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

NTP Genetically Modified Model Report on the

Toxicology and Carcinogenesis Study of Phenolphthalein (CASRN 77-09-8) in Genetically Modified Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice (Feed Study)

NTP GMM 12

DECEMBER 2007

NTP REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDY OF PHENOLPHTHALEIN

(CAS NO. 77-09-8)

IN GENETICALLY MODIFIED HAPLOINSUFFICIENT p16^{Ink4a}/p19^{Arf} MICE

(FEED STUDY)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
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U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

FOREWORD

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

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

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

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

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SUMMARY

Background

Phenolphthalein was formerly used as a laxative. Phenolphthalein is known to cause cancer in rats and mice. We tested phenolphthalein in a genetically modified mouse strain that lacks two tumor suppressor genes as part of a study to determine if this mouse model could detect cancer-causing chemcals more rapidly than the standard 2-year rodent bioassay.

Methods

We gave groups of 15 male or 15 female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice feed containing phenolphthalein for 27 weeks. The concentrations were 200, 375, 750, 3,000 or 12,000 parts per million (ppm) of phenolphthalein; other animals receiving untreated feed served as the control groups. Tissues from 22 organs were examined for every animal.

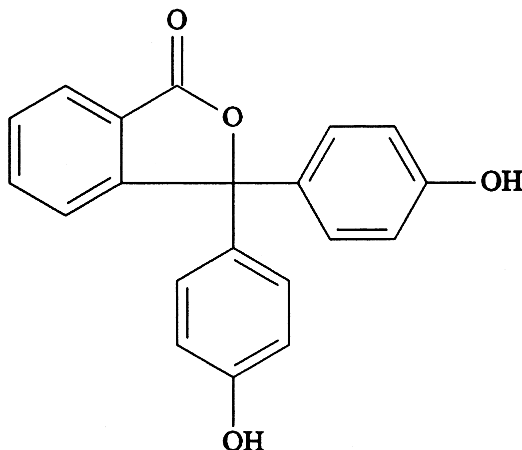
Results

Exposure to phenolphthalein caused atrophy of the testes, reduction in spermatids, and kidney nephropathy and hypertrophy in male haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice and hyperplasia of the thymus and hematopoietic cell proliferation of the spleen in male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice. However, there were no increased incidences of cancer in the exposed animals.

Conclusions

We conclude that phenolphthalein did not cause cancer in male or female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice, although it has in some other strains of mice and in rats. Phenolphthalein did cause atrophy of the testes and nephropathy and hypertrophy of the kidney in male haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice and hyperplasia of the thymus and hematopoietic cell proliferation of the spleen in male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice.

ABSTRACT



PHENOLPHTHALEIN

CAS No. 77-09-8

Chemical Formula: $C_{20}H_{14}O_4$ Molecular Weight: 318.33

Synonyms: 3,3-Bis(4-hydroxyphenyl)-1(3H)-isobenzofuranone; 3,3-bis(*p*-hydroxyphenyl)phthalide; α-(*p*-hydroxyphenyl)-α-(4-oxo-2,5-cyclohexadien-1-ylidene)-*o*-toluic acid
Trade names: Agoral®, Alophen®, Colax®, Correctol®, Dialose®, Doxidan®, Espotabs®, Evac-U-Gen®, Evac-U-Lax®, Ex-Lax®, Feen-A-Mint®, FemiLax®, Kondremul®, LaxCaps®, Lax-Pills®, Medilax®, Modane®, Phenolax®, Prulet®

Phenolphthalein was commonly used as a laxative for most of the 20th century. The use of phenolphthalein in laxatives has decreased since 1997 when the United States Food and Drug Administration (FDA) proposed to withdraw its classification as an over-the-counter drug (21 CFR, Part 310). Phenolphthalein has been previously evaluated in 2-year carcinogenicity studies by the National Toxicology Program (1996). The major route of human exposure to phenolphthalein is via ingestion, dermal contact, and inhalation of contaminated air originating from process units manufacturing the compound. In this study, the carcinogenic effects of phenolphthalein were studied in the haploinsufficient $p16^{Ink4a}$/$p19^{Arf}$ mouse model as an ongoing goal of the NTP is to seek model systems for toxicology and carcinogenesis studies, especially those that can provide mechanistic information relative to understanding an agent's mode of action. Male and female haploinsufficient $p16^{Ink4a}$/$p19^{Arf}$ mice were exposed to phenolphthalein (greater than 97% pure) in feed for 27 weeks. Genetic toxicology studies were conducted in mouse peripheral blood erythrocytes.

27-Week Study in Mice

Groups of 15 male and 15 female mice were exposed to 0, 200, 375, 750, 3,000, or 12,000 ppm phenolphthalein (equivalent to average daily doses of approximately 35, 65, 135, 540, and 2,170 mg phenolphthalein/kg body weight to males and 50, 90, 170, 680, 2,770 mg/kg to females) in feed for 27 weeks. Survival of all exposed groups of male and female mice was similar to that of the control groups. Mean body weights of males in the

12,000 ppm group were less than those of the control group after week 11. No differences in feed consumption were noted between exposed and control groups.

Atypical hyperplasia of the thymus, a premalignant change of chemically induced thymic lymphoma, occurred in exposed males and females, and the incidence was significantly increased in 12,000 ppm females. Atrophy of the seminiferous tubules in the testis, hyperplasia of the testicular interstitial (Leydig) cells, and epididymal hypospermia occurred in most 3,000 and 12,000 ppm males. Additionally, the left and right testis weights, the left epididymis weights, sperm motility, the numbers of spermatid heads per testis, and sperm heads per cauda and per gram cauda epididymis were generally significantly less in 3,000 and 12,000 ppm males than in the control group. The incidences of nephropathy were significantly increased in 3,000 and 12,000 ppm males; incidences of hypertrophy of renal tubules were significantly increased in males receiving 750 ppm or greater. Hematopoietic cell proliferation of the spleen occurred in all 12,000 ppm males, and the incidences of this lesion were significantly increased in 375, 750, and 12,000 ppm females.

Genetic Toxicology

The frequency of micronucleated erythrocytes was assessed at four time points during the 27-week study in male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice. Significant concentration-related increases in micronucleated cells were observed at all time points in male and female mice.

Conclusions

Under the conditions of this 27-week feed study, there was *no evidence of carcinogenic activity** of phenolphthalein in male or female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice exposed to 200, 375, 750, 3,000, or 12,000 ppm. Because this is a new model, there is uncertainty whether the study possessed sufficient sensitivity to detect a carcinogenic effect.

Phenolphthalein induced atypical hyperplasia, a preneoplastic lesion of the thymus, in male and female mice, hematopoietic cell proliferation of the spleen in male and female mice, and toxicity to the kidney and reproductive system in male mice.

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

Summary of the 27-Week Carcinogenesis and Genetic Toxicology Studies of Phenolphthalein in Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice

	Male	Female
Concentrations in feed	0, 200, 375, 750, 3,000, or 12,000 ppm	0, 200, 375, 750, 3,000, or 12,000 ppm
Body weights	12,000 ppm group less than the control group	Exposed groups similar to the control group
Survival rates	13/15, 15/15, 15/15, 13/15, 15/15, 14/15	15/15, 13/15, 14/15, 15/15, 14/15, 15/15
Nonneoplastic effects	Thymus: atypical hyperplasia (0/14, 0/15, 1/15, 0/14, 1/15, 0/14) Testes: germinal epithelium, atrophy (0/15, 1/15, 1/15, 0/15, 15/15, 14/15); interstitial cell, hyperplasia (0/15, 1/15, 1/15, 0/15, 15/15, 14/15) Epididymis: hypospermia (0/15, 0/15, 1/15, 0/15, 15/15, 14/15) Kidney: nephropathy (6/14, 7/15, 8/15, 6/14, 15/15, 14/15); renal tubule, hypertrophy (0/14, 0/15, 0/15, 10/14, 15/15, 14/15) Spleen: hematopoietic cell proliferation (2/14, 5/15, 3/15, 1/14, 2/15, 14/14)	Thymus: atypical hyperplasia (0/15, 0/15, 1/15, 0/15, 3/14, 5/15) Spleen: hematopoietic cell proliferation (2/15, 5/14, 9/14, 8/15, 7/15, 13/15)
Neoplastic effects	None	None
Level of evidence of carcinogenic activity	No evidence	No evidence
Genetic toxicology		
Micronucleated erythrocytes		
Mouse peripheral blood *in vivo:*	Positive in males and females at the 6.5-, 13-, 19.5-, and 27-week sampling times.	

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

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

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

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

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

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

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

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Report on phenolphthalein on August 28, 2006, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing the NTP studies:

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

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SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On August 28, 2006, the draft Report on the toxicology and carcinogenesis study of phenolphthalein received public review by the National Toxicology Program's Board of Scientific Counselors' Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. J.K. Dunnick, NIEHS, introduced the study of phenolphthalein in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice by reviewing the uses of the chemical as a laxative, the previous NTP carcinogenicity findings, the design and dose selection for the genetically modified mouse model studies, and the nonneoplastic lesions observed in the 27-week study. The proposed conclusions were *no evidence of carcinogenic activity* of phenolphthalein in male or female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice.

Dr. Deininger, the first principal reviewer, noted that the dose selection was based on the 2-year study and suggested that possibly doses could have been higher in this study.

Dr. Walker, the second principal reviewer, had no additional comments.

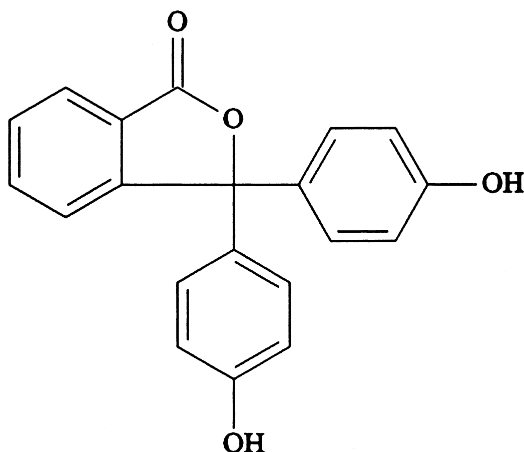
Dr. Sikka, the third principal reviewer, suggested that in discussion of the role of p53, apoptosis as well as cell cycle arrest should be mentioned.

Dr. Walker suggested that the following statement be added to the conclusion, because no neoplastic effects were seen in this study:

Because this is a new model, there is uncertainty whether this study possessed sufficient sensitivity to detect a carcinogenic effect.

Dr. Deininger moved, and Dr. Soper seconded, that the conclusions be accepted with this addition. The motion was approved unanimously with seven votes.

INTRODUCTION



PHENOLPHTHALEIN

CAS No. 77-09-8

Chemical Formula: $C_{20}H_{14}O_4$ Molecular Weight: 318.33

Synonyms: 3,3-Bis(4-hydroxyphenyl)-1(3H)-isobenzofuranone; 3,3-bis(*p*-hydroxyphenyl)phthalide; α-(*p*-hydroxyphenyl)-α-(4-oxo-2,5-cyclohexadien-1-ylidene)-*o*-toluic acid
Trade names: Agoral®, Alophen®, Colax®, Correctol®, Dialose®, Doxidan®, Espotabs®, Evac-U-Gen®, Evac-U-Lax®, Ex-Lax®, Feen-A-Mint®, FemiLax®, Kondremul®, LaxCaps®, Lax-Pills®, Medilax®, Modane®, Phenolax®, Prulet®

Chemical and Physical Properties

Phenolphthalein is a white or yellowish white, odorless, tasteless powder consisting of minute triclinic crystals (often twinned). It has a specific gravity of 1.277 at 32/4° C and a melting point range of 262° to 273° C (Weast, 1987) or 258° to 262° C (*Merck*, 1996), depending on the relative amounts of associated impurities. Although not flammable, phenolphthalein emits acrid smoke and fumes when heated to decomposition (*Sax's*, 1992). Phenolphthalein is readily soluble in alcohol (1 g dissolves in 12 to 15 mL) or ether (1 g dissolves in approximately 100 mL) and very slightly soluble in chloroform. It is almost insoluble in water; however, its solubility is increased in physiologic buffered solutions simulating intestinal contents, i.e., 7.8 mg/dL in Krebs-Ringer-bicarbonate solution, pH 7.4 (Sharaiha *et al.*, 1983). The solubility of aqueous phenolphthalein is also pH dependent and does not exceed 6 mg/dL without the addition of ethanol until the pH is above the physiologic range, i.e., greater than 9 (Fantus and Dyniewicz, 1937; Hubacher, 1945). Solutions containing phenolphthalein are colorless to pH 8.5 and pink to deep red above pH 9. Phenolphthalein is used as a laboratory reagent and an acid-base indicator in titrations of mineral and organic acids and most alkalies (*Merck*, 1996).

Production, Use, and Human Exposure

Phenolphthalein was commonly used as a laxative for most of the 20th century. In 1997, only one company produced phenolphthalein in the United States, but there were 39 suppliers of phenolphthalein in the United States. The use of phenolphthalein in laxatives has decreased since 1997 when the United States Food and Drug Administration (FDA) proposed to withdraw its classification as an over-the-counter drug (21 CFR, Part 310). In 1997, the makers of Ex-Lax® announced that they were reformulating their laxative using senna instead of phenolphthalein. The major route of human exposure to phenolphthalein is via ingestion, dermal contact, and inhalation of contaminated air originating from process units manufacturing the compound (NTP, 2004).

When phenolphthalein was available as an over-the-counter laxative, the usual daily oral laxative doses for white or yellow phenolphthalein were 30 to 270 mg for adults and children 12 years of age and older, 30 to 60 mg for children 6 to 11 years of age, and 15 to 30 mg for children 2 to 5 years of age, although the use of stimulant laxatives is generally not recommended in children younger than 6 years of age (*Fed. Regist.*, 1975; AHFS, 1995).

In making its final rule on phenolphthalein, the FDA was concerned about the improper uses of the over-the-counter laxative by the public. Laxative-cathartics, such as phenolphthalein, are habit forming (i.e., chronic usage resulting in dependence), are commonly used to self-medicate symptoms of constipation, and are frequently abused in an effort to control weight (Pietrusko, 1977; *Goodman and Gilman's*, 1990). Ten percent of college students who participated in a 1981 survey admitted to purging behavior, i.e., laxative use and self-induced vomiting (Halmi *et al.*, 1981). Purging behavior is also associated with the eating disorders anorexia nervosa and bulimia nervosa; laxative abuse has been reported in 38% to 63% of bulimia nervosa patients (Van Rooyen and Ziady, 1972; Pyle *et al.*, 1981; Johnson *et al.*, 1982; Bo-Linn *et al.*, 1983; Mitchell *et al.*, 1983; Fairburn and Cooper, 1984). A review by Cummings (1974) indicated that over 90% of chronic laxative abusers are women.

Regulatory Status

The final United States FDA rule removed phenolphthalein as an over-the-counter drug in 1999 (21 CFR, Part 310).

Absorption, Distribution, Metabolism, and Excretion

Experimental Animals

Metabolism of phenolphthalein in animals is similar to that in humans (NTP, 1996; Griffin *et al.*, 1998; Collins *et al.*, 2000; Figure 1). The drug is absorbed from the gastrointestinal tract, and the major metabolite is phenolphthalein glucuronide (PTH-G), which undergoes enterohepatic recirculation. A catechol metabolite of phenolphthalein, hydroxyphenolphthalein (PT-CAT), has been identified (Garner *et al.*, 2000a,b). These authors hypothesize that PT-CAT inhibits the enzyme catechol-*O*-methyltransferase (COMT) and, therefore, potentiates genotoxicity by either PT-CAT itself or the endogenous catechol estrogens in susceptible tissues. The concentration of uridine diphosphate glucuronosyltransferase (UDPGT; the enzyme responsible for phenolphthalein conjugation) is low in fetal or neonatal rodent tissue compared to that in the adult. Metabolism of phenolphthalein in p53$^{+/-}$ mice is similar to that in B6C3F$_1$ mice (Collins *et al.*, 2000).

Phenolphthalein is absorbed from the intestine and is excreted in the bile, urine, feces, and milk (Visek *et al.*, 1956; AHFS, 1995). Once phenolphthalein is absorbed, it is conjugated with glucuronic acid via UDPGT in the liver and intestine (Sund and Hillestad, 1982) and is distributed throughout the body in the blood and lymph (Visek *et al.*, 1956). Studies reported in the literature provide evidence for enzyme multiplicity of UDPGT. Steroidal and nonsteroidal UDPGT may have different membrane environments, and certain tissues such as the kidney and uterus have been shown to have a lower UDPGT activity for xenobiotic estrogenic compounds such as phenolphthalein (Lucier and McDaniel, 1977). Minor amounts of sulfate conjugate metabolites have also been detected in mucosal sheets isolated from the jejunum and colon of the guinea pig (Sund and Lauterbach, 1986).

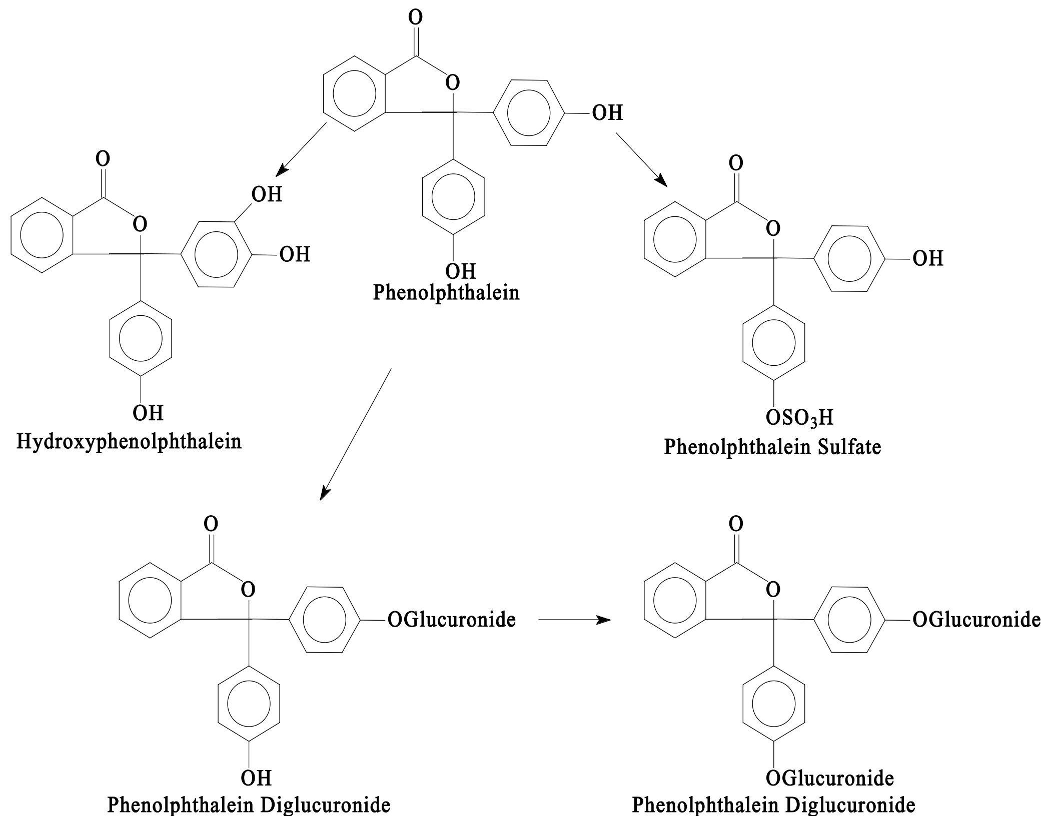


FIGURE 1
Metabolic Scheme for [^{14}C]-Phenolphthalein in F344/N Rats and B6C3F$_1$ Mice (Griffin *et al.*, 1998)

Phenolphthalein conjugation enzyme activity UDPGT is absent or very low in microsomes from fetal or neonatal rat and guinea pig liver compared to activity from adult livers (Jondorf *et al.*, 1958; Wishart, 1978a). Glucuronide conjugation activity in adult rats is stimulated by phenobarbital and unaffected by 3-methylcholanthrene (Wishart, 1978b).

Pharmacokinetic and tissue distribution studies in mice (strain not specified) and dogs (breed not specified) given 4.8 mg [^{14}C]-phenolphthalein/kg (uniformly labeled) indicated that phenolphthalein was widely and evenly distributed throughout the body, with levels of radioactivity parallel to the concentration in the blood. In dogs, approximately 50% of an oral dose was recovered in the feces and 36% in the urine after 72 hours (Visek *et al.*, 1956).

In the mouse studies (Visek *et al.*, 1956), no respiratory [^{14}C]-carbon dioxide was recovered following administration of phenolphthalein labeled with ^{14}C on the nonaromatic carbon of the lactone ring, indicating that the bonds to the labeled carbon atoms probably were not broken. Within 48 hours, approximately 96% of the administered radioactivity was recovered in the urine and feces (56% in the urine and 38% in the feces following an oral dose; 30% in the urine and 68% in the feces following an intravenous dose). At 30 minutes to 6 hours, the highest levels of radioactivity appeared in the liver, gallbladder, and small intestine. On a total organ basis, the small intestine, which plays a major role in the excretion of phenolphthalein, contained more radioactive label than did the large intestine at all measurement intervals. However, when equated on a unit weight basis, the radioactivity of the large intestine and its contents increased substantially 6 hours after dosing.

Phenolphthalein undergoes extensive first pass metabolism in the intestinal epithelium and liver, which results in almost complete conversion of phenolphthalein to its

glucuronide (Parker *et al.*, 1980). Studies by Colburn *et al.* (1979) demonstrated that 6 hours after intravenous administration of [^{3}H]-phenolphthalein to female Wistar rats, all radioactivity in the systemic circulation was present as the conjugate. In addition, a secondary peak in blood radioactivity occurred 5 to 6 hours after intravenous administration and coincided with the absorption of [^{3}H]-phenolphthalein from the intestine following bacterial β-glucuronidase hydrolysis of [^{3}H]-phenolphthalein glucuronide excreted in the bile. Hydrolysis of phenolphthalein glucuronide to the aglycone is a rate-limiting step in enterohepatic recirculation (Bergan *et al.*, 1982); pretreatment with antibiotics to suppress intestinal microflora decreased the absorption of [^{3}H]-phenolphthalein from 85% to 22% in female Wistar rats following intraduodenal administration of [^{3}H]-phenolphthalein glucuronide (Parker *et al.*, 1980).

Phenolphthalein has been used as a model compound for enterohepatic recirculation studies in rats (Colburn *et al.*, 1979), and surgical cannulation of the bile duct has been used to evaluate the extent of enterohepatic recirculation in rats and dogs. In bile duct-cannulated female Wistar rats, 95% of an intraperitoneal dose of 25 mg [^{3}H]-phenolphthalein/kg was recovered in the bile as the glucuronide within 24 hours, while only 0.2% was recovered in the urine. Administration of the same dose to intact rats resulted in recovery of 86% in the feces, predominantly as the parent drug, and 10% in the urine as the glucuronide (Parker *et al.*, 1980). In another study in female Wistar rats with biliary fistulae, phenolphthalein was completely eliminated in the bile (100%), almost entirely in the form of phenolphthalein glucuronide (98%) (Millburn *et al.*, 1967). The plasma disappearance and excretion kinetics of phenolphthalein glucuronide in the rat have been characterized in Mehendale (1990). Male Sprague-Dawley rats of the CR-1 strain were intravenously administered 3, 30, or 60 mg phenolphthalein or 3, 30, or 100 mg phenolphthalein glucuronide, and the femoral vein, artery, and common bile duct were cannulated. After administration of phenolphthalein, 99.5% of the dose was eliminated in the bile as phenolphthalein glucuronide with only trace quantities (0.5%) as phenolphthalein. Following administration of phenolphthalein glucuronide, phenolphthalein was undetectable in the bile. Biliary excretion was saturable at higher doses of both compounds.

Humans

Phenolphthalein is absorbed from the small intestine and conjugated in the liver to phenolphthalein glucuronide (PTH-G). PTH-G is excreted in bile and passes into the colon where the glucuronide is hydrolyzed by bacterial flora. In humans, approximately 70% to 90% of an ingested dose is eliminated in the feces in the free or conjugated form, and renal excretion accounts for the remaining 10% to 30% of the dose in the free or conjugated form (NTP, 1996; Collins *et al.*, 2000).

TOXICITY

Experimental Animals

The toxicity of phenolphthalein has been summarized by the National Toxicology Program (1996) and the International Agency for Research on Cancer (2000). Phenolphthalein is not acutely toxic in rodents. In 13-week toxicity studies in F344/N rats and B6C3F$_1$ mice (NTP, 1996), where animals were exposed to up to 50,000 ppm in the feed, there was no treatment-related mortality or clinical signs of toxicity or gross or microscopic lesions in rats. In mice, hypoplasia of the bone marrow occurred at exposures of 12,000 ppm or greater. This lesion was characterized by decreased amounts of hematopoietic tissue with a decreased myeloid to erythroid ratio. Splenic hematopoiesis was seen in male mice exposed to 25,000 ppm or greater. There was no evidence of a laxative effect for phenolphthalein in rats or mice.

Humans

Phenolphthalein, a drug used for most of the 20th century, was generally regarded as nontoxic and safe at therapeutic doses (approximately 1 to 2 mg/kg) (NTP, 1996; IARC, 2000). Under normal conditions, phenolphthalein has not been associated with acute toxicity in humans. However, indiscriminate use of phenolphthalein results in constipation and laxative dependence. Anecdotal cases of long-term use or overdose of phenolphthalein have been associated with abdominal pain, diarrhea, vomiting, electrolyte imbalance, dehydration, malabsorption, and muscle weakness (Heizer *et al.*, 1968; Velentzas and Ikkos, 1971; Cummings, 1974; LaRusso and McGill, 1975; Pohl and Lowe, 1978; AHFS, 1995).

One hypothesis for the laxative effects of phenolphthalein was by inhibition of the normal water absorption from the bowel, and stimulation of secretion of Na^+ and Cl^- ions accompanied by water excretion (Mason *et al.*, 2003). This phenomenon may be due to inhibition of Na^+/K^+-ATPase; recent studies show that nitric oxide generation is not required for phenolphthalein stimulated potassium release (Mason *et al.*, 2004).

Reproductive and Developmental Toxicity

Experimental Animals

In the 13-week F344/N rat and $B6C3F_1$ mouse studies, there was no evidence for reproductive toxicity in female mice or rats (NTP, 1996). However, lower epididymal weights and sperm density were seen in male mice. In a continuous breeding protocol, phenolphthalein caused lower fertility in mice at levels of 700 ppm and above.

Humans

There have been no systematic studies to evaluate the reproductive toxicity of phenolphthalein in humans (NTP, 1996; IARC, 2000).

Carcinogenicity

Experimental Animals

In the NTP (1996) 2-year study, phenolphthalein was found to be carcinogenic in F344/N rats and $B6C3F_1$ mice (Table 1).

Phenolphthalein induced thymic lymphomas in $p53^{+/-}$ mice on a C57BL/6 background (Dunnick *et al.*, 1997). This p53 mouse was the fifth backcross (N5) of $p53^{-/-}$ C57BL/6 males (N4) × inbred wild type C57BL/6 females with a null mutation introduced into a p53 gene by homologous recombination in murine embryonic stem cells by insertion of a neo cassette into the Trp 53 locus resulting in a deletion of a 450-base pair fragment containing 106 nucleotides of exon 5, and about 350 nucleotides of intron 4 (Donehower *et al.*, 1992). The "wild" p53 gene in these phenolphthalein-induced thymic lymphomas was lost (French *et al.*, 2001a,b, 2002; Hulla *et al.*, 2001a,b). The loss of p53 activity and the ability to control the cell cycle and apoptosis were considered to be part of the pathways leading to cancer.

The production of free radicals by phenolphthalein, as demonstrated in *in vitro* studies, may also take part in the events leading to cancer (Sipe *et al.*, 1997). Phenolphthalein has cell-transforming activity and is genotoxic in Syrian hamster embryo cells (Tsutsui *et al.*, 1997).

When phenolphthalein (3,000, 6,000, or 12,000 ppm in feed) was given alone for 6 months to the *ras*H2 mouse, no treatment-related tumors were seen (Koujitani *et al.*, 2000). However, in the Imaoka *et al.* (2002) study with *ras*H2 mice, 12,000 ppm phenolphthalein administered in the feed for 26 weeks promoted the development of lung tumors induced by N-ethyl-N-nitrosurea (received by intraperitoneal injection of 60 mg/kg body weight). Phenolphthalein (26-week exposure to 6,000 or 12,000 ppm in feed) did not induce tumors in a $p53^{+/-}$ mouse on a CBA or CIEA background, where the p53 gene was knocked out in exon 2 (Okamura, 2003).

Humans

No studies to adequately evaluate the possible relation between excess consumption of phenolphthalein used in weight control and cancer development have been reported (Bishop *et al.*, 1997). One study found no relationship between phenolphthalein use as a laxative and the development of adenomatous colorectal polyps (Longnecker *et al.*, 1997), and another study found no relationship between phenolphthalein use as a laxative and a risk for ovarian cancer (Cooper *et al.*, 2004).

Phenolphthalein has been shown to cause chromosomal aberrations in human cells (Biondi *et al.*, 2000). Human thymidylate synthase is inhibited by micromolar concentrations of phenolphthalein with an IC_{50} of 1.2 µM. Inhibition of thymine synthesis stops the production of DNA, disrupts progress through the cell cycle, and eventually leads to cell death (Stout *et al.*, 1999).

Phenolphthalein competes with estrogen for binding to cultured MCF-7 human breast cancer cells, with a relative binding affinity 10^{-4} that of estradiol, and it stimulates cell growth as measured by DNA and protein assays (Ravdin *et al.*, 1987). Acting as an estrogen agonist, phenolphthalein induced elevated levels of progesterone receptor in the MCF-7 cells. Growth stimulation by both phenolphthalein and estradiol was blocked by the anti-estrogen, 4-hydroxytamoxifen.

Table 1
Chemical-related Neoplasms in NTP Phenolphthalein Studies

	0 ppm	12,000 ppm	25,000 ppm	50,000 ppm
F344/N Male Rats (2-Year Feed Study; NTP, 1996)				
Adrenal Medulla				
Benign Pheochromocytoma	17/50 (34%)	34/50 (68%)	34/50 (68%)	34/50 (68%)
Kidney				
Renal Tubule Adenoma				
(Standard Evaluation)	0/50 (0%)	4/50 (8%)	2/50 (4%)	6/50 (12%)
(Standard and Extended Evaluations Combined)	1/50 (2%)	10/50 (20%)	15/50 (30%)	15/50 (30%)
Renal Tubule Adenoma or Carcinoma				
(Standard Evaluation)	0/50 (0%)	5/50 (10%)	3/50 (6%)	7/50 (14%)
(Standard and Extended Evaluations Combined)	1/50 (2%)	10/50 (20%)	16/50 (32%)	16/50 (32%)
F344/N Female Rats (2-Year Feed Study; NTP, 1996)				
Adrenal Medulla				
Benign Pheochromocytoma	3/50 (6%)	11/50 (22%)	9/50 (18%)	2/49 (4%)
Benign or Malignant Pheochromocytoma	3/50 (6%)	12/50 (24%)	10/50 (20%)	2/49 (4%)
	0 ppm	**3,000 ppm**	**6,000 ppm**	**12,000 ppm**
$B6C3F_1$ Male Mice (2-Year Feed Study; NTP, 1996)				
Histiocytic Sarcoma	1/50 (2%)	3/50 (6%)	11/50 (22%)	12/49 (24%)
Malignant Lymphoma (Thymic Origin)	0/50 (0%)	4/50 (8%)	7/50 (14%)	2/49 (4%)
$B6C3F_1$ Female Mice (2-Year Feed Study; NTP, 1996)				
Histiocytic Sarcoma	0/50 (0%)	2/50 (4%)	7/50 (14%)	7/50 (14%)
Malignant Lymphoma (Multiple Organs)	15/50 (30%)	28/50 (56%)	33/50 (66%)	25/50 (50%)
Malignant Lymphoma (Thymic Origin)	1/50 (2%)	9/50 (18%)	10/50 (20%)	7/50 (14%)

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Female $p53^{+/-}$ Mice (Feed Study; Dunnick *et al.*, 1997)						
Malignant Lymphoma (Thymic Origin)	0/19 (0%)	1/20 (5%)	0/20 (0%)	2/20 (10%)	17/20 (85%)	14/20 (70%)

Phenolphthalein is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals (NTP, 1996, 2004; IARC, 2000).

Genetic Toxicity

The mutagenicity data for phenolphthalein indicates that the chemical, although not active in bacterial gene mutation assays (Kada *et al.*, 1972; Fujita *et al.*, 1976; Bonin *et al.*, 1981; Mortelmans *et al.*, 1986) does induce chromosomal damage in mammalian cells *in vitro* when testing occurs in the presence of induced rat liver S9 enzymes (Witt *et al.*, 1995; Bishop *et al.*, 1998). A number of studies have shown induction of micronuclei by phenolphthalein in mammalian cells *in vivo*. Blood samples obtained from $B6C3F_1$ mice at the end of the NTP 13-week toxicity study with phenolphthalein showed significant increases in micronucleated erythrocytes in males and females (Dietz *et al.*, 1992; NTP, 1996).

Short-term micronucleus assays in mouse bone marrow erythrocytes confirmed the ability of the chemical to induce micronuclei (Witt *et al.*, 1995). The data from this series of short-term tests indicated that relatively high doses (greater than 2,000 mg/kg per day for at least 2 days) administered either in feed or by gavage were required to induce an increase in the frequency of micronucleated erythrocytes in $B6C3F_1$ mice. Swiss CD-1 mice appeared to be more sensitive to the effects of phenolphthalein, showing significant increases in micronucleated erythrocytes at doses of 120 mg/kg per day administered in the diet (0.1%) for a period of 14 weeks.

Holden (1998) suggested that phenolphthalein might not induce genetic damage through direct interaction with chromosomal structural components, but that the increases in micronuclei observed in some experiments were the consequence of increased erythropoiesis in response to a phenolphthalein-induced low grade chronic anemia. Although higher doses of phenolphthalein, at which increased frequencies of micronucleated cells are observed, are frequently associated with increased normochromatic erythrocyte/polychromatic erythrocyte (PCE/NCE) ratios (indicating enhanced cell proliferation), elevated micronucleus counts are also seen at lower doses in subchronic studies in the absence of detectable alterations in the percentage of PCEs in blood. Therefore, phenolphthalein is capable of inducing micronuclei in erythrocytes in the absence of signs of overt bone marrow toxicity and accelerated erythropoiesis.

Results of NTP-sponsored micronucleus studies in transgenic $p53^{+/-}$ mice showed highly significant increases in micronucleated erythrocytes in peripheral blood of treated females at all doses ranging from 200 to 12,000 ppm after 26 weeks of administration in feed; increases in the percentages of PCEs were noted at 3,000 and 12,000 ppm, but the increases were small, going from a background of 2.12% to a maximum of 3.77% (Tice *et al.*, 1998). More recently, Stoll *et al.* (2006) also reported significant increases in micronucleated polychromatic erythrocytes in blood samples obtained from male and female $p53^{+/-}$ mice administered phenolphthalein by gavage or in feed for 39 to 183 days. Furthermore, they reported positive results in the *in vitro* Syrian hamster embryo assay for cell transformation. In the NTP micronucleus studies, immunofluorescent staining techniques were used to gain additional mechanistic information on the origin of the phenolphthalein-induced micronuclei in the $p53^{+/-}$ mice. Staining for the presence of kinetochores in the micronuclei indicated that the majority of the induced micronuclei contained whole chromosomes and probably resulted from aneuploidy events (changes in chromosome number due to mitotic errors). Results of an alkaline Single Cell Gel (Comet) assay with leukocytes and hepatocytes obtained from the $p53^{+/-}$ mice at the termination of the 6-month study were judged to be equivocal, based on inconsistencies in responses obtained at different sample times during the course of the exposure period (Tice *et al.*, 1998).

Background on Genetically Altered Mice

The CDKN2A genetic locus contains two important tumor suppressor genes located on chromosomes 9, 4, and 5 in the human, mouse, and rat, respectively (NCBI, 2005). The locus is unique in that alternate splice variants produce two different tumor suppressor proteins (Sherr and Weber, 2000; Sherr and McCormick, 2002; Lowe and Sherr, 2003). The $p16^{Ink4a}$ and $p19^{Arf}$ variants have exons 2 and 3 in common but use different exon 1 (alpha and beta). Expression of these two splice variants is conserved across mammalian species. Mouse $p19^{Arf}$ and human $p14^{ARF}$ polypeptides are approximately 50% identical, and mouse $p16^{Ink4}$ and human $p16^{Ink4a}$ proteins are approximately 72% identical (Quelle *et al.*, 1995).

The two proteins translated from the mRNA expressed from CDKN2A are a p16-KDa protein and a p19-KDa protein (or p14-KDa protein in humans) (Serrano *et al.*, 1996). The p16 protein ($p16^{Ink4a}$, inhibitor of kinase 4a) is a cell cycle regulatory protein that binds to cyclin dependent kinase 4 or 6 (CDK4/6) and inhibits the catalytic activity of the CDK/cyclin D complex and the phosphorylation of retinoblastoma protein. Since loss of the normal function of $p16^{Ink4a}$ leads to uncontrolled cell growth, p16 is classified as a tumor suppressor gene (Serrano *et al.*, 1993). The second protein coded, $p19^{Arf}$ (Arf, alternate reading frame), induces G1 arrest and apoptosis. The 19Arf protein binds to MDM2 and neutralizes MDM2 inhibition of p53 (Sherr and Weber, 2000).

The targeted deletion of exons 2 and 3 of the Cdkn2a gene by a homologous recombination resulted in the elimination of both $p16^{Ink4a}$ and $p19^{Arf}$ proteins (Serrano *et al.*, 1996). Homozygous null $Cdkn2a^{-/-}$ (or $Cdkn2a^{-/-}$) mice are viable and fertile (Serrano *et al.*, 1996). On inspection, these animals appear normal until about 2 months of age, but histological analysis of the spleen shows a mild proliferative expansion of the white pulp and the presence of numerous megakaryocytes and lymphoblasts in the red pulp. The $p16^{-/-}$ mice develop tumors at an average age of 29 weeks. Lymphomas and fibrosarcomas are two common types of tumors seen in this $Cdkn2a^{-/-}$ mouse. In contrast, the $Cdkn2a^{+/-}$ mouse does not usually develop any obvious tumors or display compromised health until after 36 weeks (Serrano *et al.*, 1996).

Deletions in the Cdkn2a gene predispose both rodents and humans to cancer at multiple organ sites (Sharpless and DePinho, 1999). The complete loss of Cdkn2a gene(s) function is observed in approximately 10% of small cell lung tumors, 30% of esophageal tumors, 55% of gliomas, 100% of pancreatic tumors, and 20% of head and neck tumors.

Transition from G1 to S phase in the mammalian cell cycle is under complex regulatory control, and one G1-S regulatory pathway involves $p16^{Ink4a}$ protein. $P16^{Ink4a}$ inhibits the cdk4/cyclin D1 complex, preventing cdk4 from phosphorylating pRb, and thus ensuring that pRb maintains G1 arrest. Disruption of this pathway, by $p16^{Ink4a}$ gene mutations, perturbs the cell cycle (Serrano *et al.*, 1993) and in the case of these Cdkn2a genetically altered mice (Serrano *et al.*, 1993) results in more cell proliferation (Figure 2).

Serrano *et al.* (1996) report that treatment with DMBA and UV light causes an earlier onset of fibrosarcoma and lymphoma in the $p16^{-/-}$ mouse (8 to 10 weeks) and the $p16^{-/+}$ mouse (7 to 20 weeks).

Study Rationale

The purpose of this phenolphthalein study and the companion benzene and glycidol studies (NTP, 2007a,b) was to determine if a mouse with a deletion at the p16 gene locus (CDKN2), a locus that codes for two tumor suppressor genes, would enable the identification of carcinogenic chemicals in a shorter time frame and with fewer animals than the traditional 2-year NTP cancer study. These three chemicals were all multisite carcinogens in the NTP 2-year bioassays (NTP 1986, 1990, 1996). This study reports the findings from the phenolphthalein study.

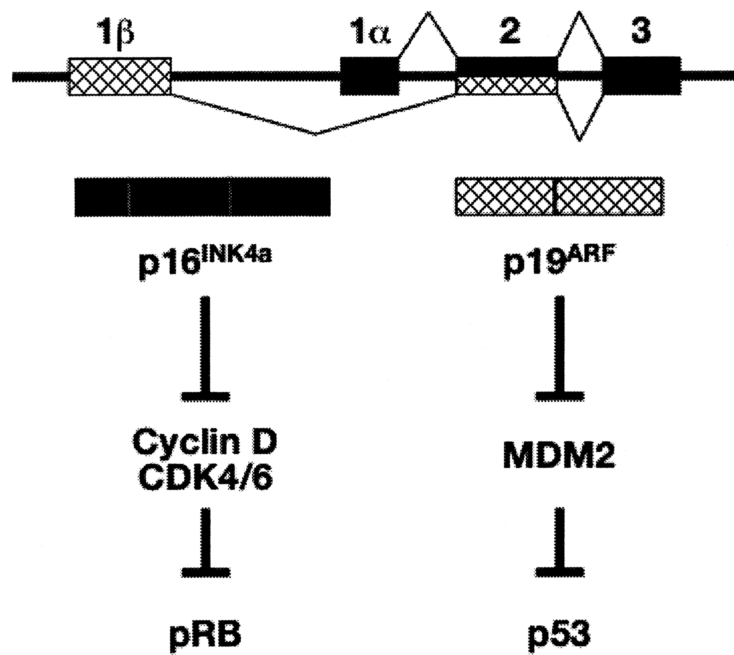


FIGURE 2

The *Ink4a/Arf* Locus. The open reading frames $p16^{Ink4a}$ (in black) and $p19^{Arf}$ (in crosshatch) are shown. Each has a unique first exon that then splices to a common second exon, but in alternate reading frames. $P16^{Ink4a}$ inhibits cdk4/6 activity producing retinoblastoma phosphorylation, which induces cell cycle arrest. $P19^{Arf}$ inhibits MDM2-mediated degradation of p53 (Sharpless, 2005).

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF PHENOLPHTHALEIN

Phenolphthalein was obtained from Aldrich Chemical Company (Milwaukee, WI) in one lot (13427LF). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC), Galbraith Laboratories, Inc. (Knoxville, TN), and the study laboratory, Battelle Columbus Operations (Columbus, OH) (Appendix E). Reports on analyses performed in support of the phenolphthalein study are on file at the National Institute of Environmental Health Sciences.

The chemical, a light brown/yellowish-white powder, was identified as phenolphthalein by the analytical chemistry laboratory using direct probe mass spectrometry and infrared, ultraviolet/visible, and proton nuclear magnetic resonance spectroscopy and by the study laboratory using infrared spectroscopy. The melting point range was consistent with a literature value (*Merck*, 1989).

The moisture content of lot 13427LF was determined by Galbraith Laboratories, Inc., using Karl Fischer titration; this laboratory also performed elemental analyses of lot 13427LF. The purity of lot 13427LF was determined by the analytical chemistry laboratory and by the study laboratory using high-performance liquid chromatography (HPLC). For lot 13427LF, Karl Fischer titration indicated 0.507% water. Elemental analyses for carbon, hydrogen, and oxygen were consistent with the theoretical values for phenolphthalein. HPLC analyses indicated one major peak and one impurity of 0.4%. The overall purity of lot 13427LF was determined to be greater than 99%.

Stability studies of the bulk chemical were performed by the analytical chemistry laboratory using HPLC. These studies indicated that phenolphthalein was stable as a bulk chemical for at least 14 days when stored protected from light at temperatures up to 60° C. To ensure stability, the bulk chemical was stored in the original shipping containers at room temperature, protected from light. Stability was monitored during the study using HPLC; no degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared approximately every 4 weeks by mixing phenolphthalein with feed (Table E2). A premix was prepared by hand and then blended with additional feed in a Patterson-Kelley twin-shell blender for 15 minutes using an intensifier bar for the initial 5 minutes. Formulations were stored in plastic buckets at –20° C or below for up to 42 days, with the exception of formulations prepared on November 29, 1999, which were originally stored at room temperature for 2 days and then transferred to –20° C storage.

Homogeneity studies of the 200 and 12,000 ppm dose formulations were performed by the analytical chemistry and study laboratories using HPLC. Stability studies of the 200 ppm dose formulation were performed by the analytical chemistry laboratory using HPLC. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in sealed glass bottles protected from light at –20° C and for at least 7 days under simulated animal room conditions (exposed to light and air) in the absence of animal excreta.

Periodic analyses of the dose formulations of phenolphthalein were conducted by the study laboratory using HPLC. All 15 dose formulations analyzed and used in the study were within 10% of the target concentrations; the dose formulations prepared on November 29, 1999, were not adversely affected by storage at room temperature for 2 days (Table E3). Animal room samples of these dose formulations were also analyzed; two of five animal room samples were within 10% of the target concentrations. Degradation was attributed to unavoidable excreta in the feeders.

27-Week Study

Study Design

Groups of 15 male and 15 female mice were fed diets containing 0, 200, 375, 750, 3,000, or 12,000 ppm phenolphthalein for 27 weeks. The exposure concentrations selected for use in the 27-week phenolphthalein study were chosen to overlap those used for $p53^{+/-}$ mice in a phenolphthalein feed study (Dunnick *et al.*, 1997) and for $B6C3F_1$ mice in a 2-year feed study of phenolphthalein (NTP, 1996).

Source and Specification of Animals

Male and female heterozygous B6.129-Cdkn2a^{tm1Rdp} N2 (i.e., haploinsufficient p16^{Ink4a}/p19^{Arf}) mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY) for use in the 27-week study. The N1 male mice homozygous null for the Cdkn2a deletion were backcrossed to inbred C57BL/6 females from Taconic Laboratory to produce male and female B6.129-Cdkn2^{atm1Rdp} haploinsufficient or p16^{Inka}/p19$^{Arf+/-}$ mice (Serrano *et al.*, 1996). The genetic background of these mice was: 80% C57BL/6, 19% 129/Sv, and 1% SJL. This line, designated 5003 by Taconic Laboratory, was embryo cryopreserved in 2003. Upon receipt, the mice were 4 weeks old. Animals were quarantined for 14 days and were 6 weeks old on the first day of the study. Before the study began, five male and five female mice were randomly selected for parasite evaluation and gross observation for evidence of disease. Blood samples were collected from four male and four female sentinel animals at 5 weeks and at study termination. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b). All results were negative.

Animal Maintenance

Mice were housed individually. Feed and water were available *ad libitum*. Cages and racks were rotated every 2 weeks. Further details of animal maintenance are given in Table 2.

Clinical Examinations and Pathology

All mice were observed twice daily. Clinical findings were recorded weekly. The mice were weighed initially, weekly, and at the end of the study.

At the end of the 27-week study, samples were collected from all male mice for sperm motility evaluations. The parameters evaluated are listed in Table 2. 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. Modified Tyrode's buffer 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 mice. The heart, right kidney, liver, lung, right testis, and thymus were weighed. 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, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin for microscopic examination. Histopathologic examinations were performed on all mice. Table 2 lists the tissues and organs routinely examined.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy; the slide and tissue counts were verified; and the histotechnique was evaluated. For the 27-week study, a quality assessment pathologist evaluated slides from all tumors and all potential target organs, which included the epididymis, kidney, ovary, spleen, testis, and thymus.

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

TABLE 2
Experimental Design and Materials and Methods in the 27-Week Feed Study of Phenolphthalein

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

Strain and Species
Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice (Heterozygous B6.129-$Cdkn2a^{tm1Rdp}$ N3)

Animal Source
Taconic Laboratory Animals and Services (Germantown, NY)

Time Held Before Study
14 days

Average Age When Studies Began
6 weeks

Date of First Exposure
December 8, 1999

Duration of Exposure
7 days/week for 27 weeks

Date of Last Exposure
June 7-9, 2000

Necropsy Dates
June 7-9, 2000

Average Age at Necropsy
32 to 33 weeks

Size of Study Groups
15 males and 15 females

Method of Distribution
Animals were distributed randomly into groups of approximately equal initial mean body weights.

Animals per Cage
1

Table 2
Experimental Design and Materials and Methods in the 27-Week Feed Study of Phenolphthalein

Method of Animal Identification
Tail tattoo and ear tag

Diet
Irradiated NTP-2000 pelleted feed (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*

Water
Tap water (City of Columbus municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*

Cages
Polycarbonate (Lab Products, Inc., Maywood, NJ), changed weekly

Racks
Stainless steel (Lab Products, Inc., Maywood, NJ), changed every 2 weeks

Bedding
Irradiated Sani-Chips® (P.J. Murphy Forest Products Corp., Montville, NJ), changed weekly

Cage Filters
DuPont 2024 spun-bonded polyester (Snow Filtration Co., Cincinnati, OH), changed every 2 weeks

Animal Room Environment
Temperature: 72° ± 3° F
Relative humidity: 50% ± 15%
Room fluorescent light: 12 hours/day
Room air changes: at least 10/hour

Exposure Concentrations
0, 200, 375, 750, 3,000, or 12,000 ppm in feed, available *ad libitum*

Type and Frequency of Observation
Observed twice daily; animals were weighed initially, weekly, and at the end of the study; clinical findings were recorded weekly.

Method of Sacrifice
CO_2 asphyxiation

Necropsy
Necropsies were performed on all animals. Organs weighed were the heart, right kidney, liver, lung, right testis, and thymus.

Histopathology
Histopathology was performed on all mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, large intestine (colon, cecum, rectum), small intestine (duodenum, ileum, jejunum), heart, kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, ovary, pituitary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis, thymus, thyroid gland, urinary bladder, and uterus.

Sperm Motility
At the end of the study, sperm samples were collected from all male mice for sperm motility evaluations. Spermatid and sperm measurements were evaluated. The left cauda, left epididymis, and left testis were weighed.

Statistical Methods

Survival Analyses

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

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions are presented in Tables A1, A2, A3, and A4 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. The Fisher exact test, a procedure based on the overall proportion of affected animals, was used to determine significance (Gart *et al.*, 1979).

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between exposed 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). 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. Average severity values were analyzed for significance with the Mann-Whitney U test (Hollander and Wolfe, 1973). Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for simultaneous equality of measurements across exposure concentrations.

Quality Assurance Methods

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

Genetic Toxicology

Mouse Peripheral Blood Micronucleus Test Protocol

A detailed discussion of this assay is presented by MacGregor *et al.* (1990). At 6.5, 13, 19.5, and 27 weeks, peripheral blood samples were obtained from male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes (NCEs) in each of up to 15 animals per dose group. In addition, the percentage of polychromatic erythrocytes among 1,000 total erythrocytes was determined for each animal as a measure of phenolphthalein-induced 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 exposure groups using a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each exposed group and the control group (ILS, 1990). In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the

trend test P value is less than or equal to 0.025 or if the P value for any single exposed group is less than or equal to 0.025 divided by the number of exposed groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Results of the interim samplings and the final sample at 27 weeks were accepted without repeat tests, because additional test data were not available. Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, reproducibility of any effects observed, and the magnitudes of those effects.

RESULTS

Mice

27-Week Study

Survival

Estimates of 27-week survival probabilities for male and female mice are shown in Table 3. Survival of all exposed groups of male and female mice was similar to that of the control groups.

Body Weights, Feed and Compound Consumption, Organ Weights, Clinical Findings, and Sperm Evaluation

Mean body weights of males in the 12,000 ppm group were less than those of the control group after week 11 (Figure 3 and Table 4). Mean body weights of exposed females were similar to those of the control group throughout the study (Figure 3 and Table 5). No differences in feed consumption were noted between exposed and control groups (Tables F1 and F2). Dietary concentrations of 200, 375, 750, 3,000, and 12,000 ppm delivered average daily doses of approximately 35, 65, 135, 540, and 2,170 mg/kg to males and 50, 90, 170, 680, and 2,770 mg/kg to females. No clinical findings related to phenolphthalein exposure were observed in males or females.

No exposure-related changes in organ weights were observed for females (Table C2). Compared to the control group, the absolute and relative right and left testis weights of 3,000 and 12,000 ppm males were significantly less (Tables C1 and D1). The left epididymis and left testis weights of 3,000 and 12,000 ppm males and the left cauda epididymis weight of 12,000 ppm males were significantly less than those of the controls.

In 12,000 ppm males, sperm motility and the numbers of spermatid heads per testis and sperm heads per cauda and per gram cauda epididymis were significantly less than those of the control groups; except for motility, these parameters were also significantly less in the 3,000 ppm group.

Table 3
Survival of Haploinsufficient p16[Ink4a]/p19[Arf] Mice in the 27-Week Feed Study of Phenolphthalein

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Male						
Animals initially in study	15	15	15	15	15	15
Moribund	1	0	0	0	0	0
Natural deaths	1	0	0	2	0	1
Animals surviving to study termination	13	15	15	13	15	14
Percent probability of survival at end of study[a]	87	100	100	87	100	93
Mean survival (days)[b]	165	183	183	163	183	173
Survival analysis[c]	P=1.000N	P=0.464N	P=0.464N	P=1.000	P=0.464N	P=0.984N
Female						
Animals initially in study	15	15	15	15	15	15
Moribund	0	0	1	0	0	0
Natural deaths	0	2	0	0	1	0
Animals surviving to study termination	15	13	14	15	14	15
Percent probability of survival at end of study	100	87	93	100	93	100
Mean survival (days)	184	162	173	184	174	184
Survival analysis	P=0.746N	P=0.464	P=1.000	—[d]	P=1.000	—

[a] Kaplan-Meier determinations
[b] Mean of all deaths (uncensored, censored, and terminal sacrifice)
[c] The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed group columns. A negative trend or lower mortality in an exposed group is indicated by **N**.
[d] Value of statistic cannot be computed.

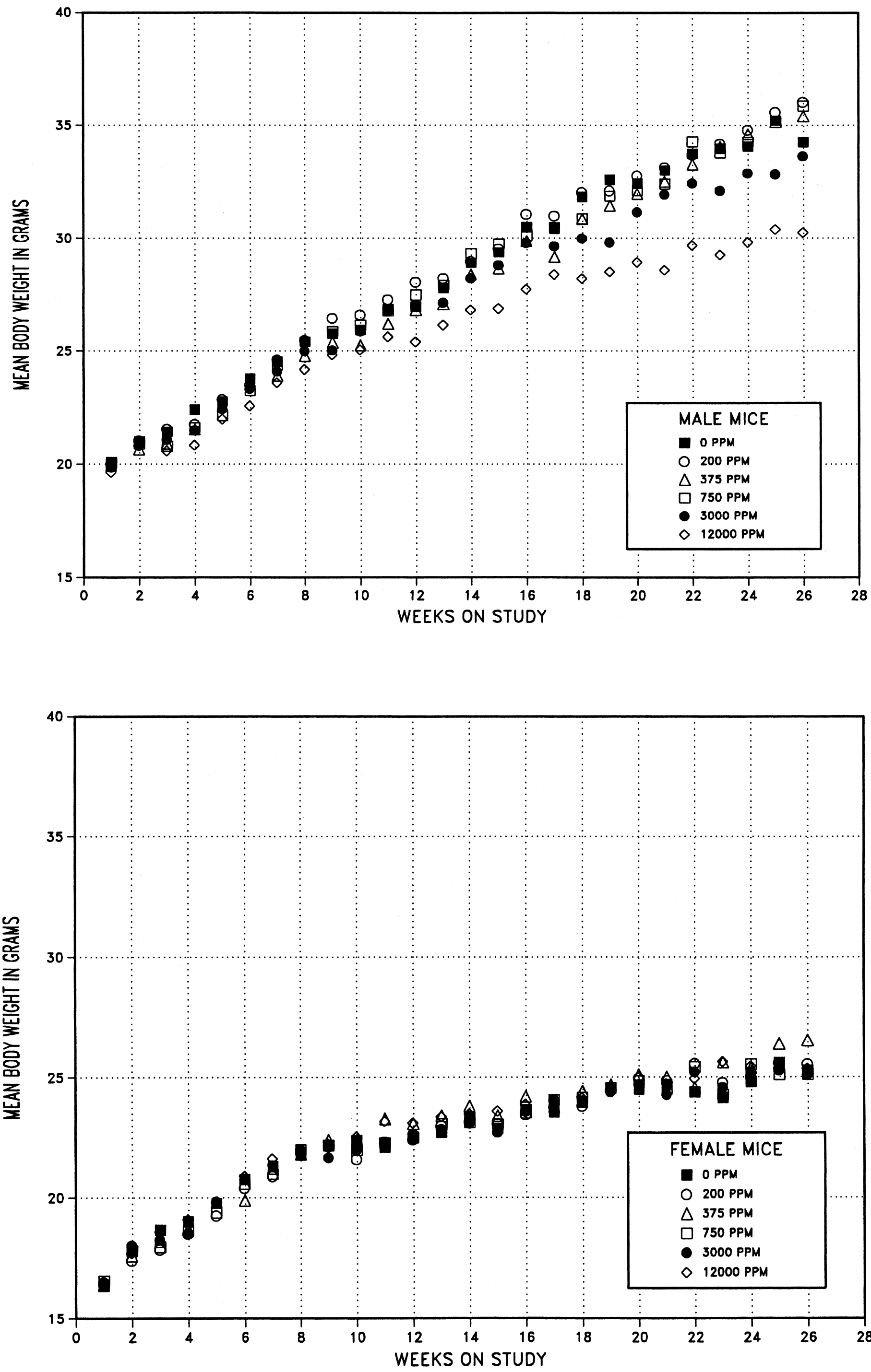


FIGURE 3
Growth Curves for Male and Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice Exposed to Phenolphthalein in Feed for 27 Weeks

Table 4
Mean Body Weights and Survival of Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein

Weeks on Study	0 ppm		200 ppm			375 ppm			750 ppm		
	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	20.1	15	20.0	100	15	19.9	99	15	20.0	100	15
2	21.0	15	21.0	100	15	20.7	99	15	20.9	100	15
3	21.4	15	21.6	101	15	20.9	98	15	20.8	97	15
4	22.4	14	21.8	97	15	21.5	96	15	21.6	96	14
5	22.8	14	22.9	100	15	22.6	99	15	22.2	97	14
6	23.8	14	23.5	99	15	23.5	99	15	23.3	98	14
7	24.5	14	24.6	100	15	23.9	98	15	24.4	100	13
8	25.4	14	25.5	100	15	24.8	98	15	25.4	100	13
9	25.8	14	26.4	102	15	25.4	98	15	25.9	100	13
10	25.9	14	26.6	103	15	25.2	97	15	26.2	101	13
11	26.9	14	27.3	102	15	26.2	97	15	26.8	100	13
12	27.0	13	28.0	104	15	26.8	99	15	27.5	102	13
13	27.8	13	28.2	101	15	27.1	98	15	27.9	100	13
14	28.9	13	29.0	100	15	28.4	98	15	29.3	101	13
15	29.4	13	29.5	100	15	28.7	98	15	29.8	101	13
16	30.5	13	31.1	102	15	29.9	98	15	30.1	99	13
17	30.4	13	31.0	102	15	29.2	96	15	30.5	100	13
18	31.8	13	32.0	101	15	30.9	97	15	30.9	97	13
19	32.6	13	32.1	99	15	31.4	96	15	31.9	98	13
20	32.4	13	32.8	101	15	32.0	99	15	32.1	99	13
21	33.0	13	33.1	100	15	32.5	99	15	32.4	98	13
22	33.7	13	33.7	100	15	33.3	99	15	34.3	102	13
23	34.0	13	34.2	101	15	34.1	100	15	33.8	99	13
24	34.1	13	34.8	102	15	34.6	102	15	34.3	101	13
25	35.2	13	35.6	101	15	35.2	100	15	35.1	100	13
26	34.3	13	36.0	105	15	35.4	103	15	35.9	105	13
Mean for weeks											
1-13	24.2		24.4	101		23.7	98		24.1	99	
14-27	32.3		32.7	101		32.0	99		32.3	100	

Table 4
Mean Body Weights and Survival of Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein

Weeks on Study	3,000 ppm			12,000 ppm		
	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	19.9	99	15	19.6	98	15
2	20.8	99	15	20.8	99	15
3	21.1	99	15	20.6	96	15
4	21.5	96	15	20.8	93	15
5	22.4	98	15	22.0	97	15
6	23.3	98	15	22.6	95	14
7	24.1	98	15	23.6	96	14
8	25.0	98	15	24.2	95	14
9	25.0	97	15	24.8	96	14
10	25.9	100	15	25.0	97	14
11	26.8	100	15	25.6	95	14
12	27.0	100	15	25.4	94	14
13	27.1	98	15	26.2	94	14
14	28.2	98	15	26.8	93	14
15	28.8	98	15	26.9	92	14
16	29.8	98	15	27.7	91	14
17	29.6	97	15	28.4	93	14
18	30.0	94	15	28.2	89	14
19	29.8	91	15	28.5	87	14
20	31.1	96	15	28.9	89	14
21	31.9	97	15	28.6	87	14
22	32.4	96	15	29.7	88	14
23	32.1	94	15	29.3	86	14
24	32.9	97	15	29.8	87	14
25	32.8	93	15	30.4	86	14
26	33.6	98	15	30.3	88	14
Mean for weeks						
1-13	23.8	98		23.2	96	
14-27	31.0	96		28.7	89	

TABLE 5
Mean Body Weights and Survival of Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein

Weeks on Study	0 ppm		200 ppm			375 ppm			750 ppm		
	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	16.4	15	16.4	100	15	16.4	100	15	16.5	101	15
2	17.8	15	17.4	98	15	17.6	99	15	17.8	100	15
3	18.7	15	17.8	95	14	18.2	97	14	17.9	96	15
4	19.0	15	18.5	97	13	18.7	98	14	18.6	98	15
5	19.8	15	19.3	98	13	19.8	100	14	19.4	98	15
6	20.8	15	20.4	98	13	19.9	96	14	20.6	99	15
7	21.3	15	20.9	98	13	21.2	100	14	21.0	99	15
8	21.8	15	21.9	101	13	21.8	100	14	22.0	101	15
9	22.2	15	22.1	100	13	22.4	101	14	22.2	100	15
10	22.4	15	21.6	96	13	22.4	100	14	22.0	98	15
11	22.1	15	22.3	101	13	23.3	105	14	22.3	101	15
12	22.5	15	22.4	100	13	23.1	103	14	22.5	100	15
13	22.7	15	23.0	101	13	23.4	103	14	23.1	102	15
14	23.4	15	23.1	99	13	23.8	102	14	23.1	99	15
15	22.9	15	23.1	101	13	23.4	102	14	23.1	101	15
16	23.7	15	23.5	99	13	24.2	102	14	23.5	99	15
17	23.6	15	23.8	101	13	23.8	101	14	24.1	102	15
18	24.0	15	23.8	99	13	24.4	102	14	24.2	101	15
19	24.5	15	24.4	100	13	24.7	101	14	24.6	100	15
20	24.5	15	25.0	102	13	25.1	102	14	24.8	101	15
21	24.7	15	24.8	100	13	25.0	101	14	24.5	99	15
22	24.4	15	25.6	105	13	24.6	101	14	25.4	104	15
23	24.2	15	24.8	103	13	25.6	106	14	24.3	100	15
24	24.8	15	25.0	101	13	25.4	102	14	25.5	103	15
25	25.6	15	25.6	100	13	26.4	103	14	25.1	98	15
26	25.3	15	25.5	101	13	26.5	105	14	25.1	99	15
Mean for weeks											
1-13	20.6		20.3	99		20.6	100		20.5	99	
14-27	24.3		24.5	101		24.8	102		24.4	101	

TABLE 5
Mean Body Weights and Survival of Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein

Weeks on Study	3,000 ppm			12,000 ppm		
	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	16.5	101	15	16.5	101	15
2	18.0	101	15	17.7	99	15
3	18.2	97	15	18.6	100	15
4	18.5	97	15	19.1	101	15
5	19.8	100	14	19.8	100	15
6	20.8	100	14	20.9	101	15
7	21.4	101	14	21.6	101	15
8	22.0	101	14	22.0	101	15
9	21.7	98	14	22.2	100	15
10	22.0	98	14	22.5	100	15
11	22.3	101	14	23.2	105	15
12	22.7	101	14	23.1	103	15
13	22.8	100	14	23.4	103	15
14	23.1	99	14	23.5	100	15
15	22.7	99	14	23.6	103	15
16	23.6	100	14	23.9	101	15
17	24.1	102	14	24.0	102	15
18	24.0	100	14	24.1	100	15
19	24.4	100	14	24.5	100	15
20	24.7	101	14	25.0	102	15
21	24.3	98	14	24.7	100	15
22	25.2	103	14	24.9	102	15
23	24.6	102	14	25.6	106	15
24	25.2	102	14	25.5	103	15
25	25.3	99	14	25.4	99	15
26	25.1	99	14	25.4	100	15
Mean for weeks						
1-13	20.5	100		20.8	101	
14-27	24.3	100		24.6	101	

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of nonneoplastic lesions in the thymus, testis, epididymis, kidney, and spleen. Summaries of the incidences of neoplasms and nonneoplastic lesions are presented in Tables A1, A2, A3, and A4.

Thymus: Atypical hyperplasia was observed in 375, 3,000, and 12,000 ppm females, and the incidence in 12,000 ppm females was significantly greater than that in the controls (Tables 6 and A4). One 375 ppm male and one 3,000 ppm male had atypical hyperplasia (Tables 6 and A2). This lesion was characterized by the presence of a thymic lobule that had a moderate cell density, loss of corticomedullary organizational distinction, and sheets of large lymphocytes or other mononuclear cells. Atypical hyperplasia of the thymus has been described in $B6C3F_1$ and p53 deficient mice (Dunnick *et al.*, 1997) and characterized as a distinct proliferation of large, immature lymphocytes confined to one or both lobes of the thymus accompanied by an absence of distinct thymic corticomedullary junction. Often the affected lobe(s) were smaller than normal, and the lymphocytes did not extend beyond the thymic capsule. Atypical hyperplasia is sometimes preceded by thymic atrophy and believed to be a premalignant change of chemically-induced thymic lymphoma.

Testis and epididymis: Significantly increased incidences of atrophy of the seminiferous tubules and coinciding hyperplasia of the testicular interstitial (Leydig) cells and epididymal hypospermia occurred in 3,000 and 12,000 ppm males (Tables 6 and A2). Testis atrophy was characterized by smaller testis profiles, decreased numbers of germinal epithelial cells, individualization or separation of germinal epithelial cells, vacuolization of germinal epithelial cell cytoplasm, and syncytial cell formation. The epididymis from affected testicles had hypospermia (decrease intraluminal sperm) with decreased lumen profiles. The lumens sometimes contained desquamated germinal epithelial cells and increased hyaline protein.

Kidney: Significantly increased incidences of minimal to mild nephropathy occurred in 3,000 and 12,000 ppm males, and the severity was slightly increased in the 12,000 ppm group (Tables 6 and A2). Nephropathy was consistent with the spontaneous condition described in various mouse strains and characterized by cortical foci of regenerative tubular epithelium (basophilic tubules with nuclear crowding) with varying degrees of chronic interstitial inflammation and fibrosis, tubular dilation, tubular epithelial cell atrophy, protein casts, desquamated renal tubular epithelium, and eosinophilic tubular debris.

There were increased incidences of minimal hypertrophy of renal tubules in the 750 ppm or greater male groups. Hypertrophy of renal tubules was characterized as occasional, scattered individual or clusters of enlarged tubular epithelium. Hypertrophy is an uncommon lesion in rodents that is sometimes observed as part of advanced nephropathy (which was not evident in this study) and not considered a preneoplastic or proliferative change (G. Hard, personal communication). In this setting, it was likely an adaptive physiological response, possibly related to altered sodium ion exchange.

Spleen: Significantly increased incidences of hematopoietic cell proliferation occurred in 12,000 ppm males and 375, 750, and 12,000 ppm females (Tables 6, A2, and A4). Increased numbers of hematopoietic cells, primarily erythroid series, composed the hematopoietic cell proliferation. Most instances were graded as minimal and consisted of increased cell density with no increase in splenic size. A few mice had slightly higher severity grades sometimes accompanied by an increased overall size of the splenic profile.

GENETIC TOXICOLOGY

The frequency of micronucleated normochromatic erythrocytes (NCEs) was assessed in male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice at 6.5, 13, 19.5, and 27 weeks of exposure to phenolphthalein. At all four time points, a significant increase in micronucleated NCEs was observed in both sexes (Table B1). At the 6.5-week sampling time, the mean micronucleus frequencies of the two highest exposure groups (3,000 and 12,000 ppm) of males and females were significantly increased over the controls (Table B1). The trend test was positive ($P<0.001$). At all subsequent sampling times, only the 12,000 ppm groups showed significant increases in micronucleated NCEs compared to the controls. No significant alterations in the percentage of polychromatic erythrocytes were seen in male or female mice at any sampling time, indicating an absence of phenolphthalein-induced toxicity to the bone marrow.

Table 6
Incidences of Selected Nonneoplastic Lesions in Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Male						
Thymus[a]	14	15	15	14	15	14
Hyperplasia, Atypical[b]	0	0	1 (2.0)[c]	0	1 (3.0)	0
Testes	15	15	15	15	15	15
Germinal Epithelium, Atrophy	0	1 (1.0)	1 (4.0)	0	15** (1.6)	14** (2.6)
Interstitial Cell, Hyperplasia	0	1 (1.0)	1 (2.0)	0	15** (1.5)	14** (1.8)
Epididymis	15	15	15	15	15	15
Hypospermia	0	0	1 (4.0)	0	15** (1.1)	14** (3.0)
Kidney	14	15	15	14	15	15
Nephropathy	6 (1.0)	7 (1.0)	8 (1.0)	6 (1.0)	15** (1.0)	14** (1.4)
Renal Tubule, Hypertrophy	0	0	0	10** (1.0)	15** (1.0)	14** (1.0)
Spleen	14	15	15	14	15	14
Hematopoietic Cell Proliferation	2 (1.0)	5 (1.4)	3 (2.0)	1 (1.0)	2 (1.0)	14** (1.0)
Female						
Thymus	15	15	15	15	14	15
Hyperplasia, Atypical	0	0	1 (3.0)	0	3 (2.0)	5* (2.6)
Spleen	15	14	14	15	15	15
Hematopoietic Cell Proliferation	2 (1.0)	5 (1.0)	9** (1.1)	8* (1.0)	7 (1.3)	13** (1.0)

* 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 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

DISCUSSION AND CONCLUSIONS

Phenolphthalein is a bisphenolic compound that was used as a laxative for most of the 20th century. NTP studies have shown that phenolphthalein exposure causes carcinogenic effects in F344/N rats and $B6C3F_1$ mice after a 2-year exposure period and in haploinsufficient p53 mice after a 6-month exposure period (NTP, 1996; Dunnick *et al.*, 1997).

No treatment-related neoplasms were identified in this study. However, a putative precursor lesion, atypical hyperplasia of the thymus, occurred at increased incidences in exposed haploinsufficient $p16^{Ink4a}/p19^{Arf}$ female (9/74) and male (2/73) mice. Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice remain relatively free of spontaneous tumors at 8 months of age, the time frame in which this study was completed. However, by 11 months of age (Appendix G), tumors including malignant lymphoma begin to occur spontaneously. Atypical hyperplasia of the thymus is an uncommon lesion in mice and reported in only a few strains, including B6.129-p53 deficient (N5) and $B6C3F_1$ mice. Atypical hyperplasia of lymphocytes and/or malignant lymphoma involving the thymus were induced by phenolphthalein in haploinsufficient p53 and $B6C3F_1$ mice (NTP, 1996; Dunnick *et al.*, 1997) and by benzene in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice (NTP, 2007a). The cases of atypical hyperplasia of the thymus occurring in exposed haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice in this study were morphologically similar to those previously described. Since atypical hyperplasia is considered a premalignant change of chemically induced thymic lymphoma (Dunnick, *et al.*, 1997), it is possible that with more time, some cases of atypical hyperplasia in this study may have evolved into malignant lymphoma.

Phenolphthalein was negative in the *Salmonella* mutagenicity assay (NTP, 1996), but it has been shown to induce micronucleated erythrocytes in a number of studies in different strains of mice (Dietz *et al.*, 1992; Witt *et al.*, 1995; Tice *et al.*, 1998; Stoll *et al*., 2006). Although significant increases in the frequencies of micronucleated erythrocytes were seen at each of four sampling time points in the 6-month study with phenolphthalein-exposed haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice, the response was diminished compared with the magnitude of the response induced by phenolphthalein in haploinsufficient p53 (Tice *et al.*, 1998; Figure 4) or $B6C3F_1$ mice (Dietz *et al.*, 1992). The majority of chemicals that induce micronuclei in mice *in vivo* have been shown to be rodent carcinogens (Witt *et al.*, 2000), and phenolphthalein was carcinogenic in both haploinsufficient p53 and $B6C3F_1$ mice.

Exposure of haploinsufficient p53 mice to phenolphthalein in the diet induced an exposure concentration-related increase in the incidence of lymphoma and highly significant increases in the frequency of micronucleated erythrocytes (Dunnick *et al.*, 1997; Tice *et al.*, 1998; Figure 4). In haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice in the present study, phenolphthalein induced increased incidences of atypical hyperplasia in the thymus and small but significant increases in micronucleated erythrocytes (Figure 4). It may be that the duration of the present study was insufficient to detect an increase in the incidences of lymphoma in this mouse model, or the magnitude of the difference in the micronucleus response between the two mouse models [haploinsufficient $p16^{Ink4a}/p19^{Arf}$ (80% C57Bl/6 background) and haploinsufficient p53 (97% C57Bl/6 background)] is biologically significant and predictive of the tumor response.

Ink4a inhibits Cdk4/Cdk6 and phosphorylation of Rb resulting in G1/S arrest in response to DNA damage (Shapiro *et al.*, 1998, 2000). Thus, $p16^{Ink4a}$ deficiency compromises the ability to arrest the cell cycle in response to direct DNA damage and repair. A decrease in Arf may allow Mdm2 to bind more readily to p53, allowing for degradation of p53, and suppression of p53's ability to promote G1/S arrest and induce apoptosis and mitotic spindle checkpoint functions (Kurokawa *et al.*, 1999; Weber *et al.*, 1999). The indirect effect on p53 function may not be as critical to maintaining cell function when it is mediated through loss of $p19^{Arf}$ function in this mouse model as direct loss of p53 function in the p53 haploinsufficient mouse. Thus, the haploinsufficiency in the $p16^{Ink4a}/p19^{Arf}$ deficient mouse compromises both the Ink4a-Rb and the Arf-p53 signaling

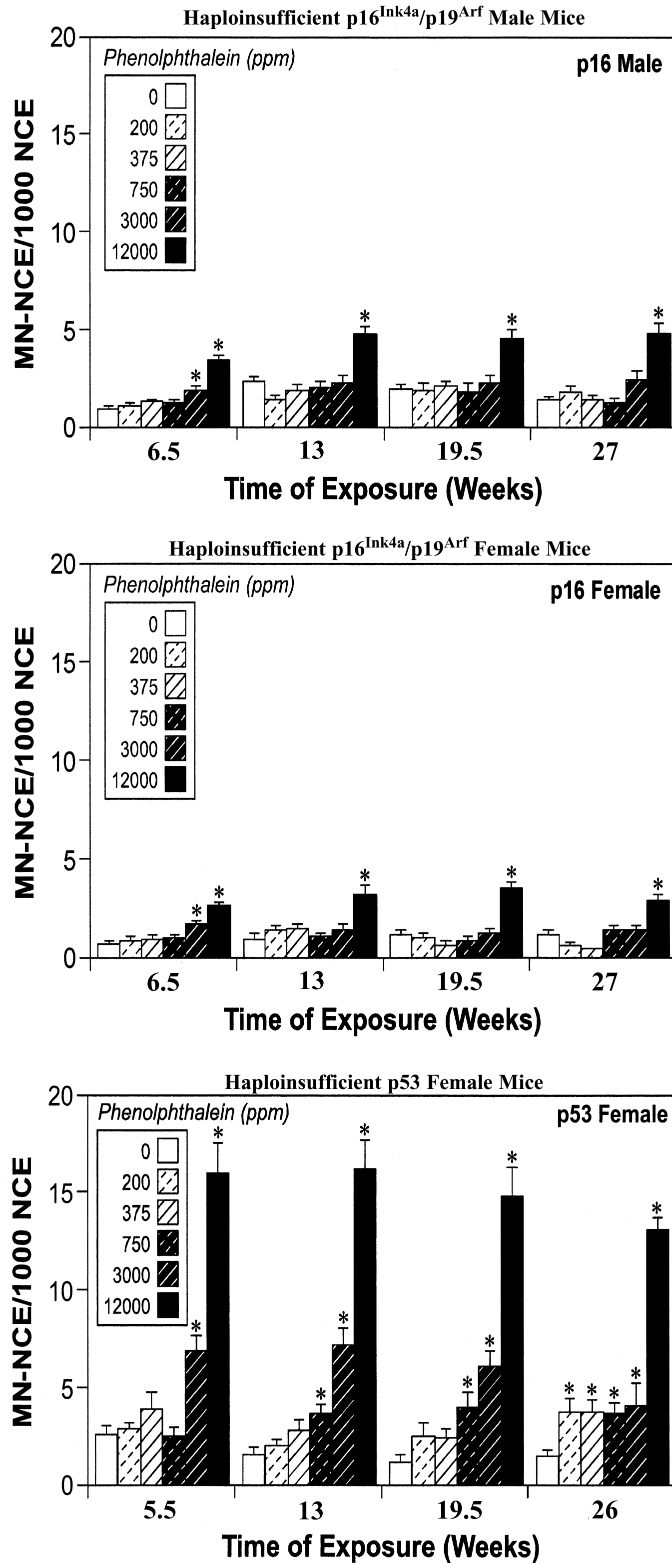


FIGURE 4
Micronucleus Results Comparison Between Haploinsufficient p16^{Ink4a}/p19^{Arf} Male and Female Mice and Haploinsufficient p53 Female Mice Exposed to Phenolphthalein

pathways and, thus, affects both the G1/S cell cycle checkpoint through p16^{Ink4a} regulation of Rb and p19^{Arf} regulation of p53 through Mdm2 and, to a lesser extent, the mitotic spindle checkpoints in response to DNA and mitotic spindle damage through Arf-p53.

The p53 tumor suppressor protein is critical for both the G1/S cell cycle and apoptosis and mitotic spindle checkpoint functions, and loss of p53 function may increase aneuploidy (non-disjunction and chromosome duplication) by failing to resolve anaphase bridges (centromere division) (Cross *et al.*, 1995; Schwartz and Rotter, 1998; Meek, 2000; Fang *et al.*, 2006). The observed magnitude of the difference in micronuclei and the specific induction of kinetechore positive micronuclei in the phenolphthalein studies in the haploinsufficient p53 mouse may be explained by the critical role of p53 in the mitotic spindle checkpoint and prevention of phenolphthalein-induced aneuploidy (Tice *et al.*, 1998). Thus, the mode of action of phenolphthalein-induced DNA and chromosomal damage that results in non-disjunction and aneuploidy may be less influenced by the p16-RB and p19Arf-p53 pathway functions than the p53 function, and/or differences in genetic composition may explain the biological differences observed between these two mouse models.

In the current study, phenolphthalein induced other toxic effects in the haploinsufficient p16^{Ink4a}/p19^{Arf} mouse including nephropathy and reproductive toxicity in male mice. Phenolphthalein-induced toxicity to the male reproductive system has previously been observed in B6C3F$_1$ mice after 13-week and 2-year exposures (NTP, 1996). In the current haploinsufficient p16^{Ink4a}/p19^{Arf} mouse study, phenolphthalein induced male target organ toxicity at 3,000 or 12,000 ppm, as demonstrated by lower epididymis and testis weights and decreased sperm motility and numbers at 12,000 ppm. In the 13-week B6C3F$_1$ mouse study, lower epididymal weights and sperm density were seen at doses of 12,000 ppm or greater (NTP, 1996). In the 2-year B6C3F$_1$ mouse study, degeneration of the testis was seen in all exposed groups (3,000, 6,000, and 12,000 ppm). In a continuous breeding protocol, phenolphthalein caused lower fertility in CD-1 mice at concentrations of 700 ppm or greater (NTP, 1996).

Phenolphthalein administered in the feed for 2 years induced significant increases in the incidences of histiocytic sarcoma and lymphoma of thymic origin in male and female B6C3F$_1$ mice; malignant lymphoma and benign ovarian sex cord stromal tumors in female B6C3F$_1$ mice; benign pheochromocytomas of the adrenal medulla in male and female F344/N rats; and renal tubule adenoma in male F344/N rats (NTP, 1996). Phenolphthalein induced thymic lymphomas in haploinsufficient p53 female mice (Dunnick *et al.*, 1997). Phenolphthalein induced preneoplastic lesions in the thymus of haploinsufficient p16^{Ink4a}/p19^{Arf} mice in the current study.

CONCLUSIONS

Under the conditions of this 27-week feed study, there was *no evidence of carcinogenic activity** of phenolphthalein in male or female haploinsufficient p16^{Ink4a}/p19^{Arf} mice exposed to 200, 375, 750, 3,000, or 12,000 ppm. Because this is a new model, there is uncertainty whether the study possessed sufficient sensitivity to detect a carcinogenic effect.

Phenolphthalein induced atypical hyperplasia, a preneoplastic lesion of the thymus, in male and female mice, hematopoietic cell proliferation of the spleen in male and female mice, and toxicity to the kidney and reproductive system in male mice.

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

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APPENDIX A SUMMARY OF LESIONS IN HAPLOINSUFFICIENT p16^{Ink4a}/p19^{Arf} MICE IN THE 27-WEEK FEED STUDY OF PHENOLPHTHALEIN

TABLE A1 Summary of the Incidence of Neoplasms in Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein 48

TABLE A2 Summary of the Incidence of Nonneoplastic Lesions in Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein 50

TABLE A3 Summary of the Incidence of Neoplasms in Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein 52

TABLE A4 Summary of the Incidence of Nonneoplastic Lesions in Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein 54

Table A1
Summary of the Incidence of Neoplasms in Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein[a]

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Disposition Summary						
Animals initially in study	15	15	15	15	15	15
Early deaths						
Moribund	1					
Natural deaths	1			2		1
Survivors						
Terminal sacrifice	13	15	15	13	15	14
Animals examined microscopically	15	15	15	15	15	15
Alimentary System						
Intestine large, colon	(14)	(15)	(15)	(14)	(15)	(14)
Intestine large, rectum	(14)	(15)	(15)	(14)	(15)	(14)
Intestine large, cecum	(14)	(15)	(15)	(14)	(15)	(14)
Intestine small, duodenum	(14)	(15)	(15)	(14)	(15)	(14)
Intestine small, jejunum	(14)	(15)	(15)	(14)	(15)	(14)
Intestine small, ileum	(14)	(15)	(15)	(14)	(15)	(14)
Liver	(15)	(15)	(15)	(14)	(15)	(14)
Histiocytic sarcoma			1 (7%)			
Stomach, forestomach	(14)	(15)	(15)	(15)	(15)	(14)
Stomach, glandular	(14)	(15)	(15)	(14)	(15)	(14)
Cardiovascular System						
Heart	(15)	(15)	(15)	(15)	(15)	(15)
Endocrine System						
Adrenal cortex	(14)	(15)	(15)	(14)	(15)	(14)
General Body System						
None						
Genital System						
Epididymis	(15)	(15	(15)	(15)	(15)	(15)
Hematopoietic System						
Bone marrow	(15)	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma			1 (7%)	1 (7%)		
Lymph node	(11)	(11)	(9)	(10)	(15)	(12)
Mediastinal, histiocytic sarcoma			1 (11%)			
Lymph node, mandibular	(14)	(15)	(15)	(14)	(15)	(13)
Lymph node, mesenteric	(14)	(15)	(15)	(14)	(15)	(14)
Spleen	(14)	(15)	(15)	(14)	(15)	(14)
Thymus	(14)	(15)	(15)	(14)	(15)	(14)

TABLE A1
Summary of the Incidence of Neoplasms in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Feed Study of Phenolphthalein

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Integumentary System						
Skin	(15)	(15)	(15)	(14)	(15)	(15)
Musculoskeletal System						
None						
Nervous System						
Brain	(15)	(15)	(15)	(14)	(15)	(14)
Respiratory System						
Lung	(15)	(15)	(15)	(14)	(15)	(15)
Histiocytic sarcoma			1 (7%)			
Special Senses System						
None						
Urinary System						
Kidney	(14)	(15)	(15)	(14)	(15)	(15)
Urinary bladder	(14)	(15)	(15)	(14)	(15)	(14)
Systemic Lesions						
Multiple organs[b]	(15)	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma			1 (7%)	1 (7%)		
Lymphoma malignant	1 (7%)					
Neoplasm Summary						
Total animals with primary neoplasms[c]	1		1	1		
Total primary neoplasms	1		1	1		
Total animals with malignant neoplasms	1		1	1		
Total malignant neoplasms	1		1	1		

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

Table A2
Summary of the Incidence of Nonneoplastic Lesions in Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein[a]

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Disposition Summary						
Animals initially in study	15	15	15	15	15	15
Early deaths						
Moribund	1					
Natural deaths	1			2		1
Survivors						
Terminal sacrifice	13	15	15	13	15	14
Animals examined microscopically	15	15	15	15	15	15
Alimentary System						
Liver	(15)	(15)	(15)	(14)	(15)	(14)
Hematopoietic cell proliferation			1 (7%)			
Inflammation, chronic	11 (73%)	8 (53%)	7 (47%)	9 (64%)	10 (67%)	5 (36%)
Mesentery		(1)				
Fat, necrosis		1 (100%)				
Stomach, glandular	(14)	(15)	(15)	(14)	(15)	(14)
Epithelium, cyst				1 (7%)		
Cardiovascular System						
None						
Endocrine System						
Adrenal cortex	(14)	(15)	(15)	(14)	(15)	(14)
Hyperplasia, focal				4 (29%)	1 (7%)	
Hypertrophy, focal	4 (29%)	3 (20%)	1 (7%)	1 (7%)	2 (13%)	
Thyroid gland	(14)	(15)	(15)	(14)	(15)	(15)
Infiltration cellular, polymorphonuclear			1 (7%)			
General Body System						
None						
Genital System						
Epididymis	(15)	(15	(15)	(15)	(15)	(15)
Hypospermia			1 (7%)		15 (100%)	14 (93%)
Inflammation, chronic				1 (7%)		
Inflammation, granulomatous			1 (7%)			
Mineralization		1 (7%)				
Testes	(15)	(15)	(15)	(15)	(15)	(15)
Germinal epithelium, atrophy		1 (7%)	1 (7%)		15 (100%)	14 (93%)
Interstitial cell, hyperplasis		1 (7%)	1 (7%)		15 (100%)	14 (93%)

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

Table A2
Summary of the Incidence of Nonneoplastic Lesions in Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Hematopoietic System						
Bone marrow	(15)	(15)	(15)	(15)	(15)	(15)
Hyperplasia		1 (7%)				
Lymph node, mandibular	(14)	(15)	(15)	(14)	(15)	(13)
Hyperplasia, lymphoid	2 (14%)	1 (7%)	2 (13%)		3 (20%)	1 (8%)
Lymph node, mesenteric	(14)	(15)	(15)	(14)	(15)	(14)
Atrophy		1 (7%)	1 (7%)	1 (7%)		
Hyperplasia, lymphoid			1 (7%)		2 (13%)	1 (7%)
Spleen	(14)	(15)	(15)	(14)	(15)	(14)
Hematopoietic cell proliferation	2 (14%)	5 (33%)	3 (20%)	1 (7%)	2 (13%)	14 (100%)
Infiltration cellular, mononuclear cell	1 (7%)					
Pigmentation						1 (7%)
Capsule, fibrosis						1 (7%)
Lymphoid follicle, atrophy				1 (7%)		
Thymus	(14)	(15)	(15)	(14)	(15)	(14)
Atrophy, diffuse				1 (7%)		
Hyperplasia, atypical			1 (7%)		1 (7%)	
Hyperplasia, lymphoid			1 (7%)			
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(15)	(15)	(15)	(14)	(15)	(15)
Inflammation, chronic		2 (13%)		2 (14%)	1 (7%)	
Alveolar epithelium, hyperplasia						1 (7%)
Special Senses System						
None						
Urinary System						
Kidney	(14)	(15)	(15)	(14)	(15)	(15)
Nephropathy	6 (43%)	7 (47%)	8 (53%)	6 (43%)	15 (100%)	14 (93%)
Renal tubule, accumulation, hyaline droplet			1 (7%)			
Renal tubule, hypertrophy				10 (71%)	15 (100%)	14 (93%)

Table A3
Summary of the Incidence of Neoplasms in Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein[a]

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Disposition Summary						
Animals initially in study	15	15	15	15	15	15
Early deaths						
Moribund			1			
Natural deaths		2			1	
Survivors						
Terminal sacrifice	15	13	14	15	14	15
Animals examined microscopically	15	15	15	15	15	15
Alimentary System						
Liver	(15)	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)				
Pancreas		(1)				
Histiocytic sarcoma		1 (100%)				
Stomach, glandular	(15)	(14)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)				
Cardiovascular System						
None						
Endocrine System						
Adrenal medulla	(15)	(14)	(15)	(15)	(15)	(15)
Pheochromocytoma, benign					1 (7%)	
General Body System						
None						
Genital System						
Ovary	(15)	(14)	(15)	(15)	(14)	(15)
Histiocytic sarcoma		1 (7%)	2 (13%)			
Periovarian tissue, hystiocytic sarcoma						1 (7%)
Uterus	(15)	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)				
Sarcoma					1 (7%)	
Hematopoietic System						
Bone marrow	(15)	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)	1 (7%)	1 (7%)		
Lymph node	(13)	(10)	(13)	(14)	(13)	(13)
Deep cervical, histiocytic sarcoma		1 (10%)				
Lymph node, mandibular	(15)	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)				

TABLE A3
Summary of the Incidence of Neoplasms in Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Hematopoietic System (continued)						
Spleen	(15)	(14)	(14)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)				
Thymus	(15)	(15)	(15)	(15)	(14)	(15)
Histiocytic sarcoma		1 (7%)				
Integumentary System						
None						
Musculoskeletal System						
Bone		(1)				(1)
Histiocytic sarcoma		1 (100%)				
Skeletal muscle					(1)	
Histiocytic sarcoma					1 (100%)	
Nervous System						
None						
Respiratory System						
Lung	(15)	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)			1 (7%)	
Special Senses System						
None						
Urinary System						
Kidney	(15)	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)			1 (7%)	
Systemic Lesions						
Multiple organs[b]	(15)	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)	2 (13%)	1 (7%)	1 (7%)	1 (7%)
Neoplasm Summary						
Total animals with primary neoplasms[c]		1	2	1	2	1
Total primary neoplasms		1	2	1	4	1
Total animals with benign neoplasms					1	
Total malignant neoplasms					1	
Total animals with malignant neoplasms		1	2	1	1	1
Total malignant neoplasms		1	2	1	3	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 A4
Summary of the Incidence of Nonneoplastic Lesions in Female Haploinsufficient p16[Ink4a]/p19[Arf] Mice in the 27-Week Feed Study of Phenolphthalein[a]

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Disposition Summary						
Animals initially in study	15	15	15	15	15	15
Early deaths						
Moribund			1			
Natural deaths		2			1	
Survivors						
Terminal sacrifice	15	13	14	15	14	15
Animals examined microscopically	15	15	15	15	15	15
Alimentary System						
Intestine small, jejunum	(15)	(14)	(15)	(15)	(15)	(15)
Infiltration cellular, polymorphonuclear			1 (7%)			
Liver	(15)	(15)	(15)	(15)	(15)	(15)
Basophilic focus						1 (7%)
Inflammation, chronic	10 (67%)	8 (53%)	12 (80%)	10 (67%)	7 (47%)	8 (53%)
Necrosis, focal	1 (7%)					1 (7%)
Stomach, forestomach	(15)	(14)	(15)	(15)	(15)	(15)
Ulcer					1 (100%)	
Stomach, glandular	(15)	(14)	(15)	(15)	(15)	(15)
Hemorrhage					1 (7%)	
Cardiovascular System						
None						
Endocrine System						
Thyroid gland	(15)	(14)	(15)	(15)	(15)	(15)
Inflammation, granulomatous	1 (7%)					
General Body System						
None						
Genital System						
Ovary	(15)	(14)	(15)	(15)	(14)	(15)
Cyst	1 (7%)	1 (7%)			1 (7%)	1 (7%)
Uterus	(15)	(15)	(15)	(15)	(15)	(15)
Endometrium, hyperplasia, cystic	15 (100%)	12 (80%)	14 (93%)	15 (100%)	13 (87%)	14 (93%)
Vagina						(1)
Epithelium, hyperplasia						1 (100%)
Hematopoietic System						
Lymph node, mandibular	(15)	(15)	(15)	(15)	(15)	(15)
Atrophy		2 (13%)			2 (13%)	
Hematopoietic cell proliferation		1 (7%)				
Hemorrhage		1 (7%)				
Hyperplasia, lymphoid	4 (27%)	2 (13%)	2 (13%)		1 (7%)	6 (40%)

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

Table A4
Summary of the Incidence of Nonneoplastic Lesions in Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Hematopoietic System (continued)						
Lymph node, mesenteric	(15)	(14)	(15)	(15)	(15)	(15)
Atrophy	1 (7%)	1 (7%)	1 (7%)		2 (13%)	2 (13%)
Hematopoietic cell proliferation			1 (7%)			
Hyperplasia, lymphoid			1 (7%)			
Infiltration cellular, polymorphonuclear		1 (7%)				
Spleen	(15)	(14)	(14)	(15)	(15)	(15)
Hematopoietic cell proliferation	2 (13%)	5 (36%)	9 (64%)	8 (53%)	7 (47%)	13 (87%)
Lymphoid follicle, atrophy		1 (7%)			2 (13%)	
Lymphoid follicle, hyperplasia			1 (7%)			
Thymus	(15)	(15)	(15)	(15)	(14)	(15)
Atrophy, diffuse		1 (7%)	1 (7%)		1 (7%)	
Hyperplasia, atypical			1 (7%)		3 (21%)	5 (33%)
Hyperplasia, lymphoid	1 (7%)					
Integumentary System						
None						
Musculoskeletal System						
Bone		(1)				(1)
Hyperplasia		1 (100%)				
Nervous System						
Brain	(15)	(15)	(15)	(15)	(15)	(15)
Hemorrhage		1 (7%)				
Neuron, necrosis					1 (7%)	
Respiratory System						
Lung	(15)	(15)	(15)	(15)	(15)	(15)
Inflammation, chronic	2 (13%)	1 (7%)	1 (7%)	3 (20%)	1 (7%)	
Special Senses System						
None						
Urinary System						
Kidney	(15)	(15)	(15)	(15)	(15)	(15)
Hydronephrosis					1 (7%)	1 (7%)
Nephropathy	10 (67%)	6 (40%)	8 (53%)	8 (53%)	7 (47%)	11 (73%)
Renal tubule, mineralization	1 (7%)					
Ureter						(1)
Cyst						1 (100%)

APPENDIX B
GENETIC TOXICOLOGY

Table B1 **Frequency of Micronuclei in Peripheral Blood Erythrocytes of Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice Following Administration of Phenolphthalein in Feed for up to 27 Weeks** **58**

Table B1
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice Following Administration of Phenolphthalein in Feed for up to 27 Weeks[a]

Compound	Dose (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	P Value[c]	PCEs[b] (%)
Male					
6.5 Weeks					
Feed[d]		14	0.89 ± 0.09		1.757 ± 0.09
Phenolphthalein	200	15	0.97 ± 0.15	0.3854	1.813 ± 0.13
	375	15	1.20 ± 0.14	0.1271	1.700 ± 0.11
	750	13	1.19 ± 0.17	0.1400	1.785 ± 0.11
	3,000	15	1.83 ± 0.18	0.0011	2.240 ± 0.12
	12,000	14	3.32 ± 0.28	0.0000	2.121 ± 0.14
			P=0.000[e]		
13 Weeks					
Feed		13	2.23 ± 0.26		3.346 ± 0.34
Phenolphthalein	200	15	1.33 ± 0.23	0.9944	3.200 ± 0.36
	375	15	1.83 ± 0.25	0.8520	3.527 ± 0.34
	750	13	1.96 ± 0.30	0.7489	4.338 ± 0.49
	3,000	15	2.20 ± 0.35	0.5308	3.113 ± 0.30
	12,000	14	4.68 ± 0.44	0.0000	2.521 ± 0.23
			P=0.000		
19.5 Weeks					
Feed		13	1.88 ± 0.24		2.392 ± 0.16
Phenolphthalein	200	15	1.77 ± 0.39	0.5966	2.247 ± 0.16
	375	15	2.03 ± 0.24	0.3832	2.233 ± 0.19
	750	13	1.73 ± 0.42	0.6215	2.177 ± 0.21
	3,000	15	2.17 ± 0.40	0.2900	2.527 ± 0.13
	12,000	14	4.46 ± 0.49	0.0000	2.743 ± 0.19
			P=0.000		
27 Weeks					
Feed		13	1.35 ± 0.15		2.469 ± 0.16
Phenolphthalein	200	15	1.73 ± 0.31	0.1788	2.440 ± 0.22
	375	15	1.30 ± 0.27	0.5473	3.540 ± 1.12
	750	13	1.19 ± 0.23	0.6520	2.154 ± 0.21
	3,000	15	2.37 ± 0.43	0.0140	1.920 ± 0.08
	12,000	14	4.68 ± 0.57	0.0000	2.921 ± 0.29
			P=0.000		

Table B1
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice Following Administration of Phenolphthalein in Feed for up to 27 Weeks

Compound	Dose (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs	P Value	PCEs (%)
Female					
6.5 Weeks					
Feed		15	0.60 ± 0.16		2.307 ± 0.16
Phenolphthalein	200	13	0.81 ± 0.17	0.1764	1.977 ± 0.12
	375	14	0.89 ± 0.18	0.0977	1.714 ± 0.10
	750	15	0.93 ± 0.16	0.0701	1.940 ± 0.11
	3,000	14	1.61 ± 0.20	0.0001	2.107 ± 0.14
	12,000	15	2.57 ± 0.20	0.0000	2.213 ± 0.12
			P=0.000		
13 Weeks					
Feed		15	0.87 ± 0.26		2.013 ± 0.23
Phenolphthalein	200	13	1.31 ± 0.22	0.1018	1.877 ± 0.13
	375	14	1.39 ± 0.21	0.0652	2.250 ± 0.19
	750	15	0.97 ± 0.18	0.3732	1.693 ± 0.08
	3,000	14	1.29 ± 0.33	0.1088	1.907 ± 0.17
	12,000	15	3.13 ± 0.45	0.0000	2.180 ± 0.17
			P=0.000		
19.5 Weeks					
Feed		15	1.07 ± 0.25		2.153 ± 0.18
Phenolphthalein	200	13	0.96 ± 0.22	0.6216	2.315 ± 0.25
	375	14	0.57 ± 0.19	0.9504	2.307 ± 0.21
	750	15	0.80 ± 0.23	0.8026	1.787 ± 0.08
	3,000	14	1.18 ± 0.24	0.3744	2.257 ± 0.20
	12,000	15	3.40 ± 0.38	0.0000	2.073 ± 0.14
			P=0.000		
27 Weeks					
Feed		15	1.07 ± 0.22		2.333 ± 0.12
Phenolphthalein	200	13	0.50 ± 0.17	0.9909	2.523 ± 0.18
	375	14	0.36 ± 0.11	0.9992	2.700 ± 0.20
	750	15	1.30 ± 0.25	0.2029	2.553 ± 0.27
	3,000	14	1.32 ± 0.20	0.1869	4.057 ± 1.48
	12,000	15	2.83 ± 0.33	0.0000	2.793 ± 0.29
			P=0.000		

[a] Study was performed at SITEK Research Laboratories, Inc. 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 controls, significant at P≤0.005 (ILS, 1990)
[d] Control
[e] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test, significant at P≤0.025 (ILS, 1990)

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

TABLE C1 **Organ Weights and Organ-Weight-to-Body-Weight Ratios for Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein** **62**

Table C1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein[a]

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
Male						
n	13	15	15	13	15	14
Necropsy body wt	35.7 ± 0.8	36.4 ± 1.1	36.1 ± 0.9	36.6 ± 0.7	34.8 ± 0.8	31.3 ± 0.5**
Heart						
Absolute	0.226 ± 0.010	0.230 ± 0.009	0.210 ± 0.008	0.247 ± 0.010	0.213 ± 0.009	0.201 ± 0.008
Relative	6.340 ± 0.261	6.393 ± 0.305	5.821 ± 0.191	6.743 ± 0.232	6.125 ± 0.243	6.439 ± 0.285
R. Kidney						
Absolute	0.278 ± 0.004	0.293 ± 0.009	0.289 ± 0.010	0.284 ± 0.006	0.268 ± 0.006	0.251 ± 0.005*
Relative	7.820 ± 0.143	8.074 ± 0.149	7.983 ± 0.168	7.758 ± 0.085	7.730 ± 0.182	8.029 ± 0.146
Liver						
Absolute	1.734 ± 0.057	1.827 ± 0.082	1.751 ± 0.070[b]	1.851 ± 0.057	1.769 ± 0.058	1.700 ± 0.052
Relative	48.573 ± 1.123	50.083 ± 1.364	48.312 ± 1.128[b]	50.458 ± 0.812	50.905 ± 1.292	54.316 ± 1.257**
Lung						
Absolute	0.247 ± 0.011	0.239 ± 0.005	0.228 ± 0.006	0.283 ± 0.010*	0.262 ± 0.011	0.238 ± 0.012
Relative	6.922 ± 0.258	6.651 ± 0.253	6.335 ± 0.193	7.748 ± 0.257	7.568 ± 0.354	7.650 ± 0.410
R. Testis						
Absolute	0.128 ± 0.003	0.125 ± 0.002	0.126 ± 0.002[b]	0.124 ± 0.004	0.063 ± 0.003**	0.046 ± 0.003**
Relative	3.592 ± 0.114	3.485 ± 0.113	3.436 ± 0.051[b]	3.382 ± 0.085	1.815 ± 0.087**	1.473 ± 0.090**
Thymus						
Absolute	0.039 ± 0.002	0.035 ± 0.002	0.039 ± 0.003	0.039 ± 0.002	0.036 ± 0.002	0.031 ± 0.002
Relative	1.104 ± 0.064	0.961 ± 0.067	1.077 ± 0.060	1.065 ± 0.043	1.052 ± 0.071	0.988 ± 0.062
Female						
n	15	13	14	15	14	15
Necropsy body wt	25.7 ± 0.4	25.7 ± 0.4	27.2 ± 0.8	25.9 ± 0.4	26.0 ± 0.3	26.0 ± 0.3
Heart						
Absolute	0.154 ± 0.003	0.153 ± 0.006	0.155 ± 0.003	0.162 ± 0.006	0.169 ± 0.008	0.152 ± 0.004
Relative	6.001 ± 0.150	5.973 ± 0.199	5.749 ± 0.156	6.259 ± 0.274	6.536 ± 0.349	5.855 ± 0.127
R. Kidney						
Absolute	0.201 ± 0.002	0.192 ± 0.006	0.199 ± 0.006	0.201 ± 0.004	0.207 ± 0.005	0.208 ± 0.005[b]
Relative	7.841 ± 0.162	7.481 ± 0.167	7.368 ± 0.213	7.739 ± 0.094	7.990 ± 0.193	8.041 ± 0.174[b]
Liver						
Absolute	1.268 ± 0.028	1.230 ± 0.037	1.331 ± 0.044	1.272 ± 0.043	1.311 ± 0.027	1.324 ± 0.026
Relative	49.294 ± 0.918	47.848 ± 0.927	49.004 ± 0.871	48.946 ± 1.179	50.525 ± 1.089	50.961 ± 0.995
Lung						
Absolute	0.240 ± 0.010	0.245 ± 0.010	0.251 ± 0.012	0.259 ± 0.013	0.237 ± 0.009	0.223 ± 0.008
Relative	9.347 ± 0.413	9.532 ± 0.328	9.284 ± 0.450	9.957 ± 0.456	9.124 ± 0.349	8.598 ± 0.337
Thymus						
Absolute	0.040 ± 0.003	0.041 ± 0.002	0.039 ± 0.001	0.039 ± 0.003	0.038 ± 0.002[c]	0.033 ± 0.002
Relative	1.536 ± 0.116	1.593 ± 0.093	1.442 ± 0.060	1.481 ± 0.078	1.446 ± 0.081[c]	1.259 ± 0.085

* Significantly different ($P \leq 0.05$) from the 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] n=14
[c] n=13

APPENDIX D
REPRODUCTIVE TISSUE EVALUATIONS

TABLE D1 Summary of Reproductive Tissue Evaluations for Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein 64

Table D1
Summary of Reproductive Tissue Evaluations for Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Feed Study of Phenolphthalein[a]

	0 ppm	200 ppm	375 ppm	750 ppm	3,000 ppm	12,000 ppm
n	13	15	15	13	15	14
Weights (g)						
Necropsy body weight	35.7 ± 0.8	36.4 ± 1.1	36.1 ± 0.9	36.6 ± 0.7	34.8 ± 0.8	31.3 ± 0.5**
L. Cauda epididymis	0.0145 ± 0.0007	0.0153 ± 0.0008	0.0153 ± 0.0009	0.0160 ± 0.0008	0.0128 ± 0.0005	0.0112 ± 0.0004**
L. Epididymis	0.0491 ± 0.0016	0.0494 ± 0.0014	0.0482 ± 0.0023	0.0498 ± 0.0019	0.0396 ± 0.0018**	0.0320 ± 0.0016**
L. Testis	0.1222 ± 0.0035	0.1202 ± 0.0019	0.1125 ± 0.0070	0.1181 ± 0.0029	0.0604 ± 0.0032**	0.0408 ± 0.0022**
Spermatid measurements						
Spermatid heads (10^6/testis)	18.90 ± 1.02[b]	18.39 ± 0.80[c]	18.65 ± 1.50[c]	18.93 ± 0.52[b]	6.34 ± 0.88**[c]	2.63 ± 0.44**[d]
Epididymal spermatozoal measurements						
Sperm heads (10^6/g cauda epididymis)	814 ± 31	910 ± 95	780 ± 74	813 ± 56	275 ± 16**	100 ± 24**
Sperm heads (10^6/cauda)	11.62 ± 0.38	13.22 ± 0.69	11.96 ± 1.17	12.87 ± 0.97	3.57 ± 0.27**	1.07 ± 0.25**
Sperm motility (%)	82.71 ± 1.31	82.90 ± 1.00	78.36 ± 5.68	79.05 ± 1.35	81.79 ± 1.17	35.97 ± 10.88**

** Significantly different ($P \leq 0.01$) from the control group by Williams' test (body weights and tissue weights) or Shirley's test (spermatid and epididymal spermatozoal measurements)

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

[b] n=12

[c] n=14

[d] n=13

APPENDIX E
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

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Figure E1 Infrared Absorption Spectrum of Phenolphthalein 68
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Table E2 Preparation and Storage of Dose Formulations in the 27-Week Feed Study of Phenolphthalein 70
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CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF PHENOLPHTHALEIN

Phenolphthalein was obtained from Aldrich Chemical Company (Milwaukee, WI) in one lot (13427LF). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC), Galbraith Laboratories, Inc. (Knoxville, TN), and the study laboratory, Battelle Columbus Operations (Columbus, OH). Reports on analyses performed in support of the phenolphthalein study are on file at the National Institute of Environmental Health Sciences.

Lot 13427LF, a light brown/yellowish-white powder, was identified as phenolphthalein by the analytical chemistry laboratory using direct probe mass spectrometry and infrared (IR), ultraviolet/visible, and proton nuclear magnetic resonance (NMR) spectroscopy and by the study laboratory using IR spectroscopy; the spectra were consistent with the structure of phenolphthalein, showed absorption maxima consistent with literature values, matched spectra of a frozen reference standard from the same lot, or matched literature spectra (*EPA/NIH*, 1978; *Aldrich*, 1981, 1985; *Merck*, 1989). Representative IR and proton NMR spectra are presented in Figures E1 and E2. The melting point range (264° to 265° C) was consistent with a literature value (*Merck*, 1989).

The moisture content of lot 13427LF was determined by Galbraith Laboratories, Inc., using Karl Fischer titration; this laboratory also performed elemental analyses of lot 13427LF. The purity of lot 13427LF was determined by the analytical chemistry laboratory using high-performance liquid chromatography (HPLC) by system A (Table E1) and by the study laboratory using HPLC by system B.

For lot 13427LF, Karl Fischer titration indicated 0.507% water. Elemental analyses for carbon, hydrogen, and oxygen were consistent with the theoretical values for phenolphthalein. HPLC analyses by system A indicated one major peak and one impurity of 0.4%. The overall purity of lot 13427LF was determined to be greater than 99%.

Stability studies of the bulk chemical were performed by the analytical chemistry laboratory using HPLC by system B. These studies indicated that phenolphthalein was stable as a bulk chemical for at least 14 days when stored protected from light at temperatures up to 60° C. To ensure stability, the bulk chemical was stored in the original shipping containers at room temperature, protected from light. Stability was monitored during the study using HPLC by system B; no degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared approximately every 4 weeks by mixing phenolphthalein with feed (Table E2). A premix was prepared by hand and then blended with additional feed in a Patterson-Kelley twin-shell blender for 15 minutes using an intensifier bar for the initial 5 minutes. Formulations were stored in plastic buckets at less than or equal to –20° C for up to 42 days, with the exception of formulations prepared on November 29, 1999, which were originally stored at room temperature for 2 days and then transferred to –20° C storage.

Homogeneity studies of the 200 and 12,000 ppm dose formulations were performed by the analytical chemistry and study laboratories using HPLC by system C (Table E1). Stability studies of the 200 ppm dose formulation were performed by the analytical chemistry laboratory using HPLC by system C. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in sealed glass bottles protected from light at –20° C and for at least 7 days under simulated animal room conditions (exposed to light and air) in the absence of animal excreta.

Periodic analyses of the dose formulations of phenolphthalein were conducted by the study laboratory using HPLC by system B. All 15 dose formulations analyzed and used in the study were within 10% of the target concentrations; the dose formulations prepared on November 29, 1999, were not adversely affected by storage at room temperature for 2 days (Table E3). Animal room samples of these dose formulations were also analyzed; two of five animal room samples were within 10% of the target concentrations. Degradation was attributed to unavoidable excreta in the feeders.

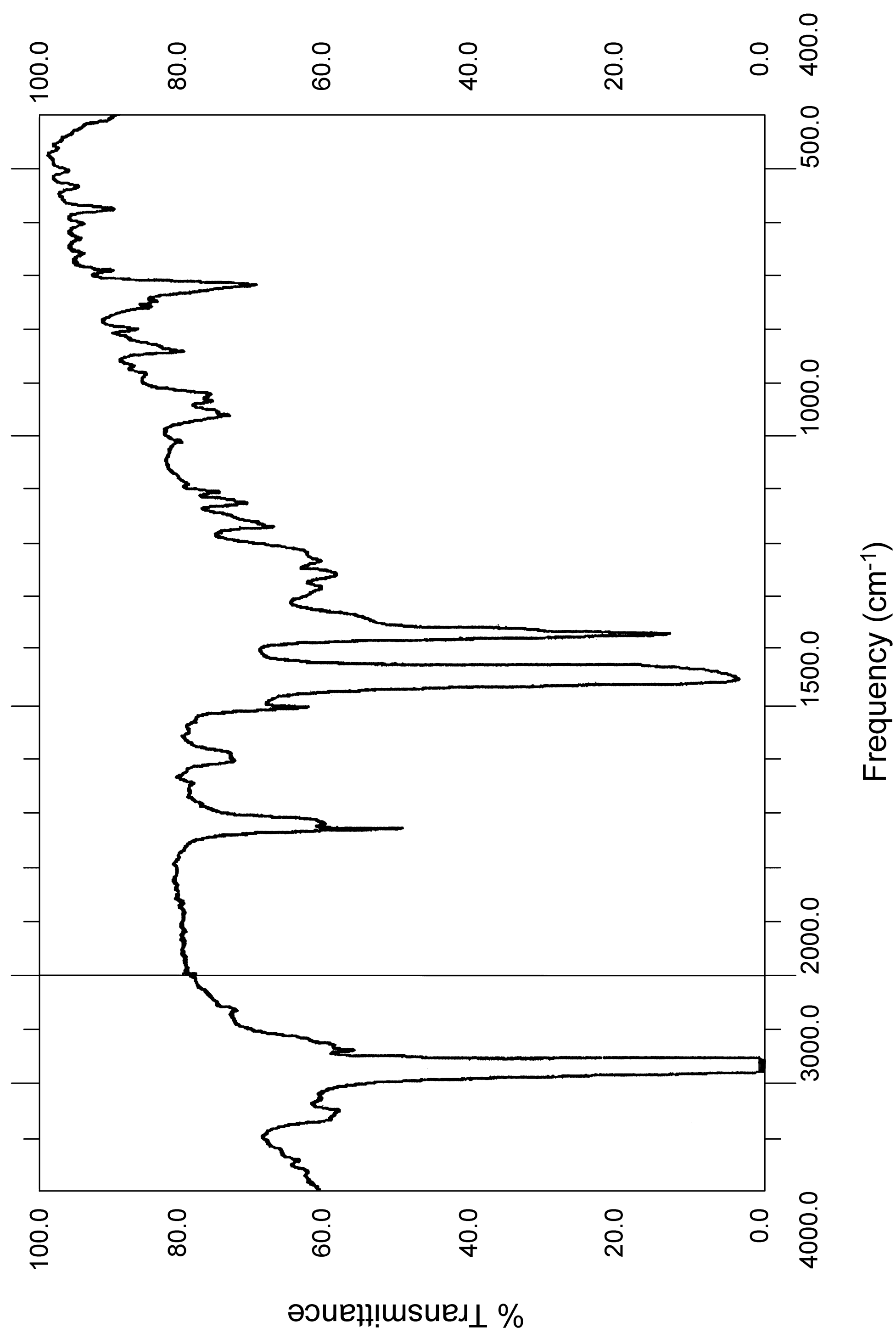

FIGURE E1
Infrared Absorption Spectrum of Phenolphthalein

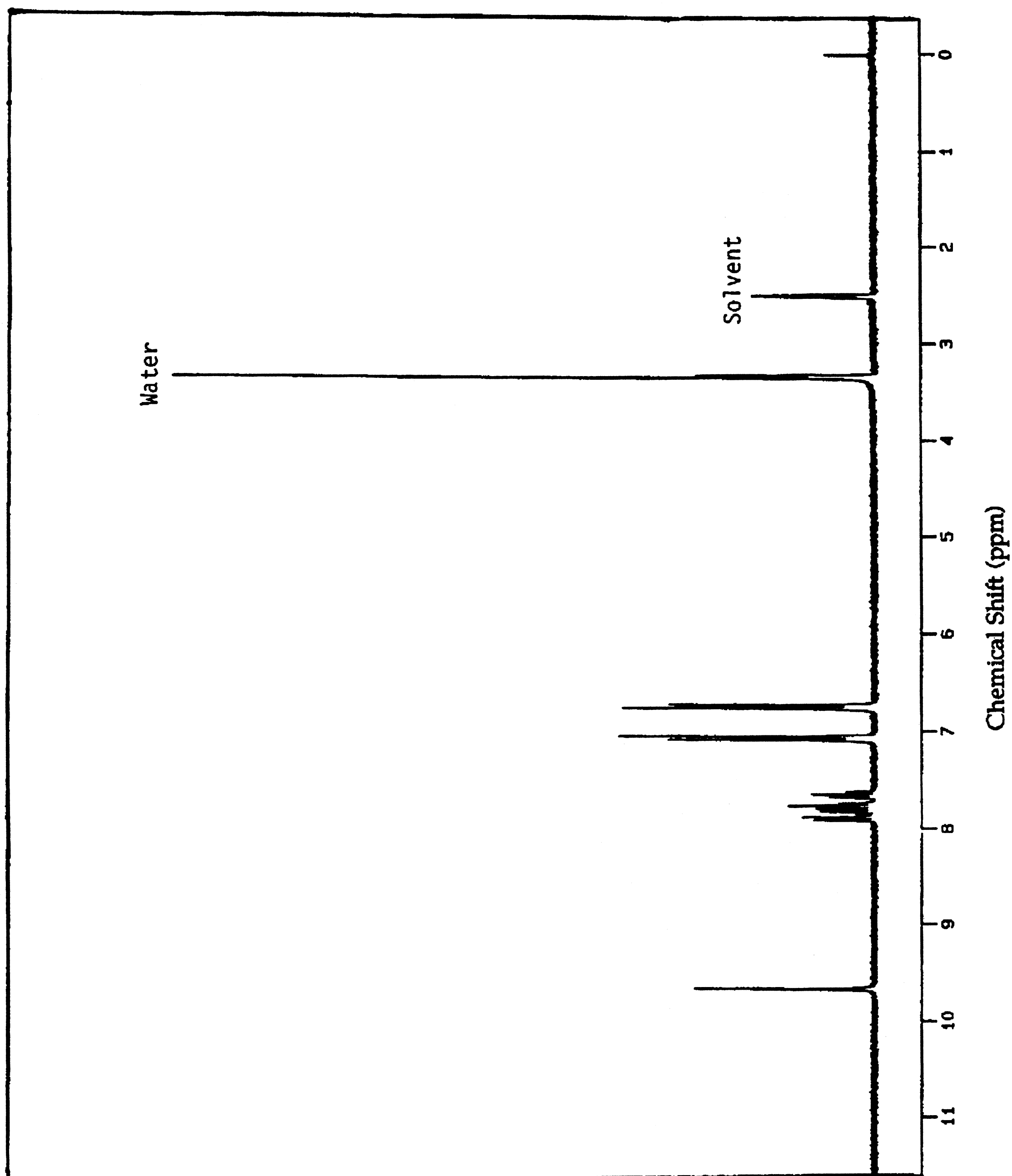

FIGURE E2
Proton Nuclear Magnetic Resonance Spectrum of Phenolphthalein

TABLE E1
High-Performance Liquid Chromatography Systems Used in the 27-Week Feed Study of Phenolphthalein[a]

Detection System	Column	Solvent System
System A		
Ultraviolet (254 nm) light	Primesphere™ C_{18}-HC, 150 mm × 3.2 mm, 5 µm (Phenomenex, Torrance, CA)	A) Acetonitrile and B) 0.00016 M monobasic potassium phosphate, pH 4.5, adjusted with phosphoric acid; linear gradient from 10% A:90% B to 90% A:10% B in 15 minutes at a flow rate of 0.5 mL/minute, held for 10 minutes
System B		
Ultraviolet (254 nm) light	Primesphere™ C_{18}-HC, 150 mm × 3.2 mm, 5 µm or Inertsil™ ODS-2, 150 mm × 3.0 mm, 5 µm (Phenomenex)	Acetonitrile:0.02 M monobasic potassium phosphate (40:60); pH 4.5, adjusted with phosphoric acid; flow rate 0.5 mL/minute; propiophenone as internal standard
System C		
Ultraviolet (254 nm) light	Symmetry® C_{18}, 150 mm × 3.9 mm, 5 µm (Waters Corporation, Milford, MA)	Acetonitrile:0.02 M monobasic potassium phosphate (45:55); pH 4.5, adjusted with phosphoric acid; flow rate 0.5 mL/minute; propiophenone as internal standard

[a] The high-performance liquid chromatographs were manufactured by Waters Corporation (Milford, MA).

TABLE E2
Preparation and Storage of Dose Formulations in the 27-Week Feed Study of Phenolphthalein

Preparation
A premix of feed and phenolphthalein was prepared in a stainless steel beaker, then layered into the remaining feed and blended in a Patterson-Kelley twin-shell blender for 15 minutes with the intensifier bar on for the first 5 minutes. The dose formulations were prepared approximately every 4 weeks.

Chemical Lot Number
13427LF

Maximum Storage Time
42 days

Storage Conditions
Dose formulations were stored in amber glass bottles sealed with Teflon®-lined lids at –20° C with the exception of formulations prepared on November 29, 1999, which were originally stored at room temperature for 2 days and then transferred to –20° C storage.

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

TABLE E3
Results of Analyses of Dose Formulations Administered to Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Feed Study of Phenolphthalein

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
November 29, 1999	November 30, 1999	200	188.6	–6
		375	368.9	–2
		750	743.8	–1
		3,000	3,012	0
		12,000	11,900	–1
	December 3, 1999[b]	200	191.4	–4
		375	367.1	–2
		750	727.3	–3
		3,000	3,053	+2
		12,000	11,700	–3
	January 7-8, 2000[c]	200	115.2	–42
		375	226.0	–40
		750	601.7	–20
		3,000	3,199	+7
		12,000	11,040	–8
February 21, 2000	February 22, 2000	200	186.8	–7
		375	349.3	–7
		750	706.8	–6
		3,000	2,941	–2
		12,000	12,240	+2
May 15, 2000	May 16, 2000	200	183.1	–8
		375	347.4	–7
		750	720.1	–4
		3,000	2,959	–1
		12,000	12,240	+2

[a] Results of duplicate analyses
[b] Results of reanalysis of dose formulations originally stored at room temperature for 2 days before being transferred to –20° C storage
[c] Animal room samples

APPENDIX F
FEED AND COMPOUND CONSUMPTION IN THE 27-WEEK FEED STUDY OF PHENOLPHTHALEIN

TABLE F1 **Feed and Compound Consumption by Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein 74**

TABLE F2 **Feed and Compound Consumption by Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein 76**

Table F1
Feed and Compound Consumption by Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein

	0 ppm		200 ppm			375 ppm			750 ppm		
Weeks	Feed (g)[a]	Body Weight (g)	Feed (g)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g)	Body Weight (g)	Dose (mg/kg)	Feed (g)	Body Weight (g)	Dose (mg/kg)
2	4.0	21.0	4.0	21.0	38	4.2	20.7	77	4.1	20.9	146
3	4.1	21.4	4.1	21.6	38	4.2	20.9	75	3.9	20.8	142
4	4.2	22.4	4.0	21.8	37	4.0	21.5	70	4.3	21.6	150
5	4.0	22.8	4.4	22.9	38	4.1	22.6	68	4.1	22.2	138
6	4.0	23.8	4.2	23.5	36	4.5	23.5	71	4.5	23.3	143
7	4.2	24.5	4.3	24.6	35	4.3	23.9	68	4.3	24.4	134
8	4.4	25.4	4.6	25.5	36	4.7	24.8	72	4.8	25.4	142
9	4.4	25.8	4.8	26.4	37	4.9	25.4	73	4.4	25.9	127
10	4.5	25.9	4.7	26.6	36	4.4	25.2	65	4.5	26.2	130
11	4.6	26.9	5.0	27.3	36	4.8	26.2	69	4.9	26.8	137
12	4.7	27.0	5.0	28.0	36	5.0	26.8	70	5.0	27.5	136
13	4.6	27.8	4.8	28.2	34	4.6	27.1	64	4.9	27.9	133
14	4.6	28.9	5.0	29.0	35	5.0	28.4	66	5.0	29.3	128
15	4.7	29.4	5.1	29.5	34	4.7	28.7	61	4.8	29.8	121
16	4.6	30.5	5.0	31.1	32	4.9	29.9	61	4.9	30.1	123
17	5.0	30.4	5.1	31.0	33	4.8	29.2	62	5.4	30.5	132
18	5.4	31.8	5.3	32.0	33	5.4	30.9	66	5.3	30.9	130
19	5.3	32.6	5.4	32.1	34	5.5	31.4	65	5.6	31.9	133
20	4.9	32.4	5.3	32.8	32	5.3	32.0	62	5.5	32.1	129
21	5.3	33.0	5.3	33.1	32	5.2	32.5	60	5.7	32.4	132
22	5.2	33.7	5.8	33.7	34	5.4	33.3	61	5.8	34.3	127
23	5.5	34.0	5.6	34.2	33	5.7	34.1	63	5.8	33.8	128
24	5.4	34.1	5.7	34.8	33	5.5	34.6	59	5.8	34.3	127
25	5.5	35.2	5.7	35.6	32	5.7	35.2	60	5.6	35.1	119
26	5.2	34.3	5.6	36.0	31	5.6	35.4	59	5.6	35.9	117
Mean for weeks											
1-13	4.3	24.6	4.5	24.8	36	4.5	24.0	70	4.5	24.4	138
14-26	5.1	32.3	5.4	32.7	33	5.3	31.9	62	5.4	32.3	127

TABLE F1
Feed and Compound Consumption by Male Haploinsufficient p16[Ink4a]/p19[Arf] Mice in the 27-Week Feed Study of Phenolphthalein

	3,000 ppm			12,000 ppm		
Weeks	Feed (g)	Body Weight (g)	Dose (mg/kg)	Feed (g)	Body Weight (g)	Dose (mg/kg)
2	4.1	20.8	596	4.2	20.8	2,436
3	3.9	21.1	556	4.2	20.6	2,437
4	4.2	21.5	586	4.0	20.8	2,330
5	4.2	22.4	562	4.2	22.0	2,313
6	4.4	23.3	570	4.1	22.6	2,205
7	4.1	24.1	506	4.4	23.6	2,237
8	4.5	25.0	541	4.4	24.2	2,191
9	4.6	25.0	549	4.3	24.8	2,092
10	4.5	25.9	527	4.4	25.0	2,115
11	5.0	26.8	558	4.9	25.6	2,280
12	4.7	27.0	518	4.6	25.4	2,156
13	4.4	27.1	488	4.8	26.2	2,220
14	4.9	28.2	525	4.6	26.8	2,068
15	4.9	28.8	506	4.5	26.9	2,009
16	4.9	29.8	492	4.4	27.7	1,907
17	5.3	29.6	538	5.1	28.4	2,142
18	5.4	30.0	543	4.9	28.2	2,091
19	5.5	29.8	550	5.0	28.5	2,093
20	5.8	31.1	559	5.2	28.9	2,156
21	5.6	31.9	529	5.1	28.6	2,136
22	6.0	32.4	552	5.2	29.7	2,102
23	5.9	32.1	554	5.4	29.3	2,232
24	6.0	32.9	544	5.2	29.8	2,074
25	5.7	32.8	525	5.3	30.4	2,107
26	5.9	33.6	524	5.1	30.3	2,023
Mean for weeks						
1-13	4.4	24.2	546	4.4	23.5	2,251
14-26	5.5	31.0	534	5.0	28.7	2,088

[a] Grams of feed consumed per animal per day
[b] Milligrams of phenolphthalein consumed per kilogram body weight per day

TABLE F2
Feed and Compound Consumption by Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice in the 27-Week Feed Study of Phenolphthalein

	0 ppm		200 ppm			375 ppm			750 ppm		
Weeks	Feed (g)[a]	Body Weight (g)	Feed (g)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g)	Body Weight (g)	Dose (mg/kg)	Feed (g)	Body Weight (g)	Dose (mg/kg)
2	4.0	17.8	4.0	17.4	46	4.2	17.6	90	4.1	17.8	175
3	4.4	18.7	4.2	17.8	47	4.0	18.2	82	3.8	17.9	160
4	4.0	19.0	4.2	18.5	45	3.9	18.7	78	4.0	18.6	161
5	4.1	19.8	4.0	19.3	41	4.2	19.8	80	4.0	19.4	154
6	4.1	20.8	4.3	20.4	42	4.3	19.9	81	4.5	20.6	165
7	4.4	21.3	4.2	20.9	40	4.7	21.2	83	4.3	21.0	152
8	5.2	21.8	4.9	21.9	45	5.2	21.8	89	4.8	22.0	164
9	4.7	22.2	5.2	22.1	47	5.7	22.4	95	4.5	22.2	151
10	5.1	22.4	5.3	21.6	49	5.3	22.4	89	5.2	22.0	178
11	5.2	22.1	5.5	22.3	50	5.7	23.3	93	4.8	22.3	163
12	5.1	22.5	5.6	22.4	50	5.9	23.1	96	4.8	22.5	159
13	5.1	22.7	5.9	23.0	51	5.4	23.4	87	5.2	23.1	170
14	5.5	23.4	6.1	23.1	53	6.1	23.8	96	5.2	23.1	167
15	5.1	22.9	5.9	23.1	51	5.5	23.4	88	4.8	23.1	156
16	5.3	23.7	6.1	23.5	52	5.4	24.2	83	5.1	23.5	163
17	5.4	23.6	5.7	23.8	48	5.4	23.8	84	5.4	24.1	168
18	5.8	24.0	5.8	23.8	49	5.5	24.4	84	5.3	24.2	164
19	5.9	24.5	6.1	24.4	50	6.0	24.7	91	6.1	24.6	185
20	5.4	24.5	6.5	25.0	52	5.6	25.1	84	5.8	24.8	174
21	5.4	24.7	5.9	24.8	48	5.0	25.0	75	5.4	24.5	167
22	5.7	24.4	6.1	25.6	48	5.2	24.6	80	5.6	25.4	165
23	5.8	24.2	5.7	24.8	46	6.0	25.6	88	5.4	24.3	167
24	5.7	24.8	6.0	25.0	48	5.6	25.4	83	5.9	25.5	173
25	6.2	25.6	6.3	25.6	50	6.3	26.4	89	5.8	25.1	172
26	6.3	25.3	6.1	25.5	48	6.1	26.5	86	5.6	25.1	166
Mean for weeks											
1-13	4.6	20.9	4.8	20.6	46	4.9	21.0	87	4.5	20.8	163
14-26	5.6	24.3	6.0	24.5	49	5.7	24.8	85	5.5	24.4	168

TABLE F2
Feed and Compound Consumption by Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 27-Week Feed Study of Phenolphthalein

	3,000 ppm			12,000 ppm		
Weeks	Feed (g)	Body Weight (g)	Dose (mg/kg)	Feed (g)	Body Weight (g)	Dose (mg/kg)
2	4.3	18.0	724	4.2	17.7	2,840
3	3.9	18.2	637	4.3	18.6	2,782
4	3.9	18.5	636	4.2	19.1	2,644
5	4.1	19.8	619	4.5	19.8	2,731
6	4.5	20.8	648	4.6	20.9	2,646
7	4.6	21.4	649	4.7	21.6	2,622
8	4.6	22.0	623	5.0	22.0	2,714
9	4.6	21.7	639	5.2	22.2	2,800
10	5.0	22.0	683	5.1	22.5	2,700
11	4.8	22.3	646	5.4	23.2	2,779
12	4.9	22.7	654	5.3	23.1	2,750
13	5.0	22.8	663	5.5	23.4	2,829
14	5.2	23.1	677	5.4	23.5	2,741
15	5.3	22.7	695	5.6	23.6	2,830
16	5.7	23.6	721	5.7	23.9	2,861
17	5.4	24.1	678	5.6	24.0	2,778
18	5.9	24.0	737	5.5	24.1	2,755
19	5.9	24.4	726	5.8	24.5	2,862
20	6.0	24.7	727	6.1	25.0	2,927
21	5.5	24.3	682	5.3	24.7	2,568
22	6.0	25.2	709	5.5	24.9	2,664
23	5.6	24.6	687	6.2	25.6	2,908
24	6.1	25.2	726	6.1	25.5	2,873
25	6.2	25.3	731	5.9	25.4	2,798
26	6.0	25.1	719	6.0	25.4	2,850
Mean for weeks						
1-13	4.5	20.8	652	4.8	21.2	2,736
14-26	5.7	24.3	709	5.7	24.6	2,801

[a] Grams of feed consumed per animal per day
[b] Milligrams of phenolphthalein consumed per kilogram body weight per day

APPENDIX G
HISTORICAL CONTROL INCIDENCES

TABLE G1 Historical Incidences of Neoplasms
in Control Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice 80

TABLE G2 Historical Incidences of Nonneoplastic Lesions
in Control Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice 81

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TABLE G4 Historical Incidences of Nonneoplastic Lesions
in Control Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice 84

Table G1
Historical Incidences of Neoplasms in Control Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice[a]

	Benzene	Phenolphthalein	Total
27 Weeks			
Lung			
Alveolar/Bronchiolar Adenoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Alveolar/Bronchiolar Carcinoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Alveolar/Bronchiolar Adenoma or Carcinoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Histiocytic Sarcoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Malignant Lymphoma	0/15 (0%)	1/15 (7%)	1/30 (3%)
	Aspartame	**Glycidol**	**Total**
40 Weeks			
Lung			
Alveolar/Bronchiolar Adenoma	0/15 (0%)	1/15 (7%)	1/30 (3%)
Alveolar/Bronchiolar Carcinoma	0/15 (0%)	2/15 (13%)	2/30 (7%)
Alveolar/Bronchiolar Adenoma or Carcinoma	0/15 (0%)	3/15 (20%)	3/30 (10%)
Histiocytic Sarcoma	2/15 (13%)	2/15 (13%)	4/30 (13%)
Malignant Lymphoma	0/15 (0%)	2/15 (13%)	2/30 (7%)

[a] Data as of July 6, 2006

Table G2
Historical Incidences of Nonneoplastic Lesions in Control Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice[a]

	Benzene	Phenolphthalein	Total
27 Weeks			
Kidney			
Casts Protein	1/15 (7%)	0/14 (0%)	1/29 (3%)
Nephropathy	1/15 (7%)	6/14 (43%)	7/29 (24%)
Liver			
Inflammation, Chronic	3/15 (20%)	11/15 (73%)	14/30 (47%)
Spleen			
Hematopoietic Cell Proliferation	0/15 (0%)	2/14 (14%)	2/29 (7%)
Adrenal Cortex			
Subcapsular Hyperplasia	0/15 (0%)	0/14 (0%)	0/29 (0%)
Lung			
Inflammation	0/15 (0%)	0/15 (0%)	0/30 (0%)
Inflammation, Chronic	2/15 (13%)	0/15 (0%)	2/30 (7%)
Stomach, Glandular			
Mineralization	0/15 (0%)	0/14 (0%)	0/29 (0%)
Muscularis Mineralization	0/15 (0%)	0/14 (0%)	0/29 (0%)
Heart			
Myocardium Degeneration	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Necrosis	0/15 (0%)	0/15 (0%)	0/30 (0%)

Table G2
Historical Incidences of Nonneoplastic Lesions in Control Male Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice

	Aspartame	Glycidol	Total
40 Weeks			
Kidney			
Casts Protein	0/15 (0%)	0/15 (0%)	0/30 (0%)
Nephropathy	2/15 (13%)	4/15 (27%)	6/30 (20%)
Liver			
Inflammation, Chronic	0/15 (0%)	6/15 (40%)	6/30 (20%)
Spleen			
Hematopoietic Cell Proliferation	0/15 (0%)	4/15 (27%)	4/30 (13%)
Adrenal Cortex			
Subcapsular Hyperplasia	2/15 (13%)	1/15 (7%)	3/30 (10%)
Lung			
Inflammation	0/15 (0%)	3/15 (20%)	3/30 (10%)
Inflammation, Chronic	0/15 (0%)	0/15 (0%)	0/30 (0%)
Stomach, Glandular			
Mineralization	0/15 (0%)	10/15 (67%)	10/30 (33%)
Muscularis Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Heart			
Myocardium Degeneration	0/15 (0%)	2/15 (13%)	2/30 (7%)
Myocardium Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Necrosis	0/15 (0%)	0/15 (0%)	0/30 (0%)

[a] Data as of July 6, 2006

Table G3
Historical Incidences of Neoplasms in Control Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice[a]

	Benzene	Phenolphthalein	Total
27 Weeks			
Lung			
Alveolar/Bronchiolar Adenoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Alveolar/Bronchiolar Carcinoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Alveolar/Bronchiolar Adenoma or Carcinoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Histiocytic Sarcoma	3/15 (20%)	0/15 (0%)	3/30 (10%)
Malignant Lymphoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
	Aspartame	**Glycidol**	**Total**
40 Weeks			
Lung			
Alveolar/Bronchiolar Adenoma	1/15 (7%)	0/15 (0%)	1/30 (3%)
Alveolar/Bronchiolar Carcinoma	0/15 (0%)	0/15 (0%)	0/30 (0%)
Alveolar/Bronchiolar Adenoma or Carcinoma	1/15 (7%)	0/15 (0%)	1/30 (3%)
Histiocytic Sarcoma	5/15 (33%)	9/15 (60%)	14/30 (47%)
Malignant Lymphoma	0/15 (0%)	0/15 (0%)	0/30 (0%)

[a] Data as of July 6, 2006

Table G4
Historical Incidences of Nonneoplastic Lesions in Control Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice[a]

	Benzene	Phenolphthalein	Total
27 Weeks			
Kidney			
Casts Protein	5/15 (33%)	0/15 (0%)	5/30 (17%)
Nephropathy	3/15 (20%)	10/15 (67%)	13/30 (43%)
Liver			
Inflammation, Chronic	10/15 (67%)	10/15 (67%)	20/30 (67%)
Spleen			
Hematopoietic Cell Proliferation	5/15 (33%)	2/15 (13%)	7/30 (23%)
Uterus			
Endometrium Hyperplasia Cystic	12/15 (80%)	15/15 (100%)	27/30 (90%)
Ovary			
Cyst	1/14 (7%)	1/15 (7%)	2/29 (7%)
Lung			
Inflammation	0/15 (0%)	0/15 (0%)	0/30 (0%)
Inflammation, Chronic	2/15 (13%)	2/15 (13%)	4/30 (13%)
Stomach, Glandular			
Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Muscularis Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Heart			
Myocardium Degeneration	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Necrosis	0/15 (0%)	0/15 (0%)	0/30 (0%)

Table G4
Historical Incidences of Nonneoplastic Lesions in Control Female Haploinsufficient p16^{Ink4a}/p19^{Arf} Mice

	Aspartame	Glycidol	Total
40 Weeks			
Kidney			
Casts Protein	0/15 (0%)	0/15 (0%)	0/30 (0%)
Nephropathy	1/15 (7%)	12/15 (80%)	13/30 (43%)
Liver			
Inflammation, Chronic	1/15 (7%)	11/15 (73%)	12/30 (40%)
Spleen			
Hematopoietic Cell Proliferation	5/15 (33%)	9/15 (60%)	14/30 (47%)
Uterus			
Endometrium Hyperplasia Cystic	14/15 (93%)	13/15 (87%)	27/30 (90%)
Adrenal Cortex			
Subcapsular Hyperplasia	14/15 (93%)	15/15 (100%)	29/30 (97%)
Ovary			
Cyst	0/15 (0%)	4/15 (27%)	4/30 (13%)
Lung			
Inflammation	0/15 (0%)	8/15 (53%)	8/30 (27%)
Inflammation, Chronic	0/15 (0%)	0/15 (0%)	0/30 (0%)
Stomach, Glandular			
Mineralization	0/15 (0%)	4/15 (27%)	4/30 (13%)
Muscularis Mineralization	0/15 (0%)	0/15 (0%)	0/30 (0%)
Heart			
Myocardium Degeneration	0/15 (0%)	0/15 (0%)	0/30 (0%)
Myocardium Mineralization	0/15 (0%)	1/15 (7%)	1/30 (3%)
Myocardium Necrosis	0/15 (0%)	0/15 (0%)	0/30 (0%)

[a] Data as of July 6, 2006

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