLE 11
Incidences of Selected Nonneoplastic Lesions in Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Female				
Kidney	50	50	50	49
Glomerulopathy, Hyaline	0	17** (1.0)	49** (2.2)	48** (3.3)
Nephropathy	42 (1.2)	44 (1.3)	49** (2.9)	48** (3.4)
Liver	50	50	50	47
Hepatocyte, Cellular Alteration, Diffuse	0	4 (2.0)	45** (1.5)	43** (1.9)
Fatty Change	7 (1.3)	25** (1.4)	35** (1.9)	11* (2.0)
Bile Duct, Cyst	1 (3.0)	6 (2.3)	38** (2.3)	13** (1.8)
Bile Duct, Hyperplasia	5 (1.0)	4 (1.3)	49** (3.2)	43** (3.7)
Hepatocyte, Necrosis	4 (1.5)	2 (1.5)	20** (1.7)	15** (2.0)
Oval Cell, Hyperplasia	0	0	45** (1.7)	43** (1.8)
Portal, Fibrosis	0	3 (1.0)	28** (1.3)	35** (1.4)
Basophilic Focus	44	21**	3**	2**
Clear Cell Focus	8	12	4	1
Nose	50	50	50	41
Olfactory Epithelium, Degeneration	2 (1.0)	40** (1.2)	48** (2.0)	37** (2.1)
Olfactory Epithelium, Metaplasia, Respiratory	1 (1.0)	8* (1.1)	46** (1.7)	36** (2.0)
Inflammation	12 (1.2)	22* (1.7)	39** (2.2)	26** (2.5)
Glands, Dilatation	0	6* (1.2)	6* (1.5)	2 (1.0)
Pancreas	50	50	49	46
Acinus, Atrophy	11 (1.4)	12 (1.2)	15 (1.1)	17** (1.5)
Acinus, Hyperplasia	7 (1.6)	1* (1.0)	1* (1.0)	2 (1.0)
Ovary	50	50	49	46
Atrophy	5 (1.2)	5 (2.2)	4 (2.8)	16** (2.3)
Lymph Node, Mesenteric	50	50	50	45
Hemorrhage	2 (1.5)	0	1 (1.0)	18** (2.2)
Bone Marrow	50	50	50	50
Hyperplasia	8 (3.1)	8 (3.3)	20* (3.0)	38** (2.9)

* Significantly different ($P \leq 0.05$) from the vehicle control group by 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

A variety of histochemical stains were used to further characterize the glomerular lesions, including a Congo Red for amyloid, Periodic Acid Schiff (PAS) for glycoproteins, Masson's Trichrome for collagen, and Jones' Methenamine Silver (JMS) for basement membranes. The material expanding the mesangia was negative with Congo Red for amyloid and was positive with PAS and Masson's Trichrome (Plate 4) in the more severely affected rats. A silver stain did not reveal increased basement membrane material or basement membrane 'spikes,' although basement membrane splitting was occasionally present (Plate 5).

Affected glomeruli were variably intensely positive when stained immunohistochemically for the presence of IgG, IgM, and IgA and were negative for complement protein 3. The staining pattern was multifocal within an affected glomerulus, with both the mesangium and capillary loops displaying regional positivity (Plate 6). Transmission electron microscopy was performed on the renal cortices to further characterize the lesions. Ultrastructurally, the glomerular tuft was markedly expanded by amorphous, finely granular, variably dense material occasionally containing small, smooth-margined vacuoles (Electron Micrograph 1). This material was consistently located on the endothelial surface of the capillary loops and within the mesangium but was never visualized within the glomerular basement membrane or on the subepithelial surface. In severely affected glomeruli, the dense material often completely obliterated the mesangium and capillary loops (Electron Micrograph 2).

Nephropathy consisted of multifocal to diffuse regenerative renal tubules surrounded by a thickened basement membrane, variable thickening of the glomerular mesangium, tubule protein casts, and chronic interstitial inflammatory infiltrates with fibrosis. The nephropathy was severe in the majority of the high dose rats of both sexes and was consistent with end-stage renal disease.

Liver: The incidences of diffuse hepatocyte cellular alteration were significantly increased in 37.5 mg/kg and 75 mg/kg stop-exposure males and 75 mg/kg and 150 mg/kg stop-exposure females (Tables 11, A3, and B4). Hepatocyte cellular alteration was the term used to describe a constellation of lesions in the liver. These lesions included a slight decrease in cell size, increased cytoplasmic basophilia and loss of cytoplasmic glycogen and lipid, and an increase in nuclear size with coarsely clumped chromatin and increased prominence and often number of nucleoli (Plate 7). Karyomegaly was present occasionally. Variable numbers of apoptotic hepatocytes, characterized by a shrunken, rounded, hypereosinophilic cellular appearance with pyknotic nuclei, were also present in the affected animals; this change was not diagnosed separately.

Significant increases in the incidences of other liver lesions occurred and included fatty change in 18.75 and 37.5 mg/kg males and all dosed groups of females; bile duct cyst, hepatocyte necrosis, oval cell hyperplasia, and bile duct hyperplasia in the two highest dosed groups of both males and females; and portal fibrosis in 75 mg/kg stop-exposure males and 75 mg/kg and 150 mg/kg stop-exposure females (Tables 11, A3, and B4).

The incidences of basophilic focus were significantly decreased in all dosed groups, and the incidence of clear cell focus was significantly decreased in 75 mg/kg stop-exposure males. The incidence of eosinophilic focus was significantly increased in 37.5 mg/kg males (Tables 11, A3, and B4).

Nose: In 37.5 mg/kg and 75 mg/kg stop-exposure males and all dosed groups of females, incidences of olfactory epithelium degeneration were significantly increased (Tables 11, A3, and B4). All dosed groups of females had significantly increased incidences of respiratory metaplasia of the olfactory epithelium and nasal inflammation, and 37.5 and 75 mg/kg females had significantly increased incidences of glandular dilatation.

Pancreas: Incidences of acinar atrophy in 18.75 mg/kg and 75 mg/kg stop-exposure males and 150 mg/kg stop-exposure females were significantly increased compared to those in the vehicle controls (Tables 11, A3, and B4). The incidences of acinar hyperplasia were decreased in all dosed groups.

Stomach: In male rats, incidences of forestomach inflammation and ulcer were significantly increased in the 37.5 mg/kg and 75 mg/kg stop-exposure groups (Tables 11 and A3). Incidences of mineralization, epithelial hyperplasia, and perforation were significantly increased in 75 mg/kg stop-exposure males. In the glandular stomach, the incidence of inflammation was increased in 75 mg/kg stop-exposure males.

Ovary: The incidence of ovarian atrophy was significantly increased in 150 mg/kg stop-exposure females (Tables 11 and B4). This lesion was characterized by a decrease in the number of follicles, corpora hemorrhagica, and corpora lutea.

Other Findings: The incidences of mesenteric lymph node hemorrhage in 150 mg/kg stop-exposure females and bone marrow hyperplasia in 75 mg/kg and 150 mg/kg stop-exposure females were significantly increased (Tables 11 and B4).

In all dosed groups of females, the incidences of pituitary gland pars distalis adenoma were significantly less than that in the vehicle control group (vehicle control, 27/50; 37.5 mg/kg, 12/50; 75 mg/kg, 9/50; 150 mg/kg stop-exposure, 3/50; Tables B1 and B2) and below the historical control ranges for corn oil gavage studies [106/200 (53.0% ± 3.8%), range 50%-58%] and all study routes [744/1,344 (55.4% ± 11.1%), range 32%-73%].

The incidences of mammary gland fibroadenoma were significantly decreased in 75 mg/kg and 150 mg/kg stop-exposure females (21/50, 27/50, 8/50, 0/50; Tables B1 and B2); fibroadenoma incidences in these groups were below the historical ranges for corn oil gavage studies [88/200 (44.0% ± 7.8%), range 34%-52%] and all study routes [701/1,350 (51.9% ± 14.8%), range 24%-86%].

In females, significantly decreased incidences of thyroid gland C-cell hyperplasia occurred in the 75 mg/kg and 150 mg/kg stop-exposure groups (39/50, 32/50, 19/50, 3/49; Table B4). The incidence of thyroid gland C-cell adenoma was significantly decreased in the 150 mg/kg stop-exposure group (8/50, 6/50, 3/50, 0/49; Table B2).

Lesions Considered Secondary to Renal Disease: The incidences of a number of nonneoplastic lesions considered secondary to renal disease were significantly increased (Tables 12, A3, and B4).

TABLE 12
Incidences of Nonneoplastic Lesions Considered Secondary to Renal Disease in Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Male				
Bone[a]	50	50	50	50
Fibrous Osteodystrophy[b]	0	0	2 (3.0)[c]	34** (2.3)
Parathyroid Gland	49	44	45	48
Hyperplasia	0	0	3 (2.3)	36** (2.5)
Cecum	50	50	49	50
Inflammation	0	0	6* (1.2)	21** (1.7)
Mineralization	0	0	0	4* (4.5)
Ulcer	0	0	0	3* (3.0)
Glandular Stomach	50	50	50	50
Mineralization	0	1 (2.0)	4 (2.0)	21** (1.7)
Blood Vessel	50	50	50	50
Mineralization	0	0	1 (2.0)	24** (2.0)
Heart	50	50	50	50
Mineralization	0	0	1 (3.0)	23** (1.6)
Adrenal Medulla	50	50	50	50
Hyperplasia	22 (1.7)	19 (1.6)	30 (1.9)	31** (1.8)
Lung	50	50	50	50
Interstitium, Mineralization	0	1 (1.0)	2 (1.5)	16** (1.6)
Kidney	50	50	50	50
Cortex, Mineralization	1 (1.0)	2 (1.0)	2 (1.5)	21** (2.1)

TABLE 12
Incidences of Nonneoplastic Lesions Considered Secondary to Renal Disease in Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Female				
Bone	50	50	50	50
Fibrous Osteodystrophy	0	0	1 (2.0)	27** (2.9)
Parathyroid Gland	46	47	49	49
Hyperplasia	0	1 (1.0)	11** (2.0)	31** (2.9)
Cecum	50	50	49	45
Inflammation	0	1 (1.0)	3 (1.0)	12** (1.8)
Glandular Stomach	50	50	49	46
Mineralization	1 (2.0)	3 (1.3)	1 (1.0)	13** (2.2)
Blood Vessel	50	50	50	50
Mineralization	0	1 (2.0)	0	20** (2.3)
Heart	50	50	50	50
Inflammation	0	0	0	4* (2.5)
Mineralization	0	1 (2.0)	1 (1.0)	11** (2.0)
Adrenal Medulla	50	50	50	49
Hyperplasia	6 (1.2)	11 (1.1)	17* (1.6)	27** (1.7)
Lung	50	50	50	50
Hemorrhage	0	0	0	12** (2.4)
Interstitium, Mineralization	0	0	0	16** (1.7)
Kidney	50	50	50	49
Cortex, Mineralization	0	1 (2.0)	1 (2.0)	26** (2.0)

* Significantly different ($P \leq 0.05$) from the vehicle control group by 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

MICE
2-WEEK STUDY

Four of five females and one of five males in the 300 mg/kg groups died or were determined to be moribund by study day 5 (Table 13). Two of five early deaths were moribund terminations. Three of five died spontaneously with no dosing accidents. All early deaths were attributed to liver toxicity. Final mean body weights and body weight gains of the dosed groups were similar to those of the vehicle controls. Clinical findings were observed only in 300 mg/kg mice and included thinness, lethargy, and ruffled fur.

Absolute and relative liver weights of 300 mg/kg males and relative liver weights of 150 mg/kg males and females were significantly greater than those of the vehicle controls (Table H3). Other organ weight changes were not considered to be chemical related.

The only macroscopic finding was pale discoloration of the liver in three 300 mg/kg female mice.

TABLE 13
Survival and Body Weights of Mice in the 2-Week Gavage Study of Pulegone

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	20.7 ± 0.8	22.2 ± 1.0	1.5 ± 0.3	
18.75	5/5	21.0 ± 0.8	23.4 ± 0.9	2.3 ± 0.1	105
37.5	5/5	20.8 ± 0.4	22.6 ± 0.5	1.8 ± 0.2	102
75	5/5	20.5 ± 0.4	22.2 ± 0.6	1.7 ± 0.5	100
150	5/5	20.7 ± 0.7	23.0 ± 0.8	2.3 ± 0.3	103
300	4/5[c]	21.0 ± 0.3	21.9 ± 0.2	1.2 ± 0.1	98
Female					
0	5/5	17.4 ± 0.4	18.3 ± 0.4	0.9 ± 0.6	
18.75	5/5	17.4 ± 0.3	19.4 ± 0.5	2.0 ± 0.3	106
37.5	5/5	17.3 ± 0.5	19.1 ± 0.5	1.8 ± 0.1	104
75	5/5	17.0 ± 0.6	18.8 ± 0.5	1.8 ± 0.2	102
150	5/5	17.3 ± 0.6	18.6 ± 0.3	1.3 ± 0.3	101
300	1/5[c]	16.5 ± 0.3	17.6	1.1	96

[a] Number of animals surviving at 2 weeks/number initially in group

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

[c] All early deaths occurred on or before study day 5.

Microscopically, the incidences of cytoplasmic vacuolization and diffuse fatty change in 300 mg/kg females were significantly increased (Table 14). "Cytoplasmic vacuolization" was used for hepatocytes with a fine lace-like vacuolization. "Fatty change" was used as a separate diagnosis for the presence of fat vacuoles in hepatocytes that exceeded the size of nuclei, even though this may have been a progression from the fine cytoplasmic vacuolization that was also noted. Fatty change in this study was described as diffuse, meaning that virtually all lobules were affected with centrilobular to midzonal distribution within affected lobules. The incidences of necrosis were increased in 300 mg/kg males and females, and the increase in males was significant. Hemorrhage, which was not diagnosed separately, accompanied necrosis of the liver in three of four affected female mice and one of five affected male mice, while inflammation accompanied necrosis in three of four affected female mice and three of five affected male mice. "Hepatocyte degeneration" was used for hepatocytes with pale rarefied cytoplasm usually accompanied by a few inflammatory cells in the kidney; this lesion occurred in two 300 mg/kg males. Bile duct hyperplasia was found in three males and one female administered 300 mg/kg, and mineralization was found in three 300 mg/kg females.

Dose Selection Rationale: Based on mortality and liver lesions at 300 mg/kg in the 2-week study, doses selected for the 3-month gavage study in mice were 9.375, 18.75, 37.5, 75, and 150 mg/kg.

TABLE 14
Incidences of Nonneoplastic Lesions of the Liver in Mice in the 2-Week Gavage Study of Pulegone

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg
Male						
Number Examined Microscopically	5	0	0	0	5	5
Cytoplasmic Vacuolization[a]	0				0	1 (2.0)[b]
Necrosis	1 (1.0)				0	5* (1.6)
Fatty Change, Diffuse	0				0	1 (2.0)
Hepatocyte Degeneration	0				0	2 (1.0)
Bile Duct Hyperplasia	0				0	3 (2.0)
Inflammation	0				1 (2.0)	3 (1.7)
Female						
Number Examined Microscopically	5	0	0	0	5	5
Cytoplasmic Vacuolization	0				0	4* (3.0)
Necrosis	1 (1.0)				0	4 (2.3)
Mineralization	0				0	3 (1.7)
Fatty Change, Diffuse	0				0	4* (1.5)
Bile Duct Hyperplasia	0				0	1 (1.0)
Inflammation	0				0	3 (2.0)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Fisher exact test

[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

All mice survived to the end of the study (Table 15). Final mean body weights and mean body weight gains of dosed mice were similar to those of the vehicle controls (Table 15 and Figure 5). No chemical-related clinical findings were reported.

No changes in the clinical chemistry endpoints in mice were considered toxicologically or biologically relevant to the administration of pulegone (Table F2).

On day 4, treatment-related increases in reduced, oxidized, and total glutathione concentrations were observed in the male and female 150 mg/kg groups compared to their respective vehicle control groups (Table G2). In addition, in 75 mg/kg females on day 4, the mean reduced and total glutathione concentrations were significantly increased but the increase in oxidized glutathione concentration was not statistically significant. At week 14, mean reduced glutathione concentrations were significantly increased in 37.5 mg/kg or greater females and 75 and 150 mg/kg males, mean oxidized glutathione concentrations were significantly increased in 75 and 150 mg/kg females and in 150 mg/kg males, and total glutathione concentrations were significantly increased in 37.5 mg/kg or greater males and all dosed groups of females. The percentage change from the vehicle control for both the oxidized and reduced glutathione concentrations in the same dose groups and sex was of the same magnitude, resulting in no change in the reduced glutathione/oxidized glutathione ratio at any dose.

TABLE 15
Survival and Body Weights of Mice in the 3-Month Gavage Study of Pulegone

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	23.3 ± 0.5	37.3 ± 0.8	14.0 ± 0.6	
9.375	10/10	23.5 ± 0.5	36.9 ± 1.3	13.4 ± 0.9	99
18.75	10/10	23.4 ± 0.4	38.1 ± 0.8	14.7 ± 0.6	102
37.5	10/10	23.5 ± 0.5	36.7 ± 0.9	13.2 ± 0.6	98
75	10/10	23.6 ± 0.5	36.5 ± 1.5	12.9 ± 1.1	98
150	10/10	23.2 ± 0.5	37.1 ± 1.1	13.9 ± 0.7	99
Female					
0	10/10	18.7 ± 0.2	29.1 ± 0.7	10.4 ± 0.5	
9.375	10/10	18.5 ± 0.3	29.6 ± 0.8	11.1 ± 0.8	102
18.75	10/10	18.7 ± 0.3	29.4 ± 0.7	10.7 ± 0.7	101
37.5	10/10	18.8 ± 0.4	31.6 ± 1.5	12.8 ± 1.2	109
75	10/10	18.5 ± 0.3	29.8 ± 0.9	11.3 ± 0.8	103
150	10/10	18.7 ± 0.2	28.1 ± 0.7	9.4 ± 0.5	96

[a] Number of animals surviving at 3 months/number initially in group

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

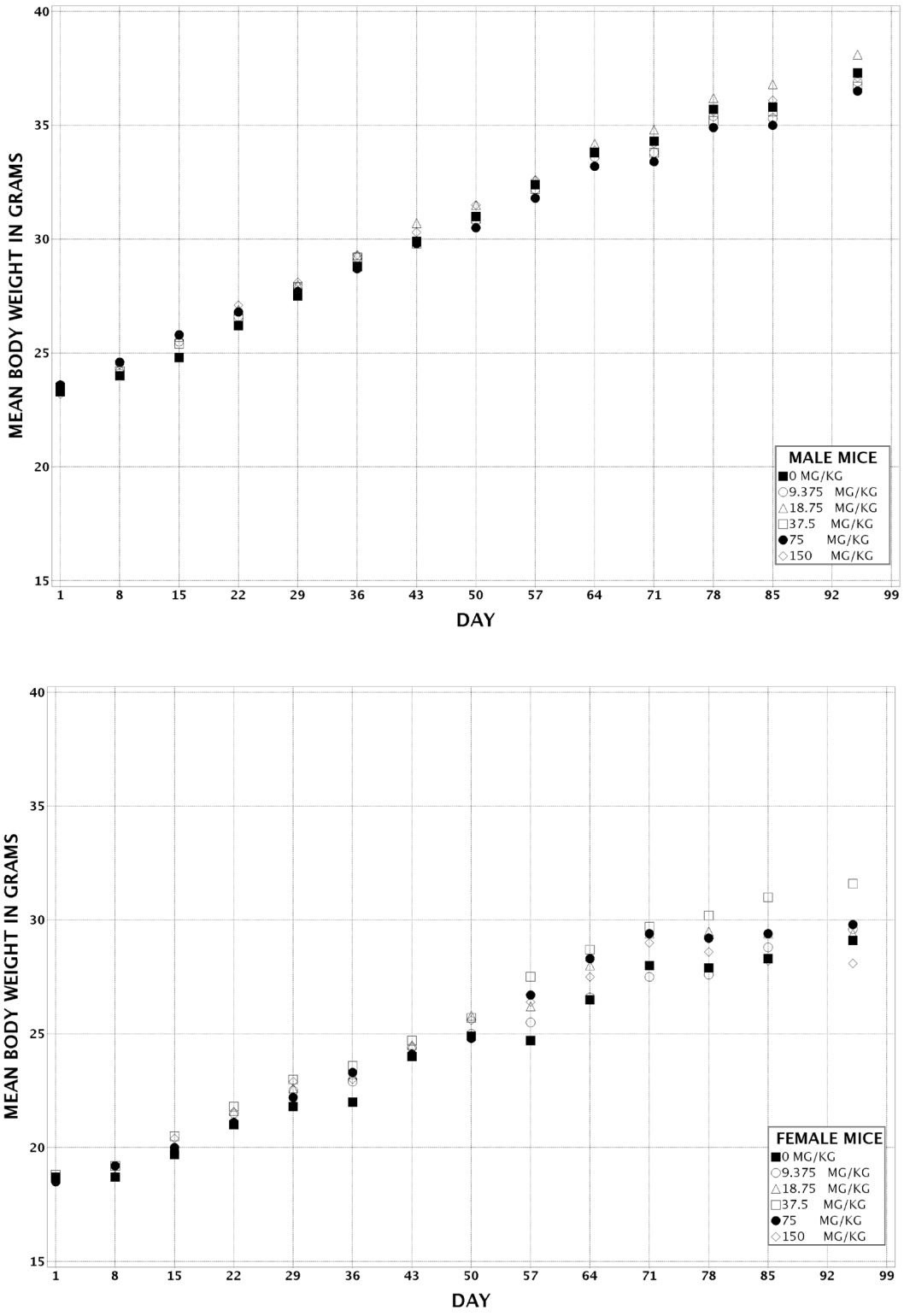


FIGURE 5
Growth Curves for Mice Administered Pulegone by Gavage for 3 Months

Absolute and relative liver weights of 150 mg/kg males and 75 and 150 mg/kg females and the relative liver weight of 75 mg/kg males and absolute liver weights of 18.75 and 37.5 mg/kg females were significantly greater than those of the vehicle controls (Table H4). Other organ weight changes were sporadic and not considered to be chemical related.

No significant differences were observed in the sperm parameters of male mice or in the estrous cyclicity of female mice administered 37.5, 75, or 150 mg/kg when compared to the vehicle controls (Tables I3 and I4). Statistically significant increases in the absolute and relative testicular spermatid counts of approximately 14% were not considered to be biologically significant in the absence of histopathology or other supportive information.

No histopathologic lesions were observed that could be attributed to the administration of pulegone.

Dose Selection Rationale: Based on the lack of mortality, effects on body weights, and lesions attributable to pulegone administration, doses selected for the 2-year gavage study in mice were 37.5, 75, and 150 mg/kg.

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 16 and in the Kaplan-Meier survival curves (Figure 6). Survival of all dosed groups was similar to that of the vehicle controls.

TABLE 16
Survival of Mice in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Male				
Animals initially in study	50	50	50	50
Accidental deaths[a]	0	2	0	0
Moribund	7	8	7	8
Natural deaths	5	4	1	1
Animals surviving to study termination	38	36	42	41
Percent probability of survival at end of study[b]	76	75	84	82
Mean survival (days)[c]	691	692	717	697
Survival analysis[d]	P=0.387N	P=1.000	P=0.373N	P=0.594N
Female				
Animals initially in study	50	50	50	50
Accidental death[a]	0	0	0	1
Missing[a]	1	0	0	0
Moribund	9	3	5	9
Natural deaths	5	6	7	3
Animals surviving to study termination	35	41	38	37
Percent probability of survival at end of study	71	82	76	76
Mean survival (days)	705	709	695	689
Survival analysis	P=1.000	P=0.352N	P=0.857N	P=0.869N

[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 results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. A negative trend or a lower mortality in a dose group is indicated by **N**.

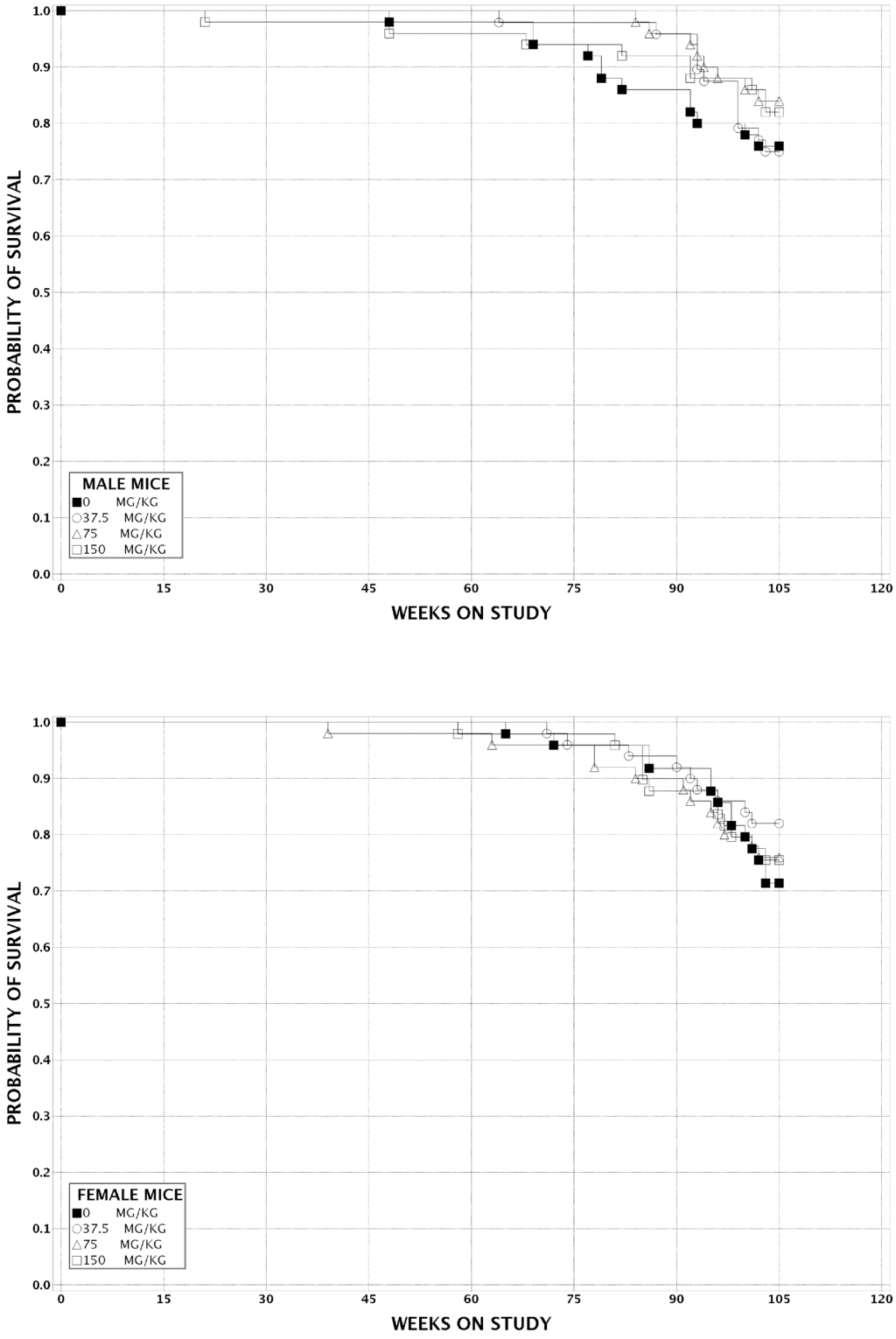


FIGURE 6
Kaplan-Meier Survival Curves for Mice Administered Pulegone by Gavage for 2 Years

Body Weights and Clinical Findings

Mean body weights of 37.5 and 75 mg/kg groups were similar to those of the vehicle controls throughout the study; those of 150 mg/kg males and females were less after weeks 25 and 33, respectively (Tables 17 and 18; Figure 7). There were no clinical findings related to the administration of pulegone.

TABLE 17
Mean Body Weights and Survival of Male Mice in the 2-Year Gavage Study of Pulegone

Days on Study	Vehicle Control		37.5 mg/kg			75 mg/kg			150 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	21.8	50	21.9	100	50	21.8	100	50	22.0	101	50
8	23.1	50	23.1	100	50	23.2	100	50	23.6	102	50
15	24.5	50	24.4	99	50	24.8	101	50	24.3	99	50
22	25.5	50	25.8	101	50	25.8	101	50	25.7	101	50
29	27.0	50	27.2	101	50	27.2	101	50	26.8	100	50
36	28.1	50	28.2	100	50	28.4	101	50	28.1	100	50
43	28.9	50	29.0	100	50	28.8	100	50	28.5	99	50
50	30.5	50	30.4	100	50	30.4	100	50	30.4	100	50
57	31.1	50	31.0	100	50	30.9	99	50	30.8	99	50
64	32.2	50	32.0	99	49	32.2	100	50	31.7	98	50
71	33.3	50	32.7	98	49	33.0	99	50	31.9	96	50
78	34.5	50	34.3	99	49	34.2	99	50	33.3	97	50
85	34.9	50	35.0	100	49	34.5	99	50	34.1	98	50
113	38.4	50	38.6	101	49	38.2	100	50	37.2	97	50
141	41.8	50	41.7	100	49	41.0	98	50	39.5	95	49
169	44.6	50	44.8	101	49	44.2	99	50	41.6	93	49
197	45.9	50	46.5	101	49	45.4	99	50	41.3	90	49
225	47.3	50	47.3	100	49	46.3	98	50	41.7	88	49
253	49.1	50	49.2	100	49	48.5	99	50	42.8	87	49
281	50.3	50	50.1	100	49	49.3	98	50	44.3	88	49
309	50.9	50	51.8	102	49	50.7	100	50	44.8	88	49
337	51.7	49	52.1	101	49	51.3	99	50	44.8	87	48
365	53.0	49	53.1	100	49	53.1	100	50	44.7	84	48
393	52.4	49	52.9	101	49	52.5	100	50	44.3	84	48
421	52.9	49	53.1	100	49	53.3	101	50	43.8	83	48
449	53.7	49	53.5	100	47	54.0	101	50	45.0	84	48
477	53.4	48	53.7	101	47	54.4	102	50	45.8	86	47
505	53.8	47	54.1	101	47	53.8	100	50	45.3	84	47
533	54.2	47	54.7	101	47	55.4	102	50	46.0	85	47
561	55.3	44	53.9	97	47	55.0	99	50	46.3	84	47
589	56.0	43	54.5	98	47	55.2	99	49	46.9	84	46
617	56.2	43	54.3	97	46	54.2	96	48	47.3	84	46
645	56.4	41	53.7	95	46	52.9	94	47	45.6	81	44
673	56.6	40	56.1	99	42	53.7	95	44	42.9	76	44
701	55.5	39	55.8	100	38	52.9	95	43	42.3	76	44
Mean for weeks											
1-13	28.9		28.8	100		28.9	100		28.6	99	
14-52	46.7		46.9	101		46.1	99		42.0	90	
53-101	54.6		54.1	99		53.9	99		45.1	83	

TABLE 18
Mean Body Weights and Survival of Female Mice in the 2-Year Gavage Study of Pulegone

Days on Study	Vehicle Control		37.5 mg/kg			75 mg/kg			150 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	18.2	50	18.4	101	50	18.3	101	50	18.5	102	50
8	18.5	50	18.9	102	50	18.9	102	50	19.3	104	49
15	19.8	50	20.2	102	50	20.3	103	50	20.4	103	49
22	20.7	50	20.7	100	50	21.2	103	50	21.4	103	49
29	21.6	50	21.6	100	50	22.0	102	50	22.2	103	49
36	23.0	50	23.0	100	50	22.9	100	50	22.5	98	49
43	22.4	50	23.1	103	50	22.7	101	50	22.9	102	49
50	24.2	50	24.2	100	50	24.1	100	50	24.7	102	49
57	24.2	50	24.4	101	50	24.2	100	50	24.4	101	49
64	26.1	50	26.1	100	50	25.9	99	50	25.6	98	49
71	26.7	50	26.6	100	50	26.9	101	50	27.4	103	49
78	27.2	50	26.9	99	50	27.0	99	50	27.9	102	49
85	27.5	50	27.0	98	50	27.5	100	50	27.4	100	49
113	31.7	50	31.6	100	50	31.4	99	50	32.0	101	49
141	33.5	50	33.6	100	50	33.6	100	50	33.8	101	49
169	37.9	50	36.4	96	50	37.7	100	50	36.9	97	49
197	39.2	50	38.7	99	50	40.3	103	50	38.2	98	49
225	41.3	50	41.3	100	50	41.5	101	50	38.7	94	49
253	44.0	50	44.0	100	50	43.5	99	50	39.4	90	49
281	46.4	50	46.1	99	50	46.4	100	49	41.7	90	49
309	48.4	50	48.4	100	50	48.4	100	49	43.5	90	49
337	51.3	50	50.7	99	50	52.1	102	49	43.6	85	49
365	54.0	49	53.4	99	50	55.1	102	49	43.8	81	49
393	54.4	49	54.1	99	50	55.1	101	49	43.7	80	49
421	54.9	49	54.9	100	50	55.4	101	49	43.0	78	48
449	55.6	49	56.1	101	50	54.4	98	48	44.1	79	48
477	59.8	48	60.2	101	50	58.5	98	48	46.3	78	48
505	61.4	47	60.2	98	49	59.1	96	48	44.8	73	48
533	62.3	47	61.2	98	48	60.9	98	48	46.5	75	48
561	62.4	47	61.6	99	48	60.9	98	46	46.9	75	48
589	63.2	47	63.6	101	47	62.4	99	45	48.2	76	45
617	64.9	45	64.8	100	47	62.6	96	45	47.7	74	43
645	64.7	45	64.7	100	44	61.4	95	43	46.6	72	43
673	62.7	42	64.2	103	43	58.0	93	41	44.3	71	41
701	60.4	39	60.5	100	42	60.6	100	39	44.5	74	39
Mean for weeks											
1-13	23.1		23.2	101		23.2	101		23.4	102	
14-52	41.5		41.2	99		41.7	100		38.6	94	
53-101	60.1		60.0	100		58.8	98		45.4	76	

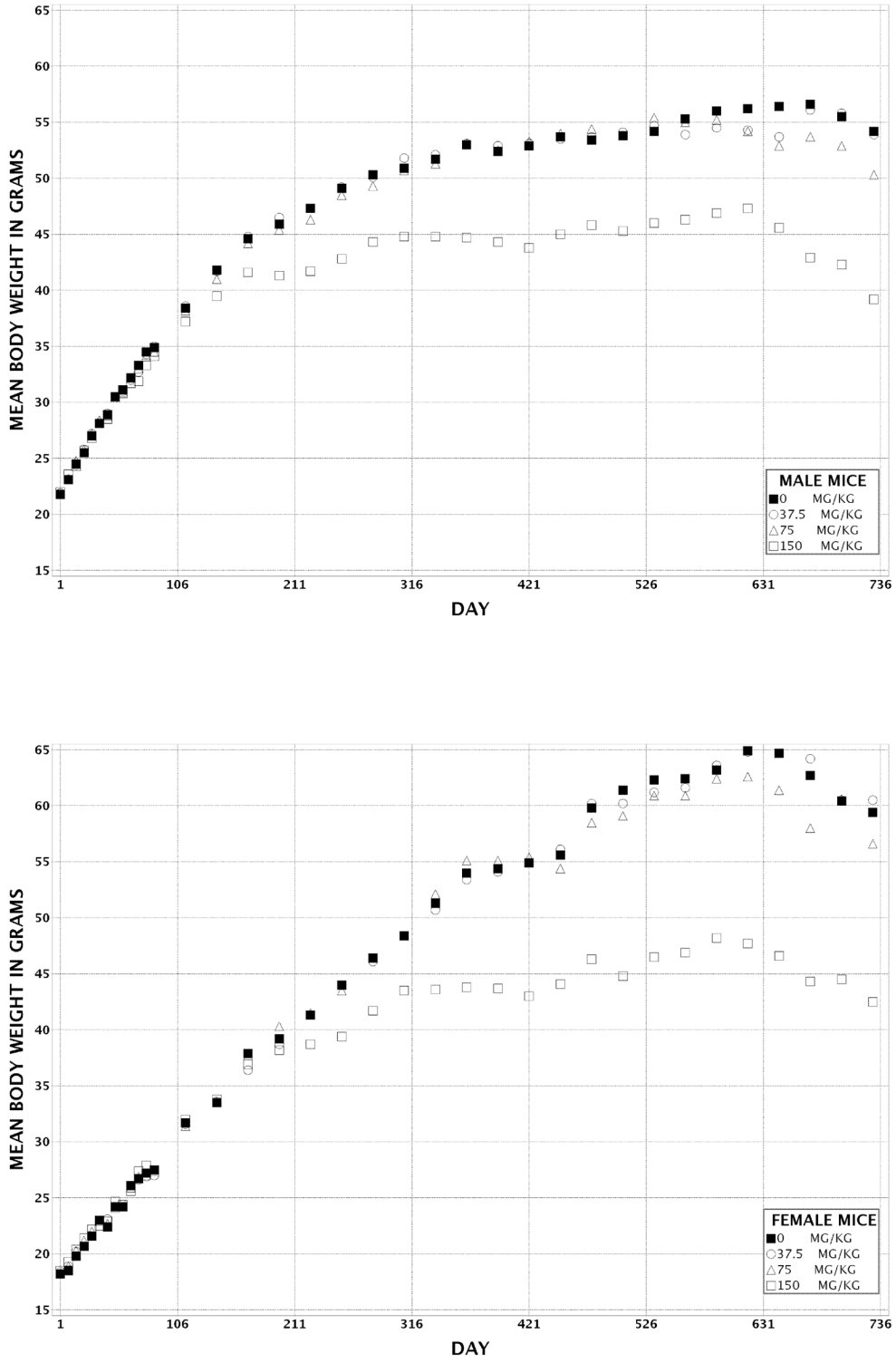


FIGURE 7
Growth Curves for Mice Administered Pulegone by Gavage for 2 Years

Pathology and Statistical Analyses

This section describes the statistically significant and biologically noteworthy changes in the incidences of hepatocellular adenoma, renal hyaline glomerulopathy, osteoma, and osteosarcoma. Various other dose-related increases or decreases in the incidences of other nonneoplastic lesions are also described. 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 C for male mice and Appendix D for female mice.

Liver: The incidences of multiple hepatocellular adenoma were significantly increased in all dosed groups of males, and the incidences of hepatocellular adenoma (including multiple) and hepatoblastoma (including multiple) were significantly increased in 75 mg/kg males (Tables 19, C1, and C2). The incidences of hepatocellular adenoma in 37.5 and 75 mg/kg males exceeded the historical control range for corn oil gavage studies and for all routes (Tables 19 and C3). The combined incidence of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma occurred with a positive trend and was significantly increased in 75 mg/kg males; the incidence in 75 mg/kg males exceeded the historical control range for corn oil gavage studies. The incidence of hepatocellular adenoma was significantly increased in 150 mg/kg females, and the incidences in 37.5 and 150 mg/kg females exceeded the historical control range for corn oil gavage studies (Tables 19, D1, D2, and D3). The combined incidence of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma in 150 mg/kg females was significantly increased and exceeded the historical control ranges for corn oil gavage studies and all routes (Tables 19, D1, D2, and D3). Histologically, hepatocellular adenomas were variably sized nodular lesions composed of well differentiated, neoplastic hepatocytes that typically compressed the adjacent hepatic parenchyma. Portal areas and central veins were typically absent.

The incidences of clear cell focus in all dosed groups, eosinophilic focus in 75 and 150 mg/kg mice, and mixed cell focus in 75 mg/kg females and 150 mg/kg males and females were significantly increased (Tables 19, C4, and D4). The incidences of focal fatty change were significantly increased in males and females administered 75 and 150 mg/kg, but those of diffuse fatty change were significantly decreased in all dosed groups of males and 150 mg/kg females. The incidences of centrilobular hepatocyte hypertrophy were significantly increased in all dosed groups except 37.5 mg/kg females. The incidences of intravascular hepatocytes were increased in 75 and 150 mg/kg mice; these hepatocytes proliferated within the wall of the central veins (Plate 8). The incidences of other liver lesions that were significantly increased in 150 mg/kg mice included necrosis, pigmentation (females), bile duct cyst, bile duct hyperplasia, and oval cell hyperplasia.

The incidences of inflammation in 75 and 150 mg/kg females and tension lipidosis in 150 mg/kg females were significantly decreased (Tables 19 and D4).

TABLE 19
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Male				
Number Examined Microscopically	50	50	50	50
Clear Cell Focus[a]	15	27**	28*	34**
Eosinophilic Focus	7	12	20**	36**
Mixed Cell Focus	18	20	19	34**
Fatty Change, Focal	3 (2.0)[b]	8 (1.4)	20** (1.2)	23** (1.2)
Fatty Change, Diffuse	38 (1.3)	27** (1.1)	21** (1.0)	3** (3.3)
Necrosis	1 (1.0)	8* (1.6)	5 (1.6)	26** (1.6)
Bile Duct, Cyst	0	0	3 (2.0)	14** (1.9)
Bile Duct, Hyperplasia	0	0	1 (1.0)	35** (1.5)
Centrilobular, Hepatocyte, Hypertrophy	0	11** (1.1)	23** (1.2)	46** (1.7)
Oval Cell, Hyperplasia	1 (1.0)	0	1 (1.0)	36** (1.4)
Vein, Intravascular Hepatocyte	3 (1.0)	1 (1.0)	15** (1.0)	47** (2.1)
Hepatocellular Adenoma, Multiple	6	19**	27**	18**
Hepatocellular Adenoma (includes multiple)[c]				
Overall rate[d]	22/50 (44%)	31/50 (62%)	35/50 (70%)	28/50 (56%)
Adjusted rate[e]	47.5%	65.4%	72.7%	60.3%
Terminal rate[f]	17/38 (45%)	24/36 (67%)	33/42 (79%)	25/41 (61%)
First incidence (days)	479	428	654	638
Poly-3 test[g]	P=0.175	P=0.058	P=0.008**	P=0.150
Hepatocellular Carcinoma, Multiple	2	2	2	3
Hepatocellular Carcinoma (includes multiple)[h]	13	11	18	15
Hepatoblastoma, Multiple	0	0	2	0
Hepatoblastoma (includes multiple)[i]	1	3	7*	2
Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma[j]				
Overall rate	29/50 (58%)	37/50 (74%)	42/50 (84%)	36/50 (72%)
Adjusted rate	60.0%	76.3%	85.3%	77.5%
Terminal rate	20/38 (53%)	26/36 (72%)	36/42 (86%)	33/41 (81%)
First incidence (days)	479	428	588	638
Poly-3 test	P=0.038	P=0.064	P=0.004	P=0.051
Female				
Number Examined Microscopically	49	50	50	50
Clear Cell Focus	0	6*	23**	32*
Eosinophilic Focus	3	7	10*	31**
Mixed Cell Focus	4	8	16**	20**
Fatty Change, Focal	1 (1.0)	2 (1.5)	20** (1.2)	12** (1.4)
Fatty Change, Diffuse	36 (1.5)	31 (1.1)	34 (1.4)	3** (2.7)
Inflammation	40 (1.2)	40 (1.1)	14** (1.0)	31* (1.1)
Necrosis	5 (2.2)	2 (2.0)	4 (2.5)	27** (1.3)
Pigmentation	0	0	0	46** (1.5)
Tension Lipidosis	5	5	4	0*
Bile Duct, Cyst	0	0	4 (2.0)	38** (2.6)

TABLE 19
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Female (continued)				
Number Examined Microscopically	49	50	50	50
Bile Duct, Hyperplasia	0	0	2 (1.0)	47** (2.2)
Centrilobular, Hepatocyte, Hypertrophy	0	4 (1.3)	12** (1.1)	29** (1.2)
Oval Cell, Hyperplasia	0	0	3 (1.3)	46** (1.5)
Vein, Intravascular Hepatocyte	0	2 (1.0)	20** (1.1)	46** (2.2)
Hepatocellular Adenoma, Multiple	6	8	3	12
Hepatocellular Adenoma (includes multiple)[k]				
Overall rate	13/49 (27%)	15/50 (30%)	13/50 (26%)	27/50 (54%)
Adjusted rate	28.5%	31.8%	28.5%	59.0%
Terminal rate	10/35 (29%)	12/41 (29%)	10/38 (26%)	24/37 (65%)
First incidence (days)	663	645	631	566
Poly-3 test	P<0.001	P=0.455	P=0.590N	P=0.002
Hepatocellular Carcinoma, Multiple	0	0	1	0
Hepatocellular Carcinoma (includes multiple)[l]	5	1	4	8
Hepatoblastoma, Multiple	0	0	0	1
Hepatoblastoma (includes multiple)[m]	0	1	2	2
Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma[n]				
Overall rate	17/49 (35%)	15/50 (30%)	15/50 (30%)	33/50 (66%)
Adjusted rate	37.2%	31.8%	32.7%	70.4%
Terminal rate	13/35 (37%)	12/41 (29%)	11/38 (29%)	26/37 (70%)
First incidence (days)	663	645	631	566
Poly-3 test	P<0.001	P=0.371N	P=0.410N	P<0.001

* 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
[c] Historical incidence for 2-year gavage studies with corn oil vehicle control groups (mean ± standard deviation): 121/250 (48.4% ± 4.6%), range 44%-54%; all routes: 751/1,447 (51.9% ± 12.7%), range 24%-72%
[d] Number of animals with neoplasm per number of animals with liver examined microscopically
[e] Poly-3 estimated neoplasm incidence after adjustment for intercurrent morality
[f] Observed incidence at terminal kill
[g] Beneath the vehicle control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons 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 lower incidence in a dose group is indicated by **N**.
[h] Historical incidence for corn oil gavage studies: 72/250 (28.8% ± 9.0%), range 16%-40%; all routes: 430/1,447 (29.7% ± 8.7%), range 16%-52%
[i] Historical incidence for corn oil gavage studies: 10/250 (4.0% ± 3.2%), range 0%-8%; all routes: 51/1,447 (3.5% ± 6.4%), range 0%-34%
[j] Historical incidence for corn oil gavage studies: 165/250 (66.0% ± 7.2%), range 58%-76%; all routes: 995/1,447 (68.8% ± 11.6%), range 46%-92%
[k] Historical incidence for corn oil gavage studies: 41/247 (16.6% ± 8.1%), range 6%-27%; all routes: 395/1,495 (26.4% ± 14.9%), range 2%-62%
[l] Historical incidence for corn oil gavage studies: 14/247 (5.7% ± 3.0%), range 2%-10%; all routes: 138/1,495 (9.2% ± 6.5%), range 0%-28%
[m] Historical incidence for corn oil gavage studies: 0/247; all routes: 4/1,495 (0.3% ± 0.7%), range 0%-2%
[n] Historical incidence for corn oil gavage studies: 51/247 (20.7% ± 10.4%), range 8%-35%; all routes: 483/1,495 (32.3% ± 16.9%), range 6%-64%

Kidney: Incidences of hyaline glomerulopathy were significantly increased in all dosed groups of males and 75 and 150 mg/kg females (Tables 20, C4, and D4). Hyaline glomerulopathy was characterized by a thickening of the glomerular mesangium and, to a lesser degree, the capillary loops by amorphous eosinophilic material, resulting in significant glomerular enlargement in the more markedly affected animals (Plate 9). A variety of histochemical stains were used to further characterize the lesions, including an alkaline Congo Red for amyloid, Lieb's Cresyl Violet for amyloid, Bennhold's Congo Red for amyloid, PAS for glycoproteins, Masson's Trichrome for collagen, and JMS for basement membranes. The material expanding the mesangium was negative for amyloid and was positive with PAS and Masson's Trichrome in the more severely affected mice (Plates 10 and 11). The JMS stain did not reveal increased basement membrane material or basement membrane 'spikes,' although basement membrane splitting was occasionally present (Plate 12). Many affected glomeruli, particularly those graded as mild or moderate, also displayed mesangiolysis and intraglomerular hemorrhage, which was not diagnosed separately (Plate 13). Immunohistochemical staining was performed for immunoglobulins (IgG, IgM, and IgA) and complement protein 3. There was positive staining of the mesangium with anti-IgM antibody (Plate 14). The other stains were considered negative.

Electron microscopy was performed on the renal cortices to further characterize the lesions. In a kidney with mild lesions, there were irregular dense deposits expanding the glomerular mesangium and extending into the capillary subendothelial space; the glomerular basement membrane was irregularly thickened, even at some distance from the dense deposits (Electron Micrograph 3). The material was finely granular, amorphous, and variably dense and was suggestive of immune complex deposition.

In a kidney with marked lesions, the ultrastructural changes included large, well circumscribed accumulations of closely packed tubules in longitudinal and cross-sectional arrays (Electron Micrograph 4). These tubules were highly structured, nonbranching, curvilinear, fibrillary deposits forming discrete, variably sized, principally extracellular bundles. These bundles of fibrils were oriented in parallel rows, often swirling

TABLE 20
Incidences of Nonneoplastic Lesions of the Kidney in Mice in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Male				
Number Examined Microscopically	50	50	50	50
Glomerulopathy, Hyaline[a]	1 (1.0)[b]	19** (1.1)	30** (1.1)	44** (1.9)
Cyst	9 (1.4)	3 (1.0)	4 (1.3)	0**
Mineralization	30 (1.0)	34 (1.0)	41* (1.1)	38 (1.0)
Nephropathy	45 (1.2)	45 (1.3)	49 (1.4)	49 (1.9)
Glomerulus, Congestion	9 (1.6)	14 (1.4)	17 (1.4)	44** (2.0)
Female				
Number Examined Microscopically	49	50	50	50
Glomerulopathy, Hyaline	0	3 (1.0)	15** (1.3)	41** (1.5)
Mineralization	1 (1.0)	0	3 (1.0)	20** (1.0)
Nephropathy	13 (1.4)	19 (1.2)	12 (1.2)	25** (1.2)
Glomerulus, Congestion	5 (1.2)	2 (1.0)	12 (1.1)	37** (1.3)

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

with a characteristic "fingerprint" pattern. These structures markedly expanded the glomeruli and obliterated the capillary lumens.

In 75 mg/kg males and 150 mg/kg females, incidences of mineralization were significantly increased (Tables 20, C4, and D4). The incidence of nephropathy in 150 mg/kg females and the severity of nephropathy in 150 mg/kg males were increased. Incidences of congestion of the glomerulus were significantly increased in 150 mg/kg males and females. The incidence of cyst was significantly decreased in 150 mg/kg males.

Osteoma and Osteosarcoma: One 150 mg/kg male and one 75 mg/kg female had nasal osteoma; no nasal osteomas have been seen in historical control mice (Tables 21, C1, and D1). In the bone, an osteoma was seen in one 75 mg/kg female and an osteosarcoma was seen in one 75 mg/kg female and one 150 mg/kg female. When all organs are combined, the incidences of osteoma and osteoma or osteosarcoma (combined) in 75 mg/kg females exceed the historical control ranges for corn oil gavage studies and all study routes.

Nose: The incidences of olfactory epithelial degeneration were significantly increased in all dosed groups of females and in 75 and 150 mg/kg males (Tables 22, C4, and D4). Incidences of inflammation, nerve atrophy, and olfactory epithelium metaplasia were significantly greater in 150 mg/kg males and females than in the vehicle control groups.

Forestomach: Incidences of squamous hyperplasia and inflammation were significantly increased in 75 and 150 mg/kg males and 150 mg/kg females (Tables 22, C4, and D4). In 75 and 150 mg/kg males, incidences of ulcer were significantly increased as well.

Other Findings: The incidence of mineralization of the heart was significantly increased in 150 mg/kg females, and the severity was increased in 150 mg/kg males (Tables 22, C4, and D4). Incidences of corneal inflammation in 150 mg/kg females and ovarian atrophy in 75 and 150 mg/kg females were also increased.

The incidences of hypertrophy of the adrenal cortex were significantly decreased in all dosed groups of males as was the incidence of pancreatic islet hyperplasia in 150 mg/kg males (Tables 22 and C4).

In the spleen of female mice, the incidence of hematopoietic cell proliferation was significantly increased in the 150 mg/kg group compared to that of vehicle controls (Tables 22 and D4).

Table 21
Incidences of Osteoma and Osteosarcoma in Mice in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Male				
Nose[a]	50	50	50	50
Osteoma[b,c]	0	0	0	1
Female				
Bone	49	50	50	50
Osteoma[d]	0	0	1	0
Osteosarcoma[e]	0	0	1	1
Osteoma or Osteosarcoma[f]	0	0	2	1
Nose	49	50	50	50
Osteoma[g]	0	0	1	0
All Organs	49	50	50	50
Osteoma[d]	0	0	2	0
Osteoma or Osteosarcoma[f]				
Overall rate[h]	0/49 (0%)	0/50 (0%)	3/50 (6%)	1/50 (2%)
Adjusted rate[i]	0.0%	0.0%	6.5%	2.2%
Terminal rate[j]	0/35 (0%)	0/41 (0%)	2/38 (5%)	1/37 (3%)
First incidence (days)	—[l]	—	439	729 (T)
Poly-3 test[k]	P=0.234	—[m]	P=0.122	P=0.498

(T) Terminal sacrifice
[a] Number of animals microscopically examined (nose) or number necropsied (bone, all organs)
[b] Number of animals with neoplasm
[c] Historical incidence for 2-year gavage studies with corn oil vehicle control groups (mean ± standard deviation): 0/250; all routes: 0/1,445
[d] Historical incidence for corn oil gavage studies: 0/248; all routes: 1/1,498 (0.1% ± 0.4%), range 0%-2%
[e] Historical incidence for corn oil gavage studies: 1/248 (0.4% ± 0.9%), range 0%-2%; all routes: 7/1,498 (0.5% ± 1.0%), range 0%-4%
[f] Historical incidence for corn oil gavage studies: 1/248 (0.4% ± 0.9%), range 0%-2%; all routes: 8/1,498 (0.5% ± 1.0%), range 0%-4%
[g] Historical incidence for corn oil gavage studies: 0/248; all routes: 0/1,495
[h] Number of animals with neoplasm per number of animals necropsied
[i] Poly-3 estimated neoplasm incidence after adjustment for intercurrent morality
[j] Observed incidence at terminal kill
[k] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for differential morality in animals that do not reach terminal sacrifice.
[l] Not applicable; no neoplasms in animal group
[m] Value of statistic cannot be computed.

TABLE 22
Incidences of Selected Nonneoplastic Lesions in Mice in the 2-Year Gavage Study of Pulegone

	Vehicle Control		37.5 mg/kg		75 mg/kg		150 mg/kg	
Male								
Nose[a]	50		50		50		50	
Inflammation[b]	2	(1.5)[c]	3	(1.7)	2	(2.0)	22**	(1.4)
Nerve, Atrophy	1	(1.0)	3	(1.7)	3	(1.0)	45**	(1.8)
Olfactory Epithelium, Degeneration	3	(2.0)	3	(2.0)	11*	(1.4)	46**	(1.7)
Olfactory Epithelium, Metaplasia	1	(2.0)	5	(1.4)	3	(1.0)	44**	(1.9)
Forestomach	50		50		50		50	
Hyperplasia, Squamous	7	(2.0)	10	(2.3)	27**	(2.1)	41**	(2.6)
Inflammation	3	(1.7)	9	(2.0)	24**	(1.7)	39**	(2.1)
Ulcer	0		3	(1.3)	9**	(1.9)	22**	(2.0)
Heart	50		50		50		50	
Mineralization	1	(2.0)	1	(1.0)	1	(1.0)	4	(2.8)
Adrenal Cortex	50		50		50		50	
Hypertrophy	17	(1.6)	9*	(1.6)	8*	(1.3)	4**	(1.3)
Pancreatic Islets	50		50		50		50	
Hyperplasia	20	(1.4)	17	(1.5)	21	(1.2)	2**	(1.0)
Female								
Nose	49		50		50		50	
Inflammation	2	(1.5)	1	(1.0)	4	(1.8)	27**	(1.8)
Nerve, Atrophy	0		1	(2.0)	2	(1.0)	49**	(2.7)
Olfactory Epithelium, Degeneration	0		5*	(1.8)	22**	(1.6)	48**	(1.9)
Olfactory Epithelium, Metaplasia	1	(1.0)	2	(1.0)	4	(1.8)	49**	(2.8)
Forestomach	49		50		50		50	
Hyperplasia, Squamous	13	(2.1)	1**	(1.0)	10	(1.9)	26**	(2.1)
Inflammation	10	(1.9)	0**		7	(1.9)	20*	(2.1)
Ulcer	8	(2.1)	0**		4	(2.0)	10	(2.0)
Heart	49		50		50		50	
Mineralization	2	(3.0)	0		2	(3.0)	8*	(1.9)
Eye	49		50		49		50	
Cornea, Inflammation	2	(2.0)	0		0		9*	(2.2)
Ovary	49		49		50		50	
Atrophy	11	(3.3)	10	(3.3)	24**	(3.5)	39**	(3.9)
Spleen	49		50		50		50	
Hematopoietic Cell Proliferation	24	(1.6)	29	(1.4)	25	(1.4)	40**	(1.5)

* Significantly different ($P \leq 0.05$) from the vehicle control group by 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

GENETIC TOXICOLOGY

Pulegone (doses up to 3,333 µg/plate in the first study and 3,500 µg/plate in the second study) was not mutagenic in either of two independent bacterial mutagenicity assays, with or without exogenous metabolic activation (Tables E1 and E2). Bacterial strains tested in the first study included *Salmonella typhimurium* strains TA97, TA98, TA100, and TA1535, with and without 10% or 30% hamster or rat liver S9. Strains tested in the second study, conducted on the same lot of pulegone that was tested in the 2-year rodent bioassay, included *S. typhimurium* strains TA98 and TA100 and *Escherichia coli* strain WP2 *uvrA*/pKM101, with and without 10% rat liver S9.

A second study using the same lot of pulegone that was tested in the 2-year rodent bioassay (12.5 to 1,500 µg/plate) was positive for mutagenicity in *S. typhimurium* strain TA98 and *E. coli* strain WP2 *uvrA*/pKM101 when tested in the presence of 10% rat liver S9 (Table E3). The lowest concentration that produced a mutagenic response was 500 µg/plate in both strains.

In vivo, no significant increases in the frequencies of micronucleated normochromatic erythrocytes were seen in peripheral blood of male or female B6C3F1 mice administered pulegone (9.375 to 150 mg/kg) for 3 months by gavage (Table E4). The percentage of micronucleated polychromatic erythrocytes (reticulocytes) in the peripheral blood of male and female mice was not significantly changed by pulegone administration, suggesting an absence of chemical-associated bone marrow toxicity.

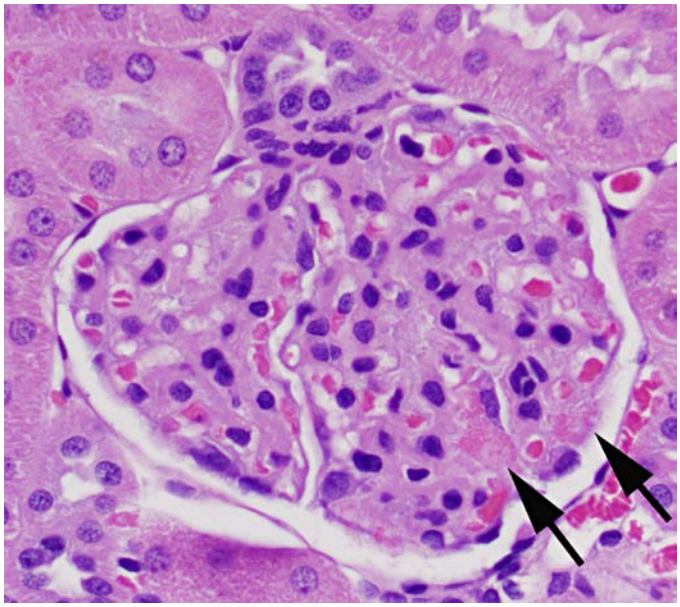


PLATE 1
Hyaline glomerulopathy in the kidney of a male F344/N rat administered 150 mg/kg pulegone by gavage for 3 months. There are small, round, eosinophilic globules present within the glomerular mesangium (arrows). (H&E)

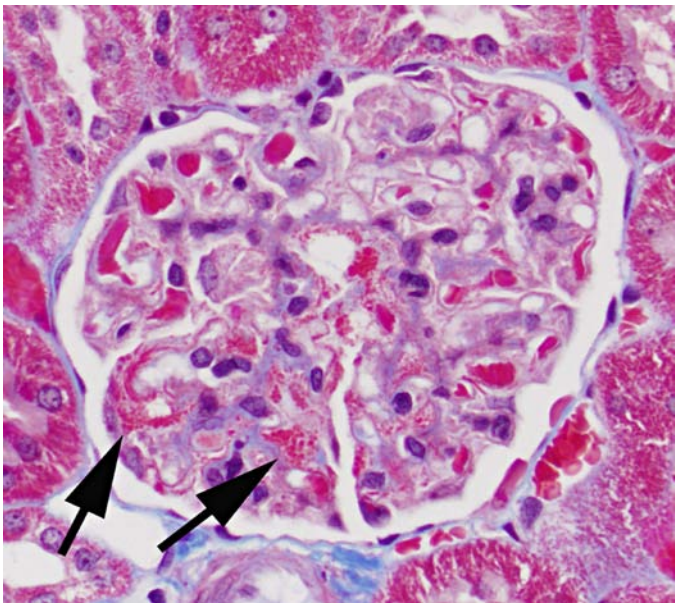


PLATE 2
Hyaline glomerulopathy in the kidney of a male F344/N rat administered 150 mg/kg pulegone by gavage for 3 months. The small, round, intraglomerular globules stain red with Masson's Trichrome (arrows).

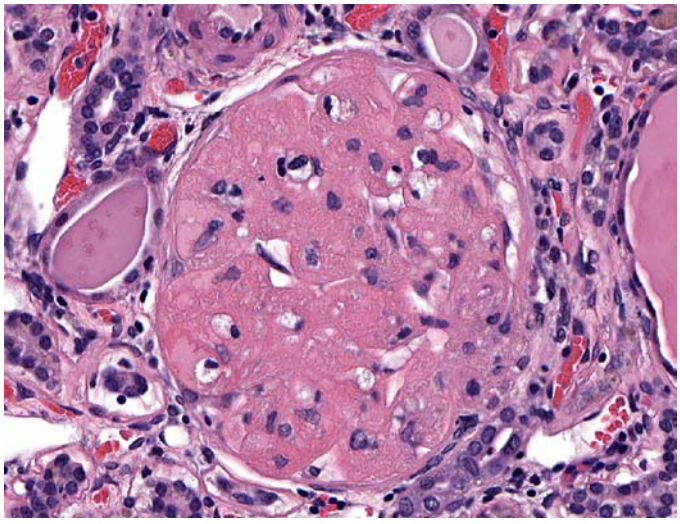


PLATE 3
Hyaline glomerulopathy in the kidney of a female F344/N rat administered 150 mg/kg pulegone by gavage for 60 weeks in the 2-year study. This lesion was characterized histologically by a thickening of the glomerular mesangium and, to a lesser degree, the capillary loops by amorphous eosinophilic material, resulting in significant glomerular enlargement in the more markedly affected animals. (H&E)

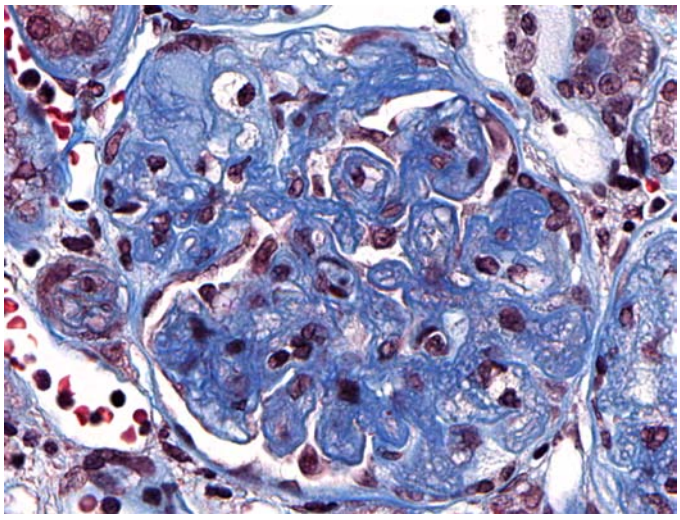


PLATE 4
Hyaline glomerulopathy in the kidney of a female F344/N rat administered 150 mg/kg pulegone by gavage for 60 weeks in the 2-year study. The material expanding the glomerular mesangium stained blue with Masson's Trichrome.

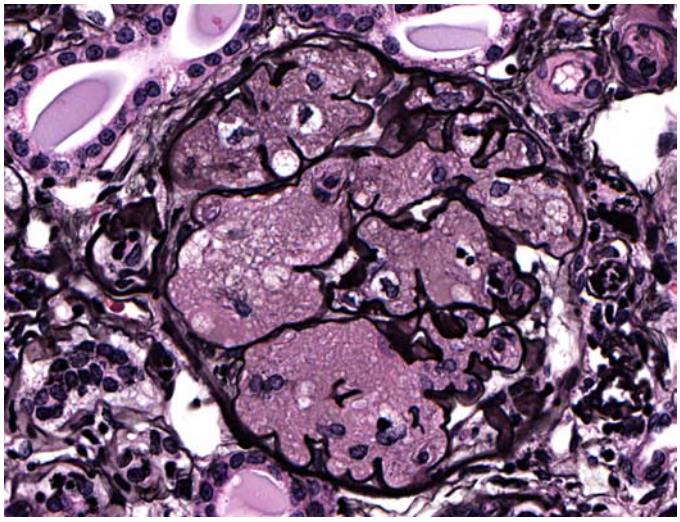


PLATE 5
Hyaline glomerulopathy in the kidney of a female F344/N rat administered 150 mg/kg pulegone by gavage for 60 weeks in the 2-year study. The basement membranes are not increased in thickness. (Jones' Methenamine Silver)

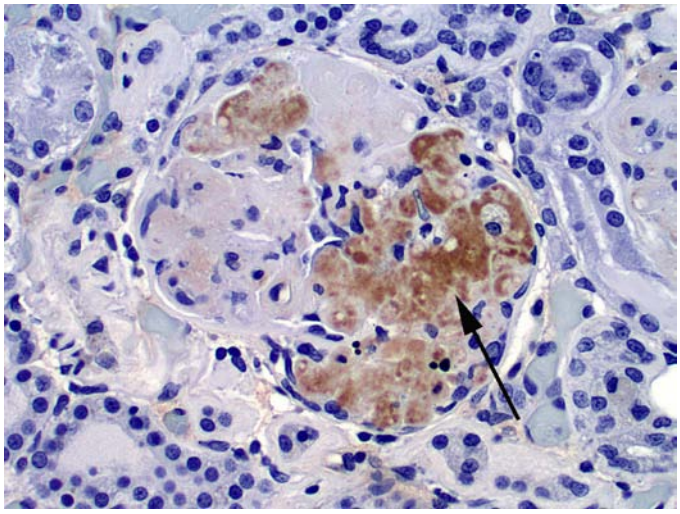


PLATE 6
Hyaline glomerulopathy in the kidney of a female F344/N rat administered 150 mg/kg pulegone by gavage for 60 weeks in the 2-year study. Immunohistochemical staining with anti-IgM antibody revealed segmental granular mesangial deposits (arrow).

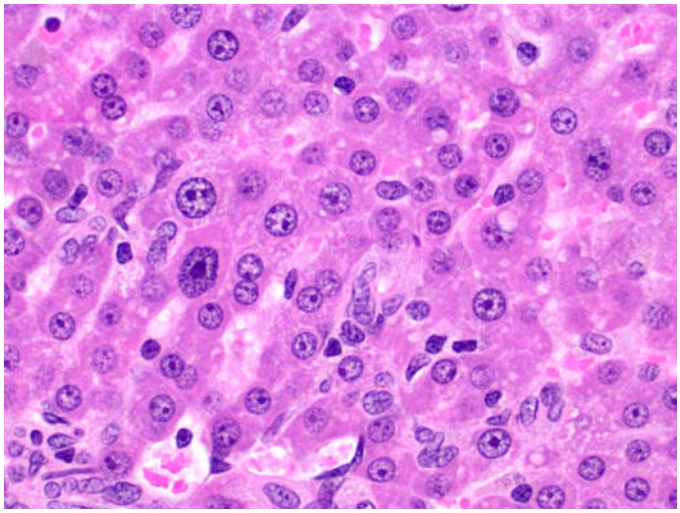


PLATE 7
Hepatocyte cellular alteration in the liver of a female F344/N rat administered 150 mg/kg pulegone by gavage for 60 weeks in the 2-year study. This term was used to describe a constellation of lesions in the liver that included a slight decrease in cell size, increased cytoplasmic basophilia and loss of cytoplasmic glycogen and lipid, and an increase in nuclear size with coarsely clumped chromatin and increased prominence and often number of nucleoli. Karyomegaly was occasionally present. (H&E)

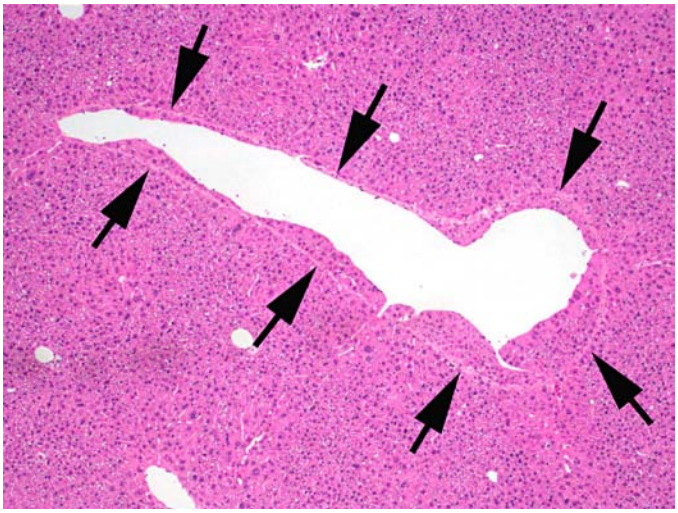


PLATE 8
Intravascular hepatocytes in the liver of a male B6C3F1 mouse administered 150 mg/kg pulegone by gavage for 2 years. Hepatocytes have proliferated within the wall (arrows) of a central vein. (H&E)

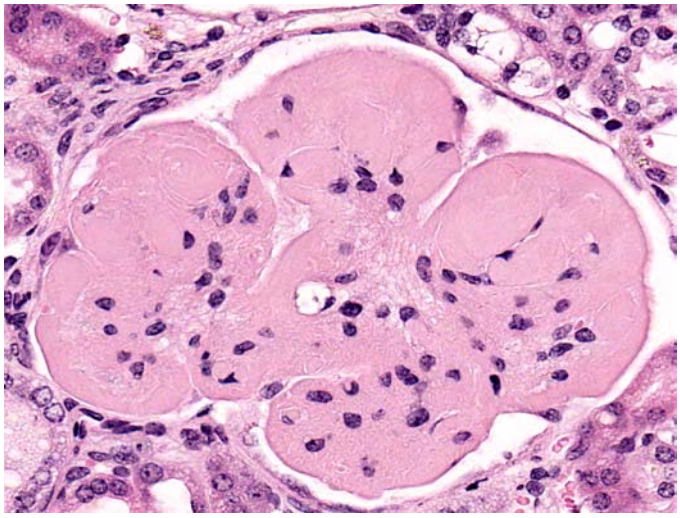


PLATE 9
Hyaline glomerulopathy in the kidney of a female B6C3F1 mouse administered 75 mg/kg pulegone by gavage for 2 years. This lesion was characterized by a thickening of the glomerular mesangium and, to a lesser degree, the capillary loops by amorphous eosinophilic material, resulting in significant glomerular enlargement in the more markedly affected animals. (H&E)

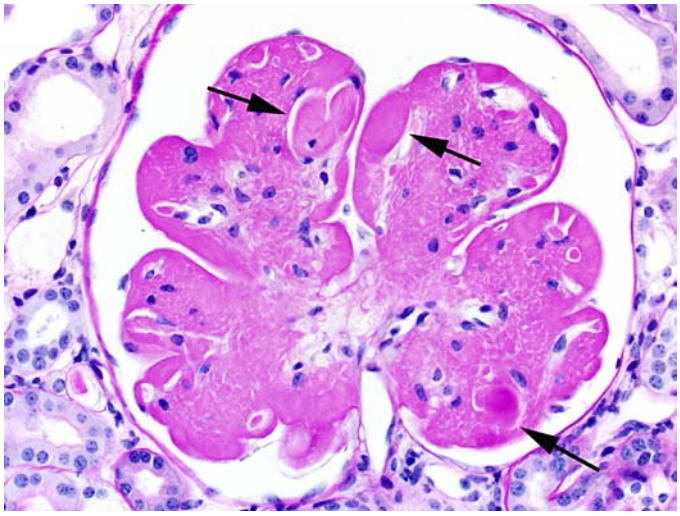


PLATE 10
Hyaline glomerulopathy in the kidney of a female B6C3F1 mouse administered 75 mg/kg pulegone by gavage for 2 years. The material expanding the mesangium is positive for Periodic Acid Schiff stain. Intracapillary thrombi can be identified (arrows).

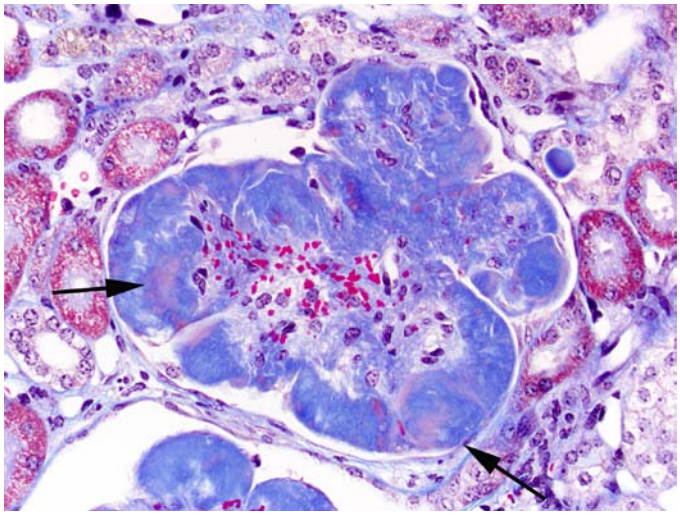


PLATE 11
Hyaline glomerulopathy in the kidney of a female B6C3F1 mouse administered 75 mg/kg pulegone by gavage for 2 years. The material expanding the mesangium stains blue with Masson's Trichrome. Organizing thrombi show a pale eosinophilic staining (arrows). Intraglomerular hemorrhage is also present.

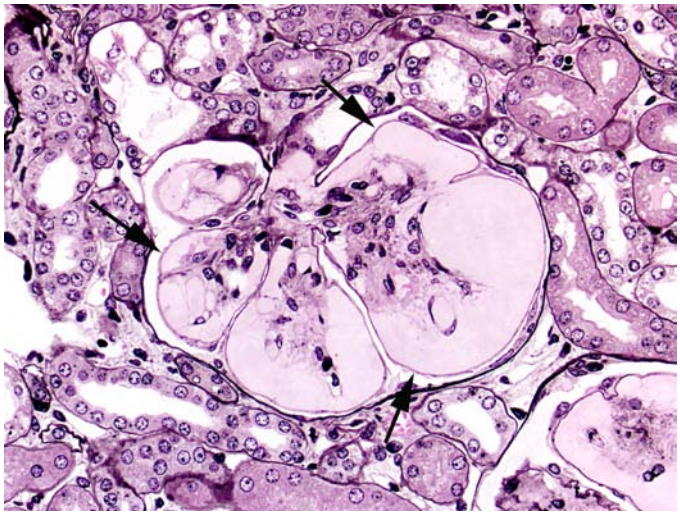


PLATE 12
Hyaline glomerulopathy in the kidney of a female B6C3F1 mouse administered 75 mg/kg pulegone by gavage for 2 years. Jones' Methenamine Silver stain reveals basement membranes of normal thickness (arrow).

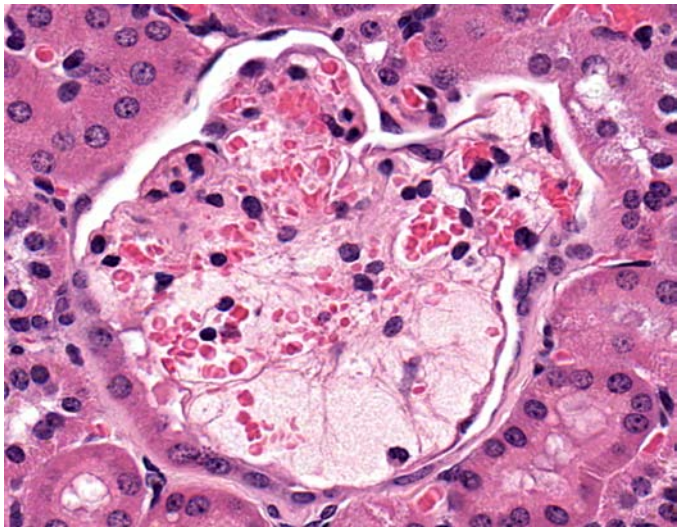


PLATE 13
Hyaline glomerulopathy in the kidney of a female B6C3F1 mouse administered 150 mg/kg pulegone by gavage for 2 years. Many affected glomeruli also displayed mesangiolysis with intraglomerular hemorrhage. (H&E)

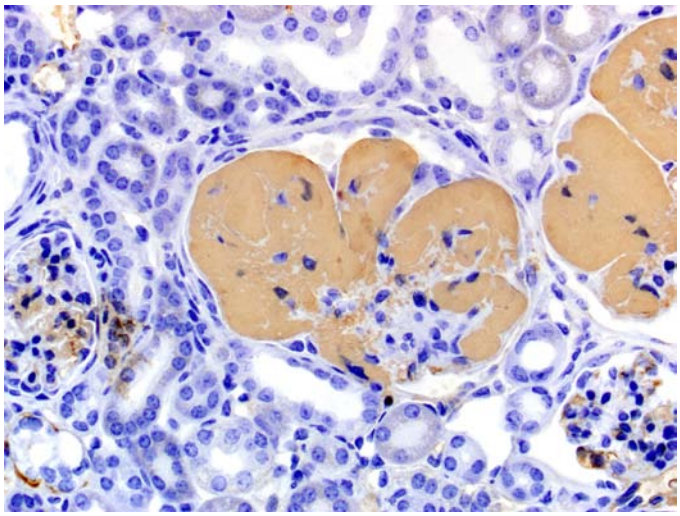
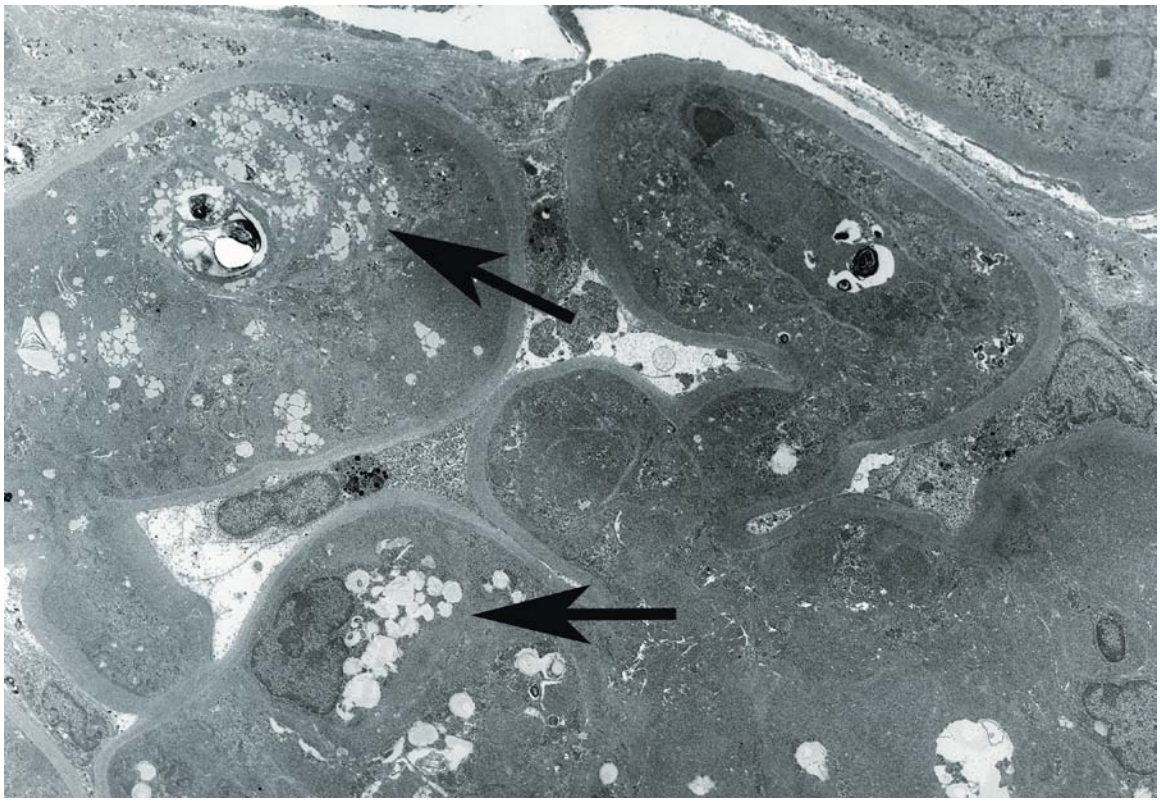
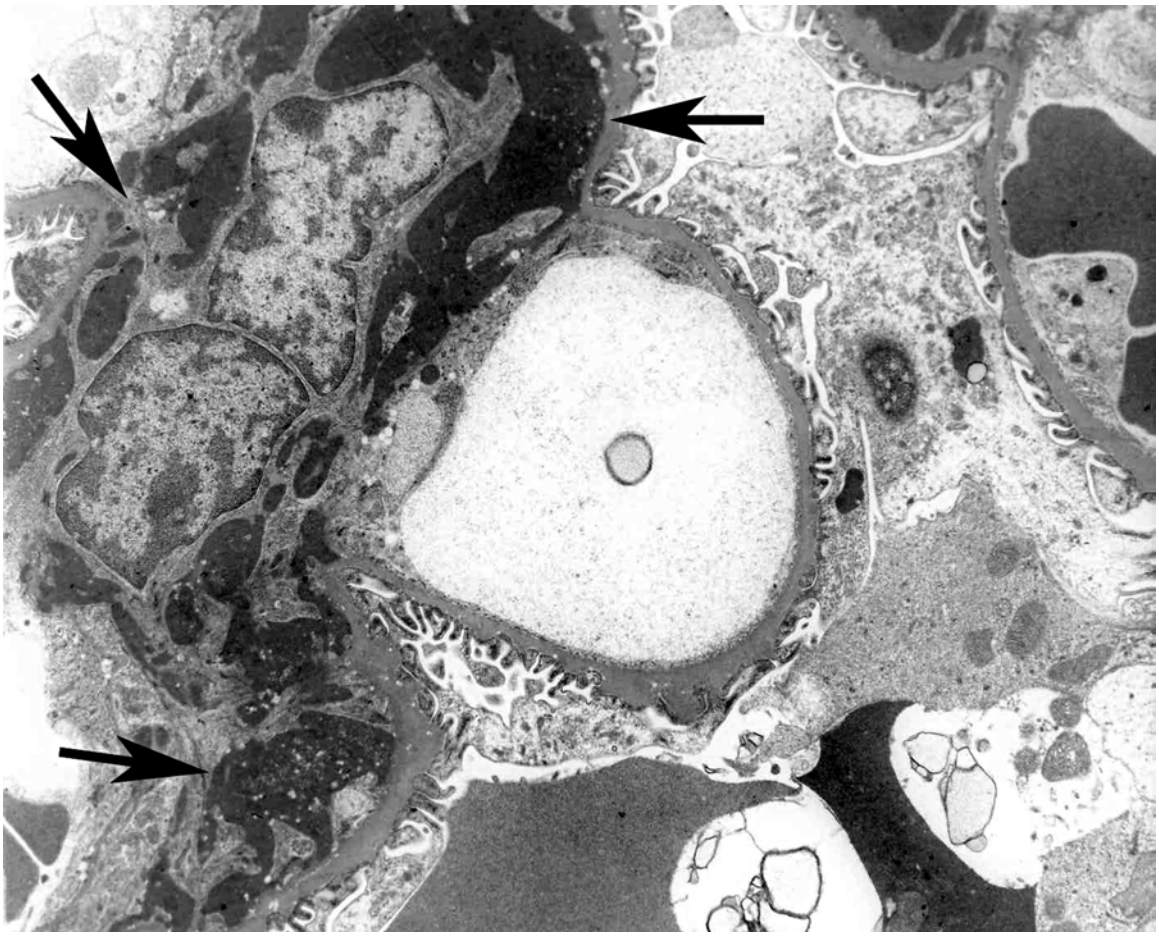


PLATE 14
Hyaline glomerulopathy in the kidney of a female B6C3F1 mouse administered 75 mg/kg pulegone by gavage for 2 years. Immunohistochemical staining with anti-IgM antibody revealed positive diffuse staining of the material expanding the mesangium.

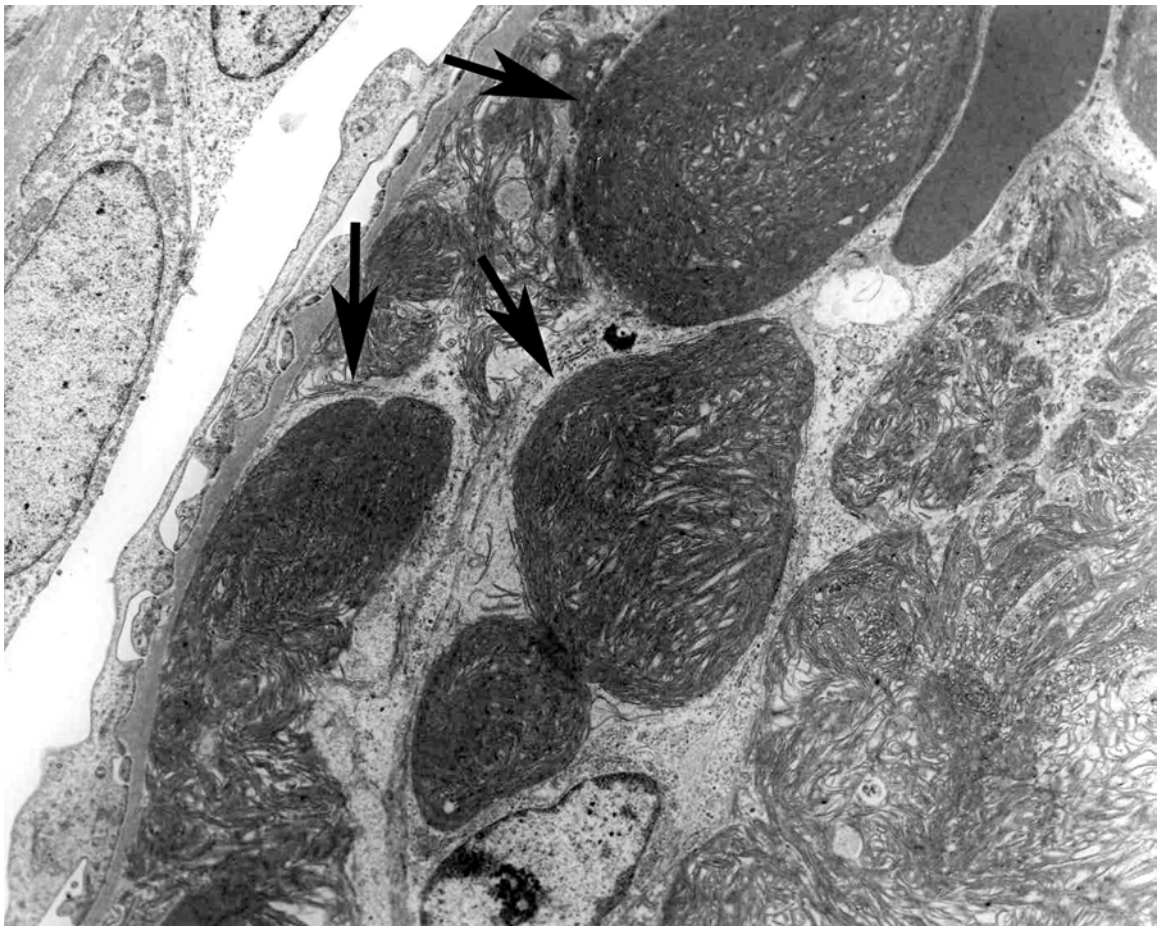


ELECTRON MICROGRAPH 1
Electron microscopy was performed on the renal cortices to further characterize the lesions of rats in the 2-year study. The glomerular tuft is expanded by amorphous, finely granular, variably dense material occasionally containing small smooth-margined vacuoles (arrows). Transmission electron microscopy.

ELECTRON MICROGRAPH 2
Higher magnification of a renal cortex illustrating the amorphous, finely granular and homogeneous nature of the material (arrows). Transmission electron microscopy.



ELECTRON MICROGRAPH 3
Electron microscopy was performed on the renal cortices of mice in the 2-year study to further characterize the lesions. In a kidney with mild hyaline glomerulopathy, there were irregular dense deposits (arrows) expanding the glomerular mesangium and extending into the capillary subendothelial space, and the glomerular basement membrane was irregularly thickened. Transmission electron microscopy.



ELECTRON MICROGRAPH 4
In a kidney of a 2-year mouse with marked hyaline glomerulopathy, the ultrastructural changes included large, well-circumscribed accumulations of closely packed tubules in longitudinal and cross-sectional arrays (arrows). These tubules were highly structured, nonbranching, curvilinear, fibrillary deposits forming discrete, variably sized, principally extracellular bundles. These bundles of fibrils were oriented in parallel rows, often swirling with a characteristic "fingerprint" pattern. These structures markedly expanded the glomeruli and obliterated the capillary lumens. Transmission electron microscopy.

DISCUSSION AND CONCLUSIONS

Pulegone is a monoterpene that is contained in essential oils produced by members of the mint (*Lamiaceae*) family (Lawrence, 2006). It is used as a mint-flavored additive in many food products and is found in a number of essential oils including the herbal remedy, pennyroyal oil, which has been employed in aromatherapy and as an emmenagogue (menstrual flow stimulant) and an abortifacient in humans (Lawrence, 2006). Published animal toxicity studies have shown it to be primarily a hepatotoxicant and to a lesser extent a lung and kidney toxicant (Gordon *et al.*, 1982; Thorup *et al.*, 1983a). In humans, pulegone (primarily from pennyroyal oil) has been associated with severe liver and kidney damage that has, in a fraction of cases, resulted in fatalities (Sullivan *et al.*, 1979; Anderson *et al.*, 1996; Bakerink *et al.*, 1996). Pulegone was nominated for study based on widespread human exposure, its use as a flavoring agent, and the absence of carcinogenicity data. To address the nomination, the NTP performed 2-week, 3-month, and 2-year studies in male and female F344/N rats and B6C3F1 mice, in addition to absorption, distribution, metabolism, and excretion and toxicokinetic studies published elsewhere (Chen *et al.*, 2001; 2003; Ferguson *et al.*, 2007).

In rats, doses of 300 and 600 mg/kg in the 2-week study caused high mortality; all rats in the lower dose groups survived to the end of the study. Minimal to mild liver necrosis was observed in 150 mg/kg male rats and 300 mg/kg female rats. Based on these results, 150 mg/kg was selected as the high dose in the 3-month rat study. In the 3-month rat study, one 150 mg/kg female died. Decreases in body weight were observed in 75 and 150 mg/kg male rats and 150 mg/kg female rats. Hepatic necrosis was observed in 150 mg/kg male rats but not in females administered the same dose. Based on these results, 75 mg/kg (males) and 150 mg/kg (females) were selected as the high doses for the 2-year rat study. In the 2-year rat study, dosing of the 75 mg/kg males and 150 mg/kg females was stopped during week 60 due to increased mortality and compromised body weight gains; these groups are referred to as the "stop-exposure" groups. The stop-exposure animals continued to exhibit high rates of mortality after dosing was stopped. The suspected cause of death was renal failure, which manifested histologically as hyaline glomerulopathy and severe chronic progressive nephropathy (nephropathy). Based on the severe renal pathology and high mortality, a retrospective review of the rat kidneys from the 3-month study was performed. This review identified minimal hyaline glomerulopathy in the 75 mg/kg males and the 150 mg/kg males and females. Hyaline glomerulopathy has never been reported in rats and has never been identified in a NTP bioassay. Other renal pathology assessments of the 3-month rat study revealed increased kidney weights of both males and females (18.75 mg/kg and greater groups). None of these observations from the 3-month study indicated that doses of 75 mg/kg in males and 150 mg/kg in females would lead to the early deaths and severely compromised body weight gains that were observed in these groups in the 2-year study. Based upon analyses of sperm parameters, vaginal cytology, and weights and histopathology of the reproductive organs of the 3-month study animals, there was no evidence of pulegone toxicity to the reproductive system of either rats or mice.

In the 2-week mouse study, doses of 300 mg/kg caused increases in mortality, clinical signs of toxicity, increased liver weights, and liver lesions, whereas 150 mg/kg had little to no effect on these parameters. Due to the adverse effects observed at 300 mg/kg, 150 mg/kg was selected as the high dose for the 3-month study in mice. The results of the 3-month study in mice were largely unremarkable. For this reason, the high dose selected for the 2-year study in mice was 150 mg/kg.

Overall, the mice were more resistant to the toxic effects of pulegone than rats. In the 3-month rat study, doses of 75 and 150 mg/kg in males and 150 mg/kg in females produced significant decreases in terminal body weights. In addition, the incidences of hepatic hypertrophy were significantly increased in 75 and 150 mg/kg male rats and 150 mg/kg female rats. In contrast, 3 months of dosing at 150 mg/kg in mice had no effect on body weight and produced no histopathologic changes in the liver. In the 2-year mouse study, despite lower body weight gains in the 150 mg/kg groups and a

significant increase in hepatic neoplastic transformation, there was no dose-related effect on survival. By comparison, doses as low as 37.5 mg/kg in male rats and 150 mg/kg in female rats caused decreased survival in the 2-year study. Both mice and rats experienced treatment-related increases in the incidences of hyaline glomerulopathy; however, rats developed end-stage renal disease with associated mortality, and mice did not. This may have been related to the compounded effects of severe nephropathy.

In the 2-year rat study, there were no treatment-related increases in the incidences of neoplasms in males; however, there were significant increases in the incidences of urinary bladder papilloma and papilloma or carcinoma (combined) in 150 mg/kg stop-exposure females, which were considered clear evidence of carcinogenic activity. A number of variables influenced this conclusion. 1) No urinary bladder papillomas or carcinomas have been observed in 1,347 control animals from previous studies; in approximately 200 of the most recent NTP chronic studies, only four chemicals have produced treatment-related increases in urinary bladder papilloma or carcinoma (combined) in female F344/N rats [1-amino-2,4-dibromoanthraquinone (NTP, 1996), anthraquinone (NTP, 2005), chloroprene (NTP, 1998), and *o*-nitroanisole (NTP, 1993)]. 2) A continuum of dose-related benign or malignant cancer was observed (papillomas and carcinomas). 3) Despite the significant decrease in survival in the 150 mg/kg stop-exposure group, a significant increase in the incidence of urinary bladder neoplasms was observed in this group (the survival-adjusted rate was greater than 20%, potentially indicating a robust carcinogenic effect).

Mechanisms of bladder carcinogenesis have been studied in some detail. Exposure to a number of genotoxic agents (particularly aromatic amines) has been associated with bladder cancer in both rodents and humans (Johansson and Cohen, 1997; Cohen, 1998). In addition, chronic irritation and regenerative hyperplasia of the bladder epithelium brought on by infection (chronic bacterial cystitis and schistosomiasis) or long-standing crystalline precipitates have been associated with increased risk of bladder cancer (Johansson and Cohen, 1997; Cohen, 1998). As reported here, pulegone was mutagenic as measured by the Ames assay. There were no indications of urinary tract infection in female rats, and dose-related urolithiasis and uroepithelium hyperplasia were not observed in animals in the 2-week, 3-month, or 2-year studies. Hence, the dose-related increase in the incidences of urinary bladder neoplasms observed in female rats is most likely related to the mutagenic activity of pulegone. It is currently uncertain what role the severe kidney disease (hyaline glomerulopathy and nephropathy) observed in the high dose animals played in the pathogenesis of the bladder tumors. In humans, chronic kidney disease has been associated with increased risk of bladder cancer (Wong *et al.*, 2009). Despite the lack of a similar association in rats between kidney disease and bladder cancer, it can be hypothesized that, considering the rapid onset of hyaline glomerulopathy in the dosed animals (meaning they lived a large fraction of their life with severely compromised renal function) and the unique nature of the glomerular lesion, the changes in kidney function may lead to changes in urine composition (e.g., growth factors) that are potentially related to the carcinogenic changes observed in the bladder (Cohen *et al.*, 2007). Further studies will be needed to determine if there is an interaction between kidney disease and the mutagenic activity of pulegone in the pathogenesis of bladder cancer.

In the 2-year studies, the manifestation of hyaline glomerulopathy exhibited a clear dose-response relationship, affecting many dosed male and female mice, most 75 mg/kg and 150 mg/kg stop-exposure female rats, and approximately 50% of the 75 mg/kg stop-exposure male rats. Hyaline glomerulopathy was irreversible as evidenced by the continued accelerated rate of death in the stop-exposure rats after pulegone administration was halted. In the 3-month rat study, hyaline glomerulopathy consisted of numerous, small, round, eosinophilic globules apparently confined to the glomerular mesangium. In the 2-year mouse and rat studies, hyaline glomerulopathy was characterized by accumulations of an amorphous eosinophilic material within the glomerulus. This lesion has never been observed in NTP studies and has never been reported in the rat; although it has been documented in B6C3F1 mice (Wojcinski *et al.*, 1991), its manifestation has not been associated with chemical administration. In humans, there are only a handful of glomerular diseases with Congo Red-negative immunoglobulin-derived organized deposits (Jennette and Heptinstall, 2007). When organized glomerular deposits are grouped by their ultrastructural appearance, the diagnosis of immunotactoid glomerulopathy (ITG) is reserved for cases with larger, parallel tubules. Patients with ITG have no associated systemic disease. With ITG there is mesangial expansion by eosinophilic, Congo Red (amyloid stain)-negative, PAS (glycoprotein stain)-positive, and Trichrome Blue-positive material with a subepithelial and subendothelial location via light microscopy. There can be a range from a slight increase in glomerular deposits to massive deposits with glomerular distortion. Electron microscopy reveals extracellular deposits that are elongated, nonbranching

fibrils or tubules that do not show periodicity or substructure. Granular unorganized deposits are also seen separately from the organized deposits or intermixed with them. The composition of the deposits is uncertain but immunohistochemistry indicates they contain immunoglobulin and complement C3 as principal components. These light and electron microscopy findings are similar in both mice and humans. The etiology and pathogenesis of ITG are unknown, although recent experiments in the CD2-associated protein (CD2ap) knockout mouse, which develops a similar lesion (mesangial deposits) as observed in ITG, suggest that defects in podocyte function involved in the clearance of filtered and retained immunoglobulin may in part be responsible for the pathogenesis of the disease (Shih *et al.*, 1999; Schwartz *et al.*, 2002). A separate study has noted an ITG-like pathology in neurotrophin-1/B cell-stimulating factor-3 over-expressing mice (Senaldi *et al.*, 2002). The presumed cause of the pathology in these mice was high circulating levels of immunoglobulins that overwhelmed the glomerular filtration apparatus. Notably, there were some indications of increased total serum protein in rats from the 3-month study of pulegone; however, levels of immunoglobulins were not specifically measured. Although ITG has not been associated with pulegone exposure in humans, toxic doses of pennyroyal oil lead to renal damage in humans (Anderson *et al.*, 1996).

Renal disease can lead to a number of secondary pathologies. As it progresses, hyperphosphatemia develops due to decreased glomerular filtration rates (Figure 8). The increased phosphate levels in the blood coupled with a decreased calcitrol production by the diseased kidney leads to decreased levels of ionized calcium in the blood. The physiologic response to decreased blood calcium is parathyroid gland hyperplasia (Drüeke, 2000) and increased parathyroid hormone (PTH) secretion, which in turn causes increased bone reabsorption and increased calcium absorption from the intestines, which can lead to hypercalcemia. Increased bone reabsorption can also lead to fibrous osteodystrophy (Hruska and Teitelbaum, 1995). As renal failure advances, hypercalcemia can develop, leading to soft tissue mineralization. Furthermore, hypercalcemia and PTH-induced increases in vitamin D_3 can have proliferative effects on the adrenal medulla (Rosol *et al.*, 2001). For these reasons, the increased incidences of fibrous osteodystrophy, parathyroid gland hyperplasia, soft tissue mineralization (heart, glandular stomach, blood vessel, and lung) and the associated inflammation, and the adrenal medulla hyperplasia observed in rats in the 2-year study were considered to be secondary to the renal failure. Hyperparathyroidism, administration of recombinant parathyroid hormone, and chronic hypercalcemia have been associated with increased C-cell hyperplasia and C-cell adenomas (Tomita and Millard, 1992; FDA, 2002). Paradoxically, in the studies described here, a reduced incidence of C-cell hyperplasia was observed in female rats. The mechanistic underpinnings of this observation are unknown; however, it is likely that the changes in C-cell hyperplasia are related to the renal failure and the related perturbations in calcium homeostasis.

There were significantly increased incidences of liver neoplasms in male and female mice in the 2-year study that were considered clear evidence of carcinogenicity, and male and female mice exhibited extensive dose-related increases in nonneoplastic lesions and putative preneoplastic foci (eosinophilic foci). In males, hepatocellular adenoma multiplicity was significantly increased in all dosed groups, and the incidences of hepatocellular adenoma (includes multiple) and hepatoblastoma (includes multiple) were increased in 75 mg/kg males. In addition, the combined incidences of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma were significantly increased in 75 mg/kg males and nearly significantly increased in 150 mg/kg males. In control B6C3F1 mice, body weight decrements lead to a decreased rate of spontaneous liver tumors (Haseman *et al.*, 1997). After adjusting for body weight, average survival time, housing, and route of exposure, the expected incidences of liver neoplasms in male mice in the absence of a chemical effect were 33/50, 33/50, 33/50, and 22/50, whereas this study's observed incidences were 29/50, 37/50, 42/50, and 36/50. Assuming sampling variability of the estimates to be approximately ± 3 animals (with an approximate 95% confidence interval), the incidences in the vehicle control and 37.5 mg/kg groups were fairly close to what was expected, while the incidences in the 75 and 150 mg/kg groups were, respectively, nine and 14 animals greater than expected. Overall, the data and additional analysis indicate there was clear evidence of carcinogenic activity in male mice.

In female mice, the incidence of hepatocellular adenoma and the combined incidences of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma were significantly increased in the 150 mg/kg group despite a concurrent vehicle control incidence that was at the top of the historical control range for corn oil gavage studies. Body weight adjusted tumor incidence (Haseman *et al.*, 1997) indicates a nearly fivefold increase above the expected in hepatic neoplasms in the 150 mg/kg group (16/49, 15/50, 17/50, 7/50, expected;

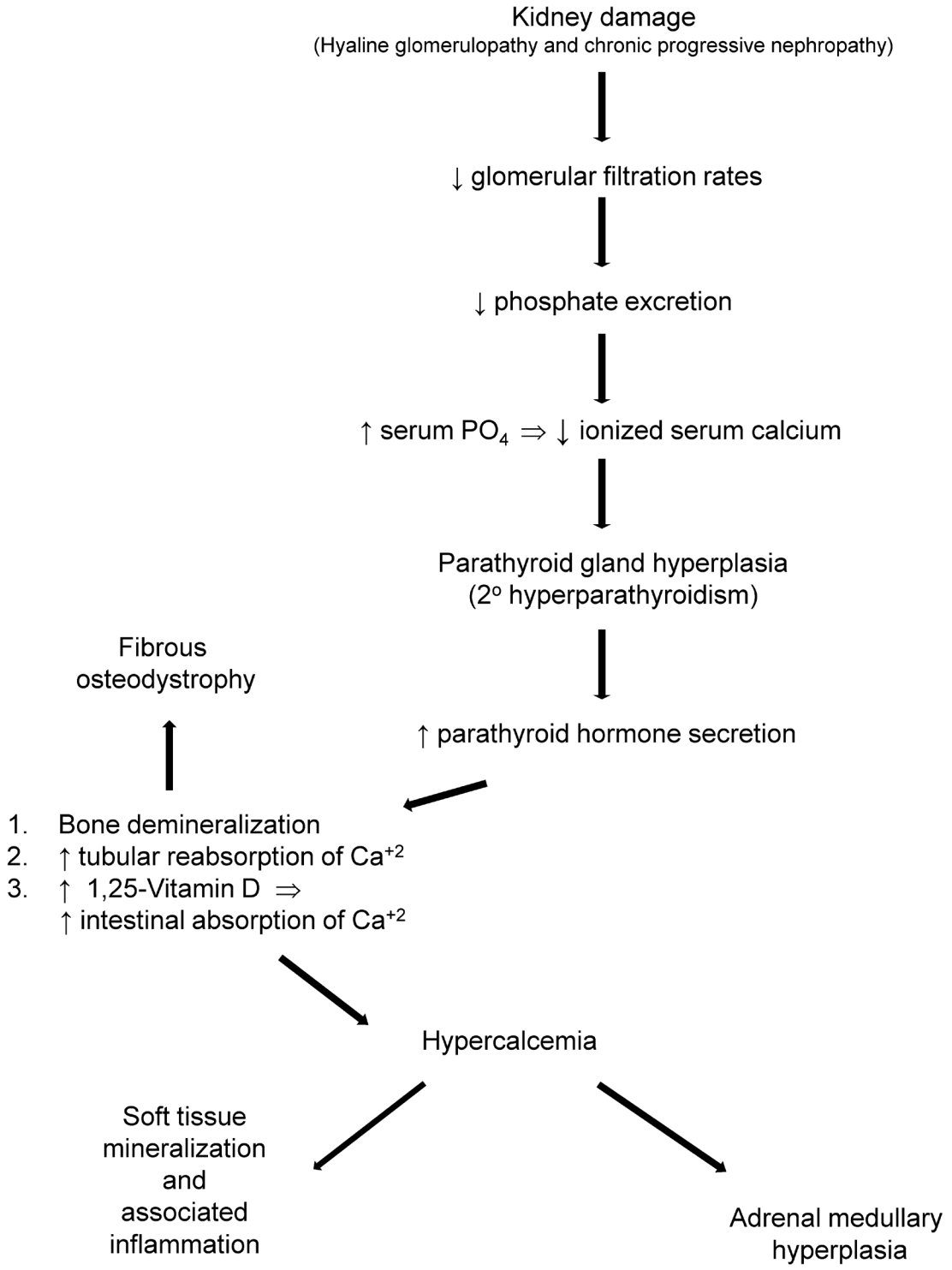


FIGURE 8
Potential Relationship Between Renal Failure and Other Lesions Observed in the 2-Year Rat Study
Biologically possible relationships substantiated by published data are shown.

17/49, 15/50, 15/50, 33/50, observed). The significant pairwise increase in hepatocellular neoplasms and body weight adjusted incidence rates of liver tumors led to the conclusion that there was clear evidence of carcinogenic activity.

The increases in the incidences of liver neoplasms observed in the mice are potentially related to the hepatotoxicity of pulegone. Pulegone is metabolized to reactive metabolites that form macromolecular adducts, in turn leading to cytotoxicity (Nelson *et al.*, 1992; Nelson, 1995). Protein adduct data from humans that have experienced pennyroyal toxicity suggest that a similar mode of pulegone-induced hepatoxicity occurs in humans (Anderson *et al.*, 1996). Gene expression studies using liver from male Sprague-Dawley rats treated with pulegone indicate an increase in the expression of transcripts related to oxidative stress that is possibly causally related to the cytotoxicity (McMillian *et al.*, 2004).

In the current studies, the liver was a major target of toxicity in both rats and mice. In addition to the neoplasms highlighted above, numerous nonneoplastic lesions were documented in the 2-week, 3-month (rats only), and 2-year studies in both sexes of rats and mice. Corresponding increases in serum alanine aminotransferase, γ-glutamyltransferase, and alkaline phosphatase were observed to a greater extent in rats than in mice in the 3-month studies. The observation of pulegone-induced liver toxicity is in agreement with previous rodent studies (Gordon *et al.*, 1982, 1987; Mizutani *et al.*, 1987; Thomassen *et al.*, 1988, 1990, 1992), and liver toxicity has been observed in humans consuming toxic doses of pennyroyal oil (Anderson *et al.*, 1996). The mechanism of pulegone-mediated hepatoxicity is related to the balance of bioactivation through oxidative metabolism and detoxification via conjugation of reactive metabolites (Nelson *et al.*, 1992). Inhibition of P450-dependent metabolism attenuates the hepatotoxicity of pulegone (Mizutani *et al.*, 1987; McClanahan *et al.*, 1989; Madyastha and Moorthy, 1989; Nelson *et al.*, 1992), whereas depletion of glutathione exacerbates the hepatotoxicity of pulegone (Gordon *et al.*, 1982). Toxicity to a certain degree is related to formation of menthofuran, the highly toxic metabolite of pulegone (Gordon *et al.*, 1982; Thomassen *et al.*, 1988). Menthofuran is hypothesized to rearrange to form the γ-ketoenal, 8-pulegone aldehyde, a soft electrophile that is hypothesized to react with sulfhydryl groups in proteins (leading to toxicity) or glutathione (leading to detoxification) (McClanahan *et al.*, 1989). The adduction of proteins is generally believed to lead to toxicity through the disruption of essential cellular processes and through the activation of the unfolded protein or endoplasmic reticulum (ER) stress response (Schröder and Kaufman, 2005; Liebler, 2008). Activation of the ER stress response pathway can result in adaptive changes [such as induction of chaperone proteins and antioxidant proteins such as members of the glutathione-S-transferase (GST) family of proteins] or alternatively cell death, by way of apoptosis or necrosis if the extent of cellular injury is too great to repair. Notably, treatment of rats with pulegone leads to the induction of genes that are integral to the ER stress response such as *Hsp*90, and a number of the GSTs (McMillian *et al.*, 2004). The relevance of this mechanism of toxicity to humans is supported by the observation of menthofuran in the blood and menthofuran-adducted hepatic microsomal proteins in a 24-year-old woman who died following ingestion of pennyroyal herbal extract and black cohosh root (Anderson *et al.*, 1996). Contrary to the findings of studies that involved highly toxic/lethal doses of pennyroyal, a recent pulegone metabolism study in human subjects that used a dose of 500 µg/kg (a dose in the estimated range of pulegone exposure in the general population) did not identify menthofuran as a major metabolite; the small amount that was identified was thought to be an artifact of the extraction process (Engel, 2003). Furthermore, pulegone metabolism studies performed in F344/N rats with doses that did not produce overt toxicity also indicated menthofuran was not a major metabolite (Chen *et al.*, 2001). A review of the latter suggests that the formation of menthofuran decreased disproportionately with decreasing doses of pulegone. Such observations are consistent with results demonstrating alterations in hepatic levels of glutathione in the 3-month studies at high, but not low, doses.

The incidences of osteoma and osteoma or osteosarcoma (combined) in all organs in 75 mg/kg female mice exceeded the historical control ranges for corn oil gavage studies and all study routes. Notably, one 150 mg/kg male mouse and one 75 mg/kg female mouse developed a nasal osteoma. No osteomas of the nose have been observed in historical control mice from NTP studies. Due to the absence of bone neoplasms in historical control mice and the observation of osteomas in both male and female dosed mice, these neoplasms may have been related to pulegone administration. Although treatment-related increases in the incidences of osteomas have not been observed before in an NTP mouse study, they have been observed in CD-1 mice following administration of sodium fluoride (Maurer *et al.*, 1993). Dose-related increased incidences of osteosarcoma in NTP studies are rare but have been observed, primarily

in rats (NCI, 1978; NTP, 1990). In humans, there are three theories on the etiology of nasal osteomas: developmental (proliferation of remnant embryonic cells), traumatic (associated with inflammation and regeneration following injury), and infectious (chronic inflammation leads to damage and regeneration of bone tissue) (Eller and Sillers, 2006). Notably, dose-related increases in the incidences of nasal epithelium inflammation were observed in female mice from the current 2-year study. Administration of recombinant parathyroid hormone causes osteosarcomas in rats (Vahle *et al.*, 2002). In humans, hyperparathyroidism is not associated with increased risk of osteosarcoma; however, elevated PTH levels may change the cytologic and histologic features of late-onset osteosarcoma (Jimenez *et al.*, 2005). Hypothetically, the secondary hyperparathyroidism due to renal damage may be related to the increase in osteogenic, histologic-type tumors in mice.

Dose-related increased incidences of nonneoplastic lesions occurred in the nose (specifically the nasal epithelium) of male and female rats and mice. Pulegone has a relatively low vapor pressure; hence, it is unlikely that volatilization of the chemical led to increased exposure of the nasal epithelium. The rodent nasal epithelium expresses high levels of xenobiotic metabolizing enzymes (Bogdanffy, 1990; Ding and Kaminsky, 2003). As discussed in the Introduction of this Technical Report, the toxicity of pulegone is dependent on metabolic activation by cytochromes P450 (Mizutani *et al.*, 1987; McClanahan *et al.*, 1989; Madyastha and Moorthy, 1989; Nelson *et al.*, 1992); hence, the toxicity in the nose may be due to increased rates of bioactivation of the chemical by enzymes expressed in the nasal epithelium. Notably, the nasal epithelium of humans expresses high levels of cytochromes P450, suggesting that similar toxicity is plausible in humans following exposure to pulegone (Ding and Kaminsky, 2003).

The incidences of mammary gland fibroadenoma and pituitary gland adenoma in female rats are known to be influenced by body weight (Haseman *et al.*, 1997). The pathophysiologic mechanism by which changes in body weight attenuate the carcinogenic process in these tissues remains largely unexplained. Expected incidence rates of mammary gland fibroadenoma and pituitary gland adenoma can be calculated for dosed groups that have reductions in body weight relative to controls. The incidences of mammary gland fibroadenoma in the current mouse study were consistent with the observed body weight effect in all dosed groups. The decreased incidences of pituitary gland adenoma in all dosed groups of female rats were greater than would be expected due to body weight loss alone. The reason for this is uncertain. Another neoplasm showing decreased incidence in female rats was thyroid gland C-cell adenoma. The decrease was consistent with a decrease in C-cell hyperplasia. C-cell adenomas are late onset neoplasms in rats and, for this reason, the decreased incidence was attributed to the decreased survival in the higher dose groups.

Dose-related increases in the incidences of ovarian atrophy occurred in both rats and mice in the 2-year studies. Pulegone had no effect on rat or mouse ovaries in the 3-month studies and did not produce changes in the estrous cycle of rats, suggesting it is unlikely that pulegone is directly toxic to the ovary or is an endocrine disruptor. Ovarian atrophy is quite common in aged rodents, and dose-dependent increases in the incidence of this lesion are typically associated with decreased body weight (Greaves, 2007). Considering the dramatic effects on body weight observed in the 75 mg/kg and 150 mg/kg stop-exposure groups in the female rat study and the 150 mg/kg group in the female mouse study, it is likely that the increases in the incidences of ovarian atrophy were related to decreased body weight.

In the 2-year studies, increases in the incidences of forestomach hyperplasia were observed in male rats and male and female mice. The results are consistent with the occurrence of forestomach lesions observed in the 3-month studies. The forestomach lesions may be related to the stress the animals experienced during this study as also evidenced by the decreased mean body weights in the high dose groups compared to the controls (Kim *et al.*, 2002).

In rats, statistically significant increases in pancreatic acinar atrophy, bone marrow hyperplasia, and mesenteric lymph node hemorrhage were observed. The pancreas acinar atrophy, although significant in certain dose groups, exhibited an inconsistent dose response and therefore was considered an inconclusive finding. Bone marrow hyperplasia was attributed to ongoing inflammation in the nose, and the increase in mesenteric lymph node hemorrhage was likely a secondary effect of kidney disease which can lead to increased capillary fragility (Broscious and Castagnola, 2006).

In mice, statistically significant increases in corneal inflammation and splenic hematopoietic cell proliferation were observed. The increased corneal inflammation, although statistically significant at the highest dose, did not exhibit an increase in severity between the

treated and control animals. The increases in hematopoietic cell proliferation in the spleen was attributed to the chronic inflammation in the forestomach and nose.

CONCLUSIONS

Under the conditions of these 2-year gavage studies, there was *no evidence of carcinogenic activity** of pulegone in male F344/N rats administered 18.75, 37.5, or 75 (stop-exposure) mg/kg. There was *clear evidence of carcinogenic activity* of pulegone in female F344/N rats based on increased incidences of urinary bladder neoplasms. There was *clear evidence of carcinogenic activity* of pulegone in male and female B6C3F1 mice based on increased incidences of hepatocellular neoplasms (adenomas in both sexes and hepatoblastomas in males). Osteomas and osteosarcomas in female B6C3F1 mice may have been related to pulegone administration.

A unique renal lesion, hyaline glomerulopathy, was observed in all dosed groups of male and female mice and female rats and in 37.5 mg/kg and 75 mg/kg stop-exposure male rats. In rats, renal failure secondary to hyaline glomerulopathy and nephropathy contributed to the decreased survival in the 75 mg/kg stop-exposure males and 150 mg/kg stop-exposure females.

Pulegone administration was also associated with the occurrence of nonneoplastic lesions in the liver and nose of rats and mice and in the forestomach of male and female mice and male rats.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 13. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 15.

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

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

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death		1		
Moribund	8	10	12	35
Natural deaths	3	7	10	13
Survivors				
Terminal sacrifice	39	32	28	2
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Intestine large, cecum	(50)	(50)	(49)	(50)
Intestine large, colon	(50)	(50)	(50)	(50)
Osteosarcoma, metastatic, spleen			1 (2%)	
Intestine large, rectum	(50)	(50)	(50)	(50)
Intestine small, duodenum	(50)	(50)	(50)	(50)
Carcinoma				1 (2%)
Intestine small, ileum	(50)	(50)	(49)	(50)
Osteosarcoma, metastatic, spleen			1 (2%)	
Intestine small, jejunum	(50)	(50)	(50)	(50)
Lipoma		1 (2%)		
Liver	(50)	(50)	(50)	(50)
Hepatocellular adenoma	1 (2%)		1 (2%)	1 (2%)
Osteosarcoma, metastatic, spleen			1 (2%)	
Mesentery	(9)	(11)	(11)	(4)
Carcinoma, metastatic, kidney		1 (9%)		
Osteosarcoma, metastatic, spleen			1 (9%)	
Oral mucosa	(0)	(1)	(3)	(0)
Pharyngeal, squamous cell carcinoma		1 (100%)		
Pancreas	(50)	(50)	(50)	(50)
Adenoma	2 (4%)	3 (6%)		
Carcinoma, metastatic, kidney		1 (2%)		
Osteosarcoma, metastatic, spleen			1 (2%)	
Salivary glands	(50)	(49)	(50)	(50)
Schwannoma malignant, metastatic, skin		1 (2%)		
Stomach, forestomach	(50)	(50)	(50)	(50)
Stomach, glandular	(50)	(50)	(50)	(50)
Tongue	(0)	(1)	(2)	(0)
Squamous cell papilloma		1 (100%)	2 (100%)	
Tooth	(0)	(2)	(0)	(0)
Odontoma		1 (50%)		
Cardiovascular System				
Blood vessel	(50)	(50)	(50)	(50)
Heart	(50)	(50)	(50)	(50)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Adenoma	2 (4%)		1 (2%)	

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

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Endocrine System (continued)				
Adrenal medulla	(50)	(50)	(50)	(50)
Pheochromocytoma benign	8 (16%)	12 (24%)	12 (24%)	2 (4%)
Pheochromocytoma benign, multiple		1 (2%)		
Pheochromocytoma malignant		2 (4%)	1 (2%)	
Islets, pancreatic	(50)	(50)	(50)	(50)
Adenoma	2 (4%)			
Parathyroid gland	(49)	(44)	(45)	(48)
Adenoma	1 (2%)			
Pituitary gland	(50)	(50)	(50)	(50)
Squamous cell carcinoma, metastatic, nose	1 (2%)			
Pars distalis, adenoma	11 (22%)	14 (28%)	17 (34%)	1 (2%)
Thyroid gland	(50)	(50)	(44)	(47)
C-cell, adenoma	8 (16%)	7 (14%)	4 (9%)	1 (2%)
Follicular cell, adenoma		4 (8%)	2 (5%)	1 (2%)
General Body System				
None				
Genital System				
Epididymis	(50)	(50)	(50)	(49)
Osteosarcoma, metastatic, spleen			1 (2%)	
Preputial gland	(50)	(50)	(50)	(50)
Adenoma	3 (6%)	5 (10%)	2 (4%)	1 (2%)
Carcinoma	1 (2%)			
Prostate	(50)	(50)	(50)	(49)
Seminal vesicle	(50)	(50)	(50)	(49)
Osteosarcoma, metastatic, spleen			1 (2%)	
Testes	(50)	(50)	(50)	(49)
Bilateral, interstitial cell, adenoma	41 (82%)	37 (74%)	38 (76%)	26 (53%)
Interstitial cell, adenoma	5 (10%)	8 (16%)	5 (10%)	18 (37%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Lymph node	(2)	(8)	(3)	(6)
Mediastinal, alveolar/bronchiolar carcinoma, metastatic, lung		1 (13%)		
Pancreatic, osteosarcoma, metastatic, spleen			1 (33%)	
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Spleen	(50)	(50)	(50)	(50)
Osteosarcoma			1 (2%)	
Thymus	(50)	(48)	(48)	(47)
Schwannoma malignant, metastatic, skin		1 (2%)		
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Fibroadenoma	1 (2%)	3 (6%)	1 (2%)	1 (2%)
Skin	(50)	(50)	(50)	(50)
Basal cell adenoma			1 (2%)	1 (2%)
Basal cell carcinoma		2 (4%)		

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

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Integumentary System (continued)				
Skin (continued)	(50)	(50)	(50)	(50)
Fibrous histiocytoma				1 (2%)
Keratoacanthoma	3 (6%)		1 (2%)	
Schwannoma malignant	1 (2%)			
Squamous cell carcinoma		1 (2%)		
Pinna, squamous cell papilloma				1 (2%)
Subcutaneous tissue, fibroma	3 (6%)	2 (4%)	3 (6%)	
Subcutaneous tissue, fibrous histiocytoma			1 (2%)	
Subcutaneous tissue, schwannoma, malignant	2 (4%)	2 (4%)	1 (2%)	
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Maxilla, osteosarcoma		1 (2%)		
Skeletal muscle	(0)	(0)	(1)	(0)
Perimysium, osteosarcoma, metastatic, spleen			1 (100%)	
Nervous System				
Brain	(50)	(50)	(50)	(50)
Astrocytoma malignant	1 (2%)	1 (2%)		
Meningioma malignant			1 (2%)	
Squamous cell carcinoma, metastatic, nose	1 (2%)			
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma		1 (2%)	3 (6%)	
Alveolar/bronchiolar carcinoma	1 (2%)	1 (2%)	1 (2%)	
Carcinoma, metastatic, kidney		1 (2%)		
Meningioma malignant, metastatic, brain			1 (2%)	
Squamous cell carcinoma			1 (2%)	
Alveolus, pheochromocytoma malignant, metastatic, adrenal medulla		1 (2%)		
Nose	(50)	(50)	(46)	(50)
Squamous cell carcinoma	1 (2%)			
Trachea	(50)	(50)	(50)	(50)
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Squamous cell carcinoma, metastatic, nose	1 (2%)			
Harderian gland	(50)	(50)	(50)	(50)
Squamous cell carcinoma, metastatic, nose	1 (2%)			
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Carcinoma		1 (2%)		
Renal tubule, adenoma	1 (2%)		2 (4%)	2 (4%)
Urinary bladder	(50)	(50)	(50)	(49)

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

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Leukemia mononuclear	4 (8%)	6 (12%)	6 (12%)	
Mesothelioma benign		2 (4%)	1 (2%)	
Mesothelioma malignant	2 (4%)		1 (2%)	1 (2%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	47	49	49	45
Total primary neoplasms	105	120	110	59
Total animals with benign neoplasms	47	48	48	44
Total benign neoplasms	92	100	95	56
Total animals with malignant neoplasms	10	16	14	3
Total malignant neoplasms	13	18	14	3
Total animals with metastatic neoplasms	3	4	2	1
Total metastatic neoplasms	15	7	10	7

[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 Pulegone

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate[a]	8/50 (16%)	13/50 (26%)	12/50 (24%)	2/50 (4%)
Adjusted rate[b]	17.4%	29.7%	27.0%	10.1%
Terminal rate[c]	6/39 (15%)	10/32 (31%)	7/28 (25%)	2/2 (100%)
First incidence (days)	699	611	608	727 (T)
Poly-3 test[d]	P=0.168	P=0.130	P=0.199	P=0.370N
Adrenal Medulla: Benign or Malignant Pheochromocytoma				
Overall rate	8/50 (16%)	15/50 (30%)	13/50 (26%)	2/50 (4%)
Adjusted rate	17.4%	33.8%	29.3%	10.1%
Terminal rate	6/39 (15%)	10/32 (31%)	8/28 (29%)	2/2 (100%)
First incidence (days)	699	611	608	727 (T)
Poly-3 test	P=0.117	P=0.059	P=0.138	P=0.370N
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	0/50 (0%)	1/50 (2%)	3/50 (6%)	0/50 (0%)
Adjusted rate	0.0%	2.3%	6.9%	0.0%
Terminal rate	0/39 (0%)	1/32 (3%)	3/28 (11%)	0/2 (0%)
First incidence (days)	—[e]	727 (T)	727 (T)	—
Poly-3 test	P=0.056	P=0.488	P=0.109	—[f]
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	1/50 (2%)	2/50 (4%)	4/50 (8%)	0/50 (0%)
Adjusted rate	2.2%	4.6%	9.2%	0.0%
Terminal rate	1/39 (3%)	1/32 (3%)	4/28 (14%)	0/2 (0%)
First incidence (days)	727 (T)	709	727 (T)	—
Poly-3 test	P=0.108	P=0.479	P=0.163	P=0.643N
Mammary Gland: Fibroadenoma				
Overall rate	1/50 (2%)	3/50 (6%)	1/50 (2%)	1/50 (2%)
Adjusted rate	2.2%	7.0%	2.3%	4.9%
Terminal rate	0/39 (0%)	2/32 (6%)	0/28 (0%)	0/2 (0%)
First incidence (days)	632	701	659	587
Poly-3 test	P=0.585	P=0.282	P=0.748	P=0.559
Pancreas: Adenoma				
Overall rate	2/50 (4%)	3/50 (6%)	0/50 (0%)	0/50 (0%)
Adjusted rate	4.4%	7.0%	0.0%	0.0%
Terminal rate	2/39 (5%)	3/32 (9%)	0/28 (0%)	0/2 (0%)
First incidence (days)	727 (T)	727 (T)	—	—
Poly-3 test	P=0.223N	P=0.472	P=0.249N	P=0.444N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	11/50 (22%)	14/50 (28%)	17/50 (34%)	1/50 (2%)
Adjusted rate	24.0%	31.7%	37.6%	5.0%
Terminal rate	10/39 (26%)	10/32 (31%)	10/28 (36%)	0/2 (0%)
First incidence (days)	692	580	594	650
Poly-3 test	P=0.095	P=0.280	P=0.116	P=0.095N

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

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Preputial Gland: Adenoma				
Overall rate	3/50 (6%)	5/50 (10%)	2/50 (4%)	1/50 (2%)
Adjusted rate	6.5%	11.3%	4.6%	4.9%
Terminal rate	2/39 (5%)	2/32 (6%)	1/28 (4%)	0/2 (0%)
First incidence (days)	653	451	724	497
Poly-3 test	P=0.462N	P=0.333	P=0.527N	P=0.601N
Preputial Gland: Adenoma or Carcinoma				
Overall rate	4/50 (8%)	5/50 (10%)	2/50 (4%)	1/50 (2%)
Adjusted rate	8.7%	11.3%	4.6%	4.9%
Terminal rate	3/39 (8%)	2/32 (6%)	1/28 (4%)	0/2 (0%)
First incidence (days)	653	451	724	497
Poly-3 test	P=0.319N	P=0.474	P=0.365N	P=0.485N
Skin: Malignant Schwannoma				
Overall rate	3/50 (6%)	2/50 (4%)	1/50 (2%)	0/50 (0%)
Adjusted rate	6.5%	4.6%	2.3%	0.0%
Terminal rate	0/39 (0%)	1/32 (3%)	0/28 (0%)	0/2 (0%)
First incidence (days)	627	672	641	—
Poly-3 test	P=0.243N	P=0.531N	P=0.326N	P=0.330N
Skin: Keratoacanthoma				
Overall rate	3/50 (6%)	0/50 (0%)	1/50 (2%)	0/50 (0%)
Adjusted rate	6.6%	0.0%	2.3%	0.0%
Terminal rate	3/39 (8%)	0/32 (0%)	0/28 (0%)	0/2 (0%)
First incidence (days)	727 (T)	—	697	—
Poly-3 test	P=0.183N	P=0.130N	P=0.324N	P=0.327N
Skin: Squamous Cell Papilloma or Keratoacanthoma				
Overall rate	3/50 (6%)	0/50 (0%)	1/50 (2%)	1/50 (2%)
Adjusted rate	6.6%	0.0%	2.3%	5.0%
Terminal rate	3/39 (8%)	0/32 (0%)	0/28 (0%)	1/2 (50%)
First incidence (days)	727 (T)	—	697	727 (T)
Poly-3 test	P=0.183N	P=0.130N	P=0.324N	P=0.609N
Skin: Squamous Cell Papilloma, Keratoacanthoma, or Squamous Cell Carcinoma				
Overall rate	3/50 (6%)	1/50 (2%)	1/50 (2%)	1/50 (2%)
Adjusted rate	6.6%	2.3%	2.3%	5.0%
Terminal rate	3/39 (8%)	1/32 (3%)	0/28 (0%)	1/2 (50%)
First incidence (days)	727 (T)	727 (T)	697	727 (T)
Poly-3 test	P=0.215N	P=0.327N	P=0.324N	P=0.609N
Skin: Basal Cell Carcinoma or Squamous Cell Carcinoma				
Overall rate	0/50 (0%)	3/50 (6%)	0/50 (0%)	0/50 (0%)
Adjusted rate	0.0%	7.0%	0.0%	0.0%
Terminal rate	0/39 (0%)	3/32 (9%)	0/28 (0%)	0/2 (0%)
First incidence (days)	—	727 (T)	—	—
Poly-3 test	P=0.616	P=0.108	—	—

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

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Skin: Squamous Cell Papilloma, Keratoacanthoma, Basal Cell Adenoma, Basal Cell Carcinoma, or Squamous Cell Carcinoma				
Overall rate	3/50 (6%)	3/50 (6%)	2/50 (4%)	2/50 (4%)
Adjusted rate	6.6%	7.0%	4.6%	10.0%
Terminal rate	3/39 (8%)	3/32 (9%)	1/28 (4%)	1/2 (50%)
First incidence (days)	727 (T)	727 (T)	697	673
Poly-3 test	P=0.440N	P=0.634	P=0.523N	P=0.508
Skin (Subcutaneous Tissue): Fibroma				
Overall rate	3/50 (6%)	2/50 (4%)	3/50 (6%)	0/50 (0%)
Adjusted rate	6.6%	4.6%	6.7%	0.0%
Terminal rate	3/39 (8%)	1/32 (3%)	1/28 (4%)	0/2 (0%)
First incidence (days)	727 (T)	605	455	—
Poly-3 test	P=0.578	P=0.523N	P=0.650	P=0.327N
Skin (Subcutaneous Tissue): Fibroma or Fibrous Histiocytoma				
Overall rate	3/50 (6%)	2/50 (4%)	4/50 (8%)	1/50 (2%)
Adjusted rate	6.6%	4.6%	9.0%	4.9%
Terminal rate	3/39 (8%)	1/32 (3%)	1/28 (4%)	0/2 (0%)
First incidence (days)	727 (T)	605	455	561
Poly-3 test	P=0.407	P=0.523N	P=0.487	P=0.601N
Testes: Adenoma				
Overall rate	46/50 (92%)	45/50 (90%)	43/50 (86%)	44/49 (90%)
Adjusted rate	97.8%	93.5%	88.7%	98.3%
Terminal rate	39/39 (100%)	29/32 (91%)	26/28 (93%)	2/2 (100%)
First incidence (days)	627	451	455	360
Poly-3 test	P=0.049N	P=0.290N	P=0.071N	P=0.784
Thyroid Gland (C-Cell): Adenoma				
Overall rate	8/50 (16%)	7/50 (14%)	4/44 (9%)	1/47 (2%)
Adjusted rate	17.2%	16.1%	10.2%	5.1%
Terminal rate	4/39 (10%)	6/32 (19%)	2/28 (7%)	0/2 (0%)
First incidence (days)	627	605	594	388
Poly-3 test	P=0.232N	P=0.558N	P=0.271N	P=0.218N
Thyroid Gland (Follicular Cell): Adenoma				
Overall rate	0/50 (0%)	4/50 (8%)	2/44 (5%)	1/47 (2%)
Adjusted rate	0.0%	9.2%	5.2%	5.3%
Terminal rate	0/39 (0%)	3/32 (9%)	2/28 (7%)	0/2 (0%)
First incidence (days)	—	632	727 (T)	605
Poly-3 test	P=0.166	P=0.054	P=0.200	P=0.349
All Organs: Mononuclear Cell Leukemia				
Overall rate	4/50 (8%)	6/50 (12%)	6/50 (12%)	0/50 (0%)
Adjusted rate	8.7%	13.6%	13.4%	0.0%
Terminal rate	2/39 (5%)	4/32 (13%)	1/28 (4%)	0/2 (0%)
First incidence (days)	627	199	598	—
Poly-3 test	P=0.292	P=0.340	P=0.349	P=0.250N

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

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
All Organs: Benign Neoplasms				
Overall rate	47/50 (94%)	48/50 (96%)	48/50 (96%)	44/50 (88%)
Adjusted rate	99.6%	99.7%	97.6%	98.0%
Terminal rate	39/39 (100%)	32/32 (100%)	28/28 (100%)	2/2 (100%)
First incidence (days)	627	451	455	360
Poly-3 test	P=0.284N	P=1.000	P=0.521N	P=0.719N
All Organs: Malignant Neoplasms				
Overall rate	10/50 (20%)	16/50 (32%)	14/50 (28%)	3/50 (6%)
Adjusted rate	21.5%	35.2%	30.1%	14.0%
Terminal rate	5/39 (13%)	9/32 (28%)	4/28 (14%)	0/2 (0%)
First incidence (days)	627	199	465	394
Poly-3 test	P=0.208	P=0.108	P=0.237	P=0.363N
All Organs: Benign or Malignant Neoplasms				
Overall rate	47/50 (94%)	49/50 (98%)	49/50 (98%)	45/50 (90%)
Adjusted rate	99.6%	99.7%	99.5%	98.4%
Terminal rate	39/39 (100%)	32/32 (100%)	28/28 (100%)	2/2 (100%)
First incidence (days)	627	199	455	360
Poly-3 test	P=0.948N	P=1.000	P=0.999N	P=0.856N

(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, pituitary gland, preputial 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; the stop-exposure group is excluded from the trend test. Beneath the dosed group incidence are the P values 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 dose group is indicated by **N**.

[e] Not applicable; no neoplasms in animal group

[f] Value of statistic cannot be computed.

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Pulegone[a]

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death		1		
Moribund	8	10	12	35
Natural deaths	3	7	10	13
Survivors				
Terminal sacrifice	39	32	28	2
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Perforation		1 (2%)		
Periesophageal tissue, inflammation		1 (2%)		
Intestine large, cecum	(50)	(50)	(49)	(50)
Inflammation			6 (12%)	21 (42%)
Mineralization				4 (8%)
Ulcer				3 (6%)
Intestine large, colon	(50)	(50)	(50)	(50)
Hemorrhage		1 (2%)		
Inflammation		1 (2%)	1 (2%)	1 (2%)
Parasite metazoan	1 (2%)		1 (2%)	
Ulcer			1 (2%)	
Intestine large, rectum	(50)	(50)	(50)	(50)
Inflammation			1 (2%)	
Parasite metazoan	2 (4%)	1 (2%)		1 (2%)
Intestine small, duodenum	(50)	(50)	(50)	(50)
Diverticulum			1 (2%)	
Erosion				2 (4%)
Inflammation			1 (2%)	1 (2%)
Ulcer				1 (2%)
Epithelium, hyperplasia				1 (2%)
Intestine small, ileum	(50)	(50)	(49)	(50)
Intestine small, jejunum	(50)	(50)	(50)	(50)
Peyer's patch, hyperplasia	1 (2%)	1 (2%)		
Liver	(50)	(50)	(50)	(50)
Angiectasis			1 (2%)	
Basophilic focus	36 (72%)	5 (10%)	2 (4%)	2 (4%)
Bile stasis				1 (2%)
Clear cell focus	22 (44%)	22 (44%)	20 (40%)	1 (2%)
Congestion		1 (2%)		
Eosinophilic focus	1 (2%)	2 (4%)	12 (24%)	3 (6%)
Fatty change	1 (2%)	10 (20%)	27 (54%)	2 (4%)
Hematopoietic cell proliferation			1 (2%)	
Hepatodiaphragmatic nodule	6 (12%)	6 (12%)	2 (4%)	3 (6%)
Inflammation	41 (82%)	36 (72%)	41 (82%)	46 (92%)

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

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Alimentary System (continued)				
Liver (continued)	(50)	(50)	(50)	(50)
Mixed cell focus	9 (18%)	3 (6%)	10 (20%)	1 (2%)
Pigmentation, bile		1 (2%)		
Thrombosis			1 (2%)	
Arteriole, inflammation		1 (2%)		
Bile duct, crystals			1 (2%)	
Bile duct, cyst			11 (22%)	9 (18%)
Bile duct, hyperplasia	29 (58%)	15 (30%)	37 (74%)	50 (100%)
Centrilobular, hepatocyte, degeneration	3 (6%)		1 (2%)	
Hepatocyte, cellular alteration, diffuse		2 (4%)	21 (42%)	46 (92%)
Hepatocyte, degeneration, cystic	1 (2%)	1 (2%)	3 (6%)	
Hepatocyte, necrosis		1 (2%)	6 (12%)	16 (32%)
Kupffer cell, pigmentation, hemosiderin			1 (2%)	
Oval cell, hyperplasia			8 (16%)	44 (88%)
Portal, fibrosis	8 (16%)	6 (12%)	13 (26%)	43 (86%)
Mesentery	(9)	(11)	(11)	(4)
Hemorrhage				1 (25%)
Inflammation		1 (9%)		
Mineralization				1 (25%)
Necrosis	7 (78%)	9 (82%)	10 (91%)	2 (50%)
Thrombosis				1 (25%)
Oral mucosa	(0)	(1)	(3)	(0)
Pharyngeal, cyst			1 (33%)	
Pharyngeal, hyperplasia			1 (33%)	
Pancreas	(50)	(50)	(50)	(50)
Hemorrhage		1 (2%)		
Inflammation			1 (2%)	
Acinus, atrophy	13 (26%)	24 (48%)	20 (40%)	18 (36%)
Acinus, atypia cellular	1 (2%)			
Acinus, hyperplasia	11 (22%)	8 (16%)	7 (14%)	1 (2%)
Duct, cyst	4 (8%)	5 (10%)	1 (2%)	5 (10%)
Salivary glands	(50)	(49)	(50)	(50)
Atrophy				1 (2%)
Basophilic focus	1 (2%)			
Hyperplasia	1 (2%)			
Stomach, forestomach	(50)	(50)	(50)	(50)
Erosion				1 (2%)
Inflammation	2 (4%)	4 (8%)	8 (16%)	23 (46%)
Mineralization		1 (2%)		4 (8%)
Perforation				5 (10%)
Ulcer		2 (4%)	7 (14%)	16 (32%)
Epithelium, hyperplasia	16 (32%)	21 (42%)	20 (40%)	23 (46%)
Stomach, glandular	(50)	(50)	(50)	(50)
Erosion			2 (4%)	
Inflammation	1 (2%)	3 (6%)	1 (2%)	6 (12%)
Mineralization		1 (2%)	4 (8%)	21 (42%)
Ulcer		1 (2%)		
Epithelium, hyperplasia			1 (2%)	
Serosa, inflammation				1 (2%)
Tongue	(0)	(1)	(2)	(0)
Tooth	(0)	(2)	(0)	(0)
Peridontal tissue, inflammation		1 (50%)		

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Cardiovascular System				
Blood vessel	(50)	(50)	(50)	(50)
Dilatation				1 (2%)
Mineralization			1 (2%)	24 (48%)
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	44 (88%)	44 (88%)	48 (96%)	50 (100%)
Mineralization			1 (2%)	23 (46%)
Artery, inflammation	1 (2%)			
Atrium, thrombosis			1 (2%)	
Endocardium, bacterium			2 (4%)	
Endocardium, inflammation			1 (2%)	
Valve, thrombosis		1 (2%)		
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Degeneration, cystic	1 (2%)		2 (4%)	
Hematopoietic cell proliferation		1 (2%)		
Hyperplasia	27 (54%)	16 (32%)	23 (46%)	18 (36%)
Hypertrophy	11 (22%)	12 (24%)	16 (32%)	9 (18%)
Vacuolization cytoplasmic		1 (2%)	1 (2%)	
Adrenal medulla	(50)	(50)	(50)	(50)
Hyperplasia	22 (44%)	19 (38%)	30 (60%)	31 (62%)
Infiltration cellular, mononuclear cell		1 (2%)		
Islets, pancreatic	(50)	(50)	(50)	(50)
Hyperplasia				1 (2%)
Parathyroid gland	(49)	(44)	(45)	(48)
Hyperplasia			3 (7%)	36 (75%)
Pituitary gland	(50)	(50)	(50)	(50)
Pars distalis, angiectasis	2 (4%)			
Pars distalis, cyst	2 (4%)		1 (2%)	1 (2%)
Pars distalis, hyperplasia	22 (44%)	24 (48%)	18 (36%)	14 (28%)
Pars distalis, pigmentation	1 (2%)			
Pars intermedia, cyst			1 (2%)	
Rathke's cleft, cyst	1 (2%)			
Thyroid gland	(50)	(50)	(44)	(47)
C-cell, hyperplasia	32 (64%)	34 (68%)	28 (64%)	20 (43%)
Follicle, cyst				1 (2%)
Follicular cell, hyperplasia	1 (2%)			
General Body System				
None				
Genital System				
Epididymis	(50)	(50)	(50)	(49)
Fibrosis				1 (2%)
Granuloma sperm	2 (4%)	2 (4%)	2 (4%)	2 (4%)
Inflammation	1 (2%)	1 (2%)	1 (2%)	2 (4%)
Preputial gland	(50)	(50)	(50)	(50)
Inflammation	9 (18%)	14 (28%)	13 (26%)	8 (16%)
Mineralization				1 (2%)
Duct, ectasia	3 (6%)	1 (2%)		1 (2%)

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Genital System (continued)				
Prostate	(50)	(50)	(50)	(49)
Atrophy			1 (2%)	
Inflammation	9 (18%)	11 (22%)	10 (20%)	3 (6%)
Mineralization				1 (2%)
Epithelium, cyst			2 (4%)	
Epithelium, hyperplasia	15 (30%)	6 (12%)	12 (24%)	3 (6%)
Seminal vesicle	(50)	(50)	(50)	(49)
Atrophy			1 (2%)	
Inflammation		1 (2%)		1 (2%)
Mineralization				1 (2%)
Epithelium, hyperplasia			1 (2%)	
Testes	(50)	(50)	(50)	(49)
Cyst	1 (2%)			
Inflammation		1 (2%)	2 (4%)	
Mineralization				1 (2%)
Germinal epithelium, atrophy	3 (6%)	4 (8%)	5 (10%)	4 (8%)
Interstitial cell, hyperplasia	1 (2%)		2 (4%)	1 (2%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hyperplasia	10 (20%)	8 (16%)	9 (18%)	6 (12%)
Lymph node	(2)	(8)	(3)	(6)
Hematopoietic cell proliferation	1 (50%)			
Deep cervical, hemorrhage				2 (33%)
Mediastinal, degeneration, cystic		2 (25%)		
Mediastinal, hemorrhage				2 (33%)
Mediastinal, hyperplasia, plasma cell		3 (38%)		
Mediastinal, infiltration cellular, histiocyte				1 (17%)
Pancreatic, degeneration, cystic				1 (17%)
Pancreatic, hemorrhage				1 (17%)
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Degeneration, cystic	1 (2%)			
Fibrosis				2 (4%)
Hemorrhage		1 (2%)		1 (2%)
Infiltration cellular, histiocyte			1 (2%)	1 (2%)
Mineralization				1 (2%)
Spleen	(50)	(50)	(50)	(50)
Congestion			1 (2%)	
Depletion cellular		1 (2%)		
Fibrosis		2 (4%)	1 (2%)	
Hyperplasia, lymphoid	3 (6%)	2 (4%)	2 (4%)	
Hyperplasia, reticulum cell			1 (2%)	
Infarct			1 (2%)	
Thrombosis			1 (2%)	
Lymphoid follicle, depletion cellular			1 (2%)	
Thymus	(50)	(48)	(48)	(47)
Atrophy			1 (2%)	1 (2%)
Ectopic parathyroid gland				1 (2%)
Hemorrhage	1 (2%)			
Inflammation		1 (2%)		
Mineralization				1 (2%)

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Hyperplasia	1 (2%)			
Inflammation	1 (2%)			
Duct, dilatation		1 (2%)	3 (6%)	
Skin	(50)	(50)	(50)	(50)
Cyst epithelial inclusion		6 (12%)	1 (2%)	1 (2%)
Inflammation, chronic active	1 (2%)	1 (2%)		
Dermis, fibrosis			1 (2%)	
Hair follicle, cyst	1 (2%)			
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Fibrous osteodystrophy			2 (4%)	34 (68%)
Skeletal muscle	(0)	(0)	(1)	(0)
Nervous System				
Brain	(50)	(50)	(50)	(50)
Hemorrhage		2 (4%)	1 (2%)	
Hydrocephalus		2 (4%)		
Cerebrum, compression			1 (2%)	
Cerebrum, necrosis			1 (2%)	
Meninges, inflammation				1 (2%)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Congestion	2 (4%)	1 (2%)		
Edema			1 (2%)	
Hemorrhage	1 (2%)		1 (2%)	1 (2%)
Inflammation		1 (2%)	1 (2%)	
Inflammation, chronic active			1 (2%)	
Metaplasia, squamous	1 (2%)			
Necrosis			1 (2%)	
Alveolar epithelium, hyperplasia	11 (22%)	9 (18%)	11 (22%)	11 (22%)
Alveolus, inflammation, histiocyte				1 (2%)
Alveolus, inflammation, histiocytic	27 (54%)	35 (70%)	37 (74%)	36 (72%)
Arteriole, inflammation		1 (2%)	1 (2%)	
Arteriole, necrosis			1 (2%)	
Interstitium, mineralization		1 (2%)	2 (4%)	16 (32%)
Serosa, hyperplasia	2 (4%)			
Vein, inflammation				1 (2%)
Nose	(50)	(50)	(46)	(50)
Inflammation	25 (50%)	23 (46%)	30 (65%)	28 (56%)
Glands, dilatation		2 (4%)	1 (2%)	1 (2%)
Goblet cell, hyperplasia	3 (6%)			
Olfactory epithelium, degeneration	1 (2%)	5 (10%)	33 (72%)	19 (38%)
Olfactory epithelium, metaplasia, respiratory	2 (4%)	3 (6%)	6 (13%)	
Respiratory epithelium, hyperplasia			1 (2%)	
Turbinate, inflammation			1 (2%)	

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg (Stop-Exposure)
Respiratory System (continued)				
Trachea	(50)	(50)	(50)	(50)
Inflammation	2 (4%)	3 (6%)	3 (6%)	3 (6%)
Peritracheal tissue, inflammation		1 (2%)		
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Inflammation				3 (6%)
Synechia				1 (2%)
Cornea, inflammation				2 (4%)
Harderian gland	(50)	(50)	(50)	(50)
Infiltration cellular, mononuclear cell				1 (2%)
Inflammation	2 (4%)		2 (4%)	
Epithelium, hyperplasia	3 (6%)		5 (10%)	6 (12%)
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Cyst			2 (4%)	7 (14%)
Glomerulopathy, hyaline			9 (18%)	24 (48%)
Hydronephrosis		1 (2%)		
Inflammation		1 (2%)	2 (4%)	
Nephropathy	45 (90%)	45 (90%)	50 (100%)	50 (100%)
Thrombosis			1 (2%)	
Cortex, mineralization	1 (2%)	2 (4%)	2 (4%)	21 (42%)
Pelvis, dilatation	1 (2%)			
Renal tubule, pigmentation	1 (2%)			
Transitional epithelium, hyperplasia	1 (2%)			
Urinary bladder	(50)	(50)	(50)	(49)
Hemorrhage		1 (2%)		
Inflammation	1 (2%)	1 (2%)		1 (2%)
Ulcer		1 (2%)		
Transitional epithelium, hyperplasia		1 (2%)		

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

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths		1		1
Moribund	13	8	7	29
Natural deaths	6	4	5	20
Survivors				
Terminal sacrifice	31	37	38	
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Intestine large, cecum	(50)	(50)	(49)	(45)
Intestine large, colon	(50)	(50)	(49)	(45)
Intestine large, rectum	(50)	(50)	(50)	(49)
Intestine small, ileum	(50)	(50)	(49)	(45)
Intestine small, jejunum	(50)	(50)	(49)	(45)
Liver	(50)	(50)	(50)	(47)
Cholangioma			1 (2%)	
Hepatocellular adenoma	1 (2%)	1 (2%)	1 (2%)	3 (6%)
Mesentery	(6)	(12)	(5)	(1)
Oral mucosa	(1)	(0)	(0)	(2)
Pancreas	(50)	(50)	(49)	(46)
Adenoma				1 (2%)
Salivary glands	(50)	(50)	(50)	(49)
Stomach, forestomach	(50)	(50)	(49)	(46)
Squamous cell papilloma	1 (2%)			
Stomach, glandular	(50)	(50)	(49)	(46)
Tongue	(0)	(1)	(1)	(0)
Squamous cell papilloma		1 (100%)	1 (100%)	
Tooth	(1)	(0)	(2)	(1)
Odontoma	1 (100%)			
Cardiovascular System				
Blood vessel	(50)	(50)	(50)	(50)
Heart	(50)	(50)	(50)	(50)
Schwannoma benign	1 (2%)			1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(49)
Adrenal medulla	(50)	(50)	(50)	(49)
Pheochromocytoma benign	3 (6%)	1 (2%)	2 (4%)	
Islets, pancreatic	(50)	(50)	(49)	(46)
Adenoma	1 (2%)	1 (2%)		

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Endocrine System (continued)				
Parathyroid gland	(46)	(47)	(49)	(49)
Adenoma	2 (4%)			
Pituitary gland	(50)	(50)	(50)	(50)
Pars distalis, adenoma	27 (54%)	12 (24%)	9 (18%)	3 (6%)
Thyroid gland	(50)	(50)	(50)	(49)
Bilateral, C-cell, adenoma	1 (2%)			
C-cell, adenoma	7 (14%)	6 (12%)	3 (6%)	
General Body System				
None				
Genital System				
Clitoral gland	(50)	(50)	(50)	(49)
Adenoma	3 (6%)	3 (6%)	2 (4%)	1 (2%)
Sarcoma		1 (2%)		
Bilateral, adenoma			1 (2%)	
Ovary	(50)	(50)	(49)	(46)
Cystadenoma		1 (2%)		
Uterus	(50)	(50)	(50)	(47)
Polyp stromal	5 (10%)	2 (4%)	5 (10%)	2 (4%)
Vagina	(0)	(0)	(1)	(0)
Sarcoma			1 (100%)	
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Lymph node	(2)	(3)	(5)	(11)
Lymph node, mesenteric	(50)	(50)	(50)	(45)
Spleen	(50)	(50)	(50)	(46)
Hemangiosarcoma	2 (4%)			
Thymus	(50)	(49)	(47)	(47)
Integumentary System				
Mammary gland	(50)	(50)	(50)	(49)
Adenoma	2 (4%)			
Carcinoma		1 (2%)		
Fibroadenoma	21 (42%)	27 (54%)	8 (16%)	
Skin	(50)	(50)	(50)	(50)
Basal cell adenoma			2 (4%)	
Keratoacanthoma				1 (2%)
Lipoma		3 (6%)		
Squamous cell papilloma	1 (2%)			1 (2%)
Subcutaneous tissue, fibroma	3 (6%)	1 (2%)		
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Mandible, osteosarcoma			1 (2%)	
Skeletal muscle	(1)	(0)	(0)	(1)

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Nervous System				
Brain	(50)	(50)	(50)	(50)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	2 (4%)	1 (2%)		
Cystic keratinizing epithelioma		1 (2%)		
Nose	(50)	(50)	(50)	(41)
Adenoma			1 (2%)	
Trachea	(50)	(50)	(50)	(50)
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Harderian gland	(50)	(50)	(50)	(50)
Zymbal's gland	(1)	(0)	(1)	(0)
Adenoma			1 (100%)	
Carcinoma	1 (100%)			
Urinary System				
Kidney	(50)	(50)	(50)	(49)
Renal tubule, adenoma			1 (2%)	1 (2%)
Urinary bladder	(50)	(49)	(50)	(47)
Transitional epithelium, carcinoma				2 (4%)
Transitional epithelium, papilloma			1 (2%)	3 (6%)
Systemic lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Histiocytic sarcoma			1 (2%)	
Leukemia mononuclear	4 (8%)	4 (8%)	11 (22%)	9 (18%)
Mesothelioma malignant	1 (2%)			
Neoplasm Summary				
Total animals with primary neoplasms[c]	44	41	38	20
Total primary neoplasms	90	67	53	28
Total animals with benign neoplasms	42	39	27	12
Total benign neoplasms	82	61	39	17
Total animals with malignant neoplasms	8	6	14	11
Total malignant neoplasms	8	6	14	11
Total animals with metastatic neoplasms	1			
Total metastatic neoplasms	6			

[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 Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate[a]	3/50 (6%)	1/50 (2%)	2/50 (4%)	0/49 (0%)
Adjusted rate[b]	7.0%	2.2%	4.2%	0.0%
Terminal rate[c]	3/31 (10%)	1/37 (3%)	1/38 (3%)	0/0 (0%)
First incidence (days)	728 (T)	728 (T)	702	—[e]
Poly-3 test[d]	P=0.375N	P=0.281N	P=0.452N	P=0.278N
Clitoral Gland: Adenoma				
Overall rate	3/50 (6%)	3/50 (6%)	3/50 (6%)	1/49 (2%)
Adjusted rate	6.9%	6.6%	6.3%	4.5%
Terminal rate	1/31 (3%)	3/37 (8%)	3/38 (8%)	0/0
First incidence (days)	495	728 (T)	728 (T)	617
Poly-3 test	P=0.545N	P=0.640N	P=0.623N	P=0.555N
Liver: Hepatocellular Adenoma				
Overall rate	1/50 (2%)	1/50 (2%)	1/50 (2%)	3/47 (6%)
Adjusted rate	2.3%	2.2%	2.1%	13.2%
Terminal rate	1/31 (3%)	1/37 (3%)	1/38 (3%)	0/0
First incidence (days)	728 (T)	728 (T)	728 (T)	470
Poly-3 test	P=0.618N	P=0.745N	P=0.737N	P=0.132
Mammary Gland: Fibroadenoma				
Overall rate	21/50 (42%)	27/50 (54%)	8/50 (16%)	0/50 (0%)
Adjusted rate	47.2%	57.8%	16.9%	0.0%
Terminal rate	14/31 (45%)	23/37 (62%)	8/38 (21%)	0/0
First incidence (days)	604	573	728 (T)	—
Poly-3 test	P<0.001N	P=0.206	P<0.001N	P<0.001N
Mammary Gland: Fibroadenoma or Adenoma				
Overall rate	22/50 (44%)	27/50 (54%)	8/50 (16%)	0/50 (0%)
Adjusted rate	49.2%	57.8%	16.9%	0.0%
Terminal rate	14/31 (45%)	23/37 (62%)	8/38 (21%)	0/0
First incidence (days)	604	573	728 (T)	—
Poly-3 test	P<0.001N	P=0.264	P<0.001N	P<0.001N
Mammary Gland: Fibroadenoma, Adenoma, or Carcinoma				
Overall rate	22/50 (44%)	28/50 (56%)	8/50 (16%)	0/50 (0%)
Adjusted rate	49.2%	59.6%	16.9%	0.0%
Terminal rate	14/31 (45%)	23/37 (62%)	8/38 (21%)	0/0
First incidence (days)	604	573	728 (T)	—
Poly-3 test	P<0.001N	P=0.210	P<0.001N	P<0.001N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	27/50 (54%)	12/50 (24%)	9/50 (18%)	3/50 (6%)
Adjusted rate	58.6%	25.8%	19.0%	12.8%
Terminal rate	17/31 (55%)	9/37 (24%)	8/38 (21%)	0/0
First incidence (days)	495	600	702	470
Poly-3 test	P<0.001N	P<0.001N	P<0.001N	P<0.001N

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Skin: Lipoma				
Overall rate	0/50 (0%)	3/50 (6%)	0/50 (0%)	0/50 (0%)
Adjusted rate	0.0%	6.6%	0.0%	0.0%
Terminal rate	0/31 (0%)	3/37 (8%)	0/38 (0%)	0/0
First incidence (days)	—	728 (T)	—	—
Poly-3 test	P=0.591N	P=0.131	—[f]	—
Skin (Subcutaneous Tissue): Fibroma				
Overall rate	3/50 (6%)	1/50 (2%)	0/50 (0%)	0/50 (0%)
Adjusted rate	7.0%	2.2%	0.0%	0.0%
Terminal rate	2/31 (7%)	0/37 (0%)	0/38 (0%)	0/0
First incidence (days)	607	652	—	—
Poly-3 test	P=0.054N	P=0.282N	P=0.103N	P=0.279N
Thyroid Gland (C-cell): Adenoma				
Overall rate	8/50 (16%)	6/50 (12%)	3/50 (6%)	0/49 (0%)
Adjusted rate	18.7%	13.1%	6.3%	0%
Terminal rate	8/31 (26%)	6/37 (16%)	2/38 (5%)	0/0
First incidence (days)	728 (T)	728 (T)	702	—
Poly-3 test	P=0.053N	P=0.334N	P=0.069N	P=0.050N
Urinary Bladder: Papilloma				
Overall rate	0/50 (0%)	0/49 (0%)	1/50 (2%)	3/47 (6%)
Adjusted rate	0.0%	0.0%	2.1%	13.3%
Terminal rate	0/31 (0%)	0/36 (0%)	1/38 (3%)	0/0
First incidence (days)	—	—	728 (T)	578
Poly-3 test	P=0.289	—	P=0.520	P=0.044
Urinary Bladder: Papilloma or Carcinoma				
Overall rate	0/50 (0%)	0/49 (0%)	1/50 (2%)	5/47 (11%)
Adjusted rate	0.0%	0.0%	2.1%	20.8%
Terminal rate	0/31 (0%)	0/36 (0%)	1/38 (3%)	0/0
First incidence (days)	—	—	728 (T)	390
Poly-3 test	P=0.289	—	P=0.520	P=0.005
Uterus: Polyp Stromal				
Overall rate	5/50 (10%)	2/50 (4%)	5/50 (10%)	2/50 (4%)
Adjusted rate	11.7%	4.3%	10.5%	8.6%
Terminal rate	4/31 (13%)	1/37 (3%)	4/38 (11%)	0/0
First incidence (days)	693	650	702	538
Poly-3 test	P=0.534N	P=0.188N	P=0.565N	P=0.513N
All Organs: Mononuclear Cell Leukemia				
Overall rate	4/50 (8%)	4/50 (8%)	11/50 (22%)	9/50 (18%)
Adjusted rate	9.3%	8.5%	22.5%	33.9%
Terminal rate	3/31 (10%)	0/37 (0%)	4/38 (11%)	0/0 (0%)
First incidence (days)	672	561	428	450
Poly-3 test	P=0.038	P=0.593N	P=0.076	P=0.014

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
All Organs: Benign Neoplasms				
Overall rate	42/50 (84%)	39/50 (78%)	27/50 (54%)	12/50 (24%)
Adjusted rate	88.4%	81.0%	56.4%	43.9%
Terminal rate	27/31 (87%)	29/37 (78%)	22/38 (58%)	0/0 (0%)
First incidence (days)	495	573	702	470
Poly-3 test	P<0.001N	P=0.229N	P<0.001N	P<0.001N
All Organs: Malignant Neoplasms				
Overall rate	8/50 (16%)	6/50 (12%)	14/50 (28%)	11/50 (22%)
Adjusted rate	17.9%	12.7%	28.3%	39.4%
Terminal rate	4/31 (13%)	1/37 (3%)	6/38 (16%)	0/0 (0%)
First incidence (days)	519	561	428	390
Poly-3 test	P=0.111	P=0.344N	P=0.172	P=0.042
All Organs: Benign or Malignant Neoplasms				
Overall rate	44/50 (88%)	41/50 (82%)	38/50 (76%)	20/50 (40%)
Adjusted rate	90.3%	83.7%	76.4%	62.0%
Terminal rate	27/31 (87%)	29/37 (78%)	27/38 (71%)	0/0 (0%)
First incidence (days)	495	561	428	390
Poly-3 test	P=0.041N	P=0.250N	P=0.053N	P<0.001N

(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, liver, pituitary gland, thyroid gland, and urinary bladder; 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; the stop-exposure group is excluded from the trend test. Beneath the dosed group incidence are the P values 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 dose group is indicated by **N**.

[e] Not applicable; no neoplasms in animal group

[f] Value of statistic cannot be computed.

TABLE B3
Historical Incidence of Urinary Bladder Neoplasms in Control Female F344/N Rats[a]

	Incidence in Controls	
Study (Study Start)	**Papilloma**	**Carcinoma**
Historical Incidence: Corn Oil Gavage Studies		
Isoeugenol (April 2002)	0/50	0/50
Kava kava extract (August 2004)	0/50	0/50
β-Myrcene (March 2002)	0/50	0/50
Pulegone (April 2003)	0/50	0/50
Total	0/200	0/200
Overall Historical Incidence: All Routes		
Total	0/1,347	0/1,347

[a] Data as of April 29, 2009

TABLE B4
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Pulegone[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths		1		1
Moribund	13	8	7	29
Natural deaths	6	4	5	20
Survivors				
Terminal sacrifice	31	37	38	
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Inflammation		1 (2%)		1 (2%)
Muscularis, degeneration		1 (2%)		
Periesophageal tissue, inflammation		1 (2%)		
Intestine large, cecum	(50)	(50)	(49)	(45)
Inflammation		1 (2%)	3 (6%)	12 (27%)
Ulcer				1 (2%)
Intestine large, colon	(50)	(50)	(49)	(45)
Inflammation				1 (2%)
Mineralization				1 (2%)
Parasite metazoan	3 (6%)	2 (4%)		
Ulcer			1 (2%)	
Intestine large, rectum	(50)	(50)	(50)	(49)
Erosion				1 (2%)
Inflammation				1 (2%)
Parasite metazoan	4 (8%)	6 (12%)	7 (14%)	1 (2%)
Intestine small, ileum	(50)	(50)	(49)	(45)
Parasite metazoan				1 (2%)
Intestine small, jejunum	(50)	(50)	(49)	(45)
Peyer's patch, hyperplasia			1 (2%)	
Liver	(50)	(50)	(50)	(47)
Angiectasis		5 (10%)		
Basophilic focus	44 (88%)	21 (42%)	3 (6%)	2 (4%)
Clear cell focus	8 (16%)	12 (24%)	4 (8%)	1 (2%)
Fatty change	7 (14%)	25 (50%)	35 (70%)	11 (23%)
Hepatodiaphragmatic nodule	3 (6%)	6 (12%)	7 (14%)	2 (4%)
Inflammation	35 (70%)	31 (62%)	37 (74%)	33 (70%)
Mixed cell focus	5 (10%)		4 (8%)	
Bile duct, cyst	1 (2%)	6 (12%)	38 (76%)	13 (28%)
Bile duct, hyperplasia	5 (10%)	4 (8%)	49 (98%)	43 (91%)
Centrilobular, hepatocyte, degeneration		1 (2%)		
Hepatocyte, cellular alteration, diffuse		4 (8%)	45 (90%)	43 (91%)
Hepatocyte, necrosis	4 (8%)	2 (4%)	20 (40%)	15 (32%)
Hepatocyte, regeneration				1 (2%)
Oval cell, hyperplasia			45 (90%)	43 (91%)
Portal, fibrosis		3 (6%)	28 (56%)	35 (74%)
Serosa, fibrosis			1 (2%)	
Serosa, hemorrhage			3 (6%)	
Serosa, inflammation			1 (2%)	

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

TABLE B4
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Alimentary System (continued)				
Mesentery	(6)	(12)	(5)	(1)
Necrosis	5 (83%)	12 (100%)	5 (100%)	1 (100%)
Oral mucosa	(1)	(0)	(0)	(2)
Pharyngeal, hyperplasia	1 (100%)			2 (100%)
Pancreas	(50)	(50)	(49)	(46)
Atypia cellular			1 (2%)	
Basophilic focus		1 (2%)		
Inflammation			2 (4%)	
Acinus, atrophy	11 (22%)	12 (24%)	15 (31%)	17 (37%)
Acinus, hyperplasia	7 (14%)	1 (2%)	1 (2%)	2 (4%)
Duct, cyst	2 (4%)	4 (8%)	3 (6%)	3 (7%)
Salivary glands	(50)	(50)	(50)	(49)
Inflammation	1 (2%)		1 (2%)	
Mineralization	1 (2%)		1 (2%)	
Stomach, forestomach	(50)	(50)	(49)	(46)
Erosion		1 (2%)		
Inflammation	3 (6%)	3 (6%)	7 (14%)	3 (7%)
Mineralization	1 (2%)			
Ulcer	3 (6%)	1 (2%)	3 (6%)	3 (7%)
Epithelium, hyperplasia	11 (22%)	7 (14%)	15 (31%)	9 (20%)
Stomach, glandular	(50)	(50)	(49)	(46)
Erosion	1 (2%)			3 (7%)
Inflammation				1 (2%)
Mineralization	1 (2%)	3 (6%)	1 (2%)	13 (28%)
Epithelium, hyperplasia				1 (2%)
Tongue	(0)	(1)	(1)	(0)
Tooth	(1)	(0)	(2)	(1)
Dysplasia			1 (50%)	
Peridontal tissue, inflammation			1 (50%)	1 (100%)
Cardiovascular System				
Blood vessel	(50)	(50)	(50)	(50)
Mineralization		1 (2%)		20 (40%)
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	48 (96%)	45 (90%)	47 (94%)	44 (88%)
Hypertrophy		1 (2%)		1 (2%)
Inflammation				4 (8%)
Mineralization		1 (2%)	1 (2%)	11 (22%)
Artery, inflammation				1 (2%)
Atrium, thrombosis			1 (2%)	1 (2%)
Endocardium, ventricle, thrombosis				1 (2%)
Valve, thrombosis				1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(49)
Degeneration, cystic	1 (2%)	4 (8%)		
Hematopoietic cell proliferation		1 (2%)		
Hemorrhage			1 (2%)	
Hyperplasia	10 (20%)	7 (14%)	6 (12%)	14 (29%)
Hypertrophy	20 (40%)	14 (28%)	34 (68%)	20 (41%)

TABLE B4
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Endocrine System (continued)				
Adrenal medulla	(50)	(50)	(50)	(49)
Hyperplasia	6 (12%)	11 (22%)	17 (34%)	27 (55%)
Mineralization	1 (2%)			
Islets, pancreatic	(50)	(50)	(49)	(46)
Parathyroid gland	(46)	(47)	(49)	(49)
Cyst	1 (2%)			
Hyperplasia		1 (2%)	11 (22%)	31 (63%)
Pituitary gland	(50)	(50)	(50)	(50)
Hemorrhage		2 (4%)		1 (2%)
Pars distalis, angiectasis			1 (2%)	1 (2%)
Pars distalis, cyst	4 (8%)			1 (2%)
Pars distalis, hyperplasia	17 (34%)	26 (52%)	24 (48%)	12 (24%)
Rathke's cleft, cyst	1 (2%)			
Thyroid gland	(50)	(50)	(50)	(49)
Inflammation				1 (2%)
C-cell, hyperplasia	39 (78%)	32 (64%)	19 (38%)	3 (6%)
Follicle, cyst	1 (2%)	1 (2%)	1 (2%)	
General Body System				
None				
Genital System				
Clitoral gland	(50)	(50)	(50)	(49)
Hyperplasia	3 (6%)	1 (2%)		1 (2%)
Inflammation	6 (12%)	7 (14%)	1 (2%)	3 (6%)
Duct, cyst	2 (4%)	2 (4%)	1 (2%)	
Ovary	(50)	(50)	(49)	(46)
Atrophy	5 (10%)	5 (10%)	4 (8%)	16 (35%)
Cyst	6 (12%)	4 (8%)	5 (10%)	5 (11%)
Hemorrhage				1 (2%)
Uterus	(50)	(50)	(50)	(47)
Hemorrhage	2 (4%)	1 (2%)		1 (2%)
Hydrometra			1 (2%)	3 (6%)
Inflammation		1 (2%)		2 (4%)
Cervix, cyst				1 (2%)
Endometrium, cyst		1 (2%)		
Endometrium, hyperplasia, cystic	1 (2%)	3 (6%)	2 (4%)	5 (11%)
Vagina	(0)	(0)	(1)	(0)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hemorrhage			2 (4%)	
Hyperplasia	8 (16%)	8 (16%)	20 (40%)	38 (76%)
Infiltration cellular, histiocyte	1 (2%)			
Myelofibrosis		1 (2%)	1 (2%)	
Lymph node	(2)	(3)	(5)	(11)
Deep cervical, hemorrhage		1 (33%)		3 (27%)
Deep cervical, hyperplasia, lymphoid			1 (20%)	1 (9%)
Deep cervical, hyperplasia, plasma cell	1 (50%)			
Deep cervical, pigmentation, hemosiderin			1 (20%)	

TABLE B4
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Hematopoietic System (continued)				
Lymph node (continued)	(2)	(3)	(5)	(11)
Mediastinal, degeneration, cystic	1 (50%)		1 (20%)	
Mediastinal, hemorrhage				2 (18%)
Mediastinal, hyperplasia, plasma cell			2 (40%)	
Pancreatic, hemorrhage				3 (27%)
Pancreatic, infiltration cellular, histiocyte			1 (20%)	
Lymph node, mesenteric	(50)	(50)	(50)	(45)
Hemorrhage	2 (4%)		1 (2%)	18 (40%)
Inflammation				2 (4%)
Necrosis, lymphoid				1 (2%)
Spleen	(50)	(50)	(50)	(46)
Accessory spleen		1 (2%)		
Fibrosis			1 (2%)	
Hematopoietic cell proliferation			1 (2%)	2 (4%)
Hemorrhage	1 (2%)			1 (2%)
Hyperplasia, lymphoid	3 (6%)		1 (2%)	2 (4%)
Necrosis, lymphoid				1 (2%)
Pigmentation, hemosiderin		1 (2%)		
Thrombosis	1 (2%)			
Capsule, hemorrhage			1 (2%)	
Thymus	(50)	(49)	(47)	(47)
Atrophy	1 (2%)			1 (2%)
Cyst		1 (2%)		
Ectopic parathyroid gland				1 (2%)
Inflammation	1 (2%)	1 (2%)		
Integumentary System				
Mammary gland	(50)	(50)	(50)	(49)
Hyperplasia		2 (4%)		
Inflammation			1 (2%)	
Mineralization		1 (2%)		
Duct, dilatation	2 (4%)	4 (8%)	1 (2%)	
Skin	(50)	(50)	(50)	(50)
Cyst epithelial inclusion	1 (2%)			
Epidermis, hyperplasia		1 (2%)		
Hair follicle, atrophy				1 (2%)
Subcutaneous tissue, inflammation		1 (2%)		
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Fibrous osteodystrophy			1 (2%)	27 (54%)
Skeletal muscle	(1)	(0)	(0)	(1)
Hemorrhage				1 (100%)
Nervous System				
Brain	(50)	(50)	(50)	(50)
Hemorrhage	1 (2%)		1 (2%)	1 (2%)
Hydrocephalus	2 (4%)	1 (2%)		
Thrombosis				1 (2%)

TABLE B4
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Bacterium				1 (2%)
Congestion			1 (2%)	
Hemorrhage				12 (24%)
Inflammation	1 (2%)	3 (6%)		
Inflammation, suppurative				1 (2%)
Pigmentation, hemosiderin				1 (2%)
Thrombosis				1 (2%)
Alveolar epithelium, hyperplasia	8 (16%)	14 (28%)	10 (20%)	12 (24%)
Alveolar epithelium, metaplasia, squamous		1 (2%)		
Alveolus, inflammation, histiocytic	40 (80%)	44 (88%)	49 (98%)	43 (86%)
Arteriole, inflammation				2 (4%)
Interstitium, mineralization				16 (32%)
Perivascular, inflammation			1 (2%)	
Serosa, inflammation		1 (2%)		
Nose	(50)	(50)	(50)	(41)
Inflammation	12 (24%)	22 (44%)	39 (78%)	26 (63%)
Thrombosis		1 (2%)	2 (4%)	2 (5%)
Glands, dilatation		6 (12%)	6 (12%)	2 (5%)
Olfactory epithelium, degeneration	2 (4%)	40 (80%)	48 (96%)	37 (90%)
Olfactory epithelium, metaplasia, respiratory	1 (2%)	8 (16%)	46 (92%)	36 (88%)
Olfactory epithelium, necrosis				1 (2%)
Respiratory epithelium, hyperplasia		1 (2%)		
Respiratory epithelium, necrosis				1 (2%)
Septum, fibrosis			1 (2%)	
Turbinate, inflammation			1 (2%)	
Trachea	(50)	(50)	(50)	(50)
Hemorrhage				1 (2%)
Inflammation	5 (10%)	1 (2%)	1 (2%)	3 (6%)
Perforation				1 (2%)
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Cataract	1 (2%)	3 (6%)	2 (4%)	1 (2%)
Hemorrhage				3 (6%)
Inflammation	1 (2%)	1 (2%)		9 (18%)
Optic nerve, atrophy				1 (2%)
Retina, atrophy	2 (4%)	4 (8%)	3 (6%)	2 (4%)
Retina, dysplasia			2 (4%)	
Harderian gland	(50)	(50)	(50)	(50)
Inflammation		1 (2%)		
Mineralization			1 (2%)	
Epithelium, hyperplasia			1 (2%)	
Zymbal's gland	(1)	(0)	(1)	(0)

TABLE B4
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg (Stop-Exposure)
Urinary System				
Kidney	(50)	(50)	(50)	(49)
Angiectasis	1 (2%)			
Cyst	2 (4%)			2 (4%)
Glomerulopathy, hyaline		17 (34%)	49 (98%)	48 (98%)
Glomerulosclerosis	1 (2%)			
Hemorrhage	1 (2%)			
Inflammation		1 (2%)	1 (2%)	2 (4%)
Nephropathy	42 (84%)	44 (88%)	49 (98%)	48 (98%)
Cortex, mineralization		1 (2%)	1 (2%)	26 (53%)
Pelvis, inflammation			1 (2%)	
Renal tubule, degeneration		2 (4%)		
Urinary bladder	(50)	(49)	(50)	(47)
Inflammation	1 (2%)			2 (4%)
Transitional epithelium, hyperplasia			2 (4%)	1 (2%)

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

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths		2		
Moribund	7	8	7	8
Natural deaths	5	4	1	1
Survivors				
Terminal sacrifice	38	36	42	41
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, liver				1 (2%)
Gallbladder	(49)	(49)	(50)	(50)
Intestine large, cecum	(50)	(50)	(50)	(50)
Intestine small, ileum	(50)	(50)	(50)	(50)
Hemangiosarcoma	1 (2%)			
Intestine small, jejunum	(50)	(50)	(50)	(50)
Carcinoma				1 (2%)
Sarcoma, metastatic, mesentery		1 (2%)		
Liver	(50)	(50)	(50)	(50)
Hemangioma			2 (4%)	
Hemangiosarcoma		1 (2%)		
Hepatoblastoma	1 (2%)	3 (6%)	5 (10%)	2 (4%)
Hepatoblastoma, multiple			2 (4%)	
Hepatocellular adenoma	16 (32%)	12 (24%)	8 (16%)	10 (20%)
Hepatocellular adenoma, multiple	6 (12%)	19 (38%)	27 (54%)	18 (36%)
Hepatocellular carcinoma	11 (22%)	9 (18%)	16 (32%)	12 (24%)
Hepatocellular carcinoma, multiple	2 (4%)	2 (4%)	2 (4%)	3 (6%)
Hepatocholangiocarcinoma				1 (2%)
Hepatocholangiocarcinoma, multiple				1 (2%)
Hepatocholangioma				1 (2%)
Sarcoma, metastatic, mesentery		1 (2%)		
Mesentery	(4)	(2)	(3)	(0)
Hepatoblastoma, metastatic, liver			1 (33%)	
Rhabdomyosarcoma, metastatic, skeletal muscle	1 (25%)			
Sarcoma		1 (50%)		
Pancreas	(50)	(50)	(50)	(50)
Hepatoblastoma, metastatic, liver			1 (2%)	
Rhabdomyosarcoma, metastatic, skeletal muscle	1 (2%)			
Sarcoma, metastatic, mesentery		1 (2%)		
Salivary glands	(50)	(50)	(50)	(50)
Stomach, forestomach	(50)	(50)	(50)	(50)
Squamous cell carcinoma	1 (2%)			
Squamous cell papilloma		2 (4%)		1 (2%)
Squamous cell papilloma, multiple				1 (2%)
Stomach, glandular	(50)	(50)	(50)	(50)
Tooth	(37)	(37)	(35)	(20)

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Cardiovascular System				
Blood vessel	(50)	(50)	(50)	(50)
Aorta, alveolar/bronchiolar carcinoma, metastatic, lung				1 (2%)
Heart	(50)	(50)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (2%)
Hemangiosarcoma, metastatic, spleen			1 (2%)	
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Hepatoblastoma, metastatic, liver			1 (2%)	
Sarcoma, metastatic, mesentery		1 (2%)		
Subcapsular, adenoma	1 (2%)	3 (6%)	3 (6%)	
Adrenal medulla	(50)	(50)	(50)	(50)
Pheochromocytoma benign	1 (2%)	1 (2%)	1 (2%)	
Pheochromocytoma malignant				1 (2%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Pituitary gland	(50)	(50)	(50)	(50)
Thyroid gland	(50)	(50)	(50)	(50)
Follicular cell, carcinoma		1 (2%)	2 (4%)	
General Body System				
None				
Genital System				
Coagulating gland	(1)	(1)	(0)	(1)
Sarcoma, metastatic, mesentery		1 (100%)		
Epididymis	(50)	(50)	(50)	(50)
Hemangiosarcoma, metastatic, spleen				1 (2%)
Rhabdomyosarcoma, metastatic, skeletal muscle	1 (2%)			
Sarcoma, metastatic, mesentery		1 (2%)		
Preputial gland	(50)	(50)	(50)	(50)
Prostate	(50)	(50)	(50)	(50)
Sarcoma, metastatic, mesentery		1 (2%)		
Seminal vesicle	(50)	(50)	(50)	(50)
Sarcoma, metastatic, mesentery		1 (2%)		
Testes	(50)	(50)	(50)	(50)
Interstitial cell, adenoma			1 (2%)	1 (2%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hemangiosarcoma			1 (2%)	
Hemangiosarcoma, metastatic, spleen	1 (2%)			1 (2%)
Hemangiosarcoma, metastatic, uncertain primary site	1 (2%)			
Lymph node	(1)	(3)	(0)	(2)
Mediastinal, hepatocholangiocarcinoma, metastatic, liver				1 (50%)
Mediastinal, sarcoma, metastatic, mesentery		1 (33%)		

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Hematopoietic System (continued)				
Lymph node, mandibular	(50)	(50)	(50)	(50)
Lymph node, mesenteric	(47)	(49)	(45)	(44)
Spleen	(50)	(50)	(50)	(50)
Hemangiosarcoma	2 (4%)	2 (4%)	1 (2%)	1 (2%)
Thymus	(49)	(46)	(45)	(42)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (2%)
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Hemangiosarcoma		1 (2%)		
Sarcoma		1 (2%)		
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Skeletal muscle	(1)	(0)	(0)	(0)
Rhabdomyosarcoma	1 (100%)			
Nervous System				
Brain	(50)	(50)	(50)	(50)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	6 (12%)	5 (10%)	6 (12%)	5 (10%)
Alveolar/bronchiolar carcinoma	3 (6%)	3 (6%)	4 (8%)	3 (6%)
Alveolar/bronchiolar carcinoma, multiple		1 (2%)		
Carcinoma, metastatic, thyroid gland			1 (2%)	
Hemangiosarcoma, metastatic, skin		1 (2%)		
Hepatoblastoma, metastatic, liver			1 (2%)	
Hepatocellular carcinoma, metastatic, liver	1 (2%)	4 (8%)	2 (4%)	5 (10%)
Hepatocholangiocarcinoma, metastatic, liver				1 (2%)
Rhabdomyosarcoma, metastatic, skeletal muscle	1 (2%)			
Sarcoma, metastatic, skin		1 (2%)		
Serosa, hepatocholangiocarcinoma, metastatic, liver				1 (2%)
Nose	(50)	(50)	(50)	(50)
Osteoma				1 (2%)
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Harderian gland	(50)	(50)	(50)	(50)
Adenoma	6 (12%)	4 (8%)	7 (14%)	7 (14%)
Adenoma, multiple			1 (2%)	
Carcinoma	1 (2%)			

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Carcinoma, metastatic, thyroid gland			1 (2%)	
Hepatoblastoma, metastatic, liver			1 (2%)	
Renal tubule, adenoma		1 (2%)		
Urethra	(0)	(0)	(1)	(0)
Transitional epithelium, carcinoma			1 (100%)	
Urinary bladder	(50)	(50)	(50)	(50)
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Histiocytic sarcoma		2 (4%)	1 (2%)	
Lymphoma malignant	1 (2%)	1 (2%)		2 (4%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	38	43	44	43
Total primary neoplasms	60	75	91	72
Total animals with benign neoplasms	30	35	37	37
Total benign neoplasms	36	47	56	45
Total animals with malignant neoplasms	19	24	28	22
Total malignant neoplasms	24	28	35	27
Total animals with metastatic neoplasms	4	7	4	7
Total metastatic neoplasms	7	15	10	14
Total animals with malignant neoplasms of uncertain primary site	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 C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Adrenal Cortex: Adenoma				
Overall rate[a]	1/50 (2%)	3/50 (6%)	3/50 (6%)	0/50 (0%)
Adjusted rate[b]	2.3%	6.7%	6.3%	0.0%
Terminal rate[c]	1/38 (3%)	3/36 (8%)	3/42 (7%)	0/41 (0%)
First incidence (days)	730 (T)	730 (T)	730 (T)	— [e]
Poly-3 test[d]	P=0.260N	P=0.312	P=0.333	P=0.492N
Harderian Gland: Adenoma				
Overall rate	6/50 (12%)	4/50 (8%)	8/50 (16%)	7/50 (14%)
Adjusted rate	13.6%	8.9%	16.6%	15.1%
Terminal rate	5/38 (13%)	4/36 (11%)	6/42 (14%)	6/41 (15%)
First incidence (days)	714	730 (T)	597	570
Poly-3 test	P=0.357	P=0.358N	P=0.455	P=0.538
Harderian Gland: Adenoma or Carcinoma				
Overall rate	7/50 (14%)	4/50 (8%)	8/50 (16%)	7/50 (14%)
Adjusted rate	15.8%	8.9%	16.6%	15.1%
Terminal rate	6/38 (16%)	4/36 (11%)	6/42 (14%)	6/41 (15%)
First incidence (days)	714	730 (T)	597	570
Poly-3 test	P=0.456	P=0.250N	P=0.572	P=0.575N
Liver: Hepatoblastoma				
Overall rate	1/50 (2%)	3/50 (6%)	7/50 (14%)	2/50 (4%)
Adjusted rate	2.3%	6.6%	14.5%	4.4%
Terminal rate	1/38 (3%)	2/36 (6%)	4/42 (10%)	2/41 (5%)
First incidence (days)	730 (T)	652	654	730 (T)
Poly-3 test	P=0.433	P=0.314	P=0.040	P=0.514
Liver: Hepatocellular Adenoma				
Overall rate	22/50 (44%)	31/50 (62%)	35/50 (70%)	28/50 (56%)
Adjusted rate	47.5%	65.4%	72.7%	60.3%
Terminal rate	17/38 (45%)	24/36 (67%)	33/42 (79%)	25/41 (61%)
First incidence (days)	479	428	654	638
Poly-3 test	P=0.175	P=0.058	P=0.008	P=0.150
Liver: Hepatocellular Carcinoma				
Overall rate	13/50 (26%)	11/50 (22%)	18/50 (36%)	15/50 (30%)
Adjusted rate	27.8%	23.8%	36.9%	32.4%
Terminal rate	8/38 (21%)	6/36 (17%)	14/42 (33%)	14/41 (34%)
First incidence (days)	535	646	588	638
Poly-3 test	P=0.247	P=0.419N	P=0.232	P=0.398
Liver: Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	13/50 (26%)	13/50 (26%)	22/50 (44%)	17/50 (34%)
Adjusted rate	27.8%	27.9%	44.7%	36.7%
Terminal rate	8/38 (21%)	7/36 (19%)	16/42 (38%)	16/41 (39%)
First incidence (days)	535	646	588	638
Poly-3 test	P=0.137	P=0.585	P=0.064	P=0.241

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	29/50 (58%)	36/50 (72%)	42/50 (84%)	35/50 (70%)
Adjusted rate	60.0%	74.7%	85.3%	75.3%
Terminal rate	20/38 (53%)	26/36 (72%)	36/42 (86%)	32/41 (78%)
First incidence (days)	479	428	588	638
Poly-3 test	P=0.060	P=0.091	P=0.004	P=0.082
Liver: Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma				
Overall rate	29/50 (58%)	37/50 (74%)	42/50 (84%)	36/50 (72%)
Adjusted rate	60.0%	76.3%	85.3%	77.5%
Terminal rate	20/38 (53%)	26/36 (72%)	36/42 (86%)	33/41 (81%)
First incidence (days)	479	428	588	638
Poly-3 test	P=0.038	P=0.064	P=0.004	P=0.051
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	6/50 (12%)	5/50 (10%)	6/50 (12%)	5/50 (10%)
Adjusted rate	13.4%	11.1%	12.6%	10.9%
Terminal rate	5/38 (13%)	4/36 (11%)	5/42 (12%)	5/41 (12%)
First incidence (days)	477	693	714	730 (T)
Poly-3 test	P=0.450N	P=0.496N	P=0.577N	P=0.484N
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	3/50 (6%)	4/50 (8%)	4/50 (8%)	3/50 (6%)
Adjusted rate	6.8%	8.8%	8.4%	6.5%
Terminal rate	3/38 (8%)	3/36 (8%)	4/42 (10%)	2/41 (5%)
First incidence (days)	730 (T)	652	730 (T)	638
Poly-3 test	P=0.509N	P=0.514	P=0.541	P=0.641N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	9/50 (18%)	9/50 (18%)	9/50 (18%)	7/50 (14%)
Adjusted rate	20.0%	19.8%	18.8%	15.1%
Terminal rate	8/38 (21%)	7/36 (19%)	8/42 (19%)	6/41 (15%)
First incidence (days)	477	652	714	638
Poly-3 test	P=0.299N	P=0.592N	P=0.547N	P=0.367N
All Organs: Hemangiosarcoma				
Overall rate	3/50 (6%)	4/50 (8%)	2/50 (4%)	1/50 (2%)
Adjusted rate	6.7%	8.8%	4.2%	2.2%
Terminal rate	2/38 (5%)	2/36 (6%)	2/42 (5%)	1/41 (2%)
First incidence (days)	640	693	730 (T)	730 (T)
Poly-3 test	P=0.151N	P=0.509	P=0.469N	P=0.294N
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	3/50 (6%)	4/50 (8%)	4/50 (8%)	1/50 (2%)
Adjusted rate	6.7%	8.8%	8.4%	2.2%
Terminal rate	2/38 (5%)	2/36 (6%)	4/42 (10%)	1/41 (2%)
First incidence (days)	640	693	730 (T)	730 (T)
Poly-3 test	P=0.196N	P=0.509	P=0.537	P=0.294N

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
All Organs: Benign Neoplasms				
Overall rate	30/50 (60%)	35/50 (70%)	37/50 (74%)	37/50 (74%)
Adjusted rate	63.7%	73.8%	76.1%	78.7%
Terminal rate	23/38 (61%)	28/36 (78%)	33/42 (79%)	33/41 (81%)
First incidence (days)	477	428	597	570
Poly-3 test	P=0.073	P=0.196	P=0.131	P=0.078
All Organs: Malignant Neoplasms				
Overall rate	19/50 (38%)	24/50 (48%)	28/50 (56%)	22/50 (44%)
Adjusted rate	39.7%	50.2%	56.5%	47.5%
Terminal rate	12/38 (32%)	13/36 (36%)	21/42 (50%)	20/41 (49%)
First incidence (days)	477	444	588	638
Poly-3 test	P=0.282	P=0.203	P=0.071	P=0.291
All Organs: Benign or Malignant Neoplasms				
Overall rate	38/50 (76%)	43/50 (86%)	44/50 (88%)	43/50 (86%)
Adjusted rate	77.4%	88.5%	88.0%	91.5%
Terminal rate	27/38 (71%)	31/36 (86%)	36/42 (86%)	39/41 (95%)
First incidence (days)	477	428	588	570
Poly-3 test	P=0.047	P=0.114	P=0.129	P=0.048

(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 are the P values 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 dose group is indicated by **N**.

[e] Not applicable; no neoplasms in animal group

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

Study (Study Start)	Incidence in Controls: Hepatocellular Adenoma	Hepatocellular Carcinoma	Hepatoblastoma	Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma
Historical Incidence: Corn Oil Gavage Studies				
Isoeugenol (May 2002)	24/50	8/50	3/50	30/50
Kava kava extract (August 2004)	27/50	20/50	0/50	38/50
β-Myrcene (April 2002)	26/50	14/50	4/50	34/50
Pulegone (April 2003)	22/50	13/50	1/50	29/50
3,3′,4,4′-Tetrachloroazobenzene (February 2003)	22/50	17/50	2/50	34/50
Total (%)	121/250 (48.4%)	72/250 (28.8%)	10/250 (4.0%)	165/250 (66.0%)
Mean ± standard deviation	48.4% ± 4.6%	28.8% ± 9.0%	4.0% ± 3.2%	66.0% ± 7.2%
Range	44%-54%	16%-40%	0%-8%	58%-76%
Overall Historical Incidence: All Routes				
Total (%)	751/1,447 (51.9%)	430/1,447 (29.7%)	51/1,447 (3.5%)	995/1,447 (68.8%)
Mean ± standard deviation	51.9% ± 12.7%	29.7% ± 8.7%	3.5% ± 6.4%	68.8% ± 11.6%
Range	24%-72%	16%-52%	0%-34%	46%-92%

[a] Data as of April 29, 2009

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths		2		
Moribund	7	8	7	8
Natural deaths	5	4	1	1
Survivors				
Terminal sacrifice	38	36	42	41
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Inflammation	2 (4%)		1 (2%)	1 (2%)
Gallbladder	(49)	(49)	(50)	(50)
Infiltration cellular, mononuclear cell	1 (2%)			
Intestine large, cecum	(50)	(50)	(50)	(50)
Hemorrhage		1 (2%)		
Intestine small, ileum	(50)	(50)	(50)	(50)
Hyperplasia, lymphoid	1 (2%)			
Inflammation		1 (2%)		
Epithelium, hyperplasia	1 (2%)			
Intestine small, jejunum	(50)	(50)	(50)	(50)
Hyperplasia, lymphoid	1 (2%)			
Liver	(50)	(50)	(50)	(50)
Angiectasis		2 (4%)		
Basophilic focus	4 (8%)	5 (10%)	4 (8%)	6 (12%)
Clear cell focus	15 (30%)	27 (54%)	28 (56%)	34 (68%)
Eosinophilic focus	7 (14%)	12 (24%)	20 (40%)	36 (72%)
Fatty change, focal	3 (6%)	8 (16%)	20 (40%)	23 (46%)
Fatty change, diffuse	38 (76%)	27 (54%)	21 (42%)	3 (6%)
Hematopoietic cell proliferation	3 (6%)		1 (2%)	2 (4%)
Hemorrhage				1 (2%)
Hepatodiaphragmatic nodule	1 (2%)			
Inflammation	24 (48%)	21 (42%)	20 (40%)	29 (58%)
Mixed cell focus	18 (36%)	20 (40%)	19 (38%)	34 (68%)
Necrosis	1 (2%)	8 (16%)	5 (10%)	26 (52%)
Pigmentation	3 (6%)		2 (4%)	1 (2%)
Tension lipidosis	5 (10%)	6 (12%)	7 (14%)	2 (4%)
Bile duct, cyst			3 (6%)	14 (28%)
Bile duct, fibrosis				2 (4%)
Bile duct, hyperplasia			1 (2%)	35 (70%)
Centrilobular, degeneration		1 (2%)		
Centrilobular, vacuolization cytoplasmic		1 (2%)	1 (2%)	
Centrilobular, hepatocyte, hypertrophy		11 (22%)	23 (46%)	46 (92%)
Oval cell, hyperplasia	1 (2%)		1 (2%)	36 (72%)
Serosa, inflammation, chronic active		1 (2%)		
Vein, intravascular hepatocyte	3 (6%)	1 (2%)	15 (30%)	47 (94%)
Mesentery	(4)	(2)	(3)	(0)
Fat, necrosis	3 (75%)	1 (50%)	3 (100%)	

[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 Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Alimentary System (continued)				
Pancreas	(50)	(50)	(50)	(50)
Cyst	1 (2%)			
Cytoplasmic alteration, focal		2 (4%)	1 (2%)	1 (2%)
Infiltration cellular, mononuclear cell	5 (10%)	5 (10%)	4 (8%)	7 (14%)
Acinus, atrophy	1 (2%)	1 (2%)		1 (2%)
Salivary glands	(50)	(50)	(50)	(50)
Atrophy			1 (2%)	
Infiltration cellular, mononuclear cell	40 (80%)	32 (64%)	30 (60%)	33 (66%)
Stomach, forestomach	(50)	(50)	(50)	(50)
Hyperplasia, squamous	7 (14%)	10 (20%)	27 (54%)	41 (82%)
Inflammation	3 (6%)	9 (18%)	24 (48%)	39 (78%)
Mineralization		1 (2%)		
Ulcer		3 (6%)	9 (18%)	22 (44%)
Artery, inflammation, chronic active	1 (2%)			
Stomach, glandular	(50)	(50)	(50)	(50)
Erosion				1 (2%)
Mineralization	2 (4%)	1 (2%)		
Epithelium, hyperplasia		1 (2%)		
Glands, cyst	4 (8%)	4 (8%)	3 (6%)	5 (10%)
Glands, hyperplasia	1 (2%)	1 (2%)		
Tooth	(37)	(37)	(35)	(20)
Dysplasia	37 (100%)	37 (100%)	35 (100%)	20 (100%)
Peridontal tissue, inflammation			1 (3%)	
Pulp, inflammation	1 (3%)	1 (3%)	1 (3%)	1 (5%)
Cardiovascular System				
Blood vessel	(50)	(50)	(50)	(50)
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	1 (2%)			
Infiltration cellular, mononuclear cell	4 (8%)		1 (2%)	1 (2%)
Inflammation	1 (2%)			
Mineralization	1 (2%)	1 (2%)	1 (2%)	4 (8%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Degeneration, cystic			1 (2%)	
Hypertrophy	17 (34%)	9 (18%)	8 (16%)	4 (8%)
Vacuolization cytoplasmic		1 (2%)		1 (2%)
Subcapsular, hyperplasia	39 (78%)	40 (80%)	47 (94%)	44 (88%)
Zona fasciculata, hyperplasia	1 (2%)			
Adrenal medulla	(50)	(50)	(50)	(50)
Hyperplasia		2 (4%)	1 (2%)	2 (4%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Hyperplasia	20 (40%)	17 (34%)	21 (42%)	2 (4%)
Pituitary gland	(50)	(50)	(50)	(50)
Cyst	1 (2%)			1 (2%)
Pars distalis, hyperplasia			1 (2%)	
Thyroid gland	(50)	(50)	(50)	(50)
Infiltration cellular, mononuclear cell	1 (2%)			1 (2%)
Follicular cell, hyperplasia				1 (2%)

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
General Body System				
None				
Genital System				
Coagulating gland	(1)	(1)	(0)	(1)
Hyperplasia	1 (100%)			
Inflammation				1 (100%)
Epididymis	(50)	(50)	(50)	(50)
Cyst		1 (2%)		
Granuloma sperm			1 (2%)	
Infiltration cellular, mononuclear cell	23 (46%)	27 (54%)	20 (40%)	20 (40%)
Inflammation	2 (4%)			
Mineralization		1 (2%)		
Preputial gland	(50)	(50)	(50)	(50)
Cyst				2 (4%)
Infiltration cellular, mononuclear cell	10 (20%)	15 (30%)	12 (24%)	7 (14%)
Inflammation	4 (8%)	4 (8%)	3 (6%)	4 (8%)
Duct, ectasia	1 (2%)	1 (2%)	3 (6%)	3 (6%)
Prostate	(50)	(50)	(50)	(50)
Infiltration cellular, mononuclear cell	23 (46%)	30 (60%)	23 (46%)	14 (28%)
Inflammation	1 (2%)		1 (2%)	1 (2%)
Epithelium, hyperplasia	6 (12%)	3 (6%)		
Seminal vesicle	(50)	(50)	(50)	(50)
Atrophy		1 (2%)		
Dilatation		1 (2%)		
Testes	(50)	(50)	(50)	(50)
Cyst		1 (2%)		
Mineralization		2 (4%)	1 (2%)	
Germinal epithelium, atrophy	2 (4%)	2 (4%)	3 (6%)	
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Atrophy, focal				1 (2%)
Hemorrhage				1 (2%)
Myelofibrosis	2 (4%)		3 (6%)	1 (2%)
Lymph node	(1)	(3)	(0)	(2)
Lymph node, mandibular	(50)	(50)	(50)	(50)
Atrophy	1 (2%)	4 (8%)	3 (6%)	2 (4%)
Hyperplasia, lymphoid	1 (2%)	1 (2%)		
Hyperplasia, plasma cell	2 (4%)	1 (2%)		
Lymph node, mesenteric	(47)	(49)	(45)	(44)
Atrophy		1 (2%)	2 (4%)	3 (7%)
Hyperplasia, lymphoid	1 (2%)			1 (2%)
Spleen	(50)	(50)	(50)	(50)
Atrophy				1 (2%)
Hematopoietic cell proliferation	29 (58%)	32 (64%)	23 (46%)	23 (46%)
Hyperplasia, lymphoid	2 (4%)	3 (6%)		
Lymphoid follicle, atrophy	1 (2%)		1 (2%)	3 (6%)
Red pulp, atrophy	1 (2%)		1 (2%)	3 (6%)
Thymus	(49)	(46)	(45)	(42)
Atrophy	36 (73%)	35 (76%)	32 (71%)	30 (71%)
Hyperplasia, lymphoid		2 (4%)		
Necrosis, lymphoid		2 (4%)		

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Inflammation		1 (2%)	2 (4%)	
Ulcer		1 (2%)	2 (4%)	
Epidermis, hyperplasia			1 (2%)	
Subcutaneous tissue, hemorrhage		1 (2%)		
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Fracture		1 (2%)		
Osteosclerosis		1 (2%)		1 (2%)
Skeletal muscle	(1)	(0)	(0)	(0)
Nervous System				
Brain	(50)	(50)	(50)	(50)
Arteriole, infiltration cellular, lymphoid		1 (2%)		
Meninges, infiltration cellular, lymphoid		1 (2%)		
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Hemorrhage	1 (2%)			
Inflammation		5 (10%)	2 (4%)	1 (2%)
Alveolar epithelium, hyperplasia	2 (4%)	7 (14%)	2 (4%)	9 (18%)
Alveolus, infiltration cellular, histiocyte	2 (4%)	4 (8%)	3 (6%)	3 (6%)
Serosa, inflammation		2 (4%)		
Nose	(50)	(50)	(50)	(50)
Inflammation	2 (4%)	3 (6%)	2 (4%)	22 (44%)
Polyp, inflammatory		2 (4%)		
Glands, cyst		1 (2%)		
Nerve, atrophy	1 (2%)	3 (6%)	3 (6%)	45 (90%)
Olfactory epithelium, degeneration	3 (6%)	3 (6%)	11 (22%)	46 (92%)
Olfactory epithelium, erosion				1 (2%)
Olfactory epithelium, metaplasia	1 (2%)	5 (10%)	3 (6%)	44 (88%)
Olfactory epithelium, necrosis				1 (2%)
Respiratory epithelium, hyperplasia	39 (78%)	38 (76%)	42 (84%)	40 (80%)
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Atrophy				1 (2%)
Cornea, inflammation	1 (2%)		1 (2%)	3 (6%)
Optic nerve, degeneration		1 (2%)		
Harderian gland	(50)	(50)	(50)	(50)
Infiltration cellular, mononuclear cell	34 (68%)	27 (54%)	23 (46%)	17 (34%)
Inflammation, chronic active		1 (2%)		
Epithelium, hyperplasia	1 (2%)	3 (6%)	1 (2%)	3 (6%)

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Accumulation, hyaline droplet	1 (2%)			
Cyst	9 (18%)	3 (6%)	4 (8%)	
Glomerulopathy, hyaline	1 (2%)	19 (38%)	30 (60%)	44 (88%)
Infarct	1 (2%)			1 (2%)
Metaplasia, osseous	3 (6%)	2 (4%)	2 (4%)	4 (8%)
Mineralization	30 (60%)	34 (68%)	41 (82%)	38 (76%)
Necrosis				1 (2%)
Nephropathy	45 (90%)	45 (90%)	49 (98%)	49 (98%)
Pigmentation			2 (4%)	
Glomerulus, congestion	9 (18%)	14 (28%)	17 (34%)	44 (88%)
Papilla, necrosis	1 (2%)			
Pelvis, inflammation	1 (2%)			2 (4%)
Renal tubule, hyperplasia	1 (2%)		1 (2%)	1 (2%)
Urethra	(0)	(0)	(1)	(0)
Urinary bladder	(50)	(50)	(50)	(50)
Infiltration cellular, mononuclear cell	28 (56%)	19 (38%)	19 (38%)	31 (62%)
Transitional epithelium, hyperplasia				1 (2%)

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

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death				1
Moribund	9	3	5	9
Natural deaths	5	6	7	3
Survivors				
Terminal sacrifice	35	41	38	37
Missing	1			
Animals examined microscopically	49	50	50	50
Alimentary System				
Esophagus	(49)	(50)	(50)	(50)
Gallbladder	(48)	(50)	(50)	(50)
Osteosarcoma, metastatic, bone			1 (2%)	
Intestine large, cecum	(49)	(50)	(50)	(50)
Leiomyoma		1 (2%)		
Intestine large, colon	(49)	(50)	(50)	(50)
Sarcoma, metastatic, skeletal muscle				1 (2%)
Intestine large, rectum	(49)	(50)	(50)	(50)
Sarcoma, metastatic, skeletal muscle				1 (2%)
Intestine small, duodenum	(49)	(50)	(50)	(50)
Osteosarcoma, metastatic, bone			1 (2%)	
Intestine small, ileum	(49)	(50)	(50)	(50)
Intestine small, jejunum	(49)	(50)	(50)	(50)
Liver	(49)	(50)	(50)	(50)
Hepatoblastoma		1 (2%)	2 (4%)	1 (2%)
Hepatoblastoma, multiple				1 (2%)
Hepatocellular adenoma	7 (14%)	7 (14%)	10 (20%)	15 (30%)
Hepatocellular adenoma, multiple	6 (12%)	8 (16%)	3 (6%)	12 (24%)
Hepatocellular carcinoma	5 (10%)	1 (2%)	3 (6%)	8 (16%)
Hepatocellular carcinoma, multiple			1 (2%)	
Hepatocholangiocarcinoma				1 (2%)
Osteosarcoma, metastatic, bone			1 (2%)	
Sarcoma, metastatic, skin		1 (2%)		
Mesentery	(6)	(12)	(11)	(2)
Osteosarcoma, metastatic, bone			1 (9%)	
Sarcoma, metastatic, skeletal muscle				1 (50%)
Sarcoma, metastatic, skin		1 (8%)		
Pancreas	(49)	(50)	(50)	(50)
Sarcoma, metastatic, skeletal muscle				1 (2%)
Sarcoma, metastatic, skin		1 (2%)		
Salivary glands	(49)	(50)	(49)	(50)
Stomach, forestomach	(49)	(50)	(50)	(50)
Squamous cell papilloma	2 (4%)	4 (8%)	1 (2%)	1 (2%)
Squamous cell papilloma, multiple			1 (2%)	
Stomach, glandular	(49)	(50)	(50)	(50)
Sarcoma, metastatic, skeletal muscle				1 (2%)
Tongue	(0)	(0)	(0)	(1)
Squamous cell carcinoma				1 (100%)
Tooth	(3)	(1)	(2)	(1)

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Cardiovascular System				
Blood vessel	(49)	(50)	(50)	(50)
Heart	(49)	(50)	(50)	(50)
Endocrine System				
Adrenal cortex	(49)	(50)	(50)	(50)
Sarcoma, metastatic, skeletal muscle				1 (2%)
Subcapsular, adenoma	1 (2%)			
Adrenal medulla	(49)	(50)	(50)	(50)
Pheochromocytoma benign	1 (2%)	2 (4%)		1 (2%)
Islets, pancreatic	(49)	(50)	(50)	(50)
Parathyroid gland	(45)	(47)	(39)	(39)
Pituitary gland	(49)	(50)	(49)	(49)
Pars distalis, adenoma	2 (4%)	2 (4%)		
Thyroid gland	(49)	(50)	(49)	(50)
Follicular cell, adenoma	1 (2%)	1 (2%)		
Follicular cell, carcinoma	1 (2%)			
General Body System				
None				
Genital System				
Clitoral gland	(47)	(50)	(49)	(49)
Ovary	(49)	(49)	(50)	(50)
Cystadenocarcinoma		1 (2%)		
Cystadenoma	1 (2%)	2 (4%)		2 (4%)
Granulosa cell tumor benign	1 (2%)			
Granulosa cell tumor malignant				1 (2%)
Hemangioma	1 (2%)		1 (2%)	
Hemangiosarcoma	1 (2%)			
Luteoma		1 (2%)		
Osteosarcoma, metastatic, bone			1 (2%)	
Teratoma benign			1 (2%)	
Uterus	(49)	(49)	(50)	(50)
Polyp stromal			3 (6%)	
Hematopoietic System				
Bone marrow	(49)	(50)	(50)	(50)
Hemangiosarcoma	1 (2%)		1 (2%)	1 (2%)
Lymph node	(1)	(2)	(5)	(2)
Inguinal, sarcoma, metastatic, skin			1 (20%)	
Mediastinal, osteosarcoma, metastatic, bone			1 (20%)	
Mediastinal, schwannoma malignant, metastatic, skin	1 (100%)			
Lymph node, mandibular	(49)	(50)	(49)	(50)
Lymph node, mesenteric	(47)	(49)	(50)	(49)
Sarcoma, metastatic, skeletal muscle				1 (2%)
Spleen	(49)	(50)	(50)	(50)
Hemangiosarcoma	1 (2%)			1 (2%)
Thymus	(48)	(49)	(49)	(46)

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Integumentary System				
Mammary gland	(49)	(50)	(50)	(50)
Adenoacanthoma	1 (2%)			
Carcinoma	1 (2%)	1 (2%)		
Skin	(49)	(50)	(50)	(50)
Fibrosarcoma	1 (2%)			1 (2%)
Fibrous histiocytoma	1 (2%)			
Sarcoma		2 (4%)	1 (2%)	1 (2%)
Schwannoma malignant	1 (2%)		1 (2%)	
Schwannoma malignant, multiple	1 (2%)	1 (2%)		
Musculoskeletal System				
Bone	(49)	(50)	(50)	(50)
Osteoma			1 (2%)	
Osteosarcoma			1 (2%)	1 (2%)
Skeletal muscle	(0)	(0)	(1)	(1)
Rhabdomyosarcoma			1 (100%)	
Sarcoma				1 (100%)
Nervous System				
Brain	(49)	(50)	(50)	(50)
Respiratory System				
Lung	(49)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	1 (2%)	5 (10%)	2 (4%)	3 (6%)
Alveolar/bronchiolar carcinoma	2 (4%)	1 (2%)	1 (2%)	3 (6%)
Alveolar/bronchiolar carcinoma, multiple			1 (2%)	
Hepatocellular carcinoma, metastatic, liver	1 (2%)			2 (4%)
Osteosarcoma, metastatic, bone			1 (2%)	
Sarcoma, metastatic, skin		1 (2%)	1 (2%)	1 (2%)
Schwannoma malignant, metastatic, skin	1 (2%)		1 (2%)	
Nose	(49)	(50)	(50)	(50)
Osteoma			1 (2%)	
Special Senses System				
Eye	(49)	(50)	(49)	(50)
Harderian gland	(49)	(50)	(49)	(50)
Adenoma	7 (14%)	6 (12%)	4 (8%)	6 (12%)
Carcinoma	1 (2%)		2 (4%)	2 (4%)
Urinary System				
Kidney	(49)	(50)	(50)	(50)
Osteosarcoma, metastatic, bone			1 (2%)	
Sarcoma, metastatic, skeletal muscle				1 (2%)
Renal tubule, adenoma	1 (2%)			
Urinary bladder	(49)	(49)	(50)	(50)

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Systemic Lesions				
Multiple organs[b]	(49)	(50)	(50)	(50)
Histiocytic sarcoma	3 (6%)	1 (2%)		3 (6%)
Leukemia granulocytic			1 (2%)	
Lymphoma malignant	6 (12%)	2 (4%)	4 (8%)	6 (12%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	40	26	34	41
Total primary neoplasms	59	50	48	73
Total animals with benign neoplasms	27	22	24	32
Total benign neoplasms	32	39	28	40
Total animals with malignant neoplasms	20	11	17	23
Total malignant neoplasms	27	11	20	33
Total animals with metastatic neoplasms	2	1	3	3
Total metastatic neoplasms	3	4	11	11

[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 Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Harderian Gland: Adenoma				
Overall rate[a]	7/49 (14%)	6/50 (12%)	4/50 (8%)	6/50 (12%)
Adjusted rate[b]	15.3%	12.7%	8.8%	13.3%
Terminal rate[c]	6/35 (17%)	4/41 (10%)	2/38 (5%)	5/37 (14%)
First incidence (days)	450	640	642	589
Poly-3 test[d]	P=0.440N	P=0.479N	P=0.266N	P=0.511N
Harderian Gland: Adenoma or Carcinoma				
Overall rate	8/49 (16%)	6/50 (12%)	6/50 (12%)	8/50 (16%)
Adjusted rate	17.4%	12.7%	13.1%	17.3%
Terminal rate	7/35 (20%)	4/41 (10%)	3/38 (8%)	5/37 (14%)
First incidence (days)	450	640	631	589
Poly-3 test	P=0.498	P=0.366N	P=0.387N	P=0.602N
Liver: Hepatocellular Adenoma				
Overall rate	13/49 (27%)	15/50 (30%)	13/50 (26%)	27/50 (54%)
Adjusted rate	28.5%	31.8%	28.5%	59.0%
Terminal rate	10/35 (29%)	12/41 (29%)	10/38 (26%)	24/37 (65%)
First incidence (days)	663	645	631	566
Poly-3 test	P<0.001	P=0.455	P=0.590N	P=0.002
Liver: Hepatocellular Carcinoma				
Overall rate	5/49 (10%)	1/50 (2%)	4/50 (8%)	8/50 (16%)
Adjusted rate	11.0%	2.1%	8.8%	17.4%
Terminal rate	3/35 (9%)	1/41 (2%)	2/38 (5%)	3/37 (8%)
First incidence (days)	681	729 (T)	631	589
Poly-3 test	P=0.076	P=0.095N	P=0.497N	P=0.285
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	17/49 (35%)	15/50 (30%)	15/50 (30%)	32/50 (64%)
Adjusted rate	37.2%	31.8%	32.7%	68.3%
Terminal rate	13/35 (37%)	12/41 (29%)	11/38 (29%)	25/37 (68%)
First incidence (days)	663	645	631	566
Poly-3 test	P<0.001	P=0.371N	P=0.410N	P=0.002
Liver: Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	5/49 (10%)	2/50 (4%)	5/50 (10%)	10/50 (20%)
Adjusted rate	11.0%	4.3%	11.0%	21.7%
Terminal rate	3/35 (9%)	2/41 (5%)	3/38 (8%)	5/37 (14%)
First incidence (days)	681	729 (T)	631	589
Poly-3 test	P=0.027	P=0.205N	P=0.627N	P=0.135
Liver: Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma				
Overall rate	17/49 (35%)	15/50 (30%)	15/50 (30%)	33/50 (66%)
Adjusted rate	37.2%	31.8%	32.7%	70.4%
Terminal rate	13/35 (37%)	12/41 (29%)	11/38 (29%)	26/37 (70%)
First incidence (days)	663	645	631	566
Poly-3 test	P<0.001	P=0.371N	P=0.410N	P<0.001
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	1/49 (2%)	5/50 (10%)	2/50 (4%)	3/50 (6%)
Adjusted rate	2.2%	10.7%	4.4%	6.7%
Terminal rate	1/35 (3%)	5/41 (12%)	1/38 (3%)	3/37 (8%)
First incidence (days)	729 (T)	729 (T)	584	729 (T)
Poly-3 test	P=0.428	P=0.109	P=0.503	P=0.302

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	2/49 (4%)	1/50 (2%)	2/50 (4%)	3/50 (6%)
Adjusted rate	4.4%	2.1%	4.4%	6.7%
Terminal rate	0/35 (0%)	1/41 (2%)	1/38 (3%)	3/37 (8%)
First incidence (days)	598	729 (T)	584	729 (T)
Poly-3 test	P=0.303	P=0.492N	P=0.693	P=0.491
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	3/49 (6%)	6/50 (12%)	3/50 (6%)	6/50 (12%)
Adjusted rate	6.6%	12.9%	6.6%	13.4%
Terminal rate	1/35 (3%)	6/41 (15%)	2/38 (5%)	6/37 (16%)
First incidence (days)	598	729 (T)	584	729 (T)
Poly-3 test	P=0.264	P=0.253	P=0.662	P=0.232
Stomach (Forestomach): Squamous Cell Papilloma				
Overall rate	2/49 (4%)	4/50 (8%)	2/50 (4%)	1/50 (2%)
Adjusted rate	4.4%	8.6%	4.4%	2.2%
Terminal rate	2/35 (6%)	4/41 (10%)	2/38 (5%)	1/37 (3%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.270N	P=0.353	P=0.694	P=0.503N
Stomach (Forestomach): Squamous Cell Papilloma or Carcinoma				
Overall rate	2/49 (4%)	4/50 (8%)	2/50 (4%)	1/50 (2%)
Adjusted rate	4.4%	8.6%	4.4%	2.2%
Terminal rate	2/35 (6%)	4/41 (10%)	2/38 (5%)	1/37 (3%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.270N	P=0.353	P=0.694	P=0.503N
Uterus: Stromal Polyp				
Overall rate	0/49 (0%)	0/50 (0%)	3/50 (6%)	0/50 (0%)
Adjusted rate	0.0%	0.0%	6.7%	0.0%
Terminal rate	0/35 (0%)	0/41 (0%)	3/38 (8%)	0/37 (0%)
First incidence (days)	—[e]	—	729 (T)	—
Poly-3 test	P=0.524	—[f]	P=0.118	—
All Organs: Hemangiosarcoma				
Overall rate	3/49 (6%)	0/50 (0%)	1/50 (2%)	2/50 (4%)
Adjusted rate	6.6%	0.0%	2.2%	4.4%
Terminal rate	2/35 (6%)	0/41 (0%)	1/38 (3%)	1/37 (3%)
First incidence (days)	716	—	729 (T)	591
Poly-3 test	P=0.558N	P=0.113N	P=0.306N	P=0.499N
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	4/49 (8%)	0/50 (0%)	2/50 (4%)	2/50 (4%)
Adjusted rate	8.9%	0.0%	4.4%	4.4%
Terminal rate	3/35 (9%)	0/41 (0%)	2/38 (5%)	1/37 (3%)
First incidence (days)	716	—	729 (T)	591
Poly-3 test	P=0.406N	P=0.056N	P=0.338N	P=0.336N
All Organs: Histiocytic Sarcoma				
Overall rate	3/49 (6%)	1/50 (2%)	0/50 (0%)	3/50 (6%)
Adjusted rate	6.6%	2.1%	0.0%	6.6%
Terminal rate	0/35 (0%)	0/41 (0%)	0/38 (0%)	1/37 (3%)
First incidence (days)	681	666	—	596
Poly-3 test	P=0.523	P=0.294N	P=0.120N	P=0.661

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
All Organs: Malignant Lymphoma				
Overall rate	6/49 (12%)	2/50 (4%)	4/50 (8%)	6/50 (12%)
Adjusted rate	13.1%	4.2%	8.9%	13.1%
Terminal rate	3/35 (9%)	0/41 (0%)	3/38 (8%)	2/37 (5%)
First incidence (days)	659	493	714	566
Poly-3 test	P=0.390	P=0.119N	P=0.377N	P=0.619N
All Organs: Osteoma or Osteosarcoma				
Overall rate	0/49 (0%)	0/50 (0%)	3/50 (6%)	1/50 (2%)
Adjusted rate	0.0%	0.0%	6.5%	2.2%
Terminal rate	0/35 (0%)	0/41 (0%)	2/38 (5%)	1/37 (3%)
First incidence (days)	—	—	439	729 (T)
Poly-3 test	P=0.234	—	P=0.122	P=0.498
All Organs: Benign Neoplasms				
Overall rate	27/49 (55%)	22/50 (44%)	24/50 (48%)	32/50 (64%)
Adjusted rate	57.5%	46.3%	51.7%	69.2%
Terminal rate	21/35 (60%)	18/41 (44%)	19/38 (50%)	28/37 (76%)
First incidence (days)	450	640	584	566
Poly-3 test	P=0.072	P=0.186N	P=0.361N	P=0.164
All Organs: Malignant Neoplasms				
Overall rate	20/49 (41%)	11/50 (22%)	17/50 (34%)	23/50 (46%)
Adjusted rate	42.6%	22.8%	35.3%	48.2%
Terminal rate	10/35 (29%)	6/41 (15%)	10/38 (26%)	13/37 (35%)
First incidence (days)	598	493	439	566
Poly-3 test	P=0.121	P=0.030N	P=0.301N	P=0.367
All Organs: Benign or Malignant Neoplasms				
Overall rate	40/49 (82%)	26/50 (52%)	34/50 (68%)	41/50 (82%)
Adjusted rate	83.1%	53.1%	70.0%	85.1%
Terminal rate	28/35 (80%)	19/41 (46%)	25/38 (66%)	30/37 (81%)
First incidence (days)	450	493	439	566
Poly-3 test	P=0.117	P<0.001N	P=0.097N	P=0.502

(T) Terminal sacrifice

[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for 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 are the P values 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 dose group is indicated by **N**.

[e] Not applicable; no neoplasms in animal group

[f] Value of statistic cannot be computed.

TABLE D3
Historical Incidence of Liver Neoplasms in Control Female B6C3F1 Mice[a]

Study (Study Start)	Incidence in Controls			
	Hepatocellular Adenoma	Hepatocellular Carcinoma	Hepatoblastoma	Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma
Historical Incidence: Corn Oil Gavage Studies				
Isoeugenol (May 2002)	11/49	3/49	0/49	13/49
Kava kava extract (August 2004)	8/50	3/50	0/50	10/50
β-Myrcene (April 2002)	6/50	1/50	0/50	7/50
Pulegone (April 2003)	13/49	5/49	0/49	17/49
3,3′,4,4′-Tetrachloroazobenzene (February 2003)	3/49	2/49	0/49	4/49
Total (%)	41/247 (16.6%)	14/247 (5.7%)	0/247	51/247 (20.7%)
Mean ± standard deviation	16.6% ± 8.1%	5.7% ± 3.0%		20.7% ± 10.4%
Range	6%-27%	2%-10%		8%-35%
Overall Historical Incidence: All Routes				
Total (%)	395/1,495 (26.4%)	138/1,495 (9.2%)	4/1,495 (0.3%)	483/1,495 (32.3%)
Mean ± standard deviation	26.4% ± 14.9%	9.2% ± 6.5%	0.3% ± 0.7%	32.3% ± 16.9%
Range	2%-62%	0%-28%	0%-2%	6%-64%

[a] Data as of April 29, 2009

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death				1
Moribund	9	3	5	9
Natural deaths	5	6	7	3
Survivors				
Terminal sacrifice	35	41	38	37
Missing	1			
Animals examined microscopically	49	50	50	50
Alimentary System				
Esophagus	(49)	(50)	(50)	(50)
Perforation				1 (2%)
Periesophageal tissue, inflammation		1 (2%)		
Gallbladder	(48)	(50)	(50)	(50)
Infiltration cellular, mononuclear cell	1 (2%)	2 (4%)		1 (2%)
Intestine large, cecum	(49)	(50)	(50)	(50)
Intestine large, colon	(49)	(50)	(50)	(50)
Intestine large, rectum	(49)	(50)	(50)	(50)
Artery, inflammation, chronic active		1 (2%)		
Intestine small, duodenum	(49)	(50)	(50)	(50)
Artery, inflammation, chronic active		1 (2%)		
Intestine small, ileum	(49)	(50)	(50)	(50)
Artery, inflammation, chronic active		1 (2%)		
Intestine small, jejunum	(49)	(50)	(50)	(50)
Artery, inflammation, chronic active		1 (2%)		
Liver	(49)	(50)	(50)	(50)
Angiectasis		1 (2%)	3 (6%)	1 (2%)
Basophilic focus	1 (2%)	3 (6%)		1 (2%)
Clear cell focus		6 (12%)	23 (46%)	32 (64%)
Eosinophilic focus	3 (6%)	7 (14%)	10 (20%)	31 (62%)
Fatty change, focal	1 (2%)	2 (4%)	20 (40%)	12 (24%)
Fatty change, diffuse	36 (73%)	31 (62%)	34 (68%)	3 (6%)
Fibrosis				2 (4%)
Hematopoietic cell proliferation	1 (2%)			1 (2%)
Inflammation	40 (82%)	40 (80%)	14 (28%)	31 (62%)
Mineralization				1 (2%)
Mixed cell focus	4 (8%)	8 (16%)	16 (32%)	20 (40%)
Necrosis	5 (10%)	2 (4%)	4 (8%)	27 (54%)
Pigmentation				46 (92%)
Tension lipidosis	5 (10%)	5 (10%)	4 (8%)	
Bile duct, cyst			4 (8%)	38 (76%)
Bile duct, hyperplasia			2 (4%)	47 (94%)
Centrilobular, hepatocyte, hypertrophy		4 (8%)	12 (24%)	29 (58%)
Oval cell, hyperplasia			3 (6%)	46 (92%)
Portal, fibrosis				1 (2%)
Vein, hypertrophy				1 (2%)
Vein, intravascular hepatocyte		2 (4%)	20 (40%)	46 (92%)

[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 Pulegone

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Alimentary System (continued)				
Mesentery	(6)	(12)	(11)	(2)
Necrosis	2 (33%)			
Artery, inflammation, chronic active		1 (8%)		
Fat, necrosis	4 (67%)	11 (92%)	10 (91%)	1 (50%)
Pancreas	(49)	(50)	(50)	(50)
Atrophy				1 (2%)
Cytoplasmic alteration, focal	1 (2%)	2 (4%)		2 (4%)
Infiltration cellular, mononuclear cell	12 (24%)	16 (32%)	15 (30%)	11 (22%)
Acinus, atrophy	1 (2%)	1 (2%)		
Artery, inflammation, chronic active		1 (2%)		
Salivary glands	(49)	(50)	(49)	(50)
Infiltration cellular, mononuclear cell	39 (80%)	29 (58%)	28 (57%)	26 (52%)
Stomach, forestomach	(49)	(50)	(50)	(50)
Hyperplasia, squamous	13 (27%)	1 (2%)	10 (20%)	26 (52%)
Inflammation	10 (20%)		7 (14%)	20 (40%)
Mineralization				1 (2%)
Ulcer	8 (16%)		4 (8%)	10 (20%)
Stomach, glandular	(49)	(50)	(50)	(50)
Mineralization			1 (2%)	
Artery, inflammation, chronic active		1 (2%)		
Glands, cyst	2 (4%)	3 (6%)	3 (6%)	2 (4%)
Tongue	(0)	(0)	(0)	(1)
Tooth	(3)	(1)	(2)	(1)
Dysplasia	3 (100%)	1 (100%)	2 (100%)	1 (100%)
Peridontal tissue, inflammation		1 (100%)		
Cardiovascular System				
Blood vessel	(49)	(50)	(50)	(50)
Mineralization			1 (2%)	
Heart	(49)	(50)	(50)	(50)
Cardiomyopathy	1 (2%)	2 (4%)	1 (2%)	5 (10%)
Infiltration cellular, mononuclear cell	1 (2%)			
Inflammation	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Mineralization	2 (4%)		2 (4%)	8 (16%)
Necrosis				2 (4%)
Ventricle, thrombosis				1 (2%)
Endocrine System				
Adrenal cortex	(49)	(50)	(50)	(50)
Hypertrophy		1 (2%)	1 (2%)	1 (2%)
Inflammation				1 (2%)
Vacuolization cytoplasmic	1 (2%)		1 (2%)	
Subcapsular, hyperplasia	49 (100%)	50 (100%)	49 (98%)	49 (98%)
Adrenal medulla	(49)	(50)	(50)	(50)
Hyperplasia	1 (2%)			
Islets, pancreatic	(49)	(50)	(50)	(50)
Hyperplasia	5 (10%)	2 (4%)	3 (6%)	1 (2%)
Parathyroid gland	(45)	(47)	(39)	(39)
Infiltration cellular, lymphocyte		1 (2%)		

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Endocrine System (continued)				
Pituitary gland	(49)	(50)	(49)	(49)
Angiectasis	3 (6%)	3 (6%)	1 (2%)	2 (4%)
Cyst				1 (2%)
Degeneration	1 (2%)			
Pars distalis, cyst		2 (4%)		
Pars distalis, hyperplasia	7 (14%)	4 (8%)	5 (10%)	4 (8%)
Thyroid gland	(49)	(50)	(49)	(50)
Infiltration cellular, mononuclear cell	1 (2%)		1 (2%)	
C-cell, hyperplasia		1 (2%)		
Follicle, cyst	1 (2%)			
Follicular cell, hyperplasia	1 (2%)			1 (2%)
General Body System				
None				
Genital System				
Clitoral gland	(47)	(50)	(49)	(49)
Infiltration cellular, mononuclear cell			1 (2%)	1 (2%)
Inflammation		1 (2%)	1 (2%)	3 (6%)
Ovary	(49)	(49)	(50)	(50)
Angiectasis	1 (2%)	1 (2%)	1 (2%)	
Atrophy	11 (22%)	10 (20%)	24 (48%)	39 (78%)
Cyst	6 (12%)	7 (14%)	9 (18%)	7 (14%)
Uterus	(49)	(49)	(50)	(50)
Angiectasis	3 (6%)			
Decidual reaction		1 (2%)		
Infiltration cellular, mononuclear cell				1 (2%)
Inflammation	1 (2%)	1 (2%)		
Metaplasia		1 (2%)		
Endometrium, hyperplasia, cystic	32 (65%)	32 (65%)	40 (80%)	35 (70%)
Hematopoietic System				
Bone marrow	(49)	(50)	(50)	(50)
Atrophy			1 (2%)	1 (2%)
Hyperplasia	2 (4%)	9 (18%)	4 (8%)	5 (10%)
Myelofibrosis	20 (41%)	17 (34%)	17 (34%)	14 (28%)
Necrosis			1 (2%)	
Lymph node	(1)	(2)	(5)	(2)
Lymph node, mandibular	(49)	(50)	(49)	(50)
Atrophy		1 (2%)		2 (4%)
Hyperplasia, lymphoid	2 (4%)		1 (2%)	2 (4%)
Pigmentation			1 (2%)	
Lymph node, mesenteric	(47)	(49)	(50)	(49)
Atrophy			2 (4%)	1 (2%)
Hyperplasia, reticulum cell			1 (2%)	
Spleen	(49)	(50)	(50)	(50)
Hematopoietic cell proliferation	24 (49%)	29 (58%)	25 (50%)	40 (80%)
Hyperplasia, lymphoid	6 (12%)	3 (6%)	9 (18%)	3 (6%)
Necrosis				1 (2%)
Pigmentation		1 (2%)	3 (6%)	2 (4%)

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Hematopoietic System (continued)				
Spleen (continued)	(49)	(50)	(50)	(50)
Lymphoid follicle, atrophy				1 (2%)
Red pulp, atrophy	1 (2%)			1 (2%)
Thymus	(48)	(49)	(49)	(46)
Atrophy	28 (58%)	21 (43%)	20 (41%)	13 (28%)
Hyperplasia, lymphoid	4 (8%)	5 (10%)	2 (4%)	1 (2%)
Integumentary System				
Mammary gland	(49)	(50)	(50)	(50)
Skin	(49)	(50)	(50)	(50)
Musculoskeletal System				
Bone	(49)	(50)	(50)	(50)
Osteosclerosis			1 (2%)	1 (2%)
Cranium, myelofibrosis			1 (2%)	
Skeletal muscle	(0)	(0)	(1)	(1)
Nervous System				
Brain	(49)	(50)	(50)	(50)
Hemorrhage				1 (2%)
Infiltration cellular, mononuclear cell	1 (2%)			
Necrosis			1 (2%)	
Hippocampus, gliosis			1 (2%)	
Respiratory System				
Lung	(49)	(50)	(50)	(50)
Inflammation	2 (4%)	4 (8%)	2 (4%)	
Mineralization			1 (2%)	
Pigmentation				1 (2%)
Alveolar epithelium, hyperplasia	1 (2%)	3 (6%)	2 (4%)	7 (14%)
Mediastinum, serosa, inflammation				1 (2%)
Nose	(49)	(50)	(50)	(50)
Inflammation	2 (4%)	1 (2%)	4 (8%)	27 (54%)
Ulcer				1 (2%)
Glands, cyst	1 (2%)			
Nerve, atrophy		1 (2%)	2 (4%)	49 (98%)
Olfactory epithelium, degeneration		5 (10%)	22 (44%)	48 (96%)
Olfactory epithelium, metaplasia	1 (2%)	2 (4%)	4 (8%)	49 (98%)
Respiratory epithelium, metaplasia	1 (2%)			
Special Senses System				
Eye	(49)	(50)	(49)	(50)
Atrophy		2 (4%)	1 (2%)	3 (6%)
Cataract	1 (2%)			1 (2%)
Anterior chamber, inflammation				2 (4%)
Choroid, sclera, inflammation			1 (2%)	1 (2%)

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

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Special Senses System (continued)				
Eye (continued)	(49)	(50)	(49)	(50)
Cornea, inflammation	2 (4%)			9 (18%)
Cornea, pigmentation				1 (2%)
Retina, degeneration	1 (2%)			
Harderian gland	(49)	(50)	(49)	(50)
Cyst	1 (2%)			
Infiltration cellular, mononuclear cell	30 (61%)	22 (44%)	17 (35%)	15 (30%)
Inflammation, chronic active				1 (2%)
Epithelium, hyperplasia	3 (6%)			4 (8%)
Urinary System				
Kidney	(49)	(50)	(50)	(50)
Accumulation, hyaline droplet	2 (4%)			
Cyst			2 (4%)	
Glomerulopathy, hyaline		3 (6%)	15 (30%)	41 (82%)
Infarct	3 (6%)	2 (4%)	1 (2%)	1 (2%)
Metaplasia, osseous	1 (2%)	2 (4%)	2 (4%)	4 (8%)
Mineralization	1 (2%)		3 (6%)	20 (40%)
Nephropathy	13 (27%)	19 (38%)	12 (24%)	25 (50%)
Pigmentation	1 (2%)	1 (2%)		
Vacuolization cytoplasmic		1 (2%)	2 (4%)	
Glomerulus, congestion	5 (10%)	2 (4%)	12 (24%)	37 (74%)
Papilla, mineralization	1 (2%)			
Papilla, necrosis	3 (6%)	1 (2%)	1 (2%)	
Pelvis, dilatation		1 (2%)		
Urinary bladder	(49)	(49)	(50)	(50)
Infiltration cellular, mononuclear cell	37 (76%)	33 (67%)	30 (60%)	30 (60%)
Artery, inflammation, chronic active		1 (2%)		

APPENDIX E
GENETIC TOXICOLOGY

GENETIC TOXICOLOGY

BACTERIAL MUTAGENICITY TEST PROTOCOL

Tests performed at BioReliance Corporation (Rockville, MD) followed protocols reported by Zeiger *et al.* (1992); in the tests conducted at SITEK Research Laboratories (Rockville, MD) and ILS, Inc. (Research Triangle Park, NC), using the same lot of pulegone (OGI01) that was tested in the 2-week, 3-month, and 2-year rodent bioassays, a slightly modified procedure was used and is described below. Pulegone was sent to the laboratory as a coded aliquot. In the tests conducted at BioReliance Corporation, pulegone was incubated with the *Salmonella typhimurium* tester strains TA97, TA98, TA100, and TA1535 either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Two concentrations, 10% and 30%, of S9 were used. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

The modified protocol used at SITEK Research Laboratories and ILS, Inc., used only 10% rat liver S9 for exogenous metabolic activation and employed *Escherichia coli* strain WP2 *uvrA*/pKM101 as a bacterial tester strain in addition to *S. typhimurium* strains TA98 and TA100. Incubation of bacterial strains with pulegone and subsequent plating were carried out as described above for the traditional protocol.

Each trial consisted of triplicate plates of concurrent positive and negative controls and at least five doses of pulegone. In both studies, the high dose was limited by toxicity. All trials were repeated, and those that were conducted with S9 activation enzymes were repeated using the same or higher concentrations of S9.

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, although positive calls are typically reserved for increases in mutant colonies that are at least twofold over background.

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 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, mature erythrocytes) per animal. In addition, the percentage of polychromatic erythrocytes (PCEs, reticulocytes) 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 study 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 judgment of the overall evidence for activity of the chemical in an assay.

RESULTS

Pulegone (doses up to 3,333 µg/plate in the first study and 3,500 µg/plate in the second study) was not mutagenic in either of two independent bacterial mutagenicity assays, with or without exogenous metabolic activation (Tables E1 and E2). Bacterial strains tested in the first study included *S. typhimurium* strains TA97, TA98, TA100, and TA1535, with and without 10% or 30% hamster or rat liver S9. Strains tested in the second study, conducted on the same lot of pulegone that was tested in the 2-year rodent bioassay, included *S. typhimurium* strains TA98 and TA100 and *E. coli* strain WP2 *uvrA*/pKM101, with and without 10% rat liver S9.

A second study using the same lot of pulegone that was tested in the 2-year rodent bioassay (12.5 to 1,500 µg/plate was positive for mutagenicity in *S. typhimurium* strain TA98 and *E. coli* strain WP2 *uvrA*/pKM101 when tested in the presence of 10% rat liver S9 (Table E3). The lowest concentration that produced a mutagenic response was 500 µg/plate in both strains.

In vivo, no significant increases in the frequencies of micronucleated NCEs were seen in peripheral blood of male or female B6C3F1 mice administered pulegone (9.375 to 150 mg/kg) for 3 months by gavage (Table E4). The percentage of PCEs (reticulocytes) in the peripheral blood of male and female mice was not significantly changed by pulegone administration, suggesting an absence of chemical-associated bone marrow toxicity.

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

Strain	Dose (µg/Plate)	Without S9	Without S9	With 10% hamster S9	With 30% hamster S9	With 10% rat S9	With 30% rat S9
TA100	0	111 ± 11	137 ± 11	168 ± 4	123 ± 9	174 ± 4	165 ± 5
	3.3		143 ± 3				
	10	115 ± 1	173 ± 8	158 ± 14	124 ± 5	168 ± 8	137 ± 13
	33	121 ± 17	153 ± 9	159 ± 7	119 ± 5	163 ± 5	157 ± 12
	100	117 ± 15	156 ± 14	163 ± 10	123 ± 8	143 ± 4	144 ± 2
	333	91 ± 9[b]	147 ± 7	163 ± 5	133 ± 11	150 ± 10	127 ± 7
	1,000	Toxic	10 ± 3[b]	3 ± 1[b]	79 ± 39[b]	Toxic	108 ± 15[b]
	2,167			1 ± 1[b]	0 ± 0[b]	Toxic	34 ± 34[b]
	3,333	Toxic					
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[c]		503 ± 28	413 ± 20	336 ± 18	494 ± 43	591 ± 42	973 ± 28
TA1535	0	11 ± 3	7 ± 1	10 ± 1	12 ± 1	13 ± 2	11 ± 1
	3.3		12 ± 1				
	10	5 ± 1	9 ± 1	11 ± 3		9 ± 3	
	33	10 ± 2	9 ± 2	14 ± 2	12 ± 2	9 ± 1	11 ± 1
	100	8 ± 2	12 ± 1	9 ± 1	8 ± 3	11 ± 0	12 ± 1
	333	4 ± 2[b]	13 ± 1	8 ± 2	13 ± 1	11 ± 1	8 ± 1
	1,000	Toxic	Toxic	4 ± 2[b]	Toxic	Toxic	0 ± 0[b]
	2,167			0 ± 0[b]	0 ± 0[b]	Toxic	0 ± 0[b]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		128 ± 6	197 ± 36	36 ± 5	67 ± 6	212 ± 28	165 ± 5
TA97	0	107 ± 3	143 ± 5	161 ± 16	121 ± 8	168 ± 14	170 ± 8
	3.3		171 ± 7				
	10	110 ± 6	168 ± 6	181 ± 3		150 ± 10	
	33	87 ± 4	167 ± 6	185 ± 2[d]	150 ± 7	186 ± 17	189 ± 14
	100	106 ± 4	165 ± 3	190 ± 3	160 ± 8	209 ± 16	184 ± 4
	333	46 ± 7[b]	145 ± 2	166 ± 14	160 ± 2	159 ± 7	140 ± 7
	1,000	Toxic	7 ± 4[b]	Toxic	Toxic	9 ± 3[b]	Toxic
	2,167			Toxic	Toxic	20 ± 16[b]	Toxic
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		749 ± 55	1,732 ± 52	522 ± 11	725 ± 17	776 ± 37	512 ± 16

TABLE E1
Mutagenicity of Pulegone in *Salmonella typhimurium*

Strain	Dose (µg/Plate)	Without S9	Without S9	With 10% hamster S9	With 30% hamster S9	With 10% rat S9	With 30% rat S9
TA98	0	13 ± 1	14 ± 2	17 ± 1	29 ± 3	16 ± 2	23 ± 2
	3.3		12 ± 2				
	10	13 ± 2	9 ± 0	14 ± 2	25 ± 3	12 ± 1	27 ± 3
	33	15 ± 3	8 ± 2	14 ± 1	31 ± 3	13 ± 1	24 ± 3
	100	13 ± 1	5 ± 2	16 ± 3	27 ± 3	9 ± 1	24 ± 2
	333	11 ± 0	14 ± 1	17 ± 1	29 ± 4	14 ± 2	21 ± 5
	1,000	Toxic	1 ± 0[b]	2 ± 1[b]	21 ± 5[b]	Toxic	6 ± 4[b]
	2,167			0 ± 0[b]	2 ± 1[b]	Toxic	8 ± 3[b]
	3,333	Toxic					
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		98 ± 4	87 ± 3	327 ± 26	335 ± 28	259 ± 7	232 ± 42

[a] Study was performed at BioReliance Corporation; the detailed protocol is presented by Zeiger *et al.* (1992). Data are presented as revertants/plate (mean ± standard error) from three plates. 0 µg/plate was the solvent control.

[b] Slight toxicity

[c] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA97), and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

[d] Contamination on plate

TABLE E2
Mutagenicity of Pulegone in Bacterial Tester Strains (Lot OGI01 at SITEK Research Laboratories)[a]

Strain	Dose (µg/Plate)	Without S9	Without S9	With 10% rat S9	With 10% rat S9	With 10% rat S9
TA100	0	64 ± 3	72 ± 6	82 ± 6	84 ± 2	
	100	64 ± 3				
	150		62 ± 5			
	200	63 ± 2	61 ± 3	79 ± 8	87 ± 3	
	250	64 ± 1	64 ± 7	88 ± 3	75 ± 6	
	400	60 ± 5	63 ± 1	87 ± 4	82 ± 1	
	500	37 ± 3	41 ± 3	65 ± 6	82 ± 2	
	1,000	0 ± 0	5 ± 0	43 ± 2	53 ± 8	
	1,500			27 ± 4	0 ± 0	
Trial summary		Negative	Negative	Negative	Negative	
Positive control[b]		694 ± 54	696 ± 6	1,170 ± 35	797 ± 62	
TA98	0	25 ± 1	23 ± 4	26 ± 3	33 ± 2	
	150	26 ± 3	23 ± 1			
	200	21 ± 4	21 ± 4			
	250	19 ± 3	23 ± 1	30 ± 3	25 ± 2	
	400	17 ± 2	20 ± 5	21 ± 2	27 ± 5	
	500	18 ± 2	25 ± 1	19 ± 3	42 ± 0	
	1,000	0 ± 0	2 ± 1	21 ± 1	32 ± 4	
	1,500			19 ± 3	25 ± 3	
	2,500			0 ± 0	1 ± 0	
Trial summary		Negative	Negative	Negative	Negative	
Positive control		573 ± 16	570 ± 37	1,315 ± 90	1,384 ± 14	
***Escherichia coli* WP2 *uvrA*/pKM101**						
	0	140 ± 4	149 ± 3	183 ± 5	175 ± 3	227 ± 4
	100					271 ± 14
	200	132 ± 2	154 ± 5			
	250	138 ± 6	167 ± 10			
	400	151 ± 10	160 ± 8	185 ± 2	168 ± 5	
	500	133 ± 4	134 ± 3	184 ± 12	161 ± 1	326 ± 10
	1,000	59 ± 7	26 ± 1	162 ± 4	136 ± 6	
	1,500	6 ± 2	17 ± 3	125 ± 15	136 ± 1	261 ± 10
	2,500			54 ± 2	53 ± 2	302 ± 25
	3,500			8 ± 2	0 ± 0	348 ± 17
Trial summary		Negative	Negative	Negative	Negative	Equivocal
Positive control		2,113 ± 68	1,894 ± 87	1,078 ± 41	1,142 ± 21	1,007 ± 7

[a] The study used a slight modification of the protocol presented by Zeiger *et al.* (1992). Data are presented as revertants/plate (mean ± standard error) from three plates. 0 µg/plate was the solvent control.

[b] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE E3
Mutagenicity of Pulegone in Bacterial Tester Strains (Lot OGI01 at ILS, Inc.)[a]

Strain	Dose (µg/Plate)	Without S9	Without S9	Without S9		
TA100	0	87 ± 8	87 ± 6	111 ± 7		
	12.5	101 ± 2	84 ± 7	140 ± 30		
	50	105 ± 9	88 ± 10	141 ± 6		
	75		91 ± 4			
	125	89 ± 9	94 ± 7	109 ± 2		
	250		90 ± 11			
	500	Toxic	66 ± 12	63 ± 32		
	750	Toxic	Toxic	Toxic		
	1,500	Toxic		Toxic		
Trial summary		Negative	Negative	Negative		
Positive control[b]		975 ± 22	792 ± 11	993 ± 20		
		With 10% rat S9	**With 10% rat S9**	**With 10% rat S9**	**With 10% rat S9**	
TA100	0	108 ± 16	106 ± 9	111 ± 6	91 ± 4	
(continued)	12.5	110 ± 6			113 ± 8	
	25			89 ± 3		
	50	100 ± 9		98 ± 6	113 ± 2	
	100			114 ± 8		
	125	106 ± 2	92 ± 3		109 ± 4	
	250		90 ± 10	95 ± 2		
	500	214 ± 14	88 ± 7	103 ± 12	145 ± 4	
	750	193 ± 17	Toxic	71 ± 2	103 ± 2	
	1,000		Toxic			
	1,500	140 ± 77[c]	Toxic	Toxic	Toxic	
Trial summary		Equivocal	Negative	Negative	Negative	
Positive control		2,110 ± 76	1,465 ± 86	2,177 ± 64	1,710 ± 14	
		Without S9	**Without S9**	**Without S9**	**With 10% rat S9**	**With 10% rat S9**
TA98	0	31 ± 4	18 ± 2	23 ± 1	32 ± 2	28 ± 3
	12.5		25 ± 3	24 ± 2	32 ± 3	30 ± 3
	25	29 ± 2				
	50	26 ± 3	22 ± 2	48 ± 14	27 ± 3	31 ± 3
	100	32 ± 5				
	125		29 ± 2	40 ± 3	34 ± 2	30 ± 2
	250	25 ± 4				
	500	24 ± 4	21 ± 4	24 ± 1	80 ± 11	45 ± 0
	750	Toxic	24 ± 4	54 ± 9	131 ± 6	69 ± 1
	1,500	Toxic	Toxic	Toxic	Toxic	Toxic
Trial summary		Negative	Negative	Equivocal	Positive	Positive
Positive control		787 ± 15	569 ± 33	637 ± 26	1,357 ± 80	1,191 ± 54

TABLE E3
Mutagenicity of Pulegone in Bacterial Tester Strains (Lot OGI01 at ILS, Inc.)

Strain	Dose (µg/Plate)	Without S9	Without S9	With 10% rat S9	With 10% rat S9
***Escherichia coli* WP2 *uvrA*/pKM101**					
	0	109 ± 5	144 ± 2	182 ± 10	197 ± 7
	12.5	129 ± 2		197 ± 14	185 ± 9
	25		150 ± 6		
	50	128 ± 9	148 ± 3	198 ± 8	185 ± 7
	100		150 ± 11		
	125	124 ± 9		172 ± 8	203 ± 6
	250		141 ± 14		
	500	111 ± 7	145 ± 2	389 ± 11	432 ± 25
	750	Toxic	Toxic	581 ± 66	606 ± 19
	1,500	Toxic	Toxic	159 ± 11	Toxic
Trial summary		Negative	Negative	Positive	Positive
Positive control		907 ± 30	913 ± 13	1,077 ± 39	1,147 ± 43

[a] The study used a slight modification of the protocol presented by Zeiger *et al.* (1992). Data are presented as revertants/plate (mean ± standard error) from three plates. 0 µg/plate was the solvent control.

[b] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

[c] Slight toxicity

TABLE E4
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice Following Administration of Pulegone by Gavage for 3 Months[a]

	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	P value[c]	PCEs (%)[b]
Male					
Corn oil[d]	0	5	0.9 ± 0.19		2.22 ± 0.20
Pulegone	9.375	5	0.6 ± 0.19	0.7808	2.18 ± 0.17
	18.75	5	1.0 ± 0.32	0.4092	2.34 ± 0.16
	37.5	5	0.6 ± 0.24	0.7808	1.96 ± 0.33
	75	5	1.1 ± 0.29	0.3273	2.48 ± 0.43
	150	5	1.0 ± 0.22	0.4092	2.44 ± 0.13
			P=0.221[e]		
Female					
Corn oil	0	5	0.8 ± 0.34		1.82 ± 0.13
Pulegone	9.375	5	0.7 ± 0.34	0.6019	2.76 ± 0.34
	18.75	5	0.6 ± 0.19	0.7036	2.10 ± 0.08
	37.5	5	0.6 ± 0.29	0.7036	2.00 ± 0.22
	75	5	0.5 ± 0.16	0.7974	2.22 ± 0.17
	150	5	0.5 ± 0.27	0.7974	2.58 ± 0.22
			P=0.801		

[a] Study was performed at SITEK Research Laboratories. The detailed protocol is presented by MacGregor *et al.* (1990). PCE=polychromatic erythrocyte; NCE=normochromatic erythrocyte.
[b] Mean ± standard error
[c] Pairwise comparison with the vehicle control group; 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 Pulegone[a]

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Male						
n	10	10	10	10	10	10
Hematology						
Hematocrit (%)						
Day 4	39.7 ± 0.4	38.5 ± 0.3	38.8 ± 0.5	39.3 ± 0.5	39.5 ± 0.5	42.0 ± 0.5*
Day 18	40.1 ± 0.5	41.9 ± 0.5	42.1 ± 0.3**	41.0 ± 0.3	41.6 ± 0.4	41.0 ± 0.4
Week 14	43.0 ± 0.3	43.7 ± 0.4	42.3 ± 0.4	41.6 ± 0.4*	40.8 ± 0.3**	39.4 ± 0.6**
Hemoglobin (g/dL)						
Day 4	13.0 ± 0.1	12.7 ± 0.1	12.7 ± 0.2	12.9 ± 0.1	13.0 ± 0.2	13.9 ± 0.2*
Day 18	13.4 ± 0.1	13.7 ± 0.1	13.9 ± 0.1	13.6 ± 0.1	13.6 ± 0.1	13.6 ± 0.1
Week 14	15.2 ± 0.1	15.4 ± 0.1	14.9 ± 0.1	14.9 ± 0.1	14.6 ± 0.1**	14.1 ± 0.2**
Erythrocytes (10^6/µL)						
Day 4	7.11 ± 0.07	6.84 ± 0.06	6.93 ± 0.11	7.01 ± 0.09	7.05 ± 0.09	7.71 ± 0.09**
Day 18	7.29 ± 0.09	7.62 ± 0.10	7.62 ± 0.08*	7.38 ± 0.07	7.41 ± 0.07	7.39 ± 0.09
Week 14	8.55 ± 0.07	8.58 ± 0.08	8.28 ± 0.06*	8.05 ± 0.08**	7.88 ± 0.06**	8.11 ± 0.13**
Reticulocytes (10^6/µL)						
Day 4	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0**
Day 18	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0*	0.5 ± 0.0**
Week 14	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0**	0.3 ± 0.0**
Nucleated erythrocytes/100 leukocytes						
Day 4	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Day 18	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0*	0.1 ± 0.0	0.1 ± 0.0
Week 14	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0**
Mean cell volume (fL)						
Day 4	55.9 ± 0.3	56.3 ± 0.2	56.0 ± 0.2	56.0 ± 0.2	56.0 ± 0.2	54.5 ± 0.2**
Day 18	55.1 ± 0.2	55.0 ± 0.2	55.3 ± 0.2	55.6 ± 0.3	56.2 ± 0.1**	55.6 ± 0.1**
Week 14	50.4 ± 0.1	50.9 ± 0.1	51.1 ± 0.1	51.7 ± 0.1**	51.7 ± 0.2**	48.6 ± 0.3
Mean cell hemoglobin (pg)						
Day 4	18.3 ± 0.1	18.6 ± 0.1	18.4 ± 0.1	18.5 ± 0.1	18.4 ± 0.1	18.0 ± 0.1
Day 18	18.4 ± 0.1	18.0 ± 0.1	18.2 ± 0.1	18.5 ± 0.1	18.4 ± 0.1	18.4 ± 0.1
Week 14	17.7 ± 0.1	17.9 ± 0.1	18.1 ± 0.1	18.5 ± 0.1**	18.6 ± 0.1**	17.4 ± 0.2
Mean cell hemoglobin concentration (g/dL)						
Day 4	32.8 ± 0.1	33.0 ± 0.1	32.8 ± 0.1	32.9 ± 0.1	33.0 ± 0.1	33.1 ± 0.1
Day 18	33.4 ± 0.2	32.8 ± 0.2	33.0 ± 0.1	33.2 ± 0.2	32.7 ± 0.2	33.1 ± 0.2
Week 14	35.2 ± 0.2	35.2 ± 0.1	35.3 ± 0.2	35.9 ± 0.2*	35.9 ± 0.1**	35.7 ± 0.1*
Platelets (10^3/µL)						
Day 4	912.5 ± 14.3	921.6 ± 15.5	890.1 ± 17.1	866.3 ± 21.1	854.4 ± 15.1*	737.7 ± 12.3**
Day 18	883.1 ± 16.0	821.3 ± 35.1	886.7 ± 24.1	879.2 ± 17.7	918.4 ± 8.5	1,028.4 ± 29.5**
Week 14	687.2 ± 36.9	674.8 ± 49.8	696.3 ± 29.6	773.3 ± 14.2	930.7 ± 17.0**	1,073.2 ± 43.1**
Leukocytes (10^3/µL)						
Day 4	8.12 ± 0.38	7.75 ± 0.25	8.11 ± 0.31	7.93 ± 0.32	8.53 ± 0.56	8.94 ± 0.36
Day 18	10.06 ± 0.37	9.33 ± 0.70	11.36 ± 0.40	10.55 ± 0.57	11.28 ± 0.31	10.64 ± 0.49
Week 14	9.64 ± 0.50	8.71 ± 0.31	9.90 ± 0.29	10.28 ± 0.87	10.04 ± 0.58	10.94 ± 0.43
Segmented neutrophils (10^3/µL)						
Day 4	0.83 ± 0.03	0.98 ± 0.08	0.83 ± 0.06	0.96 ± 0.07	0.95 ± 0.08	1.73 ± 0.16**
Day 18	0.90 ± 0.07	0.82 ± 0.13	0.94 ± 0.08	0.90 ± 0.09	0.92 ± 0.09	1.05 ± 0.09
Week 14	1.29 ± 0.16	1.05 ± 0.10	1.36 ± 0.09	1.15 ± 0.09	1.48 ± 0.09	2.25 ± 0.17**
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 18	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

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

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Male (continued)						
n	10	10	10	10	10	10
Hematology (continued)						
Lymphocytes (10^3/μL)						
Day 4	7.17 ± 0.40	6.61 ± 0.20	7.11 ± 0.29	6.81 ± 0.26	7.41 ± 0.53	7.02 ± 0.30
Day 18	8.98 ± 0.36	8.22 ± 0.71	10.23 ± 0.35	9.45 ± 0.61	10.10 ± 0.29	9.29 ± 0.51
Week 14	8.03 ± 0.41	7.31 ± 0.33	8.39 ± 0.27	8.82 ± 0.81	8.33 ± 0.54	8.49 ± 0.45
Monocytes (10^3/μL)						
Day 4	0.08 ± 0.02	0.18 ± 0.02	0.13 ± 0.03	0.10 ± 0.03	0.17 ± 0.07	0.18 ± 0.04
Day 18	0.18 ± 0.06	0.26 ± 0.04	0.13 ± 0.05	0.16 ± 0.04	0.20 ± 0.05	0.20 ± 0.07
Week 14	0.23 ± 0.04	0.31 ± 0.06	0.15 ± 0.03	0.24 ± 0.06	0.13 ± 0.03	0.19 ± 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 18	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	0.04 ± 0.02	0.01 ± 0.01	0.05 ± 0.02	0.05 ± 0.02	0.00 ± 0.00	0.02 ± 0.01
Day 18	0.03 ± 0.02	0.06 ± 0.02	0.06 ± 0.03	0.04 ± 0.02	0.05 ± 0.02	0.08 ± 0.04
Week 14	0.11 ± 0.04	0.04 ± 0.02	0.02 ± 0.01	0.09 ± 0.04	0.09 ± 0.03	0.02 ± 0.02
Clinical Chemistry						
Urea nitrogen (mg/dL)						
Day 4	7.9 ± 0.5	7.5 ± 0.2	7.4 ± 0.5	7.9 ± 0.5	7.1 ± 0.4	7.0 ± 0.4
Day 18	9.3 ± 0.3	9.9 ± 0.5	8.9 ± 0.3	9.1 ± 0.3	8.4 ± 0.3	8.8 ± 0.4
Week 14	11.9 ± 0.6	15.0 ± 0.5*	12.1 ± 0.6	12.3 ± 0.3	13.0 ± 0.5	17.0 ± 0.7**
Creatinine (mg/dL)						
Day 4	0.41 ± 0.01	0.40 ± 0.00	0.39 ± 0.01	0.39 ± 0.01	0.40 ± 0.00	0.35 ± 0.02**
Day 18	0.56 ± 0.07	0.55 ± 0.06	0.70 ± 0.07	0.61 ± 0.07	0.54 ± 0.05	0.69 ± 0.07
Week 14	0.61 ± 0.02	0.60 ± 0.01	0.63 ± 0.02	0.62 ± 0.02	0.59 ± 0.02	0.55 ± 0.02
Total protein (g/dL)						
Day 4	5.62 ± 0.05	5.46 ± 0.06	5.37 ± 0.08	5.49 ± 0.04	5.46 ± 0.07	4.87 ± 0.06**
Day 18	5.64 ± 0.05	5.81 ± 0.08	5.81 ± 0.08	5.68 ± 0.06	5.75 ± 0.05	5.96 ± 0.06**
Week 14	6.62 ± 0.07	6.79 ± 0.05	6.50 ± 0.04	6.59 ± 0.06	7.19 ± 0.07**	6.60 ± 0.07
Albumin (g/dL)						
Day 4	3.90 ± 0.04	3.73 ± 0.04*	3.67 ± 0.06**	3.76 ± 0.04*	3.73 ± 0.05*	3.39 ± 0.03**
Day 18	3.95 ± 0.04	4.08 ± 0.04	4.07 ± 0.05	4.00 ± 0.05	4.10 ± 0.02*	4.21 ± 0.03**
Week 14	4.24 ± 0.04	4.34 ± 0.02	4.27 ± 0.03	4.30 ± 0.03	4.68 ± 0.04**	4.16 ± 0.05
Alanine aminotransferase (IU/L)						
Day 4	58.3 ± 2.2	62.1 ± 2.2	63.7 ± 1.9	65.8 ± 1.5*	73.9 ± 2.6**	78.5 ± 1.9**
Day 18	50.7 ± 1.5	54.1 ± 1.4	54.2 ± 1.6	54.1 ± 1.4	59.4 ± 1.5**	79.2 ± 2.4**
Week 14	45.1 ± 1.6	45.1 ± 1.9	38.0 ± 1.1	39.6 ± 1.4	43.3 ± 1.0	63.4 ± 2.9*
Alkaline phosphatase (IU/L)						
Day 4	762.9 ± 24.7	801.3 ± 25.9	810.0 ± 23.7	803.1 ± 14.6	909.3 ± 22.9**	1,144.4 ± 42.2**
Day 18	619.8 ± 11.2	619.5 ± 12.0	660.0 ± 15.8	674.6 ± 14.2*	703.6 ± 7.8**	810.1 ± 17.3**
Week 14	249.9 ± 6.3	259.8 ± 5.1	263.2 ± 6.0	294.7 ± 6.9**	350.3 ± 5.8**	542.7 ± 14.7**
Creatine kinase (IU/L)						
Day 4	320.6 ± 35.2	349.4 ± 53.6	387.6 ± 34.8	453.7 ± 51.4	493.5 ± 51.8	297.2 ± 27.0
Day 18	165.0 ± 27.0	169.8 ± 15.9	166.8 ± 9.3	157.5 ± 8.7	194.1 ± 16.5**	195.3 ± 15.0**
Week 14	208.8 ± 23.7	186.8 ± 38.3	151.1 ± 30.8	280.6 ± 45.3	286.8 ± 51.7	370.1 ± 133.7
Sorbitol dehydrogenase (IU/L)						
Day 4	10.9 ± 0.5	10.1 ± 0.4	10.8 ± 0.5	9.8 ± 0.4	11.5 ± 0.8	16.1 ± 1.3**
Day 18	12.5 ± 0.7	14.1 ± 0.7	12.1 ± 0.5	12.0 ± 0.7	13.6 ± 0.9	14.4 ± 0.5
Week 14	18.2 ± 1.0	16.0 ± 0.8	13.4 ± 0.8**	12.8 ± 1.0**	10.9 ± 0.9**	13.9 ± 1.6**

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

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Male (continued)						
n	10	10	10	10	10	10
Clinical Chemistry (continued)						
γ-Glutamyltransferase (IU/L)						
Day 4	0.50 ± 0.17	0.20 ± 0.13	0.40 ± 0.16	0.70 ± 0.15	0.50 ± 0.17	5.70 ± 1.04**
Day 18	2.80 ± 0.53	2.80 ± 0.51	3.60 ± 0.34	3.00 ± 0.45	3.10 ± 0.38	5.90 ± 0.91*
Week 14	0.00 ± 0.00	0.10 ± 0.10	0.20 ± 0.20	0.20 ± 0.20	0.10 ± 0.10	10.30 ± 1.10**
5′-Nucleotidase (IU/L)						
Day 4	40.4 ± 0.8	39.5 ± 0.7	38.7 ± 0.9	38.5 ± 1.0	39.2 ± 0.7	43.2 ± 0.8
Day 18	42.4 ± 1.0	42.3 ± 1.3	47.1 ± 1.9	42.9 ± 1.0	39.2 ± 1.0	43.1 ± 1.5
Week 14	40.8 ± 1.4	45.5 ± 0.9	40.1 ± 1.5	37.7 ± 0.6	31.2 ± 0.4**	35.9 ± 0.6**
Bile acids (μmol/L)						
Day 4	32.3 ± 4.5	30.8 ± 3.6	33.1 ± 3.7	43.2 ± 5.3	36.1 ± 4.3	257.4 ± 44.6**
Day 18	37.3 ± 2.8	33.1 ± 3.0	44.0 ± 3.4	43.2 ± 3.2	43.1 ± 5.8	76.7 ± 11.7**
Week 14	26.9 ± 2.1	29.1 ± 2.6	27.0 ± 2.6	36.7 ± 3.2	53.6 ± 4.5**	167.4 ± 19.5**
Female						
n						
Day 4	10	10	10	10	10	10
Day 18	10	10	10	10	10	10
Week 14	10	10	10	10	10	9
Hematology						
Hematocrit (%)						
Day 4	42.7 ± 0.4	42.6 ± 0.3	42.1 ± 0.5	41.8 ± 0.4	42.0 ± 0.4	41.9 ± 0.4
Day 18	44.4 ± 0.4	44.5 ± 0.5	44.6 ± 0.5	44.2 ± 0.5	43.9 ± 0.6	45.2 ± 0.6
Week 14	40.6 ± 0.4	41.1 ± 0.3	39.7 ± 0.3	39.5 ± 0.4	38.9 ± 0.3**	37.5 ± 0.3**
Hemoglobin (g/dL)						
Day 4	13.8 ± 0.2	13.8 ± 0.1	13.5 ± 0.2	13.5 ± 0.1	13.6 ± 0.1	13.6 ± 0.1
Day 18	14.6 ± 0.2	14.6 ± 0.2	14.5 ± 0.2	14.5 ± 0.2	14.4 ± 0.2	14.8 ± 0.2
Week 14	14.7 ± 0.1	14.7 ± 0.1	14.5 ± 0.1	14.3 ± 0.1*	14.3 ± 0.1**	13.5 ± 0.1**
Erythrocytes (10^6/μL)						
Day 4	7.41 ± 0.08	7.40 ± 0.06	7.24 ± 0.10	7.21 ± 0.07	7.27 ± 0.08	7.28 ± 0.07
Day 18	7.89 ± 0.08	7.86 ± 0.11	7.84 ± 0.10	7.79 ± 0.10	7.67 ± 0.10	7.96 ± 0.12
Week 14	7.55 ± 0.06	7.64 ± 0.06	7.38 ± 0.06	7.26 ± 0.08*	6.98 ± 0.05**	7.03 ± 0.06**
Reticulocytes (10^6/μL)						
Day 4	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Day 18	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0**	0.2 ± 0.0**
Week 14	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0*	0.2 ± 0.0*	0.2 ± 0.0**	0.3 ± 0.0**
Nucleated erythrocytes/100 leukocytes						
Day 4	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Day 18	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
Week 14	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
Mean cell volume (fL)						
Day 4	57.6 ± 0.2	57.6 ± 0.2	58.2 ± 0.2	58.0 ± 0.2	57.7 ± 0.2	57.5 ± 0.2
Day 18	56.4 ± 0.2	56.7 ± 0.2	56.8 ± 0.2	56.8 ± 0.2	57.3 ± 0.1**	56.8 ± 0.1**
Week 14	53.7 ± 0.2	53.7 ± 0.2	53.9 ± 0.2	54.4 ± 0.2	55.7 ± 0.1**	53.4 ± 0.3

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

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Female (continued)						
n						
Day 4	10	10	10	10	10	10
Day 18	10	10	10	10	10	10
Week 14	10	10	10	10	10	9
Hematology (continued)						
Mean cell hemoglobin (pg)						
Day 4	18.6 ± 0.1	18.6 ± 0.1	18.7 ± 0.1	18.7 ± 0.1	18.7 ± 0.1	18.7 ± 0.1
Day 18	18.5 ± 0.1	18.6 ± 0.1	18.5 ± 0.1	18.6 ± 0.1	18.7 ± 0.1*	18.5 ± 0.1
Week 14	19.5 ± 0.1	19.2 ± 0.1	19.6 ± 0.1	19.7 ± 0.1	20.4 ± 0.1**	19.3 ± 0.2
Mean cell hemoglobin concentration (g/dL)						
Day 4	32.2 ± 0.1	32.3 ± 0.1	32.2 ± 0.1	32.3 ± 0.1	32.3 ± 0.1	32.4 ± 0.1
Day 18	32.8 ± 0.1	32.8 ± 0.1	32.6 ± 0.1	32.8 ± 0.1	32.7 ± 0.1	32.6 ± 0.1
Week 14	36.3 ± 0.1	35.7 ± 0.1	36.4 ± 0.1	36.2 ± 0.2	36.7 ± 0.2	36.1 ± 0.2
Platelets (10^3/μL)						
Day 4	903.0 ± 17.7	877.8 ± 17.9	898.1 ± 29.0	898.0 ± 22.4	887.4 ± 31.5	813.2 ± 12.8**
Day 18	877.9 ± 35.4	920.2 ± 20.8	941.4 ± 16.6	868.7 ± 26.4	869.0 ± 10.6	810.2 ± 26.7
Week 14	718.8 ± 17.3	713.3 ± 10.0	758.2 ± 14.6	744.1 ± 10.9	751.6 ± 12.5	811.7 ± 17.5**
Leukocytes (10^3/μL)						
Day 4	8.66 ± 0.31	8.42 ± 0.42	8.41 ± 0.45	8.44 ± 0.21	8.21 ± 0.28	8.65 ± 0.43
Day 18	10.70 ± 0.56	10.92 ± 0.52	11.32 ± 0.42	12.18 ± 0.67*	12.65 ± 0.52*	12.54 ± 0.38**
Week 14	7.48 ± 0.31	7.48 ± 0.34	8.24 ± 0.30	8.49 ± 0.33	8.47 ± 0.46	10.84 ± 0.31**
Segmented neutrophils (10^3/μL)						
Day 4	0.85 ± 0.06	0.78 ± 0.08	0.94 ± 0.08	0.79 ± 0.04	0.80 ± 0.07	0.80 ± 0.06
Day 18	0.96 ± 0.11	0.89 ± 0.08	0.94 ± 0.06	0.94 ± 0.07	0.97 ± 0.10	0.99 ± 0.10
Week 14	1.03 ± 0.09	1.01 ± 0.06	1.18 ± 0.15	1.05 ± 0.10	0.94 ± 0.10	1.89 ± 0.24*
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 18	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	7.71 ± 0.33	7.48 ± 0.38	7.37 ± 0.42	7.53 ± 0.22	7.29 ± 0.30	7.70 ± 0.45
Day 18	9.59 ± 0.46	9.85 ± 0.50	10.17 ± 0.41	11.12 ± 0.62*	11.50 ± 0.47*	11.43 ± 0.37**
Week 14	6.20 ± 0.33	6.31 ± 0.30	6.95 ± 0.25	7.26 ± 0.33	7.40 ± 0.44*	8.88 ± 0.34**
Monocytes (10^3/μL)						
Day 4	0.08 ± 0.03	0.15 ± 0.02	0.11 ± 0.02	0.14 ± 0.03	0.11 ± 0.03	0.16 ± 0.03
Day 18	0.14 ± 0.02	0.11 ± 0.04	0.16 ± 0.05	0.10 ± 0.02	0.13 ± 0.04	0.10 ± 0.04
Week 14	0.17 ± 0.04	0.14 ± 0.03	0.09 ± 0.02	0.10 ± 0.03	0.11 ± 0.04	0.04 ± 0.02**
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 18	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	0.02 ± 0.01	0.03 ± 0.02	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Day 18	0.03 ± 0.02	0.05 ± 0.02	0.06 ± 0.02	0.02 ± 0.01	0.05 ± 0.02	0.01 ± 0.01
Week 14	0.07 ± 0.03	0.03 ± 0.02	0.03 ± 0.02	0.11 ± 0.04	0.03 ± 0.02	0.03 ± 0.03

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

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Female (continued)						
n						
Day 4	10	10	10	10	10	10
Day 18	10	10	10	10	10	10
Week 14	10	10	10	10	10	9
Clinical Chemistry						
Urea nitrogen (mg/dL)						
Day 4	7.5 ± 0.5	8.8 ± 0.4	8.2 ± 0.6	8.2 ± 0.4	8.0 ± 0.4	6.5 ± 0.3
Day 18	10.2 ± 0.5	10.6 ± 0.5	9.3 ± 0.4	9.6 ± 0.5	9.6 ± 0.7	9.1 ± 0.4
Week 14	12.7 ± 0.4	13.8 ± 0.4	12.9 ± 0.6	13.6 ± 0.5	15.9 ± 1.2**	18.2 ± 0.4**
Creatinine (mg/dL)						
Day 4	0.43 ± 0.02	0.41 ± 0.02	0.42 ± 0.01	0.42 ± 0.01	0.42 ± 0.01	0.41 ± 0.01
Day 18	0.49 ± 0.04	0.55 ± 0.07	0.65 ± 0.06	0.51 ± 0.05	0.48 ± 0.05	0.46 ± 0.04
Week 14	0.58 ± 0.01	0.59 ± 0.02	0.58 ± 0.01	0.57 ± 0.02	0.56 ± 0.02	0.50 ± 0.00**
Total protein (g/dL)						
Day 4	5.69 ± 0.06	5.63 ± 0.06	5.62 ± 0.07	5.64 ± 0.06	5.57 ± 0.07	5.29 ± 0.07**
Day 18	6.04 ± 0.08	6.08 ± 0.07	6.09 ± 0.04	6.05 ± 0.07	6.14 ± 0.08	6.47 ± 0.09**
Week 14	6.56 ± 0.06	6.72 ± 0.07	6.76 ± 0.09	6.50 ± 0.07	6.84 ± 0.10	6.61 ± 0.05
Albumin (g/dL)						
Day 4	4.13 ± 0.03	4.09 ± 0.04	4.09 ± 0.03	4.07 ± 0.04	4.00 ± 0.04**	3.89 ± 0.05**
Day 18	4.35 ± 0.05	4.40 ± 0.05	4.41 ± 0.03	4.35 ± 0.05	4.48 ± 0.04	4.65 ± 0.05**
Week 14	4.62 ± 0.04	4.81 ± 0.05	4.85 ± 0.07*	4.67 ± 0.06	4.90 ± 0.06**	4.69 ± 0.04
Alanine aminotransferase (IU/L)						
Day 4	45.8 ± 1.0	50.6 ± 1.5	46.2 ± 1.2	53.2 ± 1.8**	57.7 ± 1.9**	73.3 ± 2.9**
Day 18	40.7 ± 1.9	41.1 ± 1.1	41.8 ± 1.3	43.5 ± 1.6	44.6 ± 1.2*	71.3 ± 4.2**
Week 14	37.8 ± 2.7	50.6 ± 6.8	35.2 ± 1.2	32.8 ± 0.9	39.7 ± 1.2	57.8 ± 1.2**
Alkaline phosphatase (IU/L)						
Day 4	573.7 ± 11.2	579.8 ± 12.3	596.8 ± 19.3	595.3 ± 18.2	645.3 ± 12.5**	848.7 ± 32.2**
Day 18	420.4 ± 7.1	437.0 ± 11.6	432.5 ± 11.3	441.3 ± 9.2	462.9 ± 8.6**	644.6 ± 28.0**
Week 14	212.9 ± 7.7	219.8 ± 5.4	214.6 ± 8.4	234.2 ± 6.1*	284.7 ± 9.0**	535.6 ± 14.8**
Creatine kinase (IU/L)						
Day 4	323.9 ± 46.6	323.0 ± 42.6	300.0 ± 37.2	283.2 ± 33.7	329.2 ± 30.2	337.6 ± 37.1
Day 18	158.7 ± 22.6	161.9 ± 15.8	182.2 ± 34.0	177.8 ± 21.0	167.2 ± 14.2	174.4 ± 20.7
Week 14	143.7 ± 28.4	120.3 ± 20.0	108.4 ± 16.7	111.9 ± 17.1	215.6 ± 48.8	172.8 ± 31.1
Sorbitol dehydrogenase (IU/L)						
Day 4	10.4 ± 0.5	10.7 ± 0.7	10.6 ± 0.4	9.7 ± 0.4	11.1 ± 0.4	13.8 ± 0.4**
Day 18	10.9 ± 0.2	12.1 ± 1.0	11.0 ± 0.3	11.2 ± 0.3	10.8 ± 0.5	12.7 ± 0.4*
Week 14	11.5 ± 1.0	16.3 ± 2.1	10.2 ± 0.8	8.7 ± 0.9	11.2 ± 1.3	10.2 ± 0.6
γ-Glutamyltransferase (IU/L)						
Day 4	0.20 ± 0.13	0.70 ± 0.15	0.30 ± 0.15	0.80 ± 0.25	0.60 ± 0.16	1.30 ± 0.15**
Day 18	0.40 ± 0.16	0.00 ± 0.00	0.40 ± 0.16	0.30 ± 0.15	0.30 ± 0.15	2.20 ± 0.33**
Week 14	0.50 ± 0.17	0.20 ± 0.13	0.30 ± 0.15	0.20 ± 0.13	0.10 ± 0.10	4.89 ± 0.48**
5′-Nucleotidase (IU/L)						
Day 4	44.9 ± 1.4	41.8 ± 0.6	44.5 ± 1.5	40.4 ± 1.1	38.7 ± 1.2**	42.3 ± 0.5
Day 18	55.6 ± 1.9	58.2 ± 3.1	55.5 ± 1.5	52.4 ± 1.8	46.2 ± 1.7**	41.5 ± 2.4**
Week 14	39.5 ± 1.0	37.8 ± 0.4	37.8 ± 1.4	35.2 ± 0.8**	31.4 ± 0.6**	29.2 ± 0.5**
Bile acids (μmol/L)						
Day 4	21.7 ± 1.9	25.2 ± 2.9	28.2 ± 3.3	33.1 ± 3.4**	29.0 ± 3.4*	38.3 ± 4.3**
Day 18	32.3 ± 2.4	29.1 ± 3.5	35.5 ± 3.1	32.1 ± 3.4	41.3 ± 6.9	75.9 ± 9.8**
Week 14	19.7 ± 1.6	27.2 ± 1.7**	30.6 ± 2.1**	27.3 ± 2.3**	45.0 ± 5.1**	87.6 ± 7.0**

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test
** $P \leq 0.01$
[a] Mean ± standard error. Statistical tests were performed on unrounded data.

TABLE F2
Clinical Chemistry Data for Mice in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
n	10	10	10	10	10	10
Male						
Urea nitrogen (mg/dL)						
Day 4	11.0 ± 0.9[b]	10.4 ± 0.5[c]	13.3 ± 0.5[b]	11.3 ± 0.7[c]	10.9 ± 0.5	10.3 ± 0.7
Week 14	17.1 ± 0.7	14.6 ± 1.1	16.9 ± 1.4	16.8 ± 0.7	17.3 ± 1.1[c]	16.7 ± 1.1
Creatinine (mg/dL)						
Day 4	0.30 ± 0.00[b]	0.30 ± 0.00[c]	0.30 ± 0.00[b]	0.30 ± 0.00[c]	0.29 ± 0.01[c]	0.30 ± 0.00
Week 14	0.27 ± 0.02[c]	0.28 ± 0.01	0.28 ± 0.01	0.29 ± 0.01	0.28 ± 0.01[c]	0.30 ± 0.01
Total protein (g/dL)						
Day 4	5.56 ± 0.06[d]	5.62 ± 0.07	5.76 ± 0.06[b]	5.54 ± 0.06[c]	5.61 ± 0.05	5.49 ± 0.06
Week 14	5.91 ± 0.09	5.94 ± 0.07	5.96 ± 0.09	5.99 ± 0.08	5.88 ± 0.07[c]	5.76 ± 0.07
Albumin (g/dL)						
Day 4	3.78 ± 0.05[d]	3.78 ± 0.06[c]	3.90 ± 0.04[b]	3.76 ± 0.04[c]	3.79 ± 0.03	3.77 ± 0.04
Week 14	3.77 ± 0.05	3.79 ± 0.03	3.80 ± 0.05	3.80 ± 0.04	3.72 ± 0.03[c]	3.78 ± 0.04
Alanine aminotransferase (IU/L)						
Day 4	53.4 ± 9.8[c]	33.2 ± 4.0	59.9 ± 17.7	38.9 ± 5.0	32.5 ± 3.8	34.5 ± 2.6
Week 14	46.9 ± 8.5	35.0 ± 3.4	38.6 ± 4.1	34.4 ± 1.7	37.2 ± 4.2	43.0 ± 4.1
Alkaline phosphatase (IU/L)						
Day 4	241.3 ± 8.2[c]	238.1 ± 5.4	229.3 ± 5.5[c]	237.3 ± 4.1	234.6 ± 4.3	205.3 ± 6.2**
Week 14	88.1 ± 2.6	91.1 ± 1.7	87.9 ± 3.2	88.1 ± 2.5	84.0 ± 2.5	78.5 ± 2.5*
Creatine kinase (IU/L)						
Day 4	479.9 ± 156.4[b]	434.8 ± 79.1[c]	333.0 ± 61.8[b]	486.8 ± 75.9[c]	424.6 ± 56.5[c]	443.9 ± 63.9
Week 14	192.8 ± 45.7[c]	157.8 ± 27.9	144.9 ± 27.4	116.6 ± 18.2	124.7 ± 24.3[c]	99.5 ± 19.3
Sorbitol dehydrogenase (IU/L)						
Day 4	29.2 ± 1.0	30.4 ± 1.1	29.0 ± 1.2	30.8 ± 1.3	29.4 ± 2.8	32.7 ± 1.3
Week 14	25.8 ± 0.9	27.9 ± 1.1	26.4 ± 1.1	27.4 ± 1.1	28.5 ± 2.4	28.1 ± 1.1
γ-Glutamyltransferase (IU/L)						
Day 4	0.00 ± 0.00[d]	0.00 ± 0.00	0.00 ± 0.00[c]	0.10 ± 0.10	0.10 ± 0.10	0.10 ± 0.10
Week 14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.10	0.00 ± 0.00	0.10 ± 0.10
5′-Nucleotidase (IU/L)						
Day 4	24.0 ± 1.0[d]	23.2 ± 0.6	25.1 ± 1.1[c]	24.9 ± 0.9	25.5 ± 0.5	30.0 ± 1.2**
Week 14	23.9 ± 1.1	24.4 ± 0.9	25.6 ± 1.4	24.9 ± 1.0	25.5 ± 1.4	29.5 ± 1.3**
Bile acids (µmol/L)						
Day 4	21.1 ± 0.5[c]	20.4 ± 0.5	21.5 ± 0.7	20.8 ± 0.5	21.1 ± 0.6	21.7 ± 0.6
Week 14	21.0 ± 0.7	21.9 ± 0.8	21.8 ± 0.5	21.6 ± 0.6	22.2 ± 0.8	22.0 ± 0.6
Female						
Urea nitrogen (mg/dL)						
Day 4	10.1 ± 0.7[b]	10.8 ± 0.5[e]	11.3 ± 0.9[f]	10.4 ± 1.1[g]	10.7 ± 0.4[b]	8.5 ± 0.3[e]
Week 14	13.8 ± 0.9[g]	15.8 ± 1.1[c]	16.6 ± 1.0	17.6 ± 0.9[d]	16.6 ± 1.4[g]	12.0 ± 0.6[d]
Creatinine (mg/dL)						
Day 4	0.30 ± 0.00[b]	0.30 ± 0.00[e]	0.30 ± 0.00[f]	0.32 ± 0.02[g]	0.32 ± 0.02[f]	0.30 ± 0.00[e]
Week 14	0.30 ± 0.00[e]	0.33 ± 0.02[c]	0.32 ± 0.01	0.33 ± 0.02[d]	0.38 ± 0.02[g]	0.33 ± 0.03[d]
Total protein (g/dL)						
Day 4	5.48 ± 0.06[d]	5.47 ± 0.06[b]	5.46 ± 0.07[b]	5.36 ± 0.04[b]	5.54 ± 0.06[d]	5.37 ± 0.12[f]
Week 14	5.96 ± 0.16[g]	6.02 ± 0.08[c]	6.07 ± 0.09	6.29 ± 0.08[c]	6.21 ± 0.10[d]	5.98 ± 0.05[d]
Albumin (g/dL)						
Day 4	4.10 ± 0.06[d]	4.10 ± 0.04[b]	4.09 ± 0.04[b]	4.03 ± 0.04[b]	4.16 ± 0.05[d]	4.07 ± 0.06[f]
Week 14	4.04 ± 0.08[g]	4.11 ± 0.05[c]	4.12 ± 0.05	4.24 ± 0.04[c]	4.26 ± 0.06[d]	4.14 ± 0.05[d]

TABLE F2
Clinical Chemistry Data for Mice in the 3-Month Gavage Study of Pulegone

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
n	10	10	10	10	10	10
Female (continued)						
Alanine aminotransferase (IU/L)						
Day 4	31.8 ± 2.8[c]	67.3 ± 28.7[c]	36.0 ± 4.3	32.2 ± 2.4[c]	34.6 ± 2.1	47.5 ± 2.2**
Week 14	31.5 ± 2.1	33.0 ± 1.8	31.5 ± 1.4	32.3 ± 2.1	34.7 ± 2.0	35.3 ± 2.0
Alkaline phosphatase (IU/L)						
Day 4	294.9 ± 6.2[c]	298.2 ± 6.7[c]	295.7 ± 6.1	285.1 ± 9.5[c]	309.8 ± 7.5	286.5 ± 10.1
Week 14	123.9 ± 5.8	123.9 ± 5.6	127.9 ± 4.4	130.2 ± 1.7	127.3 ± 4.7	122.5 ± 4.2
Creatine kinase (IU/L)						
Day 4	222.8 ± 77.4[f]	278.5 ± 20.7[e]	306.7 ± 73.9[f]	175.8 ± 42.6[g]	364.7 ± 75.1[f]	229.8 ± 33.9[e]
Week 14	71.8 ± 19.3[e]	106.1 ± 20.8[c]	68.5 ± 14.2	65.9 ± 20.6[d]	61.8 ± 11.7[g]	57.9 ± 8.0[d]
Sorbitol dehydrogenase (IU/L)						
Day 4	29.8 ± 0.8	29.4 ± 0.9	29.2 ± 1.0	29.1 ± 0.4	30.9 ± 0.6	32.5 ± 0.7*
Week 14	22.0 ± 0.9	22.5 ± 1.4	21.3 ± 1.0	23.3 ± 0.8	24.1 ± 1.7	25.3 ± 1.7
γ-Glutamyltransferase (IU/L)						
Day 4	0.00 ± 0.00[c]	0.00 ± 0.00[d]	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00	0.00 ± 0.00
Week 14	0.20 ± 0.13	0.44 ± 0.18[c]	0.30 ± 0.15	0.50 ± 0.17	0.40 ± 0.16	0.40 ± 0.16
5′-Nucleotidase (IU/L)						
Day 4	50.1 ± 2.1[c]	48.4 ± 1.5[d]	48.9 ± 1.9[d]	45.3 ± 1.4[c]	53.3 ± 2.3	61.7 ± 2.1**
Week 14	93.1 ± 4.2	87.9 ± 3.0[c]	88.4 ± 3.9	104.1 ± 8.5	102.5 ± 5.6	101.2 ± 4.8
Bile acids (µmol/L)						
Day 4	18.8 ± 0.6[c]	19.0 ± 0.8[c]	19.1 ± 0.5	20.6 ± 0.6*[c]	21.7 ± 0.6**	21.6 ± 0.8**
Week 14	19.2 ± 1.0	18.9 ± 0.4	19.3 ± 0.5	20.1 ± 0.9	19.6 ± 0.7	18.8 ± 0.6

* Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test
** P≤0.01
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=7
[c] n=9
[d] n=8
[e] n=4
[f] n=6
[g] n=5

APPENDIX G
LIVER GLUTATHIONE DATA

TABLE G1
Liver Glutathione Data for Rats in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Male						
n						
Day 4	3	2	2	1	2	5
Day 18	10	10	10	10	10	10
Week 14	10	10	10	10	10	10
Reduced glutathione (µmol/g liver)						
Day 4	3.77 ± 0.07	4.27 ± 0.11	4.36 ± 0.42	3.13[b]	4.61 ± 0.39*	5.35 ± 0.50**
Day 18	3.40 ± 0.17	3.96 ± 0.26	3.59 ± 0.23	3.73 ± 0.21	4.04 ± 0.25	6.10 ± 0.40**
Week 14	3.82 ± 0.20	4.46 ± 0.16	4.04 ± 0.16	3.95 ± 0.23	4.69 ± 0.17**	7.00 ± 0.44**
Oxidized glutathione (µmol/g liver)						
Day 4	1.91 ± 0.19	2.37 ± 0.08	2.50 ± 0.38	1.59[b]	2.32 ± 0.20	2.96 ± 0.37
Day 18	1.57 ± 0.26	1.58 ± 0.15	1.79 ± 0.06	1.99 ± 0.13*	1.95 ± 0.18*	3.14 ± 0.48**
Week 14	1.95 ± 0.20	2.02 ± 0.18	2.19 ± 0.30	1.77 ± 0.09	2.39 ± 0.14	2.42 ± 0.29
Total glutathione (µmol/g liver)						
Day 4	5.69 ± 0.17	6.64 ± 0.03	6.86 ± 0.80	4.72[b]	6.93 ± 0.20*	8.30 ± 0.87**
Day 18	4.97 ± 0.37	5.54 ± 0.37	5.38 ± 0.25	5.72 ± 0.33	5.99 ± 0.38*	9.24 ± 0.78**
Week 14	5.76 ± 0.33	6.49 ± 0.25	6.23 ± 0.39	5.72 ± 0.23	7.08 ± 0.25**	9.42 ± 0.65**
Reduced glutathione/oxidized glutathione						
Day 4	2.01 ± 0.20	1.81 ± 0.11	1.76 ± 0.10	1.97[b]	2.02 ± 0.34	1.83 ± 0.05
Day 18	2.58 ± 0.33	2.60 ± 0.18	2.02 ± 0.12	1.89 ± 0.05	2.24 ± 0.25	2.16 ± 0.20
Week 14	2.06 ± 0.13	2.44 ± 0.31	2.06 ± 0.22	2.32 ± 0.22	2.04 ± 0.18	3.13 ± 0.29
Female						
n						
Day 4	10	10	10	10	10	10
Day 18	10	10	10	10	10	10
Week 14	10	10	10	10	10	9
Reduced glutathione (µmol/g liver)						
Day 4	4.21 ± 0.26	3.89 ± 0.22	4.25 ± 0.30	4.24 ± 0.30	4.94 ± 0.31	4.63 ± 0.22
Day 18	4.50 ± 0.22	4.63 ± 0.24	4.53 ± 0.13	5.45 ± 0.31	5.60 ± 0.25**	9.91 ± 0.49**
Week 14	3.50 ± 0.15	3.86 ± 0.25	4.10 ± 0.09**	4.52 ± 0.11**	5.43 ± 0.26**	6.82 ± 0.24**
Oxidized glutathione (µmol/g liver)						
Day 4	1.83 ± 0.20	1.92 ± 0.22	1.98 ± 0.10	1.90 ± 0.24	2.12 ± 0.10	2.32 ± 0.47
Day 18	2.04 ± 0.13	2.29 ± 0.31	2.55 ± 0.35	2.53 ± 0.11*	2.70 ± 0.21*	3.63 ± 0.53**
Week 14	1.26 ± 0.15	1.99 ± 0.33	2.07 ± 0.19*	2.24 ± 0.18**	2.30 ± 0.31**	3.07 ± 0.38**
Total glutathione (µmol/g liver)						
Day 4	6.05 ± 0.41	5.81 ± 0.36	6.23 ± 0.37	6.14 ± 0.36	7.07 ± 0.39	6.95 ± 0.54
Day 18	6.53 ± 0.28	6.93 ± 0.47	7.08 ± 0.43	7.98 ± 0.35**	8.30 ± 0.36**	13.54 ± 0.68**
Week 14	4.76 ± 0.14	5.86 ± 0.47*	6.17 ± 0.20**	6.76 ± 0.18**	7.74 ± 0.43**	9.89 ± 0.49**
Reduced glutathione/oxidized glutathione						
Day 4	2.66 ± 0.46	2.22 ± 0.26	2.15 ± 0.13	1.98 ± 0.14[c]	2.34 ± 0.11	6.74 ± 4.25
Day 18	2.29 ± 0.19	2.21 ± 0.22	2.02 ± 0.23	2.19 ± 0.15	2.21 ± 0.23	2.66 ± 0.38[c]
Week 14	2.47 ± 0.13[c]	2.27 ± 0.27	2.17 ± 0.24	2.23 ± 0.31	3.28 ± 0.87	4.47 ± 2.48

* Significantly different (P≤0.05) from the vehicle control group by Shirley's test
** P≤0.01
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] No standard error calculated because fewer than two measurements were available.
[c] n=9

TABLE G2
Liver Glutathione Data for Mice in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
n	10	10	10	10	10	10
Male						
Reduced glutathione (µmol/g liver)						
Day 4	5.00 ± 0.35	4.83 ± 0.32	4.48 ± 0.29	5.01 ± 0.39	5.56 ± 0.38	6.96 ± 0.59*
Week 14	5.30 ± 0.15	5.14 ± 0.15	5.14 ± 0.14	5.76 ± 0.20	6.55 ± 0.24**	8.94 ± 0.22**
Oxidized glutathione (µmol/g liver)						
Day 4	2.12 ± 0.19	2.51 ± 0.24	2.38 ± 0.33	2.61 ± 0.35	2.46 ± 0.19	3.16 ± 0.30*
Week 14	2.51 ± 0.19	2.37 ± 0.10	3.12 ± 0.24	3.36 ± 0.30	2.90 ± 0.19	3.92 ± 0.24**
Total glutathione (µmol/g liver)						
Day 4	7.12 ± 0.51	7.33 ± 0.50	6.86 ± 0.53	7.62 ± 0.69	8.02 ± 0.54	10.12 ± 0.85**
Week 14	7.81 ± 0.19	7.51 ± 0.20	8.26 ± 0.34	9.12 ± 0.46*	9.46 ± 0.26**	12.85 ± 0.34**
Reduced glutathione/oxidized glutathione						
Day 4	2.44 ± 0.17	2.04 ± 0.18	2.14 ± 0.28	2.02 ± 0.13	2.31 ± 0.12	2.25 ± 0.13
Week 14	2.26 ± 0.24	2.20 ± 0.10	1.72 ± 0.12	1.79 ± 0.10	2.36 ± 0.20	2.37 ± 0.18
Female						
Reduced glutathione (µmol/g liver)						
Day 4	5.23 ± 0.18	4.69 ± 0.34	5.42 ± 0.24	5.81 ± 0.38	6.36 ± 0.24**	8.96 ± 0.66**
Week 14	5.01 ± 0.16	5.31 ± 0.11	5.42 ± 0.14	5.94 ± 0.18**	7.06 ± 0.17**	8.04 ± 0.30**
Oxidized glutathione (µmol/g liver)						
Day 4	2.04 ± 0.17	2.07 ± 0.15	2.53 ± 0.15	2.46 ± 0.20	2.61 ± 0.29	3.41 ± 0.31**
Week 14	2.17 ± 0.16	2.67 ± 0.15	2.62 ± 0.08	2.72 ± 0.29	3.23 ± 0.28**	3.20 ± 0.29**
Total glutathione (µmol/g liver)						
Day 4	7.28 ± 0.30	6.77 ± 0.47	7.94 ± 0.35	8.27 ± 0.51	8.97 ± 0.45**	12.37 ± 0.91**
Week 14	7.18 ± 0.25	7.98 ± 0.21*	8.04 ± 0.19*	8.66 ± 0.42**	10.29 ± 0.34**	11.24 ± 0.49**
Reduced glutathione/oxidized glutathione						
Day 4	2.69 ± 0.20	2.30 ± 0.13	2.18 ± 0.11	2.45 ± 0.16	2.70 ± 0.27	2.74 ± 0.24
Week 14	2.49 ± 0.31	2.03 ± 0.10	2.08 ± 0.06	2.34 ± 0.17	2.36 ± 0.24	2.71 ± 0.27

* Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test
** P≤0.01
[a] Mean ± standard error. Statistical tests were performed on unrounded data.

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

TABLE H1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 2-Week Gavage Study of Pulegone[a,b]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg[c]
Male					
n	5	5	5	5	0
Necropsy body wt	157 ± 5	141 ± 4	152 ± 7	137 ± 5	
Heart					
Absolute	0.63 ± 0.02	0.60 ± 0.02	0.61 ± 0.03	0.55 ± 0.02*	
Relative	4.045 ± 0.120	4.274 ± 0.174	4.028 ± 0.087	4.033 ± 0.070	
R. Kidney					
Absolute	0.75 ± 0.03	0.66 ± 0.02	0.73 ± 0.03	0.72 ± 0.02	
Relative	4.784 ± 0.056	4.671 ± 0.114	4.793 ± 0.118	5.261 ± 0.052**	
Liver					
Absolute	8.53 ± 0.35	7.81 ± 0.27	8.66 ± 0.39	8.15 ± 0.37	
Relative	54.529 ± 1.517	55.363 ± 2.053	56.845 ± 0.948	59.451 ± 1.417	
Lung					
Absolute	1.25 ± 0.06	1.06 ± 0.03	1.38 ± 0.15	0.99 ± 0.05	
Relative	8.015 ± 0.372	7.518 ± 0.324	8.923 ± 0.544	7.248 ± 0.343	
R. Testis					
Absolute	0.965 ± 0.067	0.737 ± 0.060	0.818 ± 0.071	0.746 ± 0.065	
Relative	6.195 ± 0.506	5.201 ± 0.341	5.324 ± 0.232	5.422 ± 0.368	
Thymus					
Absolute	0.431 ± 0.017	0.421 ± 0.008	0.431 ± 0.019	0.346 ± 0.015**	
Relative	2.769 ± 0.146	2.991 ± 0.120	2.846 ± 0.151	2.527 ± 0.035	
Female					
n	5	5	5	5	1
Necropsy body wt	112 ± 2	119 ± 3	112 ± 2	115 ± 2	79
Heart					
Absolute	0.49 ± 0.02	0.51 ± 0.02	0.48 ± 0.02	0.50 ± 0.01	0.37
Relative	4.341 ± 0.103	4.276 ± 0.076	4.286 ± 0.102	4.369 ± 0.059	4.609
R. Kidney					
Absolute	0.53 ± 0.00	0.60 ± 0.02**	0.56 ± 0.01	0.63 ± 0.01**	0.49
Relative	4.710 ± 0.070	5.021 ± 0.078*	5.030 ± 0.081*	5.450 ± 0.082**	6.124
Liver					
Absolute	5.10 ± 0.08	5.91 ± 0.17**	5.54 ± 0.13	6.33 ± 0.17**	4.81
Relative	45.704 ± 1.103	49.470 ± 0.205**	49.582 ± 0.656**	54.900 ± 1.132**	60.707
Lung					
Absolute	0.88 ± 0.09	0.89 ± 0.05	0.79 ± 0.04	0.88 ± 0.06	0.53
Relative	7.867 ± 0.676	7.488 ± 0.332	7.075 ± 0.374	7.632 ± 0.452	6.654
Thymus					
Absolute	0.346 ± 0.016	0.387 ± 0.013	0.330 ± 0.016	0.325 ± 0.015	0.151
Relative	3.095 ± 0.106	3.251 ± 0.109	2.956 ± 0.137	2.820 ± 0.121	1.907

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test
** ($P \leq 0.01$)
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] No data were available for 300 mg/kg males or 600 mg/kg males or females due to 100% mortality.
[c] No standard error calculated for 300 mg/kg females because fewer than two measurements were available.

TABLE H2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
Male						
n	10	10	10	10	10	10
Necropsy body wt	358 ± 5	358 ± 5	348 ± 3	353 ± 5	324 ± 9**	248 ± 3**
Heart						
Absolute	0.98 ± 0.02	0.96 ± 0.03	0.96 ± 0.02	0.94 ± 0.02	0.90 ± 0.03*	0.82 ± 0.02**
Relative	2.737 ± 0.040	2.673 ± 0.057	2.769 ± 0.039	2.656 ± 0.044	2.787 ± 0.037	3.321 ± 0.078**
R. Kidney						
Absolute	1.01 ± 0.02	1.04 ± 0.02	1.05 ± 0.01	1.11 ± 0.02*	1.09 ± 0.04	1.00 ± 0.01
Relative	2.832 ± 0.047	2.914 ± 0.044	3.030 ± 0.042**	3.143 ± 0.031**	3.349 ± 0.043**	4.041 ± 0.042**
Liver						
Absolute	12.21 ± 0.25	12.53 ± 0.29	12.35 ± 0.15	12.55 ± 0.16	13.06 ± 0.39	12.02 ± 0.24
Relative	34.150 ± 0.370	34.953 ± 0.434	35.520 ± 0.404*	35.612 ± 0.368*	40.345 ± 0.384**	48.368 ± 0.529**
Lung						
Absolute	1.68 ± 0.09	1.64 ± 0.08	1.80 ± 0.10	1.75 ± 0.09	1.55 ± 0.08	1.57 ± 0.12
Relative	4.702 ± 0.225	4.591 ± 0.207	5.173 ± 0.278	4.952 ± 0.252	4.771 ± 0.174	6.299 ± 0.445**
R. Testis						
Absolute	1.453 ± 0.018	1.424 ± 0.128	1.469 ± 0.014	1.553 ± 0.017	1.518 ± 0.031	1.411 ± 0.015
Relative	4.069 ± 0.060	3.950 ± 0.345	4.228 ± 0.062	4.412 ± 0.080	4.704 ± 0.077**	5.684 ± 0.045**
Thymus						
Absolute	0.339 ± 0.025	0.340 ± 0.008	0.326 ± 0.012	0.297 ± 0.011	0.266 ± 0.009**	0.174 ± 0.017**
Relative	0.952 ± 0.075	0.951 ± 0.026	0.938 ± 0.035	0.844 ± 0.032	0.825 ± 0.030	0.698 ± 0.061**
Female						
n	10	10	10	10	10	9
Necropsy body wt	202 ± 2	204 ± 3	195 ± 3	195 ± 2	195 ± 3	180 ± 3**
Heart						
Absolute	0.65 ± 0.01	0.64 ± 0.01	0.62 ± 0.01	0.62 ± 0.01	0.63 ± 0.01	0.62 ± 0.01
Relative	3.201 ± 0.050	3.152 ± 0.040	3.197 ± 0.047	3.161 ± 0.061	3.222 ± 0.099	3.462 ± 0.061*
R. Kidney						
Absolute	0.67 ± 0.01	0.76 ± 0.05	0.70 ± 0.01	0.69 ± 0.01	0.71 ± 0.01	0.75 ± 0.02*
Relative	3.308 ± 0.044	3.720 ± 0.220*	3.612 ± 0.035*	3.535 ± 0.049*	3.624 ± 0.055*	4.181 ± 0.059**
Liver						
Absolute	6.58 ± 0.06	6.73 ± 0.11	6.41 ± 0.14	6.58 ± 0.13	7.23 ± 0.22**	7.92 ± 0.13**
Relative	32.637 ± 0.515	33.053 ± 0.500	32.857 ± 0.334	33.822 ± 0.463	37.046 ± 0.667**	44.060 ± 0.493**
Lung						
Absolute	1.18 ± 0.04	1.22 ± 0.06	1.11 ± 0.05	1.11 ± 0.05	1.09 ± 0.03	1.23 ± 0.11
Relative	5.876 ± 0.220	6.013 ± 0.307	5.715 ± 0.252	5.696 ± 0.225	5.615 ± 0.152	6.825 ± 0.568
Thymus						
Absolute	0.251 ± 0.007	0.265 ± 0.011	0.255 ± 0.007	0.232 ± 0.007	0.236 ± 0.009	0.196 ± 0.008**
Relative	1.243 ± 0.033	1.299 ± 0.047	1.313 ± 0.040	1.190 ± 0.031	1.214 ± 0.045	1.087 ± 0.034**

* Significantly different (P≤0.05) from the vehicle control group by Williams' or Dunnett's test
** Significantly different (P≤0.01) from the vehicle control group by Williams' test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE H3
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 2-Week Gavage Study of Pulegone[a]

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg[b]
Male						
n	5	5	5	5	5	4
Necropsy body wt	22.2 ± 1.0	23.4 ± 0.9	22.6 ± 0.5	22.2 ± 0.6	23.0 ± 0.8	21.9 ± 0.2
Heart						
Absolute	0.15 ± 0.01	0.15 ± 0.00	0.14 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.14 ± 0.01
Relative	6.512 ± 0.242	6.289 ± 0.182	5.968 ± 0.150	7.158 ± 0.368	6.484 ± 0.448	6.524 ± 0.383
R. Kidney						
Absolute	0.23 ± 0.02	0.23 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.23 ± 0.01	0.23 ± 0.01
Relative	10.488 ± 0.203	9.890 ± 0.166	10.093 ± 0.172	10.003 ± 0.258	10.13 ± 0.273	10.672 ± 0.335
Liver						
Absolute	1.20 ± 0.08	1.27 ± 0.05	1.26 ± 0.04	1.26 ± 0.03	1.34 ± 0.06	1.52 ± 0.06**
Relative	53.658 ± 1.445	54.405 ± 0.688	55.826 ± 0.933	56.967 ± 1.109	58.069 ± 0.948*	69.365 ± 2.804**
Lung						
Absolute	0.16 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
Relative	7.262 ± 0.218	7.720 ± 0.525	7.715 ± 0.351	7.783 ± 0.532	7.195 ± 0.232	8.360 ± 0.399
R. Testis						
Absolute	0.102 ± 0.003	0.102 ± 0.003	0.099 ± 0.004	0.100 ± 0.002	0.103 ± 0.004	0.0910 ± 0.004
Relative	4.577 ± 0.069	4.397 ± 0.198	4.375 ± 0.144	4.499 ± 0.053	4.505 ± 0.078	4.131 ± 0.166
Thymus						
Absolute	0.047 ± 0.002	0.052 ± 0.004	0.052 ± 0.002	0.049 ± 0.002	0.050 ± 0.002	0.039 ± 0.003
Relative	2.103 ± 0.069	2.219 ± 0.178	2.323 ± 0.068	2.206 ± 0.106	2.193 ± 0.054	1.779 ± 0.145
Female						
n	5	5	5	5	5	1
Necropsy body wt	18.3 ± 0.4	19.4 ± 0.5	19.1 ± 0.5	18.8 ± 0.5	18.6 ± 0.3	17.6
Heart						
Absolute	0.12 ± 0.00	0.12 ± 0.01	0.15 ± 0.01*	0.11 ± 0.01	0.11 ± 0.00	0.11
Relative	6.491 ± 0.277	6.078 ± 0.175	7.671 ± 0.530*	5.826 ± 0.217	6.084 ± 0.166	6.193
R. Kidney						
Absolute	0.15 ± 0.00	0.16 ± 0.01	0.14 ± 0.00	0.16 ± 0.01	0.17 ± 0.01	0.18
Relative	8.373 ± 0.200	8.031 ± 0.123	7.375 ± 0.156	8.375 ± 0.193	9.065 ± 0.691	10.114
Liver						
Absolute	0.95 ± 0.03	1.02 ± 0.06	1.05 ± 0.04	1.03 ± 0.05	1.07 ± 0.04	1.13
Relative	51.657 ± 0.996	52.666 ± 1.721	54.684 ± 1.022	54.823 ± 1.903	57.418 ± 1.783*	64.148
Lung						
Absolute	0.16 ± 0.01	0.17 ± 0.01	0.21 ± 0.01**	0.14 ± 0.00	0.18 ± 0.00	0.17
Relative	8.545 ± 0.405	8.612 ± 0.472	10.885 ± 0.815**	7.686 ± 0.123	9.711 ± 0.165	9.489
Thymus						
Absolute	0.068 ± 0.003	0.066 ± 0.003	0.069 ± 0.003	0.067 ± 0.005	0.067 ± 0.004	0.068
Relative	3.700 ± 0.125	3.402 ± 0.094	3.605 ± 0.130	3.573 ± 0.242	3.641 ± 0.236	3.864

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test
** ($P \leq 0.01$)
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] No standard error calculated for 300 mg/kg females because fewer than two measurements were available.

TABLE H4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	9.375 mg/kg	18.75 mg/kg	37.5 mg/kg	75 mg/kg	150 mg/kg
n	10	10	10	10	10	10
Male						
Necropsy body wt	37.3 ± 0.8	36.9 ± 1.3	38.1 ± 0.8	36.7 ± 0.9	36.5 ± 1.5	37.1 ± 1.1
Heart						
Absolute	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.00	0.19 ± 0.00	0.20 ± 0.01	0.20 ± 0.01
Relative	5.201 ± 0.203	5.081 ± 0.204	4.935 ± 0.127	5.207 ± 0.184	5.659 ± 0.201	5.374 ± 0.229
R. Kidney						
Absolute	0.27 ± 0.01	0.27 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	0.27 ± 0.01	0.25 ± 0.01
Relative	7.175 ± 0.120	7.297 ± 0.140	6.96 ± 0.227	7.315 ± 0.219	7.521 ± 0.175	6.809 ± 0.165
Liver						
Absolute	1.57 ± 0.04	1.51 ± 0.06	1.53 ± 0.04	1.57 ± 0.03	1.62 ± 0.08	1.74 ± 0.04*
Relative	42.004 ± 0.629	40.983 ± 0.680	40.228 ± 0.656	42.944 ± 0.681	44.502 ± 0.696*	46.964 ± 0.593**
Lung						
Absolute	0.29 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.28 ± 0.01
Relative	7.902 ± 0.168	8.576 ± 0.212	7.801 ± 0.233	8.413 ± 0.278	8.545 ± 0.308	7.621 ± 0.368
R. Testis						
Absolute	0.126 ± 0.002	0.126 ± 0.002	0.127 ± 0.002	0.125 ± 0.003	0.128 ± 0.002	0.127 ± 0.003
Relative	3.385 ± 0.046	3.430 ± 0.085	3.340 ± 0.049	3.406 ± 0.092	3.549 ± 0.127	3.436 ± 0.068
Thymus						
Absolute	0.046 ± 0.002	0.046 ± 0.002	0.050 ± 0.003	0.047 ± 0.003	0.042 ± 0.003	0.048 ± 0.002
Relative	1.229 ± 0.053	1.240 ± 0.052	1.320 ± 0.050	1.281 ± 0.059	1.153 ± 0.052	1.296 ± 0.075
Female						
Necropsy body wt	29.1 ± 0.7	29.6 ± 0.8	29.4 ± 0.7	31.6 ± 1.5	29.8 ± 0.9	28.1 ± 0.7
Heart						
Absolute	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.00	0.16 ± 0.01**	0.14 ± 0.01	0.15 ± 0.00
Relative	4.701 ± 0.179	4.809 ± 0.128	4.838 ± 0.149	5.007 ± 0.297	4.771 ± 0.230	5.255 ± 0.154
R. Kidney						
Absolute	0.16 ± 0.00	0.16 ± 0.01	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00
Relative	5.356 ± 0.104	5.274 ± 0.118	5.363 ± 0.179	5.234 ± 0.176	5.296 ± 0.143	5.664 ± 0.103
Liver						
Absolute	1.16 ± 0.02	1.23 ± 0.03	1.25 ± 0.03*	1.24 ± 0.02*	1.28 ± 0.03**	1.35 ± 0.03**
Relative	40.087 ± 0.785	41.606 ± 0.755	42.588 ± 0.670	39.829 ± 1.298	42.925 ± 0.673*	48.262 ± 0.801**
Lung						
Absolute	0.25 ± 0.01	0.25 ± 0.01	0.27 ± 0.01	0.27 ± 0.02	0.26 ± 0.01	0.25 ± 0.01
Relative	8.460 ± 0.449	8.511 ± 0.392	9.128 ± 0.303	8.590 ± 0.509	8.588 ± 0.347	8.796 ± 0.426
Thymus						
Absolute	0.047 ± 0.002	0.053 ± 0.003	0.049 ± 0.002	0.056 ± 0.003*	0.055 ± 0.002	0.050 ± 0.002
Relative	1.635 ± 0.067	1.812 ± 0.101	1.674 ± 0.078	1.796 ± 0.126	1.849 ± 0.074	1.780 ± 0.072

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test
** ($P \leq 0.01$)
[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 I
REPRODUCTIVE TISSUE EVALUATIONS AND ESTROUS CYCLE CHARACTERIZATION

TABLE I1
Summary of Reproductive Tissue Evaluations for Male Rats in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg
n	10	10	10	10
Weights (g)				
Necropsy body wt	358 ± 5	348 ± 3	353 ± 5	324 ± 9**
L. Cauda epididymis	0.1517 ± 0.0070	0.1485 ± 0.0033	0.1534 ± 0.0035	0.1389 ± 0.004
L. Epididymis	0.4393 ± 0.0067	0.4391 ± 0.0083	0.4518 ± 0.0057	0.4396 ± 0.0091
L. Testis	1.5393 ± 0.0155	1.5472 ± 0.0117	1.6206 ± 0.0172**	1.5846 ± 0.0248
Spermatid measurements				
Spermatid heads (10^6/testis)	185.50 ± 5.49	172.63 ± 5.42	181.63 ± 8.08	175.38 ± 5.13
Spermatid heads (10^6/g testis)	127.6 ± 3.9	117.7 ± 3.6	118.1 ± 4.9	116.5 ± 2.3
Epididymal spermatozoal measurements				
Sperm motility (%)	78.8 ± 1.1	80.8 ± 1.0	78.6 ± 1.2	79.4 ± 0.7
Sperm (10^6/cauda epididymis)	115.4 ± 4.8	121.5 ± 7.8	114.4 ± 3.3	110.4 ± 4.3
Sperm (10^6/g cauda epididymis)	775 ± 46	815 ± 42	748 ± 26	802 ± 42

** Significantly different ($P \leq 0.01$) from the vehicle control group by Dunnett's test

[a] Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (caudal and epididymal weights) or Dunn's test (spermatid and epididymal spermatozoal measurements).

TABLE I2
Estrous Cycle Characterization for Female Rats in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	18.75 mg/kg	37.5 mg/kg	75 mg/kg
Number weighed at necropsy	10	10	10	10
Necropsy body wt (g)	202 ± 2	195 ± 3	195 ± 2	195 ± 3
Proportion of regular cycling females[b]	10/10	9/10	10/10	10/10
Estrous cycle length (days)	5.0 ± 0.0	4.8 ± 0.1[c]	4.9 ± 0.1	5.2 ± 0.2
Estrous stages (% of cycle)				
Diestrus	60.0	57.5	56.7	54.2
Proestrus	10.8	9.2	15.0	15.8
Estrus	25.0	29.2	23.3	25.8
Metestrus	4.2	4.2	5.0	4.2

[a] Necropsy body weights and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle length). By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages. The tests for equality of transition probability matrices among dose groups and between the vehicle control group and each dosed group indicated the dosed females did not have extended estrus or diestrus.

[b] Number of females with a regular cycle/number of females cycling

[c] Estrous cycle was longer than 12 days or unclear in 1 of 10 animals.

TABLE I3
Summary of Reproductive Tissue Evaluations for Male Mice in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
n	10	10	10	10
Weights (g)				
Necropsy body wt	37.3 ± 0.8	36.7 ± 0.9	36.5 ± 1.5	37.1 ± 1.1
L. Cauda epididymis	0.0165 ± 0.0004	0.0163 ± 0.0005	0.0184 ± 0.0026	0.0151 ± 0.0006
L. Epididymis	0.0440 ± 0.0006	0.0440 ± 0.0009	0.0467 ± 0.0026	0.0418 ± 0.0022
L. Testis	0.1162 ± 0.0014	0.1182 ± 0.0013	0.1188 ± 0.0013	0.1203 ± 0.0030
Spermatid measurements				
Spermatid heads (10^6/testis)	18.95 ± 0.60	22.05 ± 0.85*	22.25 ± 0.66*	21.42 ± 0.70
Spermatid heads (10^6/g testis)	171.41 ± 6.33	195.50 ± 7.47*	195.99 ± 5.83*	186.38 ± 4.38
Epididymal spermatozoal measurements				
Sperm motility (%)	78.5 ± 1.0	77.2 ± 1.3	79.0 ± 1.0	79.4 ± 1.1
Sperm (10^6/cauda epididymis)	22.6 ± 2.0	21.8 ± 1.0	22.0 ± 1.6	20.7 ± 1.3
Sperm (10^6/g cauda epididymis)	1,368 ± 117	1,340 ± 44	1,307 ± 126	1,393 ± 107

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunn's test

[a] Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body and tissue weights) or Dunn's test (epididymal spermatozoal measurements).

TABLE I4
Estrous Cycle Characterization for Female Mice in the 3-Month Gavage Study of Pulegone[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg
Number weighed at necropsy	10	10	10	10
Necropsy body wt (g)	29.1 ± 0.7	31.6 ± 1.5	29.8 ± 0.9	28.1 ± 0.7
Proportion of regular cycling females[b]	7/10	10/10	9/10	8/10
Estrous cycle length (days)	4.2 ± 0.1	4.3 ± 0.2	4.1 ± 0.1	4.0 ± 0.1
Estrous stages (% of cycle)				
Diestrus	36.7	30.0	30.0	32.5
Proestrus	0.0	0.0	0.0	0.0
Estrus	40.8	46.7	46.7	44.2
Metestrus	21.7	23.3	23.3	23.3
Uncertain diagnoses	0.8	0.0	0.0	0.0

[a] Necropsy body weights and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle length). By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages. The tests for equality of transition probability matrices among dose groups and between the vehicle control group and each dosed group indicated the dosed females did not have extended estrus or diestrus.

[b] Number of females with a regular cycle/number of females cycling

APPENDIX J
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION

Pulegone

Pulegone was obtained from TCI America (Portland, OR) in one lot (OGI01) that was used in the 2-week, 3-month, and 2-year studies. Identity analyses were conducted by the analytical chemistry laboratory at Midwest Research Institute (Kansas City, MO) and by the study laboratory at Battelle Columbus Operations (Columbus, OH). Purity and stability analyses were conducted by the analytical chemistry laboratory. Reports on analyses performed in support of the pulegone studies are on file at the National Institute of Environmental Health Sciences.

Lot OGI01 of the chemical, a pale yellow liquid, was identified as pulegone by the analytical chemistry laboratory using infrared (IR), ultraviolet/visible, and proton nuclear magnetic resonance (NMR) spectroscopy and determinations of optical rotation, boiling point, density, and vapor pressure of the test article. The study laboratory confirmed the identity of the bulk chemical by IR spectroscopy. All spectra were consistent with literature spectra (*Aldrich*, 1985, 1993, 1997) and/or the structure of pulegone. The optical rotation, boiling point, density, and vapor pressure of lot OGI01 were consistent with literature reference values for pulegone (*Merck*, 1996; *CRC*, 1995). Representative IR and proton NMR spectra are presented in Figures J1 and J2, respectively.

The moisture content of lot OGI01 was determined by the analytical chemistry laboratory using Karl Fischer titration. The purity of lot OGI01 was determined using gas chromatography (GC) by system A (Table J1) and thin-layer chromatography (TLC). TLC was performed on 20 cm × 20 cm K6F silica gel 60 precoated (250 µm) plates (Whatman Inc., Piscataway, NJ). The plates were spotted with solutions of the test article and the reference standard (vanillin) and developed in a tank containing approximately 100 mL of methanol as the solvent. The dried plates were examined using ultraviolet (254 and 366 nm) and visible light, iodine vapor, and a vanillin/sulfuric acid spray. The analytical chemistry laboratory attempted to identify impurities in lot OGI01 using GC coupled with mass spectrometry (MS) by system B.

Karl Fischer titration indicated 0.07% ± 0.01% water. TLC indicated one major spot and no impurities. GC indicated one major peak and 10 impurities with a combined area of 3.9% of the total peak area; two of the impurities had relative areas exceeding 1%. Of these, the first impurity was identified by GC/MS as isopulegone; the second can be described as a compound with structure similar to 6-(2-hydroxypropoan-2yl)-cyclohex-2-enone and a molecular weight of 134 Da but could not be unequivocally identified due to interference from the large pulegone peak. The overall purity of lot OGI01 was determined to be approximately 96%.

Stability studies of the bulk chemical were performed by the analytical chemistry laboratory using GC by system C. These studies indicated that pulegone was stable as a bulk chemical for at least 2 weeks when stored protected from light under a nitrogen headspace at temperatures up to approximately 25° C. To ensure stability, the bulk chemical was stored at room temperature in sealed amber glass bottles. Periodic reanalyses of the bulk chemical were performed by the study laboratory during the 2-week, 3-month, and 2-year studies using a GC system similar to system C, and no degradation of the bulk chemical was detected.

Corn Oil

USP-grade corn oil was obtained in multiple lots from Spectrum Chemicals and Laboratory Products (Gardena, CA) and was used as the vehicle in the 2-week, 3-month, and 2-year studies. Periodic analyses of the corn oil vehicle performed by the study laboratory using potentiometric titration demonstrated peroxide concentrations less than 3 mEq/kg.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared by mixing pulegone with corn oil to give the required concentrations (Table J2). The dose formulations were stored at room temperature in amber glass bottles sealed with Teflon®-lined lids for up to 28 days (2-week rat studies) or 35 days (all other studies).

Because all dose formulations in the studies were determined to be solutions, no homogeneity studies were required. Stability studies of a 1.4 mg/mL formulation of a lot not used in the animal studies were performed by the analytical chemistry laboratory using GC by system D (Table J1). Stability was confirmed for at least 35 days for dose formulations stored in sealed glass bottles and protected from light at room temperature, and for at least 3 hours under simulated animal room conditions. Additional stability studies of a 0.9375 mg/mL formulation conducted by the study laboratory indicated that dose formulations were stable for 39 days when stored in sealed amber glass bottles at room temperature.

Periodic analyses of the dose formulations of pulegone were conducted by the study laboratory using a GC system similar to system D. During the 2-week studies, the dose formulations were analyzed once; all seven dose formulations for rats and mice were within 10% of the target concentrations (Table J3). Animal room samples of these dose formulations were also analyzed; all five for rats and all five for mice were within 10% of the target concentrations. During the 3-month studies, the dose formulations were analyzed three times; animal room samples of these dose formulations were also analyzed (Table J4). Of the dose formulations analyzed and used during the studies, all 18 for rats and mice were within 10% of the target concentrations; all 15 animal room samples for rats and all 15 for mice were within 10% of the target concentrations. During the 2-year studies, the dose formulations were analyzed approximately every 2 to 3 months; animal room samples were also analyzed (Table J5). Of the dose formulations analyzed and used during the studies, all 33 for rats and mice were within 10% of the target concentrations; 10 of 11 animal room samples for rats and all nine for mice were within 10% of the target concentrations.

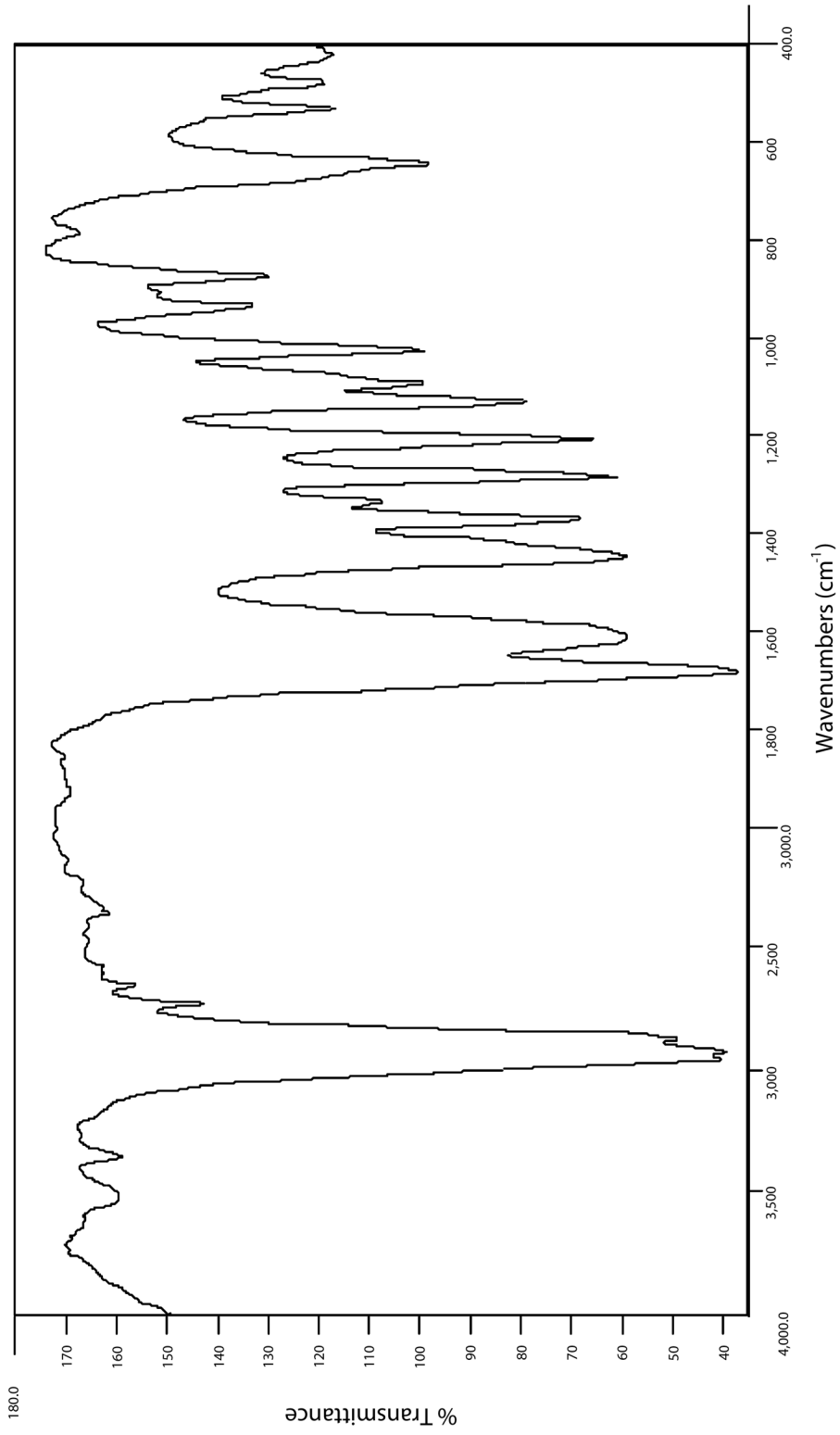

FIGURE J1
Infrared Absorption Spectrum of Pulegone

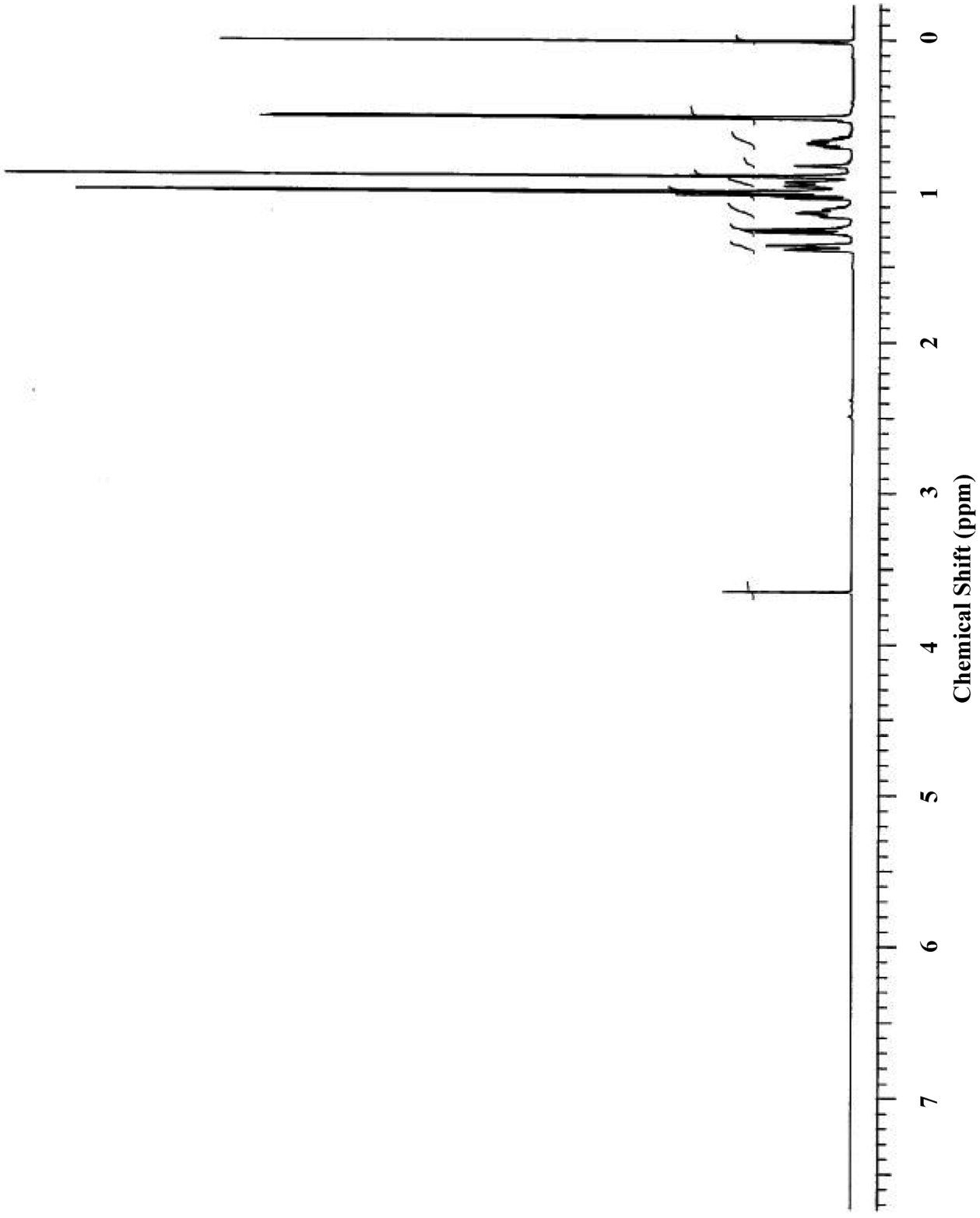

FIGURE J2
Proton Nuclear Magnetic Resonance Spectrum of Pulegone

TABLE J1
Gas Chromatography Systems Used in the Gavage Studies of Pulegone[a]

Detection System	Column	Carrier Gas	Oven Temperature Program
System A Flame ionization	J&W DB™-WAX, 30 m × 0.53 mm, 1 µm film (J&W Scientific, Folsom, CA)	Helium at 10 mL/minute	50° C for 3 minutes, then 5° C/minute to 200° C, held for 5 minutes
System B Mass spectrometry with electron impact ionization (35 to 550 amu)	J&W DB™-WAX, 30 m × 0.32 mm, 0.5 µm film (J&W Scientific)	Helium at 30 cm/second	50° C for 3 minutes, then 5° C/minute to 200° C, held for 5 minutes
System C Flame ionization	J&W DB™-WAX, 30 m × 0.53 mm, 1 µm film (J&W Scientific)	Helium at 10 mL/minute	80° C for 2 minutes, then 10° C/minute to 200° C, held for 3 minutes
System D Flame ionization	J&W DB™-WAX, 30 m × 0.53 mm, 1 µm film (J&W Scientific)	Helium at 10 mL/minute	80° C for 2 minutes, then 8° C/minute to 200° C, held for 3 minutes

[a] The gas chromatographs were manufactured by Varian, Inc. (Walnut Creek, CA) (systems A, C, and D), or Hewlett-Packard Company (Palo Alto, CA) (system B).

TABLE J2
Preparation and Storage of Dose Formulations in the Gavage Studies of Pulegone

2-Week Studies	3-Month Studies	2-Year Studies
Preparation		
The appropriate volumes of pulegone and corn oil were combined in a calibrated, clear glass mixing container. The mixing container was capped and shaken on a paint shaker for approximately 5 minutes. The dose formulations were prepared once during the studies.	The appropriate volumes of pulegone and corn oil were combined in a calibrated, clear glass mixing container. The mixing container was capped and shaken for approximately 2 minutes and inverted at least 10 times. The contents of the mixing container were then stirred with a vigorous vortex on a stir plate for 15 minutes. The dose formulations were prepared approximately monthly.	The specified volume of pulegone was measured in a graduated cylinder and poured into a calibrated glass mixing container partially filled with corn oil. The graduated cylinder was rinsed with corn oil at least three times into the mixing container. The contents of the mixing container were diluted to final volume with additional corn oil and stirred with a vigorous vortex using an overhead stirrer for approximately 15 minutes. The dose formulations were prepared approximately monthly.
Chemical Lot Number		
OGI01	OGI01	OGI01
Maximum Storage Time		
28 days (rats) or 35 days (mice)	35 days	35 days
Storage Conditions		
Stored in amber glass bottles sealed with Teflon®-lined lids at room temperature	Stored in amber glass bottles sealed with Teflon®-lined lids at room temperature	Stored in amber glass bottles sealed with Teflon®-lined lids at room temperature
Study Laboratory		
Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)

TABLE J3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Week Gavage Studies of Pulegone

Date Prepared	Date Analyzed	Target Concentration (mg/mL)[a]	Determined Concentration (mg/mL)[b]	Difference from Target (%)
Rats and Mice				
July 31, 2001	July 31-August 1, 2001	3.75	3.809	+2
		7.5	7.640	+2
		15	14.83	−1
		30	31.23	+4
		60	60.47	+1
		120	119.9	0
August 16, 2001	August 17, 2001	1.875	1.868	0
Animal Room Samples				
Rats				
July 31, 2001	September 7-8, 2001	7.5	7.604	+1
		15	14.78	−1
		30	30.90	+3
		60	60.36	+1
		120	121.5	+1
Mice				
July 31, 2001	September 7-8, 2001	3.75	3.763	0
		7.5	7.545	+1
		15	14.68	−2
		30	30.14	0
August 16, 2001	September 7-8, 2001	1.875	1.874	0

[a] The 1.875 and 3.75 mg/mL dose formulations were used for mice only. The 60 and 120 mg/mL dose formulations were used for rats only.

[b] 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; 1.875 mg/mL=18.75 mg/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.

TABLE J4
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 3-Month Gavage Studies of Pulegone

Date Prepared	Date Analyzed	Target Concentration (mg/mL)[a]	Determined Concentration (mg/mL)[b]	Difference from Target (%)
Rats and Mice				
February 7, 2002	February 11-12, 2002	0.9375	1.019	+9
		1.875	1.973	+5
		3.75	3.867	+3
		7.5	7.684	+2
		15	15.26	+2
		30	30.09	0
March 1, 2002	March 4-5, 2002	0.9375	0.9733	+4
		1.875	1.887	+1
		3.75	3.783	+1
		7.5	7.533	0
		15	15.22	+1
		30	29.82	0
April 26, 2002	April 29-30, 2002	0.9375	0.9073	−3
		1.875	1.863	−1
		3.75	3.491	−7
		7.5	7.247	−3
		15	14.61	−3
May 2, 2002	May 2, 2002	30	32.32	+8
Animal Room Samples				
Rats				
February 7, 2002	March 11-12, 2002	1.875	1.914	+2
		3.75	3.778	+1
		7.5	7.570	+1
		15	15.14	+1
		30	29.81	−1
March 1, 2002	April 9-10, 2002	1.875	1.963	+5
		3.75	3.854	+3
		7.5	7.748	+3
		15	15.21	+1
		30	29.75	−1
April 26, 2002	June 6-7, 2002	1.875	1.954	+4
		3.75	3.819	+2
		7.5	7.564	+1
		15	15.49	+3
May 2, 2002	June 6-7, 2002	30	32.17	+7

TABLE J4
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 3-Month Gavage Studies of Pulegone

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
Animal Room Samples (continued)				
Mice				
February 7, 2002	March 11-12, 2002	0.9375	0.9750	+4
		1.875	1.907	+2
		3.75	3.714	−1
		7.5	7.575	+1
		15	15.15	+1
March 1, 2002	April 9-10, 2002	0.9375	0.9738	+4
		1.875	1.770	−6
		3.75	3.849	+3
		7.5	7.640	+2
		15	15.32	+2
April 26, 2002	June 6-7, 2002	0.9375	0.9572	+2
		1.875	1.951	+4
		3.75	3.843	+2
		7.5	7.570	+1
		15	15.47	+3

[a] The 0.9375 and 30 mg/mL dose formulations were used for mice and rats only, respectively.

[b] Results of duplicate analyses. For rats, dosing volume = 5 mL/kg; 1.875 mg/mL=9.375 mg/kg, 3.75 mg/mL=18.75 mg/kg, 7.5 mg/mL=37.5 mg/kg, 15 mg/mL=75 mg/kg, 30 mg/mL=150 mg/kg. For mice, dosing volume = 10 mL/kg; 0.9375 mg/mL=9.375 mg/kg, 1.875 mg/mL=18.75 mg/kg, 3.75 mg/mL=37.5 mg/kg, 7.5 mg/mL=75 mg/kg, 15 mg/mL=150 mg/kg.

TABLE J5
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Gavage Studies of Pulegone

Date Prepared	Date Analyzed	Target Concentration (mg/mL)[a]	Determined Concentration (mg/mL)[b]	Difference from Target (%)
Rats and Mice				
March 26, 2003	March 27-28, 2003	3.75	3.968	+6
		7.5	8.247	+10
		15	16.34	+9
April 2, 2003	April 2, 2003	30	31.72	+6
June 16, 2003	June 17, 2003	3.75	3.918	+4
		7.5	7.865	+5
		15	15.80	+5
		30	31.53	+5
September 2, 2003	September 3-4, 2003	3.75	3.887	+4
		7.5	7.844	+5
		15	15.95	+6
		30	32.41	+8
November 19, 2003	November 24-25, 2003	3.75	3.898	+4
		7.5	7.861	+5
		15	15.44	+3
		30	30.51	+2
February 13, 2004	February 16-17, 2004	3.75	3.714	−1
		7.5	7.570	+1
		15	15.11	+1
		30	29.56	−1
May 7, 2004	May 10-11, 2004	3.75	3.864	+3
		7.5	7.751	+3
		15	15.26	+2
		30	30.99	+3
July 30, 2004	August 3, 2004	3.75	3.762	0
		7.5	7.576	+1
		15	15.07	+0
October 22, 2004	October 26, 2004	3.75	3.797	+1
		7.5	7.910	+5
		15	15.20	+1
January 14, 2005	January 19, 2005	3.75	3.818	+2
		7.5	7.721	+3
		15	15.51	+3

TABLE J5
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Gavage Studies of Pulegone

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
Animal Room Samples				
Rats				
March 26, 2003	May 2-3, 2003	3.75	3.942	+5
		7.5	7.983	+6
		15	15.84	+6
April 2, 2003	May 2-3, 2003	30	32.01	+7
November 19, 2003	January 6-7, 2004	3.75	4.131	+10
		7.5	8.214	+10
		15	16.36	+9
		30	33.76	+13
July 30, 2004	September 9, 2004	3.75	3.667	−2
		7.5	7.455	−1
		15	14.79	−1
Mice				
March 26, 2003	May 2-3, 2003	3.75	3.815	+2
		7.5	7.885	+5
		15	15.67	+4
November 19, 2003	January 6-7, 2004	3.75	4.109	+10
		7.5	8.205	+9
		15	16.36	+9
July 30, 2004	September 9, 2004	3.75	3.605	−4
		7.5	7.486	+0
		15	14.89	−1

[a] The 3.75 mg/mL dose formulation was used for male rats and male and female mice only. The 30 mg/mL dose formulation was used for female rats only.

[b] Results of duplicate analyses. For rats, dosing volume = 5 mL/kg; 3.75 mg/mL=18.75 mg/kg (males only), 7.5 mg/mL=37.5 mg/kg, 15 mg/mL=75 mg/kg, 30 mg/mL=150 mg/kg (females only). 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.

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

TABLE K1
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 K2
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 K3
Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	14.8 ± 0.61	13.8 – 16.1	24
Crude fat (% by weight)	8.1 ± 0.37	7.4 – 9.0	24
Crude fiber (% by weight)	9.1 ± 0.45	8.2 – 9.9	24
Ash (% by weight)	5.0 ± 0.23	4.4 – 5.4	24
Amino Acids (% of total diet)			
Arginine	0.770 ± 0.070	0.670 – 0.970	18
Cystine	0.225 ± 0.023	0.150 – 0.250	18
Glycine	0.706 ± 0.043	0.620 – 0.800	18
Histidine	0.362 ± 0.082	0.310 – 0.680	18
Isoleucine	0.542 ± 0.046	0.430 – 0.660	18
Leucine	1.087 ± 0.066	0.960 – 1.240	18
Lysine	0.712 ± 0.118	0.310 – 0.840	18
Methionine	0.407 ± 0.051	0.260 – 0.490	18
Phenylalanine	0.626 ± 0.043	0.540 – 0.720	18
Threonine	0.500 ± 0.046	0.430 – 0.610	18
Tryptophan	0.142 ± 0.024	0.110 – 0.200	18
Tyrosine	0.388 ± 0.058	0.280 – 0.540	18
Valine	0.667 ± 0.045	0.550 – 0.730	18
Essential Fatty Acids (% of total diet)			
Linoleic	3.92 ± 0.243	3.49 – 4.54	18
Linolenic	0.30 ± 0.035	0.21 – 0.35	18
Vitamins			
Vitamin A (IU/kg)	4,874 ± 129	3,230 – 8,900	24
Vitamin D (IU/kg)	1,000[a]		
α-Tocopherol (ppm)	84.2 ± 16.60	52.0 – 110.0	15
Thiamine (ppm)[b]	9.0 ± 3.66	6.4 – 25.2	24
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 (%)	0.959 ± 0.047	0.873 – 1.030	24
Phosphorus (%)	0.583 ± 0.029	0.538 – 0.641	24
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 K4
Contaminant Levels in NTP-2000 Rat and Mouse Ration[a]

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.30 ± 0.151	0.14 – 0.50	24
Cadmium (ppm)	0.07 ± 0.021	0.04 – 0.10	24
Lead (ppm)	0.08 ± 0.025	0.05 – 0.13	24
Mercury (ppm)	<0.02		24
Selenium (ppm)	0.20 ± 0.057	0.14 – 0.45	24
Aflatoxins (ppb)	<5.00		24
Nitrate nitrogen[c] (ppm)	13.6 ± 3.87	8.45 – 24.4	24
Nitrite nitrogen[c] (ppm)	<0.61		24
BHA (ppm)[d]	<1.0		24
BHT (ppm)[d]	<1.0		24
Aerobic plate count (CFU/g)	10 ± 0	10	24
Coliform (MPN/g)	3.0 ± 0	3.0	24
Escherichia coli (MPN/g)	<10		24
Salmonella (MPN/g)	Negative		24
Total nitrosoamines (ppb)[e]	4.3 ± 1.91	2.3 – 8.5	24
N-nitrosodimethylamine (ppb)[e]	2.5 ± 1.56	1.1 – 6.9	24
N-nitrosopyrrolidine (ppb)[e]	1.9 ± 0.80	0.9 – 4.1	24
Pesticides (ppm)			
α-BHC	<0.01		24
β-BHC	<0.02		24
γ-BHC	<0.01		24
δ-BHC	<0.01		24
Heptachlor	<0.01		24
Aldrin	<0.01		24
Heptachlor epoxide	<0.01		24
DDE	<0.01		24
DDD	<0.01		24
DDT	<0.01		24
HCB	<0.01		24
Mirex	<0.01		24
Methoxychlor	<0.05		24
Dieldrin	<0.01		24
Endrin	<0.01		24
Telodrin	<0.01		24
Chlordane	<0.05		24
Toxaphene	<0.10		24
Estimated PCBs	<0.20		24
Ronnel	<0.01		24
Ethion	<0.02		24
Trithion	<0.05		24
Diazinon	<0.10		24
Methyl chlorpyrifos	0.114 ± 0.129	0.020 – 0.416	24
Methyl parathion	<0.02		24
Ethyl Parathion	<0.02		24
Malathion	0.195 ± 0.386	0.020 – 1.850	24
Endosulfan I	<0.01		24
Endosulfan II	<0.01		24
Endosulfane sulfate	<0.03		24

[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 L
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.

In the 3-month studies, serum samples were collected from five male and five female sentinel rats and mice at 4 weeks and at the end of the studies. In the 2-year studies, serum samples were collected from five male (except four male rats at 18 months) and five female sentinel rats and mice at 1, 6, 12, and 18 months. In addition, at the end of the studies, serum samples were collected from seven rats (two males and five females) randomly selected from the 75 mg/kg groups, three male rats from the 37.5 mg/kg group, and five male and five female mice in the 150 mg/kg groups. Fecal samples were taken from sentinel mice at 18 months for *Helicobacter* analyses. Blood from each animal was collected and allowed to clot, and the serum was separated. The samples were processed appropriately and sent to BioReliance Corporation (Rockville, MD) for determination of antibody titers. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

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

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

RESULTS

All test results were negative.