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

NTP Genetically Modified Model Report on the

Toxicology and Carcinogenesis Study of Glycidol (CASRN 556-52-5) in Genetically Modified Haploinsufficient $p16^{INK4A}/p19^{ARF}$ Mice (Gavage study)

NTP GMM 13

NOVEMBER 2007

NTP REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDY OF GLYCIDOL
(CAS NO. 556-52-5)

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

(GAVAGE STUDY)

NATIONAL TOXICOLOGY PROGRAM
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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

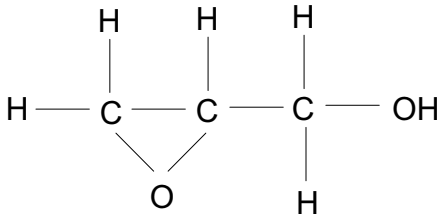
Background
Glycidol is used in the production of pharmaceuticals and other chemicals and as a stabilizer in vinyl polymers. Glycidol is known to cause cancer in rats and mice. We tested glycidol in a genetically modified mouse strain that lacks two tumor suppressor genes as part of a study to determine if this mouse model could detect cancer-causing chemicals more rapidly than the standard 2-year rodent bioassay.

Methods
We exposed groups of male or female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice by depositing solutions of glycidol in deionized water directly into the animals' stomachs through a tube five times per week for 40 weeks. The daily doses were 25, 50, 100, or 200 milligrams of glycidol per kilogram of body weight; other animals receiving only water served as the control groups. Tissues from 22 organs were examined for every animal.

Results
Exposure to glycidol caused increased rates of histiocytic sarcomas in male haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice. Male mice receiving the highest dose of glycidol also had hyperplasia of the forestomach and neuronopathy of the brain. Female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice receiving the highest dose of glycidol had alveolar/bronchiolar adenomas of the lung and squamous cell papillomas of the forestomach, as well as epithelial hyperplasia of the forestomach and neuronopathy of the brain.

Conclusions
We conclude that glycidol caused histiocytic sarcomas in male haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice and alveolar/bronchiolar adenomas of the lung in female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice. Forestomach papillomas in female mice may also have been associated with exposure to glycidol.

ABSTRACT



GLYCIDOL

CAS No. 556-52-5

Chemical Formula: $C_3H_6O_2$ Molecular Weight: 74.08

Synonyms: Allyl alcohol oxide; epihydrin alcohol; 1,2-epoxy-3-hydroxypropane; 2,3-epoxypropanol; 2,3-epoxy-1-propanol; epoxypropyl alcohol; glycide; glycidyl alcohol; 1-hydroxy-2,3-epoxypropane; 3-hydroxy-1,2-epoxypropane; 3-hydroxyl-1,2-epoxypropane; hydroxymethyl ethylene oxide; 2-(hydroxymethyl)oxirane; 2-hydroxymethyloxiran; oxiranemethanol; oxiranylmethanol; 1-propanol, 2,3-epoxy-methanol

Glycidol is used as a chemical intermediate in the pharmaceutical industry, as a stabilizer in the manufacture of vinyl polymers, and as an intermediate in the synthesis of glycerol, glycidyl ethers, and amines. Glycidol was nominated for carcinogenicity study by the United States Environmental Protection Agency. Glycidol was selected for study in the haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse because it was found to be carcinogenic in rats and mice in conventional 2-year rodent studies (NTP, 1990), but was negative in a study in $p53^{+/-}$ mice (Tennant *et al.*, 1999). Male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice received glycidol (greater than 95% pure) by gavage for 40 weeks. Genetic toxicology studies were conducted in mouse peripheral blood erythrocytes.

40-Week Study in Mice

Groups of 15 male and 15 female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice were administered 0, 25, 50, 100, or 200 mg glycidol/kg body weight in deionized water by gavage, 5 days per week for 40 weeks. Survival of 200 mg/kg male and female mice was less than that of the vehicle control groups, but the differences were not significant. Mean body weights of 200 mg/kg male mice and 50, 100, and 200 mg/kg female mice were less than those of the vehicle controls. The left testis, left epididymis, and left cauda epididymis weights were significantly decreased in 200 mg/kg males; the number of sperm heads per cauda epididymis were also significantly decreased in this group.

Enlarged spleen and foci of discolored liver were observed in 200 mg/kg male mice at necropsy. These findings corresponded to infiltration by histocytic sarcoma or extramedullary hematopoiesis. The incidences of histiocytic sarcoma were increased in dosed groups of males and in females administered 50 mg/kg or greater, and the incidences in 50 and 200 mg/kg males were significantly greater than that in the vehicle control group. In the lung, incidences of alveolar/bronchiolar adenoma were significantly increased in 100 mg/kg males and 200 mg/kg females; multiple adenomas were seen in some dosed males. Squamous cell papillomas

of the forestomach were seen in one 200 mg/kg male, one 100 mg/kg female, and three 200 mg/kg females. Significantly increased incidences of epithelial hyperplasia occurred in the forestomach of 200 mg/kg males and females. Neuronopathy, gliosis, and hemorrhage of the brain were observed at various sites in a few 200 mg/kg males and 100 and/or 200 mg/kg females.

GENETIC TOXICOLOGY

The frequency of micronucleated erythrocytes was monitored in peripheral blood of male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice in the 40-week study. No significant increases were observed at 6.5, 13, or 19.5 weeks; small but statistically significant increases were seen in both male and female mice sampled at 26 and 40 weeks.

CONCLUSIONS

Under the conditions of this 40-week gavage study, there was *clear evidence of carcinogenic activity** of glycidol in male haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice based on the occurrence of histiocytic sarcomas. The increased incidences of alveolar/bronchiolar adenomas in male mice were also considered to be related to glycidol administration. There was *some evidence of carcinogenic activity* of glycidol in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ female mice based on the occurrence of alveolar/bronchiolar adenoma. The occurrence of forestomach papillomas in female mice may also have been related to glycidol administration.

Treatment of male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice with glycidol was associated with forestomach hyperplasia and neuronopathy in the brain.

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

Summary of the 40-Week Carcinogenesis and Genetic Toxicology Studies of Glycidol in Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice

	Male	Female
Concentrations water	0, 25, 50, 100, or 200 mg/kg	0, 25, 50, 100, or 200 mg/kg
Body weights	200 mg/kg group less than vehicle control group	50, 100, and 200 mg/kg groups less than vehicle control group
Survival rates	13/15, 14/15, 13/15, 14/15, 7/15	13/15, 14/15, 12/15, 14/15, 9/15
Nonneoplastic effects	Forestomach: epithelium, hyperplasia (0/15, 1/15, 1/15, 0/15, 6/15) Brain: neuronopathy (0/15, 0/15, 0/15, 0/15, 5/15)	Forestomach: epithelium, hyperplasia (0/15, 0/15, 0/15, 1/15, 4/15) Brain: neuronopathy (0/15, 0/15, 0/15, 1/15, 4/15)
Neoplastic effects	Histiocytic sarcoma: (2/15, 6/15, 9/15, 5/15, 11/15) Lung: alveolar/bronchiolar adenoma (1/15, 0/15, 2/15, 7/15, 3/15)	Lung: alveolar/bronchiolar adenoma (0/15, 1/15, 0/15, 1/15, 4/15)
Equivocal Findings	None	Forestomach: squamous cell papilloma (0/15, 0/15, 0/15, 1/15, 3/15)
Level of evidence of carcinogenic activity	Clear evidence	Some evidence
Genetic toxicology		
Micronucleated erythrocytes		
Mouse peripheral blood *in vivo*:	Negative in males and females at the 6.5-, 13-, and 19.5-week sampling times; positive at the 26- and 40-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 glycidol 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 glycidol 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 (NIEHS), Research Triangle Park, NC.

Dr. J.K. Dunnick, NIEHS, introduced the study of glycidol in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice by reviewing the uses of the chemical, the previous NTP carcinogenicity findings, the design and dose selection for the genetically modified mouse model studies, and the nonneoplastic and neoplastic lesions observed in the 40-week study. The proposed conclusions were *clear evidence of carcinogenic activity* of glycidol in male haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice and *some evidence of carcinogenic activity* of glycidol in female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice.

Dr. Mirsalis, the first principal reviewer, felt the study was well conducted and agreed with the proposed conclusions. He asked for addition of a dose selection rationale section and for explanation of the survival statistics used.

Dr. Giesy, the second principal reviewer, had no further comments.

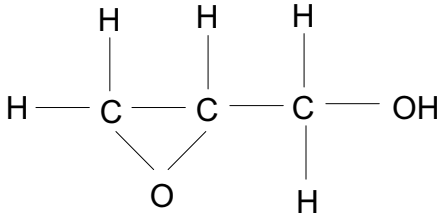
Dr. Deininger, the third principal reviewer, noted that the main evidence for this study came from histiocytic sarcomas, which have a naturally high background rate.

Dr. Dunnick noted the dose selection was included in the review of prior studies and would be more clearly identified. Dr. G.E. Kissling, NIEHS, said the survival statistics were based on the total survival time for all the animals in a group rather than on the number of animals surviving to study termination.

Dr. Sikka asked if a mechanism or known metabolite was known to be responsible for the carcinogenic activity of glycidol. Dr. Walker answered that, as an epoxide, the chemical acted directly with DNA. Dr. Walker also cautioned about use of the term "potent" in citing literature statements about the carcinogenicity of glycidol.

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

INTRODUCTION



GLYCIDOL

CAS No. 556-52-5

Chemical Formula: $C_3H_6O_2$ Molecular Weight: 74.08

Synonyms: Allyl alcohol oxide; epihydrin alcohol; 1,2-epoxy-3-hydroxypropane; 2,3-epoxypropanol; 2,3-epoxy-1-propanol; epoxypropyl alcohol; glycide; glycidyl alcohol; 1-hydroxy-2,3-epoxypropane; 3-hydroxy-1,2-epoxypropane; 3-hydroxyl-1,2-epoxypropane; hydroxymethyl ethylene oxide; 2-(hydroxymethyl)oxirane; 2-hydroxymethyloxiran; oxiranemethanol; oxiranylmethanol; 1-propanol, 2,3-epoxy-methanol

Chemical and Physical Properties

Glycidol is a viscous, colorless liquid that boils at 160° C (HSDB, 2006). It is soluble in water, alcohol, ether, acetone, benzene, and other organic solvents. At 25° C, the vapor pressure is 0.9 mm mercury. Glycidol is combustible with a flash point of 72° C and is incompatible with strong oxidizers and nitrates. It explodes when heated or in the presence of strong acids, bases, metals, and metal salts (NTP, 2004; HSDB, 2006).

Production, Use, and Human Exposure

Glycidol is used as a chemical intermediate in the pharmaceutical industry, as a stabilizer in the manufacture of vinyl polymers, and as an intermediate in the synthesis of glycerol, glycidyl ethers, and amines. Glycidol is also used as an additive for oil and synthetic hydraulic fluids, as a diluent in some epoxy resins, and as a dye-leveling agent. One domestic producer and 18 suppliers of glycidol were reported in 2000. No recent production data were found. In the past, more than 10 million pounds of glycidol compounds were produced or imported annually into the United States (NTP, 2004).

The primary routes of potential human exposures to glycidol are inhalation, eye or dermal contact, and ingestion. Occupational exposure may occur through inhalation (NTP, 2004). The National Occupational Exposure Survey conducted by the National Institute for Occupational Safety and Health (1990) from 1981 to 1983 estimated that 4,871 workers, at 88 facilities and in 10 occupations, were potentially exposed to glycidol. This estimate was derived from observations of the actual use of the compound (78% of total observations) and the use of trade name products known to contain the compound.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

Figure 1 shows the known and proposed metabolic reactions of glycidol. Because of the reactivity of epoxides, in solution glycidol can undergo several spontaneous reactions involving nucleophilic attack at the α or β carbon (March, 1978); at neutral pH and 37° C, glycidol slowly hydrolyzes to glycerol; in 0.1 M hydrochloric acid, the hydrolysis to glycerol (97.2%) and α-chlorohydrin (3-chloro-1,2-propanediol) (2.8%) occurs rapidly, with a half-life of 10 minutes. At pH 6, glycidol does not readily react with glutathione; however, at pH 7 or 8, the reaction to form *S*-(2,3-dihydroxypropyl)glutathione occurs readily. Glycidol may also directly alkylate various cellular components (Jones, 1975).

The major urinary metabolites isolated from rats administered glycidol by intraperitoneal injection are *S*-(2,3-dihydroxypropyl)glutathione, *S*-(2,3-dihydroxypropyl)cysteine, and β-chlorolactic acid. The latter compound was identified as the only radioactive urinary metabolite of glycidol isolated from rats administered [^{36}Cl]saline for 3 days before glycidol administration (Jones and O'Brien, 1980). The same urinary metabolites are found after α-chlorohydrin administration, suggesting that glycidol is converted to α-chlorohydrin by direct reaction with hydrochloric acid in the stomach. α-Chlorohydrin may then be converted to the glutathione metabolite by the action of glutathione transferase or oxidized to α-chlorolactate by the successive action of alcohol dehydrogenase and aldehyde dehydrogenase. The conversion of glycidol to glycerol by epoxide hydrase has been observed with rat liver microsomal preparations (Patel *et al.*, 1980). The oxidation of glycidol to glycidaldehyde has not been observed, but glycidaldehyde is a potential metabolite formed by the action of alcohol dehydrogenase.

Humans

No studies on the absorption, distribution, metabolism, or excretion of glycidol in humans were found in a review of the literature.

TOXICITY

Experimental Animals

The NTP (1990) conducted toxicity studies of glycidol in F344/N rats and B6C3F$_1$ mice in which the chemical was administered by oral gavage (5 days per week) at doses of 0, 37.5, 75, 150, 300, or 600 mg/kg for 16 days. All rats that received 600 mg/kg died between days 3 and 13 of the study. Edema and degeneration of the epididymal stroma, atrophy of the testis, and granulomatous inflammation of the epididymis occurred in males that received 300 mg/kg. All mice that received 600 mg/kg and three males and two females that received 300 mg/kg died by day 4 of the study. Focal demyelination in the medulla and thalamus of the brain occurred in all female mice that received 300 mg/kg.

In 13-week studies, F344/N rats received glycidol by oral gavage (5 days/week) at doses of 0, 25, 50, 100, 200, or 400 mg/kg and B6C3F$_1$ mice received doses of 0, 19, 38, 75, 150, or 300 mg/kg (NTP, 1990). All rats that received 400 mg/kg died by week 2; three males and one female that received 200 mg/kg died during weeks 11 and 12. Final mean body weights of male rats that received 50, 100, or 200 mg/kg were 85% to 96% that of vehicle controls; final mean body weights of female rats receiving the same doses were 89% to 94% that of vehicle controls. Necrosis of the cerebellum occurred in rats administered 200 or 400 mg/kg, and demyelination in the medulla of the brain, tubular degeneration and/or necrosis of the kidney, and lymphoid necrosis of the thymus occurred in rats that received 400 mg/kg. Testicular atrophy occurred in male rats administered 200 or 400 mg/kg. All mice that received 300 mg/kg died by week 2; four of 10 males and three of 10 females that received 150 mg/kg died. Mean body weights of 19, 38, 75, and 150 mg/kg males and all dosed groups of female mice surviving to the end of the studies were generally 90% to 94% those of vehicle controls. Compound-related histopathologic lesions included demyelination of the brain in male and female mice that received 150 or 300 mg/kg. Testicular atrophy was observed in male mice at all doses, and renal tubular cell degeneration was observed in male mice that received 300 mg/kg.

Humans

No toxicity studies of glycidol in humans were found in a review of the literature.

REPRODUCTIVE TOXICITY

Experimental Animals

In the 13-week studies (NTP, 1990), sperm count and sperm motility were reduced in male F344/N rats that

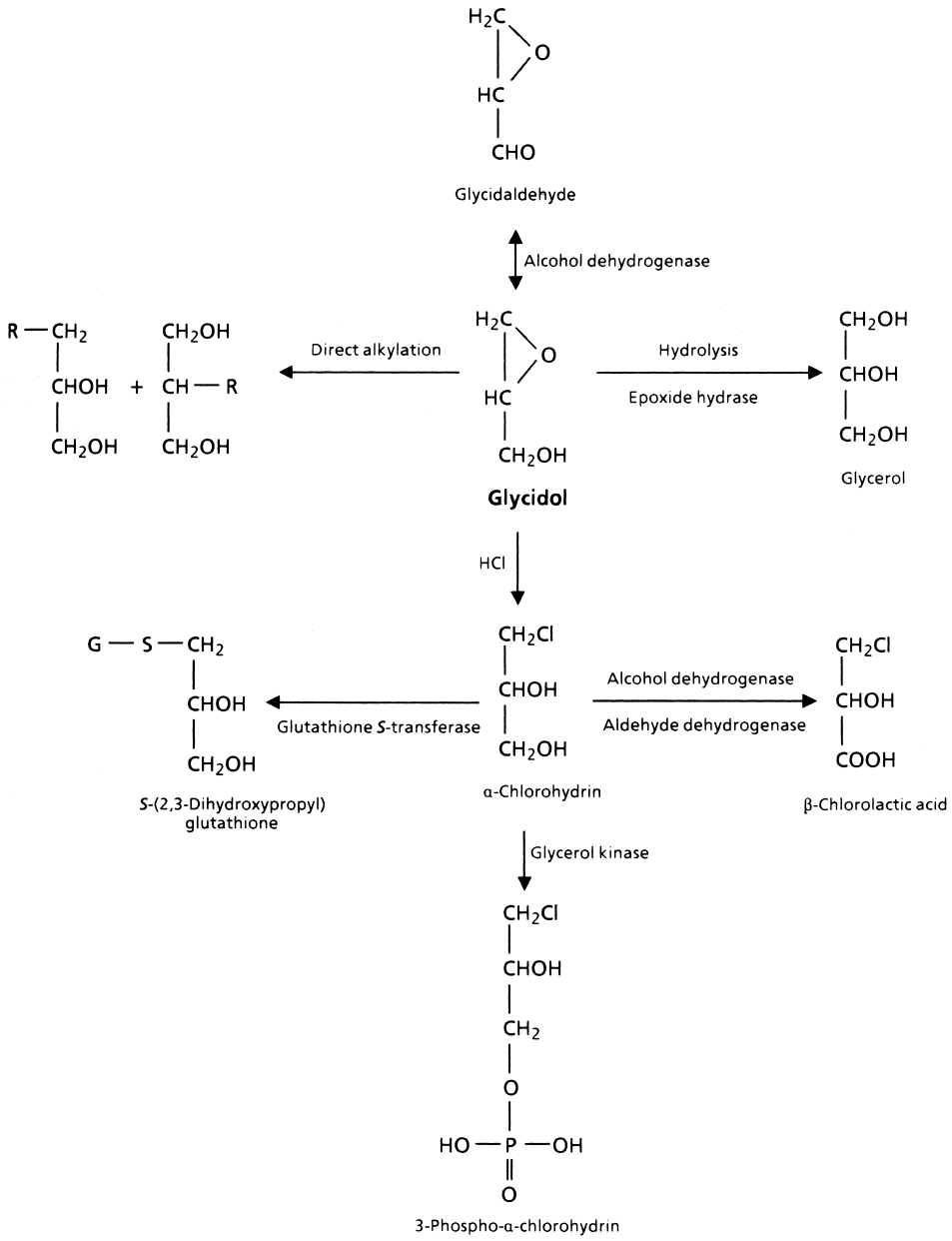


FIGURE 1
Metabolic Pathways for Glycidol (March, 1978)

received 25, 100, or 200 mg/kg, and sperm counts and sperm motility were reduced in $B6C3F_1$ mice that received 75 or 150 mg/kg. Glycidol did not affect the reproductive patterns of female rats or mice (NTP, 1990; Bishop *et al.*, 1997). In a review of the literature, the NTP reported that glycidol was teratogenic in some species of rats (Slott and Hales, 1985).

Humans

No studies of reproductive toxicity of glycidol in humans were found in a review of the literature.

CARCINOGENICITY

Experimental Animals

In a 2-year oral gavage study (5 days per week in corn oil) in F344/N rats and $B6C3F_1$ mice, glycidol was carcinogenic in several target organs (Table 1; NTP, 1990).

Glycidol did not induce skin tumors in ICR/Ha Swiss mice when the chemical was administered by skin painting three times per week for 520 days at a concentration of 5% in acetone (IARC, 2000). Glycidol did not induce tumors in Syrian golden hamsters when administered at approximately 100 mg/kg body weight twice a week for 60 weeks (IARC, 2000). Glycidol did not cause a carcinogenic effect in a 6-month $p53^{+/-}$ mouse study (Tennant *et al.*, 1999).

Humans

Glycidol is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals (NTP, 1990, 2004; IARC, 2000). No adequate exposure studies on the relationship between glycidol and cancer in humans have been reported (NTP, 2004).

GENETIC TOXICITY

Glycidol is a demonstrated potent genotoxin, *in vitro* and *in vivo*. Its mutagenicity has been reviewed by The International Agency for Research on Cancer (2000). Glycidol has been shown to induce large numbers of gene mutations in several strains of *Salmonella typhimurium* with and without liver S9 activation enzymes (Canter *et al.*, 1986), and it induced increases in chromosomal aberrations and sister chromatid exchanges in cultured Chinese hamster ovary cells (NTP, 1990). Elevated frequencies of chromosomal aberrations and sister chromatid exchanges were also observed in human lymphocytes treated with glycidol in the absence of S9 (Norppa *et al.*, 1981). Positive results were reported with glycidol in a mammalian cell gene mutation assay using mouse lymphoma $L5178Y/tk^{+/-}$ cells and conducted without S9 activation enzymes (NTP, 1990).

In vivo, glycidol was one of only a few chemicals shown to induce reciprocal translocations in male germ cells of *Drosophila melanogaster*, and it also induced sex-linked recessive lethal mutations in male *Drosophila* germ cells (Foureman *et al.*, 1994). Glycidol (150 mg/kg) induced micronuclei in bone marrow erythrocytes of male $B6C3F_1$ mice after two intraperitoneal injections (NTP, 1990). In addition, chromosomal aberrations were induced in bone marrow cells of male and female Wistar rats following intraperitoneal injection of glycidol (Thompson and Gibson, 1984).

BACKGROUND ON GENETICALLY ALTERED MICE

The CDKN2A genetic locus contains two important tumor suppressor genes located on chromosome 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 $p16^{Ink4a}$ 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).

TABLE 1
Organs with Neoplasms in F344/N Rats and B6C3F$_1$ Mice Administered Glycidol for 2 Years[a]

Site/Neoplasm[b]	Male			Female		
	Vehicle Control	37.5 mg/kg	75 mg/kg	Vehicle Control	37.5 mg/kg	75 mg/kg
Rats						
Tunica Vaginalis/Peritoneum						
Mesothelioma	3/49	34/50	39/47			
Mammary Gland						
Fibroadenoma	3/45	8/39	7/17	14/49	32/46	29/44
Adenocarcinoma				1/50	11/48	16/48
Brain						
Glioma	0/46	5/50	6/30	0/49	4/46	4/46
Oral Mucosa						
Papilloma or Carcinoma				1/46	3/37	7/26
Forestomach						
Papilloma or Carcinoma	1/46	2/50	6/32	0/47	4/38	11/30
Intestine						
Adenomatous Polyp or Adenocarcinoma	0/47	1/50	4/37			
Skin						
Sebaceous Gland Adenoma, Basal Cell Tumor, or Sebaceous Gland Adenocarcinoma	0/45	5/41	4/18			
Zymbal's Gland						
Carcinoma	1/49	3/50	6/48			
Clitoral Gland						
Adenoma, Adenocarcinoma, or Carcinoma				5/49	9/47	12/45
Thyroid Gland						
Follicular Cell Adenoma or Carcinoma	1/46	4/42	6/19	0/49	1/38	3/35
Hematopoietic System						
Leukemia				13/49	14/44	20/41

TABLE 1
Organs with Neoplasms in F344/N Rats and B6C3F$_1$ Mice Administered Glycidol for 2 Years

Site/Neoplasm	Male			Female		
	Vehicle Control	25 mg/kg	50 mg/kg	Vehicle Control	25 mg/kg	50 mg/kg
Mice						
Harderian Gland[c]						
Adenoma or Adenocarcinoma	8/46	12/41	22/44	4/46	11/43	17/43
Mammary Gland						
Adenoma, Fibroadenoma, or Carcinoma				2/50	6/50	15/50
Forestomach						
Squamous Cell Papilloma or Carcinoma	1/50	2/50	10/50			
Uterus						
Carcinoma or Adenocarcinoma				0/50	3/50	3/50
Subcutaneous Tissue						
Sarcoma or Fibrosarcoma				0/50	3/50	9/50
Skin						
Squamous Cell Papilloma or Carcinoma	0/50	0/50	4/50	0/50	0/50	2/50
Liver						
Adenoma or Carcinoma	24/50	31/50	35/50			
Lung						
Alveolar/bronchiolar Adenoma or Carcinoma	13/50	11/50	21/50			

[a] NTP, 1990
[b] A blank space indicates that the neoplasm incidence at that site and in that sex was not increased by the administration of glycidol. Neoplasm incidence is expressed as the number of neoplasm-bearing animals divided by the number of animals in each group surviving to the time the first neoplasm was observed in any of the three groups.
[c] The denominators for the incidence of harderian gland neoplasms are the actual number of harderian glands available for microscopic examination.

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 of 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 (Sharpless and DePinho, 1999).

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 ensures 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 in the $p16^{-/+}$ mouse (7 to 20 weeks).

STUDY RATIONALE

The purpose of this study of glycidol and the companion studies of benzene and phenolphthalein (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 bioassay (NTP 1986, 1990, 1996). This Report presents the findings from the glycidol study.

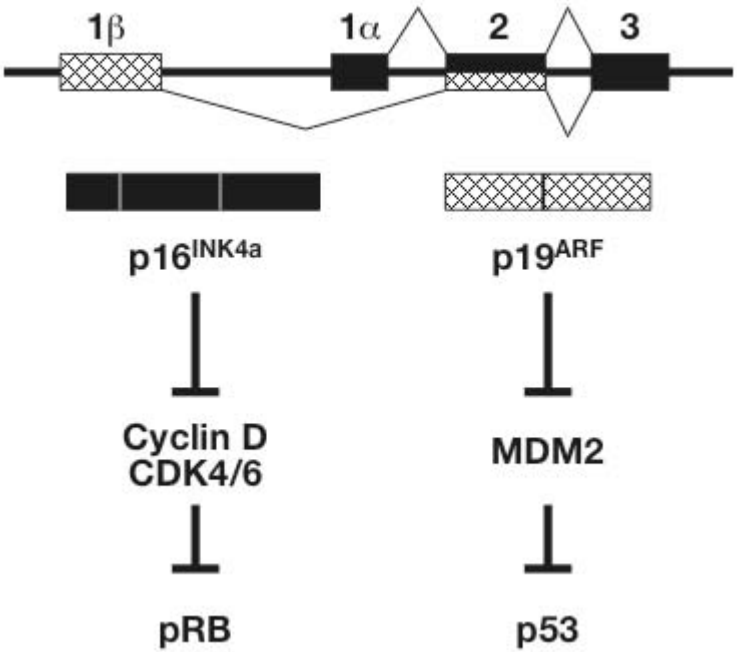


FIGURE 2
The *INK4a/ARF* Locus. The open reading frames $p16^{INK4a}$ (in black) and $p19^{ARF}$ (in crosshatch) are shown. Each has a unique first exon that then splices to a common second exon, but in alternate reading frames. $P16^{INK4a}$ inhibits cdk4/6 activity producing retinoblastoma phosphorylation, which induces cell cycle arrest. $P19^{ARF}$ inhibits MDM2-mediated degradation of p53 (Sharpless, 2005).

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF GLYCIDOL

Glycidol was obtained from Aldrich Chemical Co. (Milwaukee, WI) in one lot (01616 BS) and was used in the 40-week study. Identity and purity analyses were conducted by the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC), and the study laboratory, Battelle Columbus Operations (Columbus, OH); stability analyses were also conducted by the analytical chemistry laboratory (Appendix E). Reports on analyses performed in support of the glycidol studies are on file at the National Institute of Environmental Health Sciences.

Lot 01616 BS of the chemical, a viscous, colorless, combustible liquid (NTP, 2004), was identified as glycidol using infrared spectroscopy and proton nuclear magnetic resonance (NMR) spectroscopy by the analytical chemistry laboratory and infrared spectroscopy by the study laboratory. Spectra were consistent with the structure of glycidol, matched reference spectra (*Aldrich*, 1981, 1983, 1985), and matched the spectrum of a reference standard from the same lot.

The purity of lot 01616 BS was determined by the analytical chemistry and study laboratories using gas chromatography (GC). GC by the analytical chemistry laboratory indicated one major peak, six impurities with peak areas greater than 0.1% of the total peak area, ranging from 0.12% to 1.18%, and seven impurities with peak areas less than 0.1% of the total peak area; the purity of lot 01616 BS was determined to be greater than 96%. Impurities were identified by GC mass spectrometry and quantitated using GC flame ionization detection. Impurities with peak areas exceeding 0.1% were 1-hydroxy-2-propanone (0.6%), diglycidyl ether (0.3%), 3-methoxy-1,2-propanediol (0.5%), 2,5-dimethanol-p-dioxane (0.1%), 2,6-dimethanol-p-dioxane (2.2%), and glycidol-dimethyl ether (0.2%). GC by the study laboratory indicated one major peak and several minor impurities; the purity was determined to be 95.9% by comparison to a reference standard from the same lot. The overall purity of lot 01616 BS was determined to be greater than 95%.

Analyses of the bulk chemical were performed by the study laboratory at approximately 2 and 8 months and at the end of the study using GC. To ensure stability, the bulk chemical was protected from light in amber glass bottles capped with Teflon®-lined lids and stored at approximately 5° C. No degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

Dose formulations were prepared monthly. The dose formulations were prepared by mixing the appropriate amount of glycidol with deionized water to give the required concentrations (Table E1). Formulations were protected from light in amber glass bottles capped with Teflon®-lined lids and stored at approximately 5° C for up to 35 days.

The study laboratory conducted periodic analyses of preadministration dose formulations five times during the study using GC; postadministration formulations were also analyzed. All of the preadministration dose formulations used and analyzed were within 10% of the target concentrations; of the postadministration formulations analyzed, all 16 were more than 10% less than the target concentrations, ranging from –11% to –17%, probably due to the evaporation of glycidol during administration (Table E2).

40-WEEK STUDY

Study Design

Groups of 15 male and 15 female mice received glycidol in deionized water by gavage at doses of 0, 25, 50, 100, or 200 mg glycidol/kg body weight 5 days per week for 40 weeks. A $p53^{+/-}$ mouse study was reported to be negative when glycidol was administered by gavage at 0, 25, or 50 mg/kg body weight for 6 months (Tennant *et al.*, 1999). Thus, doses for the 40-week glycidol study in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice were selected to overlap those in the $p53^{+/-}$ mouse glycidol study (Tennant *et al.*, 1999) and the NTP (1990) 2-year gavage study in $B6C3F_1$ mice (0, 25, or 50 mg/kg) and include

higher doses to test the model over a broader dose range. A 40-week dosing period was selected (rather than a 27-week dosing period) to allow for more time for cancer development (ILSI, 2001).

Source and Specification of Animals

Male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice developed by Serrano *et al.* (1996) were obtained from Taconic Laboratory Animals and Services (Germantown, NY). To produce the mice used in these studies, the N1 male mouse homozygous null for the Cdkn2a deletion (Serrano *et al.*, 1996) was backcrossed to inbred C57BL/6 female mice from Taconic to produce male and female B6.129-$Cdkn2^{atm1Rdp}$ haploinsufficient or haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice. The genetic background of these mice was 80% C57BL/6, 19% 129/Sv, and 1% SJL. This line, designated 5003 by Taconic, was embryo cryopreserved in 2003. On receipt, the mice were 4 to 5 weeks old. Animals were quarantined for 28 days and were 8 to 9 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 up to five male and five female sentinel animals at 1, 4, and 6 months 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

Feed and water were available *ad libitum*. Mice were housed individually. Clinical findings were recorded weekly, to coincide with body weight collection, and at the end of the study. The animals were weighed initially, weekly, and at the end of the study. Details of the study design and animal maintenance are summarized in Table 2.

Clinical Examinations and Pathology

At the end of the 40-week study, samples were collected for sperm motility evaluations on all male mice. The parameters evaluated are listed in Table 2. For sperm count and motility, the left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. 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. Four sperm morphology slides were prepared for each animal evaluated. 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 animals. The heart, right kidney, liver, lungs, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin. An extended evaluation of the brain (five sections per animal) was conducted in all mice. 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 40-week study, a quality assessment pathologist evaluated slides from all tumors and all potential target organs, which included brain, forestomach, liver, and lung.

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 Gavage Study of Glycidol

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

Strain and Species
Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice

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

Time Held Before Study
28 days

Average Age When Studies Began
8 to 9 weeks

Date of First Dose
February 3, 2000

Duration of Dosing
5 days/week for 40 weeks

Date of Last Dose
November 7-9, 2000

Necropsy Dates
November 8-10, 2000

Average Age at Necropsy
48 to 49 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

Method of Animal Identification
Tail tattoo and ear tags

TABLE 2
Experimental Design and Materials and Methods in the Gavage Study of Glycidol

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 and Racks
Polycarbonate cages in stainless steel racks (Lab Products Corp., Seaford, DE), changed weekly

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)

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

Doses
0, 25, 50, 100, or 200 mg/kg in water (dosing volume 10 mL/kg body weight)

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 heart, right kidney, liver, lungs, 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 (femur and sternum), brain, large intestine (colon and cecum), small intestine (duodenum, ileum, jejunum), heart, kidney, liver, lung, lymph nodes (mandibular, 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; 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 and Analysis of Lesion Incidences

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 (Gart *et al.*, 1979) and the Cochran-Armitage trend test (Armitage, 1971; Gart *et al.*, 1979), procedures based on the overall proportion of affected animals, were used to determine significance.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). 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 dose concentrations.

QUALITY ASSURANCE METHODS

The 40-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 Protocol

A detailed discussion of this assay is presented by MacGregor *et al.* (1990). Blood samples were obtained from male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice, and smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were sent to the genetic toxicity testing laboratory (SITEK Research Laboratories, Inc.), stained with acridine orange, and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes (NCEs) per animal per dose group. In addition, the percentage of polychromatic erythrocytes among 1,000 total erythrocytes was determined for each animal as a measure of glycidol-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 dose groups using a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dose group and the vehicle control group (ILS, 1990). In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted

upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single dosed group is less than or equal to 0.025 divided by the number of dosed groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, reproducibility of any effects observed, and the magnitudes of those effects.

Evaluation Protocol

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

RESULTS

MICE

40-WEEK STUDY

Survival

Estimates of 40-week survival probabilities for male and female mice are shown in Table 3. Although survival of males and females administered 200 mg glycidol/kg body weight was less than that of the vehicle controls, the differences were not statistically significant. Survival of the other dosed groups was similar to that of the vehicle controls.

Body Weights, Clinical Findings, Organ Weights, and Sperm Evaluation

Mean body weights of 200 mg/kg males and 100 and 200 mg/kg females were less than those of the vehicle controls after weeks 4, 14, and 12, respectively (Figure 3 and Tables 4 and 5). The final mean body weights of 25 and 50 mg/kg female mice were 94% and 88% that of the vehicle controls, respectively. Treatment-related clinical findings in 200 mg/kg males and females included lethargy, abnormal breathing, and tremors.

Compared to the vehicle controls, absolute organ weights were significantly decreased in the heart and right testis of 200 mg/kg male mice and in the thymus of 200 mg/kg female mice (Table C1). There were also significant decreases in the left testis, left epididymis, and left cauda epididymis weights of 200 mg/kg males accompanied by a significant decrease in the number of sperm heads per cauda epididymis (Table D1). There were no treatment-related histopathologic abnormalities in the testis.

TABLE 3
Survival of Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Animals initially in study	15	15	15	15	15
Accidental death[a]	0	0	0	0	1
Moribund	0	0	1	0	4
Natural deaths	2	1	1	1	3
Animals surviving to study termination	13	14	13	14	7
Percent probability of survival at end of study[b]	87	93	87	93	51
Mean survival (days)[c]	272	278	269	275	251
Survival analysis[d]	P=0.006	P=0.951N	P=1.000N	P=0.984N	P=0.096
Female					
Animals initially in study	15	15	15	15	15
Moribund	2	1	2	1	4
Natural deaths	0	0	1	0	2
Animals surviving to study termination	13	14	12	14	9
Percent probability of survival at end of study	87	93	80	93	60
Mean survival (days)	267	278	275	278	257
Survival analysis	P=0.045	P=0.984N	P=0.999	P=0.984N	P=0.226

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

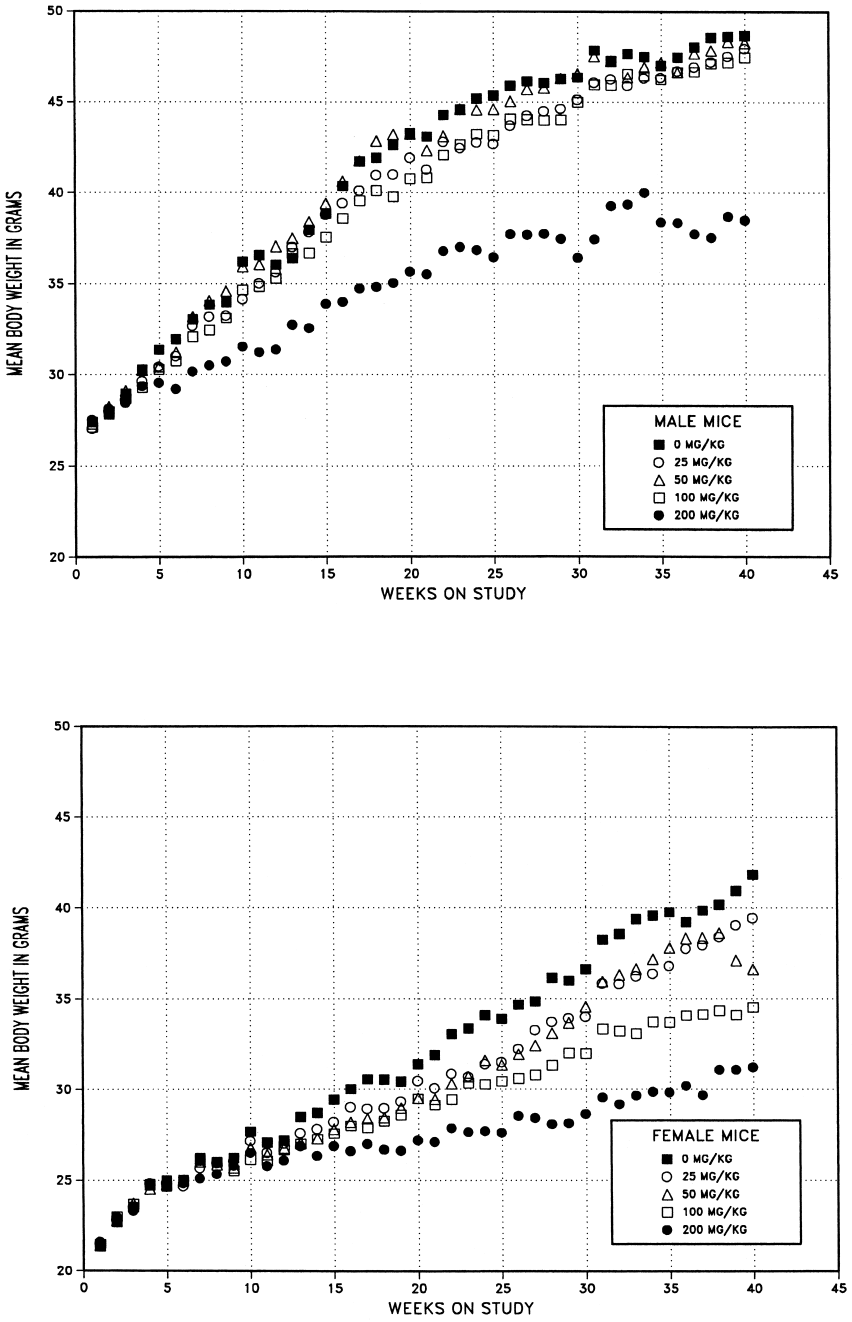


FIGURE 3
Growth Curves for Male and Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice Administered Glycidol by Gavage for 40 Weeks

TABLE 4
Mean Body Weights and Survival of Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

Weeks on Study	Vehicle Control		25 mg/kg			50 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	27.4	15	27.0	99	15	27.3	100	15
2	27.8	15	28.0	101	15	28.2	101	15
3	28.9	15	28.6	99	15	29.1	101	15
4	30.3	15	29.6	98	15	30.3	100	15
5	31.4	15	30.4	97	15	30.4	97	15
6	31.9	15	31.0	97	15	31.2	98	15
7	33.0	15	32.7	99	15	33.2	101	15
8	33.8	15	33.2	98	15	34.0	101	15
9	34.0	15	33.2	98	15	34.6	102	15
10	36.2	15	34.1	94	15	35.9	99	15
11	36.6	15	35.0	96	15	36.0	98	15
12	36.0	15	35.6	99	15	37.0	103	15
13	36.4	15	37.0	102	15	37.5	103	15
14	38.0	15	37.8	100	15	38.4	101	15
15	38.8	15	38.8	100	15	39.4	102	15
16	40.4	15	39.4	98	15	40.6	101	15
17	41.7	15	40.1	96	15	41.7	100	15
18	41.9	15	41.0	98	15	42.8	102	15
19	42.6	15	41.0	96	15	43.2	101	15
20	43.3	15	41.9	97	15	43.2	100	15
21	43.1	15	41.3	96	15	42.3	98	15
22	44.3	15	42.8	97	15	43.1	97	14
23	44.6	15	42.5	95	15	44.6	100	14
24	45.2	15	42.8	95	15	44.5	99	14
25	45.4	15	42.7	94	15	44.6	98	14
26	45.9	15	43.7	95	15	45.0	98	14
27	46.1	15	44.2	96	15	45.7	99	14
28	46.0	15	44.5	97	15	45.8	100	14
29	46.3	14	44.6	96	15	46.3	100	14
30	46.4	14	45.1	97	15	46.5	100	14
31	47.8	14	46.1	96	15	47.5	99	14
32	47.3	14	46.3	98	15	47.3	100	14
33	47.7	14	45.9	96	15	46.4	97	14
34	47.5	14	46.3	98	15	46.9	99	14
35	47.0	13	46.3	99	15	47.2	100	14
36	47.5	13	46.7	98	15	46.6	98	13
37	48.0	13	46.9	98	14	47.7	99	13
38	48.6	13	47.2	97	14	47.8	98	13
39	48.6	13	47.5	98	14	48.3	99	13
40	48.7	13	47.9	98	14	48.3	99	13
Mean for weeks								
1-13	32.6		32.0	98		32.7	100	
14-40	45.1		43.8	97		44.9	99	

TABLE 4
Mean Body Weights and Survival of Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

Weeks on Study	100 mg/kg			200 mg/kg		
	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	27.1	99	15	27.5	100	15
2	28.0	101	15	28.1	101	15
3	28.7	99	15	28.5	99	15
4	29.3	97	15	29.4	97	15
5	30.3	97	15	29.5	94	15
6	30.7	96	15	29.2	92	15
7	32.1	97	15	30.2	92	15
8	32.5	96	15	30.5	90	15
9	33.1	97	15	30.7	90	15
10	34.7	96	15	31.5	87	15
11	34.8	95	15	31.2	85	15
12	35.3	98	15	31.4	87	15
13	36.6	101	15	32.7	90	15
14	36.7	97	15	32.5	86	15
15	37.5	97	15	33.9	87	15
16	38.6	96	15	34.0	84	15
17	39.5	95	15	34.7	83	15
18	40.1	96	15	34.8	83	15
19	39.8	93	15	35.0	82	15
20	40.8	94	15	35.7	82	15
21	40.8	95	15	35.5	82	15
22	42.1	95	15	36.8	83	15
23	42.6	96	15	37.0	83	15
24	43.2	96	15	36.8	81	15
25	43.2	95	15	36.4	80	15
26	44.1	96	15	37.7	82	15
27	44.0	95	15	37.7	82	14
28	44.0	96	15	37.7	82	14
29	44.0	95	15	37.5	81	14
30	45.0	97	14	36.4	78	14
31	45.9	96	14	37.4	78	12
32	45.9	97	14	39.3	83	10
33	46.5	98	14	39.4	83	10
34	46.4	98	14	40.0	84	10
35	46.2	98	14	38.4	82	9
36	46.6	98	14	38.3	81	9
37	46.7	97	14	37.7	79	9
38	47.1	97	14	37.5	77	9
39	47.2	97	14	38.7	80	8
40	47.5	98	14	38.5	79	8
Mean for weeks						
1-13	31.8	98		30.0	93	
14-40	43.4	96		36.9	82	

TABLE 5
Mean Body Weights and Survival of Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

Weeks on Study	Vehicle Control		25 mg/kg			50 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	21.3	15	21.5	101	15	21.4	101	15
2	22.7	15	22.8	100	15	22.8	100	15
3	23.5	15	23.5	100	15	23.7	101	15
4	24.8	15	24.8	100	15	24.5	99	15
5	25.0	15	24.8	99	15	24.8	99	15
6	25.0	15	24.7	99	15	25.0	100	15
7	26.2	15	25.7	98	15	26.0	99	15
8	26.0	15	25.9	100	15	25.9	100	15
9	26.2	15	26.0	99	15	25.7	98	15
10	27.7	15	27.1	98	15	26.8	97	15
11	27.1	15	26.5	98	15	26.4	97	15
12	27.2	15	27.0	99	15	26.8	98	15
13	28.5	14	27.6	97	15	27.0	95	15
14	28.7	14	27.8	97	15	27.3	95	15
15	29.4	14	28.2	96	15	27.8	95	15
16	30.0	14	29.0	97	15	28.2	94	15
17	30.6	14	28.9	94	15	28.4	93	15
18	30.5	14	29.0	95	15	28.4	93	15
19	30.4	14	29.3	96	15	29.0	95	15
20	31.4	14	30.4	97	15	29.5	94	15
21	31.9	14	30.1	94	15	29.4	92	15
22	33.0	14	30.8	93	15	30.3	92	15
23	33.4	14	30.7	92	15	30.7	92	15
24	34.1	14	31.4	92	15	31.6	93	15
25	33.9	14	31.5	93	15	31.3	92	15
26	34.7	14	32.2	93	15	31.9	92	15
27	34.8	14	33.3	96	15	32.4	93	15
28	36.1	14	33.7	93	15	33.1	92	15
29	36.0	14	33.9	94	15	33.7	94	15
30	36.6	14	34.0	93	15	34.6	95	15
31	38.2	14	35.8	94	15	35.9	94	15
32	38.6	14	35.8	93	15	36.3	94	15
33	39.4	14	36.2	92	15	36.6	93	15
34	39.6	14	36.4	92	15	37.2	94	14
35	39.8	14	36.8	93	15	37.8	95	13
36	39.2	14	37.7	96	14	38.3	98	13
37	39.8	14	37.9	95	14	38.3	96	13
38	40.2	14	38.4	96	14	38.6	96	13
39	40.9	14	39.0	95	14	37.1	91	13
40	41.8	13	39.4	94	14	36.6	88	13
Mean for weeks								
1-13	25.5		25.2	99		25.1	99	
14-40	35.3		33.2	94		33.0	94	

TABLE 5
Mean Body Weights and Survival of Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

Weeks on Study	100 mg/kg			200 mg/kg		
	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	21.5	101	15	21.6	101	15
2	23.0	101	15	23.0	101	15
3	23.7	101	15	23.3	99	15
4	24.7	100	15	24.7	100	15
5	24.7	99	15	24.7	99	15
6	24.9	100	15	24.8	99	15
7	26.1	100	15	25.1	96	15
8	26.0	100	15	25.3	97	15
9	25.5	97	15	25.8	99	15
10	26.1	94	15	26.5	96	15
11	26.1	96	15	25.8	95	15
12	26.7	98	15	26.1	96	15
13	27.0	95	15	26.9	94	15
14	27.3	95	15	26.3	92	15
15	27.6	94	15	26.9	92	15
16	28.0	93	15	26.6	89	15
17	27.9	91	15	27.0	88	15
18	28.2	93	15	26.7	88	15
19	28.6	94	15	26.6	88	15
20	29.5	94	15	27.2	87	14
21	29.1	91	15	27.1	85	14
22	29.4	89	15	27.9	85	14
23	30.3	91	15	27.6	83	14
24	30.3	89	15	27.7	81	14
25	30.4	90	15	27.6	81	14
26	30.6	88	15	28.5	82	14
27	30.8	89	15	28.4	82	14
28	31.3	87	15	28.1	78	13
29	32.0	89	15	28.1	78	13
30	32.0	87	15	28.6	78	13
31	33.3	87	15	29.6	78	12
32	33.2	86	15	29.2	76	12
33	33.1	84	15	29.7	76	12
34	33.7	85	15	29.9	76	12
35	33.7	85	15	29.8	75	12
36	34.1	87	14	30.2	77	11
37	34.1	86	14	29.7	75	11
38	34.3	85	14	31.1	77	11
39	34.1	83	14	31.1	76	11
40	34.5	83	14	31.2	75	11
Mean for weeks						
1-13	25.1	99		24.9	98	
14-40	31.2	89		28.5	81	

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of histiocytic sarcoma and neoplasms and/or nonneoplastic lesions in the lung, forestomach, brain, heart, and thymus. Summaries of the incidences of neoplasms and nonneoplastic lesions are presented in Tables A1, A2, A3, and A4.

Gross lesions observed at necropsy included enlarged spleen and foci of discoloration in the liver in 200 mg/kg male mice.

Histiocytic sarcoma: Compared to the vehicle control and historical control incidences, the incidences of histiocytic sarcoma were increased in dosed groups of males, and the increases were significant in 50 and 200 mg/kg males (Tables 6, A1, and F1). The incidences of this neoplasm in females administered 50 mg/kg or greater were slightly increased and exceeded the historical control range (Tables 6, A3, and F3). Histiocytic sarcoma involved multiple organs including the bone marrow, heart, kidney, liver, lung, ovary, spleen, and uterus. Histiocytic sarcoma is a malignant neoplasm putatively of macrophage/histiocytic lineage and is considered a systemic neoplasm that can arise from within and spread to various organs. It is a common neoplasm in heterozygous $p16^{Ink4a}$ control mice.

Lung: The incidences of alveolar/bronchiolar adenoma in 100 mg/kg males and 200 mg/kg females were significantly greater than those in the vehicle controls (Tables 7, A1, and A3). The incidences of alveolar/bronchiolar adenoma in 50 mg/kg or greater males and 200 mg/kg females exceeded the historical control ranges (Tables 6, F1, and F3) Two 100 mg/kg males and one 200 mg/kg male had multiple alveolar/bronchiolar adenomas, and one 25 mg/kg male had multiple alveolar/bronchiolar carcinomas. Alveolar/bronchiolar adenomas were well-demarcated, mildly

TABLE 6
Incidences of Histiocytic Sarcoma in Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Histiocytic Sarcoma[a,b]					
Overall rate[c]	2/15 (13%)	6/15 (40%)	9/15 (60%)	5/15 (33%)	11/15 (73%)
Terminal rate[d]	1/13 (8%)	5/14 (36%)	7/13 (54%)	5/14 (36%)	4/7 (57%)
First incidence (days)	196	253	146	280 (T)	181
Statistical analysis[e]	P=0.005	P=0.107	P=0.010	P=0.195	P<0.001
Female					
Histiocytic Sarcoma[f]					
Overall rate	9/15 (60%)	9/15 (60%)	12/15 (80%)	10/15 (67%)	13/15 (87%)
Terminal rate	8/13 (62%)	8/14 (57%)	9/12 (75%)	9/14 (64%)	8/9 (89%)
First incidence (days)	267	239	232	239	133
Statistical analysis	P=0.076	P=0.645N	P=0.213	P=0.500	P=0.107

(T) Terminal sacrifice
[a] Histiocytic sarcoma involved multiple organs including the liver, bone marrow, ovary, spleen, heart, lung, kidney, and uterus.
[b] Historical incidence for 40-week studies with haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse vehicle control groups: 4/30 (13%), range 13%
[c] Number of animals with neoplasm per number of animals necropsied
[d] Observed incidence at terminal kill
[e] The result of the Cochran-Armitage trend test (Armitage, 1971) is in the vehicle control column, and the results of the Fisher exact pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. A lower incidence in a dosed group is indicated by **N**.
[f] Historical incidence: 14/30 (47%), range 33%-60%

TABLE 7
Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Number Examined Microscopically	15	15	15	15	15
Alveolar Epithelium, Hyperplasia, Focal[a]	0	2 (1.0)[b]	0	2 (1.0)	2 (1.5)
Alveolar/bronchiolar Adenoma, Multiple	0	0	0	2	1
Alveolar/bronchiolar Adenoma (includes multiple)[c]					
Overall rate[d]	1/15 (7%)	0/15 (0%)	2/15 (13%)	7/15 (47%)	3/15 (20%)
Terminal rate[e]	1/13 (8%)	0/14 (0%)	2/13 (15%)	7/14 (50%)	3/7 (43%)
First incidence (days)	280 (T)	—[g]	280 (T)	280 (T)	280 (T)
Statistical analysis[f]	P=0.053	P=0.500N	P=0.500	P=0.018	P=0.299
Alveolar/bronchiolar Carcinoma, Multiple	0	1	0	0	0
Alveolar/bronchiolar Carcinoma (includes multiple)[h]					
Overall rate	2/15 (13%)	1/15 (7%)	1/15 (7%)	3/15 (20%)	0/15 (0%)
Terminal rate	2/13 (15%)	1/14 (7%)	1/13 (8%)	3/14 (21%)	0/7 (0%)
First incidence (days)	280 (T)	280 (T)	280 (T)	280 (T)	—
Statistical analysis	P=0.287N	P=0.500N	P=0.500N	P=0.500	P=0.241N
Alveolar/bronchiolar Adenoma or Carcinoma[i]					
Overall rate	3/15 (20%)	1/15 (7%)	3/15 (20%)	8/15 (53%)	3/15 (20%)
Terminal rate	3/13 (23%)	1/14 (7%)	3/13 (23%)	8/14 (57%)	3/7 (43%)
First incidence (days)	280 (T)	280 (T)	280 (T)	280 (T)	280 (T)
Statistical analysis	P=0.252	P=0.299N	P=0.674N	P=0.064	P=0.674N

TABLE 7
Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female					
Number Examined Microscopically	15	15	15	15	15
Alveolar Epithelium, Hyperplasia, Focal	1 (4.0)	2 (3.0)	1 (3.0)	0	2 (1.0)
Alveolar/bronchiolar Adenoma[c]					
Overall rate	0/15 (0%)	1/15 (7%)	0/15 (0%)	1/15 (7%)	4/15 (27%)
Terminal rate	0/13 (0%)	1/14 (7%)	0/12 (0%)	1/14 (7%)	2/9 (22%)
First incidence (days)	—	281 (T)	—[j]	281 (T)	208
Statistical analysis	P=0.005	P=0.500	—[j]	P=0.500	P=0.050
Alveolar/bronchiolar Carcinoma[k]					
Overall rate	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (7%)	0/15 (0%)
Terminal rate	0/13 (0%)	0/14 (0%)	0/12 (0%)	1/14 (7%)	0/9 (0%)
First incidence (days)	—	—	—	281 (T)	—
Statistical analysis	P=0.639	—	—	P=0.500	—
Alveolar/bronchiolar Adenoma or Carcinoma[c]					
Overall rate	0/15 (0%)	1/15 (7%)	0/15 (0%)	2/15 (13%)	4/15 (27%)
Terminal rate	0/13 (0%)	1/14 (7%)	0/12 (0%)	2/14 (14%)	2/9 (22%)
First incidence (days)	—	281 (T)	—	281 (T)	208
Statistical analysis	P=0.006	P=0.500	—	P=0.241	P=0.050

(T) Terminal sacrifice
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Historical incidence for 40-week studies with haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse studies with vehicle control groups: 1/30 (3%), range 0%-7%
[d] Number of animals with neoplasm per number of animals with lung examined microscopically
[e] Observed incidence at terminal kill
[f] The result of the Cochran-Armitage trend test (Armitage, 1971) is in the vehicle control column, and the results of the Fisher exact pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. A lower incidence in a dosed group is indicated by **N**.
[g] Not applicable; no neoplasms in animal group
[h] Historical incidence: 2/30 (7%), range 0%-13%
[i] Historical incidence: 3/30 (10%), range 0%-20%
[j] Value of statistic cannot be computed
[k] Historical incidence: 0/30 (0%)

compressing, solid to papillary masses of well-differentiated cuboidal to round cells. Carcinomas were more irregular masses of pleomorphic cells with variable amounts of compression and invasion. One or two animals in most dosed groups and in the female vehicle control group had alveolar epithelial hyperplasia (Tables 7, A2, and A4). Alveolar epithelial hyperplasia consisted of focal thickening of the alveolar septa caused by increased numbers of prominent, cuboidal type-II pneumocytes, with maintenance of normal alveolar septal architecture.

Forestomach: One 200 mg/kg male, one 100 mg/kg female, and three 200 mg/kg females had squamous cell papillomas (Tables 8, A1, and A3). In male and female mice administered 200 mg/kg, the incidences of epithelial hyperplasia were significantly greater than those in the vehicle controls, and the severities were slightly increased in males (Tables 8, A2, and A4). Squamous cell papillomas consisted of small papillary projections of well-differentiated squamous epithelium overlying a thin lamina propria and protruding into the gastric lumen. Epithelial hyperplasia consisted of focal, poorly

TABLE 8
Incidences of Neoplasms and Nonneoplastic Lesions of the Forestomach in Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Number Necropsied	15	15	15	15	15
Epithelium, Hyperplasia[a]	0	1 (2.0)[b]	1 (2.0)	0	6**(2.5)
Ulcer	0	1 (2.0)	0	0	3 (1.7)
Squamous Cell Papilloma, Multiple					
Overall rate[c]	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (7%)
Terminal rate[d]	0/13 (0%)	0/14 (0%)	0/13 (0%)	0/14 (0%)	0/7 (0%)
First incidence (days)	—[f]	—	—	—	218
Statistical analysis[e]	P=0.143	—[g]	—	—	P=0.500
Female					
Number Necropsied	15	15	15	15	15
Epithelium, Hyperplasia	0	0	0	1 (1.0)	4* (1.3)
Squamous Cell Papilloma					
Overall rate	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (7%)	3/15 (20%)
Terminal rate	0/13 (0%)	0/14 (0%)	0/12 (0%)	1/14 (7%)	1/9 (11%)
First incidence (days)	—	—	—	281 (T)	278
Statistical analysis	P=0.005	—	—	P=0.500	P=0.112

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Fisher exact test
** $P \leq 0.01$
(T) Terminal sacrifice
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Number of animals with neoplasm per number of animals necropsied
[d] Observed incidence at terminal kill
[e] The result of the Cochran-Armitage trend test (Armitage, 1971) is in the vehicle control column, and the results of the Fisher exact pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns.
[f] Not applicable; no neoplasms in animal group
[g] Value of statistic cannot be computed

demarcated thickening of the stratified squamous epithelium due to increased numbers of well-differentiated squamous cells. Occasionally, the hyperplasia caused small convolutions. The incidence of ulcer was increased in male mice administered 200 mg/kg.

Brain: Neuronopathy occurred in 33% of the 200 mg/kg males and 7% and 27% of the 100 mg/kg and 200 mg/kg females, respectively (Table 9). Neuronopathy was a chronic, ongoing process that ranged from minimal to severe, depending on the location in the brain. Neuronopathy was present in the cerebrum, cerebellum, medulla, hippocampus, and/or thalamus and was accompanied by minimal to mild gliosis and hemorrhage. More specifically, neuronopathy involved the red, cerebellar roof, vestibular, and/or oculomotor nuclei. Histologically, the neuronopathy consisted of a localized minimal to marked loss of neurons resulting in vacuolization *ex vacuo* (Plates 1 and 2). Lesions were those of symmetric and asymmetric areas of shrunken or vacuolated neurons and/or glial cells, as well as clear spaces where neuronal cell bodies and processes would have been found normally. Symmetrical lesions were generally found in specific regions of the brain such as

TABLE 9
Incidences of Nonneoplastic Lesions of the Brain in Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Number Examined Microscopically	15	15	15	15	15
Neuronopathy[a]	0	0	0	0	5* (2.2)[b]
Gliosis	0	0	0	0	4* (1.2)
Hemorrhage	0	0	0	0	2 (2.7)
Female					
Number Examined Microscopically	15	15	15	15	15
Neuronopathy	0	0	0	1 (1.0)	4* (2.7)
Gliosis	0	0	0	0	4* (1.5)
Hemorrhage	0	0	0	1 (1.0)	0

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

nuclei of the cerebellum, medulla, and/or midbrain that included the red nucleus and thalamic/hypothalamic nuclei. Nuclei most often affected were the red nucleus and cerebellar roof nuclei. Asymmetric lesions were generally found in the cerebrum or hippocampus. Some neurons had one or more small vacuoles, but generally, remaining neurons were morphologically normal. The neuropil was relatively unremarkable except for rare examples of swollen axons.

The five males and two of four females with neuronopathy died before the end of the study. All five males and three of four females diagnosed with neuronopathy had clinical signs of tremors, ataxia, ruffled fur, abnormal breathing, thinness, and/or lethargy just prior to euthanasia or natural death. All of the mice with neuronopathy had histiocytic sarcoma often involving major organs such as brain, heart, lung, and kidney. The mortality and extensive disseminated histiocytic sarcoma in these mice suggest that multiorgan impairment or failure may have contributed to morbidity.

Heart: The incidence of myocardial mineralization in 200 mg/kg males was significantly greater than that in the vehicle controls (0/15, 0/15, 2/15, 1/15, 5/15; Table A2). All 200 mg/kg males with this lesion died before the end of the study.

Thymus: The incidences of atrophy in 200 mg/kg males and in 25, 100, and 200 mg/kg females were greater than those in the vehicle controls (males: 2/15, 0/15, 1/15, 0/15, 4/15; females: 0/15, 2/15, 0/15, 1/15, and 3/15; Tables A2 and A4).

GENETIC TOXICOLOGY

The frequency of micronucleated erythrocytes was monitored in peripheral blood of male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice from the 40-week study. No significant increases were observed at the 6.5, 13, or 19.5 week sampling times (Table B1). At 6.5 weeks, elevations in the frequencies of micronucleated normochromatic erythrocytes (NCEs) were observed in female mice in the 50 and 100 mg/kg groups. However, because the trend test was not significant and the increases were small (less than twice the vehicle control frequency), the results were judged to be negative. Similarly, male mice sampled at 19.5 weeks showed a small increase in micronucleated

NCEs at the high dose (200 mg/kg), but the increase was not statistically different from the vehicle control value. Therefore, despite the positive trend (P=0.002), the test was judged to be negative. In male and female mice sampled at 26 and 40 weeks of treatment, small but statistically significant increases were seen in the frequencies of micronucleated NCEs. Based on positive trend and pairwise analyses, results in male and female mice at these two time points were judged to be positive. In addition to micronucleus frequencies, the percentage of polychromatic erythrocytes (PCEs) in total erythrocytes was measured in mice at each time point; no changes with dose or time were noted until the final sample at 40 weeks. At 40 weeks, the percentage of PCEs in male mice was slightly higher than the percentages noted at earlier sampling times, and there were small but non-significant increases in percentages of PCEs at the two middle doses of 50 and 100 mg/kg. The percentage of PCEs in vehicle control females was also increased over the percentages noted at earlier sample times, and the percentages in the 50 and 100 mg/kg groups were significantly increased.

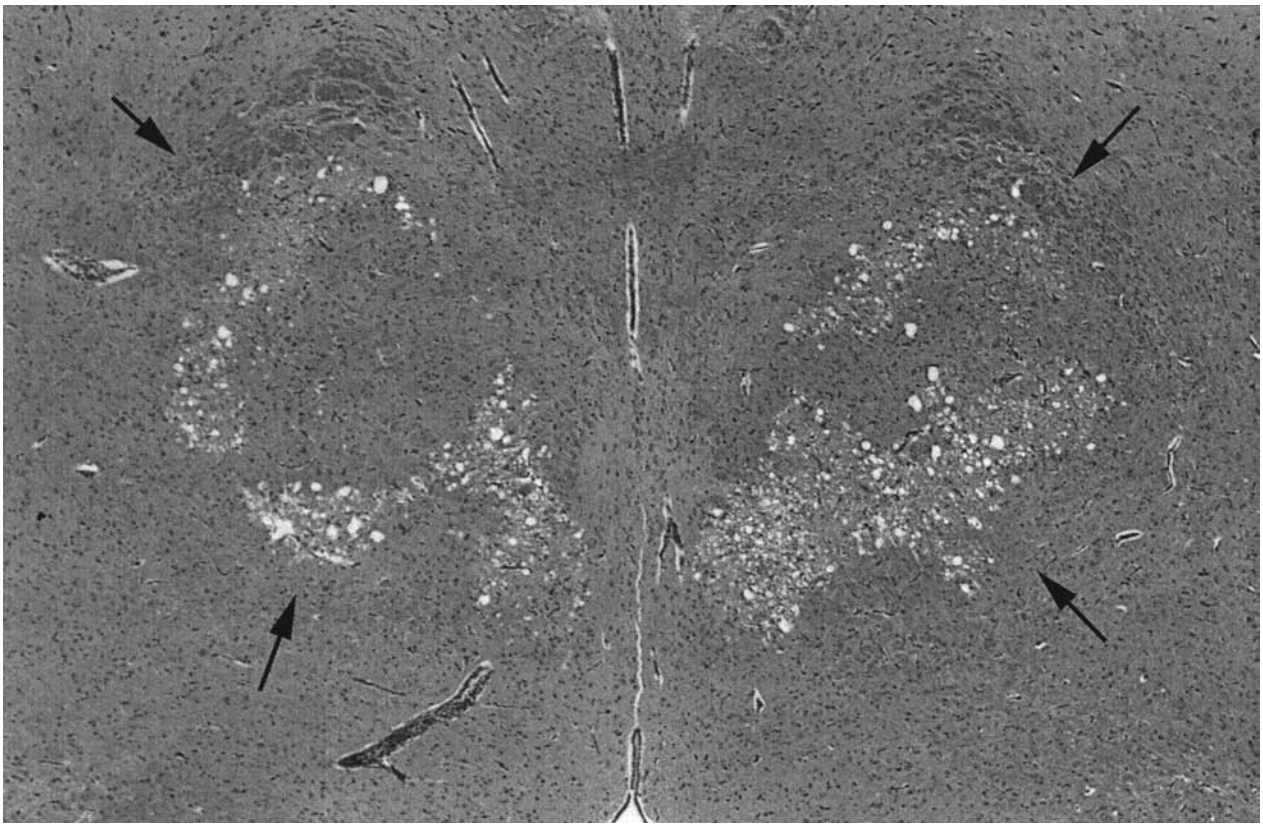


PLATE 1
Bilaterally symmetrical neuronopathy affecting the thalamus (arrows) in a female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse treated with 200 mg/kg glycidol by gavage for 40 weeks.

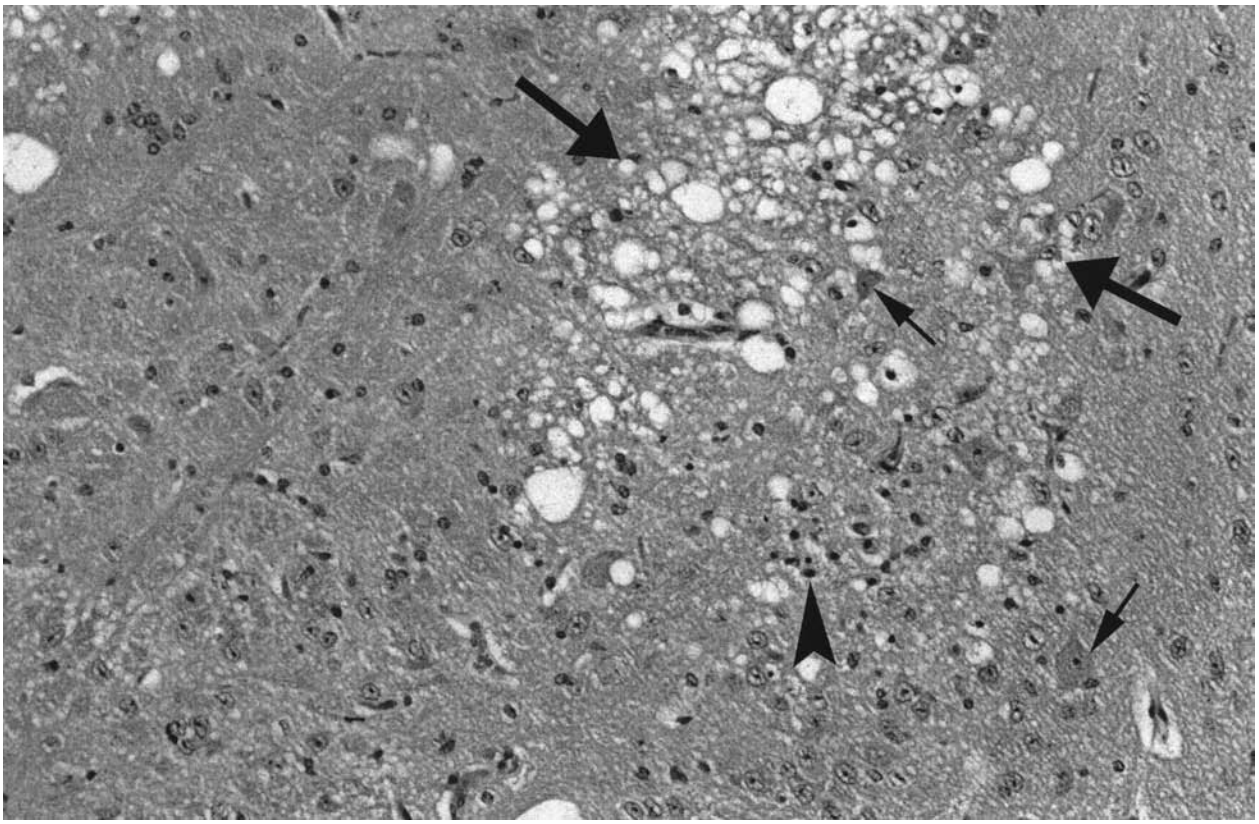


PLATE 2
Higher magnification of Plate 1 showing typical features of neuronopathy such as vacuolated neurons and glial cells (large arrows), shrunken neurons (small arrows), and pyknotic cell debris (arrowhead).

DISCUSSION AND CONCLUSIONS

In the current study, glycidol caused a detectable carcinogenic response in the haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse in a shorter time period and with fewer animals than glycidol did in the 2-year NTP studies (NTP, 1990).

Survival of male and female mice in the 25, 50, and 100 mg/kg groups was not statistically different from that of the vehicle controls, although there were fewer numbers of dosed male and female mice surviving until the end of the study. Mean body weights of male and female mice in the 200 mg/kg groups were less than those of the vehicle controls throughout most of the study. These effects were considered to be related to the toxic and carcinogenic effects of glycidol.

After 40 weeks of glycidol administration, the incidences of histiocytic sarcoma were significantly increased in male mice in the 50 and 200 mg/kg groups (13%, 40%, 60%, 33%, and 73%, respectively, for the 0, 25, 50, 100, and 200 mg/kg groups). The incidences of histiocytic sarcoma in the 25 and 100 mg/kg groups were also increased, although they were not significant by the Fisher exact test. The incidence of histiocytic sarcoma in dosed male mice was significant by the Cochran-Armitage trend test, and the incidences in all dosed groups were greater than those in the current vehicle controls and the historical controls (13% in two studies). This was considered clear evidence of carcinogenic activity. In female mice, there was a greater background for histiocytic sarcoma [current controls: 60%; historical controls: 47% (range 33% to 60%)], and there was no statistically significant increase in the incidence of histiocytic sarcoma in any dosed group.

The incidence of alveolar/bronchiolar adenoma (27%) was significantly increased in 200 mg/kg female mice. The occurrence of these lung neoplasms was considered to be some evidence for a carcinogenic effect because the effect was significant by the trend statistic and was greater than the incidence in the current (0%) and historical controls (mean 3%, range 0% to 7%; Appendix F). This was not considered to be clear evidence of a carcinogenic effect because the incidence of alveolar/bronchiolar carcinoma was not significantly increased in any dosed group by pairwise comparisons with the vehicle controls.

In male mice, the incidence of alveolar/bronchiolar adenoma was significantly increased in the 100 mg/kg group. However, because the incidence of lung neoplasms was not also increased in the 200 mg/kg group, this was not considered to be clearly related to treatment. In the 200 mg/kg male group, the decreased body weight relative to controls (i.e., 79% of controls) may have been one factor in the failure to see an increase in the incidence of lung neoplasms in this group (Seilkop, 1995).

The few forestomach squamous cell papillomas seen in dosed female mice may have been related to treatment. The irritant effects of glycidol in the forestomach, as demonstrated by the occurrence of forestomach ulcers and/or hyperplasia in glycidol treated groups, may have contributed to the development of the forestomach papillomas.

Deletion of one functional p16 gene in this mouse model affects two pathways leading to cancer — the p53 (P53, MDM2, and ARF) and Rb (Rb, P16, P14, P21, cyclin D1 CyclinE) pathways, two of the most common pathways altered in cancer (Sherr and McCormick, 2002; Lowe and Sherr, 2003). Defects in the p53 and p16 pathways are part of the multiple genetic changes that lead to lung cancer (Yokota and Kohno, 2004), and defects in these two pathways often occur simultaneously in lung cancer (Belinsky *et al.*, 1998; Toyooka *et al.*, 2003; Lubet *et al.*, 2005; Rodin and Rodin, 2005). Deficiencies in both pathways increase the rate of tumor formation in mice compared with deficiency in just one pathway (Sharpless *et al.*, 2002). Both Ink4/Arf gene alterations occur in aflatoxin- (Tam *et al.*, 2003) and urethane- (Sharpless *et al.*, 2001) induced mouse lung tumors, adding to the evidence that two tumor gene pathways are inactivated in lung cancers. Other studies suggest that when two genes are altered/mutated [e.g., K-ras and p53 (Tam *et al.*, 2003) or p53 and Ink4a/Arf (Wang *et al.*, 2003)], mice become more susceptible to formation of lung tumors (Jackson *et al.*, 2005).

Glycidol has also been shown to cause neuronopathy in $B6C3F_1$ mice and F344/N rats in 90-day gavage studies at 150 mg/kg or greater (Little and Sills, 2003) but not at lower doses (75 mg/kg or less) after 2 years of gavage (NTP, 1990). In the 90-day studies, glycidol induced neuronopathy in $B6C3F_1$ mice and F344/N rats similar to that of p16 deficient mice including acute neuronal necrosis and depletion in various specific brain nuclei (NTP, 1990; Dr. Peter Little, personal communication). The distribution and nature of the neuronal lesions was not considered consistent with those induced by ischemia/hypoxemia. In ischemia/hypoxemia, neurons in the lateral parietal cortex, CA1 region of the hippocampus, caudate/putamen, anterior lateral thalamus, and Purkinje cells are primarily affected (Ellison *et al.*, 2004). In the glycidol treated mice ($B6C3F_1$ and p16 deficient mice), lesions were seen in the oculomotor, red, cerebellar roof, vestibular, medullary, and reticular gray nuclei. In addition to these regions, glycidol induced lesions in Purkinje cells and lateral superior olivary nuclei in the rat. The findings indicate that glycidol at relatively higher doses (greater than 100 mg/kg) targets specific neuronal populations in $B6C3F_1$ mice, F344/N rats, and p16 deficient mice.

In the 2-year studies, glycidol induced gliomas in rats (NTP, 1990). However, $p16^{Ink4a}/p19^{Arf}$ gene deficiency was not sufficient for glycidol induction of brain tumors in mice. Other studies suggest that $p16^{Ink4a}/p19^{Arf}$ tumor suppressor gene deficiency needs to be combined with oncogene activation for the formation of brain tumors in mice. K-*ras* (Uhrbom *et al.*, 2002) and epidermal growth factor receptor amplification (Lachat *et al.*, 2004) are some of the other gene changes that combined with the $p16^{Ink4a}/p19^{Arf}$ tumor suppressor gene deficiency lead to gliomas in mice.

Glycidol was strongly mutagenic in a number of *Salmonella typhimurium* tester strains, and it induced gene mutations and chromosomal damage in mammalian cells *in vitro*, with over 80% of all cells showing chromosomal aberrations at concentrations of 50 µg/mL or greater (NTP, 1990); additional positive responses were seen in *Drosophila* germ cell assays and acute micronucleus tests in $B6C3F_1$ mice (NTP, 1990). Although glycidol induced increased frequencies of micronucleated erythrocytes in the haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice used in this gavage study, the increases were small and were not consistently detected until the 26-week sampling time, which is unusual considering that the circulating normochromatic erythrocyte population typically reaches steady state within about 30 days following the start of exposure. The weak, delayed micronuclei response seen with glycidol in the haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse, compared with the potent responses it induces *in vitro*, suggest that glycidol, particularly when administered by gavage, might not reach the bone marrow target site for erythrocyte micronuclei induction in amounts sufficient to produce a robust response.

This current glycidol haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mouse study detected fewer carcinogenic target sites than the 2-year glycidol $B6C3F_1$ mouse study (NTP, 1990). The carcinogenic responses in the glycidol haploinsufficient $p16^{Ink4a}/p19^{Arf}$ study were seen at 50, 100, and 200 mg/kg in the male mouse (histiocytic sarcomas and alveolar/bronchiolar adenomas at 100 mg/kg) and in female mice at 200 mg/kg (alveolar/bronchiolar adenomas). The glycidol carcinogenic response in the 2-year $B6C3F_1$ mouse study occurred at 25 and 50 mg/kg. The total glycidol dose administered in the 40-week haploinsufficient $p16^{Ink4a}/p19^{Arf}$ study at the carcinogenic dose of 50 mg/kg was 14,000 mg glycidol/kg, and the total glycidol dose administered to $B6C3F_1$ mice in the 2-year study at the carcinogenic dose of 25 mg/kg was 18,200 mg glycidol/kg.

CONCLUSIONS

Under the conditions of this 40-week gavage study, there was *clear evidence of carcinogenic activity** of glycidol in male haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice based on the occurrence of histiocytic sarcomas. The increased incidences of alveolar/bronchiolar adenomas in male mice were also considered to be related to glycidol administration. There was *some evidence of carcinogenic activity* of glycidol in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ female mice based on the occurrence of alveolar/bronchiolar adenoma. The occurrence of forestomach papillomas in female mice may also have been related to glycidol administration.

Treatment of male and female haploinsufficient $p16^{Ink4a}/p19^{Arf}$ mice with glycidol was associated with forestomach hyperplasia and neuronopathy in the brain.

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

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APPENDIX A
SUMMARY OF LESIONS
IN HAPLOINSUFFICIENT $p16^{Ink4a}/p19^{Arf}$ MICE
IN THE GAVAGE STUDY OF GLYCIDOL

TABLE A1
Summary of the Incidence of Neoplasms in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Disposition Summary					
Animals initially in study	15	15	15	15	15
Early deaths					
Accidental death					1
Moribund			1		4
Natural deaths	2	1	1	1	3
Survivors					
Terminal sacrifice	13	14	13	14	7
Animals examined microscopically	15	15	15	15	15
Alimentary System					
Intestine large, rectum	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma			1 (7%)		1 (7%)
Intestine large, cecum	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma			1 (7%)		
Intestine small, duodenum	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma			1 (7%)		2 (13%)
Intestine small, jejunum	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma			2 (13%)	1 (7%)	
Intestine small, ileum	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma					1 (7%)
Liver	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	2 (13%)	2 (13%)	4 (27%)	2 (13%)	10 (67%)
Pancreas					(1)
Histiocytic sarcoma					1 (100%)
Stomach, forestomach	(15)	(15)	(15)	(15)	(15)
Squamous cell papilloma, multiple					1 (7%)
Cardiovascular System					
Heart	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)			5 (33%)
Endocrine System					
Adrenal cortex	(15)	(15)	(15)	(14)	(15)
Histiocytic sarcoma				1 (7%)	1 (7%)
Pituitary gland	(15)	(15)	(14)	(15)	(15)
Histiocytic sarcoma					1 (7%)
General Body System					
None					
Genital System					
Epididymis	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma				2 (13%)	

TABLE A1
Summary of the Incidence of Neoplasms in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Hematopoietic System					
Bone marrow	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	2 (13%)	5 (33%)	6 (40%)	4 (27%)	8 (53%)
Lymph node	(14)	(14)	(15)	(13)	(13)
Mediastinal, histiocytic sarcoma		1 (7%)	3 (20%)		7 (54%)
Mediastinal, osteosarcoma, metastatic, skin	1 (7%)				
Lymph node, mandibular	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	1 (7%)	1 (7%)	2 (13%)		3 (20%)
Lymph node, mesenteric	(15)	(15)	(15)	(14)	(15)
Histiocytic sarcoma		1 (7%)	2 (13%)	1 (7%)	5 (33%)
Spleen	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	1 (7%)	1 (7%)	3 (20%)	1 (7%)	7 (47%)
Thymus	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)	2 (13%)	2 (13%)	4 (27%)
Integumentary System					
Skin	(15)	(15)	(15)	(15)	(15)
Osteosarcoma	1 (7%)				
Squamous cell papilloma		1 (7%)			
Musculoskeletal System					
Skeletal muscle				(1)	
Hemangiosarcoma				1 (100%)	
Nervous System					
Brain	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)	1 (7%)	1 (7%)	4 (27%)
Spinal cord				(1)	
Histiocytic sarcoma				1 (100%)	
Respiratory System					
Lung	(15)	(15)	(15)	(15)	(15)
Alveolar/bronchiolar adenoma	1 (7%)		2 (13%)	5 (33%)	2 (13%)
Alveolar/bronchiolar adenoma, multiple				2 (13%)	1 (7%)
Alveolar/bronchiolar carcinoma	2 (13%)		1 (7%)	3 (20%)	
Alveolar/bronchiolar carcinoma, multiple		1 (7%)			
Histiocytic sarcoma	1 (7%)	1 (7%)	2 (13%)	2 (13%)	6 (40%)
Osteosarcoma, metastatic, skin	1 (7%)				
Special Senses System					
Harderian gland		(1)			
Adenoma		1 (100%)			
Urinary System					
Kidney	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)	2 (13%)	2 (13%)	5 (33%)

TABLE A1
Summary of the Incidence of Neoplasms in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Systemic Lesions					
Multiple organs[b]	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	2 (13%)	6 (40%)	9 (60%)	5 (33%)	11 (73%)
Lymphoma malignant	2 (13%)			1 (7%)	
Neoplasm Summary					
Total animals with primary neoplasms[c]	7	8	12	12	12
Total primary neoplasms	8	9	12	17	15
Total animals with benign neoplasms	1	2	2	7	4
Total benign neoplasms	1	2	2	7	4
Total animals with malignant neoplasms	6	7	10	9	11
Total malignant neoplasms	7	7	10	10	11
Total animals with metastatic neoplasms	1				
Total metastatic neoplasms	2				

[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 40-week Gavage Study of Glycidol[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Disposition Summary					
Animals initially in study	15	15	15	15	15
Early deaths					
Accidental death					1
Moribund			1		4
Natural deaths	2	1	1	1	3
Survivors					
Terminal sacrifice	13	14	13	14	7
Animals examined microscopically	15	15	15	15	15
Alimentary System					
Esophagus			(1)		
Inflammation, acute			1 (100%)		
Intestine small, jejunum	(15)	(15)	(15)	(15)	(15)
Peyer's patch, hyperplasia, atypical		1 (7%)			
Peyer's patch, hyperplasia, lymphoid		1 (7%)			
Liver	(15)	(15)	(15)	(15)	(15)
Angiectasis, focal					1 (7%)
Basophilic focus		1 (7%)			
Degeneration, cystic	1 (7%)				
Eosinophilic focus					1 (7%)
Fatty change, focal				1 (7%)	
Hematopoietic cell proliferation		1 (7%)		2 (13%)	1 (7%)
Hemorrhage			1 (7%)		
Infiltration cellular, lymphoid	1 (7%)	2 (13%)		2 (13%)	1 (7%)
Inflammation, chronic	6 (40%)	9 (60%)	8 (53%)	9 (60%)	2 (13%)
Mixed cell focus				1 (7%)	
Necrosis, focal	1 (7%)	2 (13%)	2 (13%)		5 (33%)
Mesentery		(1)			
Fat, necrosis		1 (100%)			
Stomach, forestomach	(15)	(15)	(15)	(15)	(15)
Inflammation, chronic					1 (7%)
Ulcer		1 (7%)			3 (20%)
Epithelium, hyperplasia		1 (7%)	1 (7%)		6 (40%)
Stomach, glandular	(15)	(15)	(15)	(15)	(15)
Inflammation, chronic			1 (7%)		
Mineralization	10 (67%)			5 (33%)	1 (7%)
Ulcer					1 (7%)
Epithelium, hyperplasia		1 (7%)			
Glands, ectasia			1 (7%)		
Muscularis, mineralization				1 (7%)	
Cardiovascular System					
Heart	(15)	(15)	(15)	(15)	(15)
Infiltration cellular, mast cell					1 (7%)
Epicardium, inflammation, chronic	1 (7%)				
Myocardium, degeneration	2 (13%)			2 (13%)	
Myocardium, mineralization			2 (13%)	1 (7%)	5 (33%)
Myocardium, necrosis					2 (13%)

[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 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Endocrine System					
Adrenal cortex	(15)	(15)	(15)	(14)	(15)
Hyperplasia, focal	1 (7%)			1 (7%)	
Subcapsular, hyperplasia	1 (7%)	2 (13%)	2 (13%)	3 (21%)	1 (7%)
General Body System					
None					
Genital System					
Epididymis	(15)	(15)	(15)	(15)	(15)
Fibrosis		1 (7%)			
Preputial gland			(1)		
Inflammation, suppurative			1 (100%)		
Testes	(15)	(15)	(15)	(15)	(15)
Germinal epithelium, degeneration		1 (7%)			1 (7%)
Germinal epithelium, mineralization		1 (7%)			
Germinal epithelium, necrosis	1 (7%)				
Hematopoietic System					
Lymph node	(14)	(14)	(15)	(13)	(13)
Mediastinal, hyperplasia, lymphoid		1 (7%)			
Lymph node, mandibular	(15)	(15)	(15)	(15)	(15)
Hyperplasia, lymphoid		2 (13%)		1 (7%)	
Inflammation, suppurative			1 (7%)		
Necrosis					1 (7%)
Lymph node, mesenteric	(15)	(15)	(15)	(14)	(15)
Hematopoietic cell proliferation		1 (7%)			
Hyperplasia, lymphoid		2 (13%)		1 (7%)	1 (7%)
Inflammation, suppurative		1 (7%)			1 (7%)
Necrosis					1 (7%)
Spleen	(15)	(15)	(15)	(15)	(15)
Angiectasis					1 (7%)
Atrophy				1 (7%)	2 (13%)
Hematopoietic cell proliferation	4 (27%)	7 (47%)	7 (47%)	5 (33%)	5 (33%)
Thymus	(15)	(15)	(15)	(15)	(15)
Atrophy	2 (13%)		1 (7%)		4 (27%)
Integumentary System					
Skin	(15)	(15)	(15)	(15)	(15)
Ulcer					1 (7%)
Musculoskeletal System					
Bone	(15)	(15)	(15)	(15)	(15)
Hyperostosis	1 (7%)				

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Nervous System					
Brain	(15)	(15)	(15)	(15)	(15)
Mineralization	1 (7%)				
Cerebellum, gliosis					2 (13%)
Cerebellum, hemorrhage, focal					1 (7%)
Cerebellum, neuronopathy					3 (20%)
Cerebrum, hemorrhage					1 (7%)
Cerebrum, necrosis, focal					2 (13%)
Hippocampus, hemorrhage					1 (7%)
Hippocampus, necrosis			1 (7%)		1 (7%)
Medulla, gliosis					2 (13%)
Medulla, neuronopathy					3 (20%)
Thalamus, gliosis					2 (13%)
Thalamus, neuronopathy					5 (33%)
Spinal cord				(1)	
Axon, degeneration				1 (100%)	
Respiratory System					
Lung	(15)	(15)	(15)	(15)	(15)
Hemorrhage					1 (7%)
Inflammation	3 (20%)	7 (47%)	5 (33%)	4 (27%)	2 (13%)
Inflammation, granulomatous			1 (7%)		
Alveolar epithelium, hyperplasia, focal		2 (13%)		2 (13%)	2 (13%)
Bronchiole, hyperplasia			1 (7%)		
Vein, thrombosis			1 (7%)		
Special Senses System					
None					
Urinary System					
Kidney	(15)	(15)	(15)	(15)	(15)
Hydronephrosis					1 (7%)
Infarct					1 (7%)
Nephropathy	4 (27%)	5 (33%)	5 (33%)	4 (27%)	4 (27%)
Renal tubule, accumulation, hyaline droplet			1 (7%)		1 (7%)
Urinary bladder	(15)	(15)	(15)	(15)	(15)
Inflammation, chronic					1 (7%)
Transitional epithelium, hyperplasia	1 (7%)				

TABLE A3
Summary of the Incidence of Neoplasms in Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Disposition Summary					
Animals initially in study	15	15	15	15	15
Early deaths					
Moribund	2	1	2	1	4
Natural deaths			1		2
Survivors					
Terminal sacrifice	13	14	12	14	9
Animals examined microscopically	15	15	15	15	15
Alimentary System					
Intestine large, colon	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma				1 (7%)	
Intestine large, rectum	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma					1 (7%)
Intestine small, duodenum	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma			4 (27%)	1 (7%)	1 (7%)
Intestine small, jejunum	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		3 (20%)	3 (20%)	1 (7%)	
Intestine small, ileum	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		2 (13%)	2 (13%)	1 (7%)	1 (7%)
Liver	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	5 (33%)	1 (7%)	8 (53%)	5 (33%)	8 (53%)
Osteosarcoma, metastatic, uncertain primary site		1 (7%)			
Mesentery			(2)		
Histiocytic sarcoma			2 (100%)		
Stomach, forestomach	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)		1 (7%)	
Squamous cell papilloma				1 (7%)	3 (20%)
Stomach, glandular	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)	1 (7%)	2 (13%)	
Cardiovascular System					
Heart	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma		1 (7%)	2 (13%)	2 (13%)	
Endocrine System					
Adrenal cortex	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	3 (20%)		7 (47%)	2 (13%)	1 (7%)
Adrenal medulla	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	1 (7%)				
Pituitary gland	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	1 (7%)		2 (13%)	1 (7%)	1 (7%)
General Body System					
None					

TABLE A3
Summary of the Incidence of Neoplasms in Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control		25 mg/kg		50 mg/kg		100 mg/kg		200 mg/kg	
Genital System										
Ovary	(15)		(15)		(15)		(15)		(15)	
Histiocytic sarcoma	8	(53%)	5	(33%)	8	(53%)	6	(40%)	5	(33%)
Osteosarcoma, metastatic, uncertain primary site			1	(7%)						
Uterus	(15)		(15)		(15)		(15)		(15)	
Hemangiosarcoma, metastatic, spleen					1	(7%)				
Histiocytic sarcoma					4	(27%)	4	(27%)	5	(33%)
Sarcoma stromal									1	(7%)
Hematopoietic System										
Bone marrow	(15)		(15)		(15)		(15)		(15)	
Hemangiosarcoma							1	(7%)		
Histiocytic sarcoma	9	(60%)	8	(53%)	11	(73%)	8	(53%)	8	(53%)
Lymph node	(13)		(15)		(15)		(13)		(14)	
Lumbar, histiocytic sarcoma			2	(13%)	1	(7%)			2	(14%)
Mediastinal, histiocytic sarcoma	1	(8%)	1	(7%)	6	(40%)	3	(23%)	3	(21%)
Pancreatic, histiocytic sarcoma					1	(7%)				
Renal, histiocytic sarcoma			2	(13%)					1	(7%)
Lymph node, mandibular	(15)		(15)		(15)		(15)		(15)	
Histiocytic sarcoma	1	(7%)	1	(7%)	6	(40%)	2	(13%)	3	(20%)
Lymph node, mesenteric	(15)		(15)		(15)		(15)		(15)	
Histiocytic sarcoma	3	(20%)	3	(20%)	5	(33%)	3	(20%)	5	(33%)
Spleen	(15)		(15)		(15)		(15)		(15)	
Hemangiosarcoma					1	(7%)				
Histiocytic sarcoma	2	(13%)	2	(13%)	7	(47%)	3	(20%)	2	(13%)
Thymus	(15)		(15)		(15)		(15)		(15)	
Histiocytic sarcoma	1	(7%)	1	(7%)	6	(40%)	2	(13%)	2	(13%)
Integumentary System										
Skin	(15)		(15)		(15)		(15)		(15)	
Subcutaneous tissue, hemangiosarcoma	1	(7%)								
Subcutaneous tissue, histiocytic sarcoma					2	(13%)			1	(7%)
Musculoskeletal System										
Skeletal muscle									(2)	
Histiocytic sarcoma									2	(100%)
Nervous System										
Brain	(15)		(15)		(15)		(15)		(15)	
Choristoma									1	(7%)
Histiocytic sarcoma	1	(7%)			4	(27%)	4	(27%)	2	(13%)
Spinal cord									(1)	
Histiocytic sarcoma									1	(100%)

TABLE A3
Summary of the Incidence of Neoplasms in Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Respiratory System					
Lung	(15)	(15)	(15)	(15)	(15)
Alveolar/bronchiolar adenoma		1 (7%)		1 (7%)	4 (27%)
Alveolar/bronchiolar carcinoma				1 (7%)	
Histiocytic sarcoma	2 (13%)		6 (40%)	3 (20%)	6 (40%)
Osteosarcoma, metastatic, uncertain primary site		1 (7%)			
Special Senses System					
Zymbal's gland				(1)	(1)
Squamous cell carcinoma					1 (100%)
Urinary System					
Kidney	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	1 (7%)		4 (27%)	2 (13%)	3 (20%)
Urinary bladder	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma			2 (13%)	2 (13%)	2 (13%)
Systemic Lesions					
Multiple organs[b]	(15)	(15)	(15)	(15)	(15)
Histiocytic sarcoma	9 (60%)	9 (60%)	12 (80%)	10 (67%)	13 (87%)
Neoplasm Summary					
Total animals with primary neoplasms[c]	10	9	12	12	14
Total primary neoplasms	10	10	13	14	23
Total animals with benign neoplasms		1		2	7
Total benign neoplasms		1		2	7
Total animals with malignant neoplasms	10	9	12	11	14
Total malignant neoplasms	10	9	13	12	15
Total animals with metastatic neoplasms		1	1		
Total metastatic neoplasms		3	1		
Total animals with malignant neoplasms uncertain primary site		1			
Total animals with uncertain neoplasms-benign or malignant					1
Total uncertain neoplasms					1

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

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Disposition Summary					
Animals initially in study	15	15	15	15	15
Early deaths					
Moribund	2	1	2	1	4
Natural deaths			1		2
Survivors					
Terminal sacrifice	13	14	12	14	9
Animals examined microscopically	15	15	15	15	15
Alimentary System					
Liver	(15)	(15)	(15)	(15)	(15)
Angiectasis, focal				1 (7%)	2 (13%)
Eosinophilic focus					1 (7%)
Hematopoietic cell proliferation	2 (13%)	3 (20%)	1 (7%)	1 (7%)	2 (13%)
Infiltration cellular, lymphoid		3 (20%)	1 (7%)	1 (7%)	1 (7%)
Inflammation		1 (7%)			
Inflammation, chronic	11 (73%)	10 (67%)	8 (53%)	8 (53%)	7 (47%)
Mixed cell focus			1 (7%)	1 (7%)	
Necrosis, focal			5 (33%)	2 (13%)	2 (13%)
Midzonal, fatty change	1 (7%)				
Stomach, forestomach	(15)	(15)	(15)	(15)	(15)
Epithelium, hyperplasia				1 (7%)	4 (27%)
Stomach, glandular	(15)	(15)	(15)	(15)	(15)
Metaplasia, squamous			1 (7%)		
Mineralization	4 (27%)				2 (13%)
Epithelium, ectasia			1 (7%)		
Muscularis, mineralization					2 (13%)
Cardiovascular System					
Heart	(15)	(15)	(15)	(15)	(15)
Inflammation, chronic					1 (7%)
Myocardium, mineralization	1 (7%)				
Endocrine System					
Adrenal cortex	(15)	(15)	(15)	(15)	(15)
Hematopoietic cell proliferation		1 (7%)			
Hyperplasia, focal		2 (13%)			1 (7%)
Mineralization					1 (7%)
Vacuolization cytoplasmic, focal	1 (7%)				
Subcapsular, hyperplasia	15 (100%)	15 (100%)	10 (67%)	14 (93%)	15 (100%)
Pituitary gland	(15)	(15)	(15)	(15)	(15)
Necrosis					1 (7%)
Pars distalis, hyperplasia, focal	1 (7%)	1 (7%)	2 (13%)	1 (7%)	
General Body System					
None					

[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 40-Week Gavage Study of Glycidol

	Vehicle Control		25 mg/kg		50 mg/kg		100 mg/kg		200 mg/kg	
Genital System										
Clitoral gland									(1)	
Pigmentation, melanin									1	(100%)
Ovary	(15)		(15)		(15)		(15)		(15)	
Cyst	4	(27%)							2	(13%)
Uterus	(15)		(15)		(15)		(15)		(15)	
Endometrium, hyperplasia, cystic	13	(87%)	14	(93%)	10	(67%)	14	(93%)	9	(60%)
Hematopoietic System										
Bone marrow	(15)		(15)		(15)		(15)		(15)	
Hyperplasia									1	(7%)
Lymph node	(13)		(15)		(15)		(13)		(14)	
Lumbar, hyperplasia, lymphoid			1	(7%)						
Mediastinal, hyperplasia, lymphoid	2	(15%)	1	(7%)						
Pancreatic, hyperplasia, lymphoid									1	(7%)
Renal, hyperplasia, lymphoid									1	(7%)
Lymph node, mandibular	(15)		(15)		(15)		(15)		(15)	
Hyperplasia, lymphoid	1	(7%)							1	(7%)
Lymph node, mesenteric	(15)		(15)		(15)		(15)		(15)	
Hyperplasia, lymphoid							1	(7%)		
Inflammation, suppurative	1	(7%)								
Spleen	(15)		(15)		(15)		(15)		(15)	
Hematopoietic cell proliferation	9	(60%)	5	(33%)	10	(67%)	10	(67%)	10	(67%)
Thymus	(15)		(15)		(15)		(15)		(15)	
Atrophy			2	(13%)			1	(7%)	3	(20%)
Hyperplasia, lymphoid			1	(7%)						
Integumentary System										
Mammary gland	(15)		(15)		(15)		(15)		(15)	
Hyperplasia							1	(7%)		
Skin	(15)		(15)		(15)		(15)		(15)	
Subcutaneous tissue, edema									1	(7%)
Musculoskeletal System										
Bone	(15)		(15)		(15)		(15)		(15)	
Hyperostosis							1	(7%)		
Nervous System										
Brain	(15)		(15)		(15)		(15)		(15)	
Cerebellum, gliosis									1	(7%)
Cerebellum, neuronopathy							1	(7%)	3	(20%)
Cerebrum, cyst epithelial inclusion									1	(7%)
Cerebrum, hemorrhage							1	(7%)		
Medulla, gliosis									2	(13%)
Medulla, neuronopathy									3	(20%)
Thalamus, gliosis									1	(7%)
Thalamus, neuronopathy									3	(20%)

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

	Vehicle Control		25 mg/kg		50 mg/kg		100 mg/kg		200 mg/kg	
Respiratory System										
Lung	(15)		(15)		(15)		(15)		(15)	
Inflammation	8	(53%)	8	(53%)	7	(47%)	5	(33%)	3	(20%)
Inflammation, granulomatous	1	(7%)								
Alveolar epithelium, hyperplasia, focal	1	(7%)	2	(13%)	1	(7%)			1	(7%)
Arteriole, inflammation, acute	1	(7%)								
Bronchus, foreign body	1	(7%)								
Special Senses System										
None										
Urinary System										
Kidney	(15)		(15)		(15)		(15)		(15)	
Infiltration cellular, lymphoid			1	(7%)						
Metaplasia, osseous									1	(7%)
Nephropathy	12	(80%)	12	(80%)	10	(67%)	14	(93%)	9	(60%)
Renal tubule, accumulation, hyaline droplet					2	(13%)	1	(7%)	3	(20%)
Urinary bladder	(15)		(15)		(15)		(15)		(15)	
Inflammation, chronic	1	(7%)								

APPENDIX B
GENETIC TOXICOLOGY

TABLE B1
Frequency of Micronuclei in Normochromatic Erythrocytes and Percent of Polychromatic Erythrocytes in Peripheral Blood of Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice Administered Glycidol by Gavage[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	P Value[c]	PCEs (%)
6.5 Weeks					
Male					
Water[d]		15	2.13 ± 0.19		2.4
Glycidol	25	15	1.87 ± 0.24	0.7676	1.9
	50	15	2.13 ± 0.24	0.5000	2.1
	100	15	2.27 ± 0.28	0.3637	1.8
	200	15	2.77 ± 0.27	0.0583	2.4
			P=0.013[e]		
Female					
Water		15	1.13 ± 0.17		2.0
Glycidol	25	15	1.30 ± 0.22	0.2791	2.3
	50	15	2.10 ± 0.22	0.0016	2.1
	100	15	2.03 ± 0.26	0.0028	2.1
	200	15	1.77 ± 0.24	0.0208	2.6
			P=0.032		
13 Weeks					
Male					
Water		15	1.60 ± 0.21		1.9
Glycidol	25	15	1.50 ± 0.20	0.6222	1.8
	50	15	1.37 ± 0.20	0.7711	2.0
	100	15	1.83 ± 0.18	0.2450	2.0
	200	15	2.00 ± 0.24	0.1239	2.2
			P=0.036		
Female					
Water		14	1.43 ± 0.23		2.1
Glycidol	25	15	0.87 ± 0.17	0.9776	2.0
	50	15	1.23 ± 0.21	0.7406	2.4
	100	15	1.23 ± 0.25	0.7406	2.0
	200	15	1.63 ± 0.22	0.2645	2.5
			P=0.046		

TABLE B1
Frequency of Micronuclei in Normochromatic Erythrocytes and Percent of Polychromatic Erythrocytes in Peripheral Blood of Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice Administered Glycidol by Gavage

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs	P Value	PCEs (%)
19.5 Weeks					
Male					
Water		15	1.33 ± 0.24		2.8
Glycidol	25	15	1.27 ± 0.34	0.5672	2.5
	50	15	1.87 ± 0.35	0.1111	2.4
	100	15	1.57 ± 0.28	0.2874	2.4
	200	15	2.50 ± 0.34	0.0073	2.5
			P=0.002		
Female					
Water		14	0.64 ± 0.13		2.7
Glycidol	25	15	1.27 ± 0.32	0.0372	2.4
	50	15	1.13 ± 0.34	0.0727	2.3
	100	15	1.37 ± 0.22	0.0218	2.6
	200	14	1.46 ± 0.30	0.0135	2.9
			P=0.040		
26 Weeks					
Male					
Water		15	1.93 ± 0.27		2.9
Glycidol	25	15	1.80 ± 0.21	0.6474	2.9
	50	14	2.54 ± 0.23	0.0619	3.0
	100	15	2.90 ± 0.35	0.0079	2.5
	200	14	3.82 ± 0.35	0.0000	2.3
			P=0.000		
Female					
Water		14	1.07 ± 0.21		2.7
Glycidol	25	15	1.20 ± 0.19	0.3231	2.7
	50	15	1.17 ± 0.17	0.3660	3.1
	100	15	1.37 ± 0.17	0.1548	2.3
	200	14	2.07 ± 0.27	0.0014	3.1
			P=0.000		

TABLE B1
Frequency of Micronuclei in Normochromatic Erythrocytes and Percent of Polychromatic Erythrocytes in Peripheral Blood of Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice Administered Glycidol by Gavage

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs	P Value	PCEs (%)
40 Weeks					
Male					
Water		13	1.81 ± 0.41		3.1
Glycidol	25	14	1.71 ± 0.21	0.6021	3.1
	50	13	2.31 ± 0.30	0.1042	4.4
	100	14	2.61 ± 0.24	0.0243	4.6
	200	7	3.64 ± 0.61	0.0002	3.9
			P=0.000		
Female					
Water		13	1.31 ± 0.14		4.4
Glycidol	25	14	1.89 ± 0.24	0.0451	4.7
	50	12	1.88 ± 0.39	0.0553	7.0
	100	14	2.32 ± 0.26	0.0030	7.2
	200	9	2.83 ± 0.40	0.0002	4.5
			P=0.000		

[a] Study was performed at SITEK Research Laboratories. The detailed protocol is presented by MacGregor *et al.* (1990). PCE=polychromatic erythrocyte, NCE=normochromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the vehicle control group; significant at P≤0.006 (ILS, 1990)
[d] Vehicle control
[e] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test, significant at P≤0.025 (ILS, 1990)

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

TABLE C1
Organ Weights and Organ-Weight-to-Body-Weight Ratios in Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
n	13	14	13	14	7
Necropsy body wt	48.9 ± 0.9	47.5 ± 1.1	48.7 ± 0.5	47.4 ± 1.2	40.8 ± 2.4**
Heart					
Absolute	0.237 ± 0.005	0.237 ± 0.007	0.226 ± 0.006	0.227 ± 0.006	0.190 ± 0.007**
Relative	4.841 ± 0.076	5.000 ± 0.132	4.643 ± 0.118	4.788 ± 0.107	4.695 ± 0.172
R. Kidney					
Absolute	0.394 ± 0.016	0.403 ± 0.017	0.381 ± 0.009	0.392 ± 0.010	0.356 ± 0.010
Relative	8.035 ± 0.264	8.468 ± 0.260	7.824 ± 0.173	8.286 ± 0.185	8.852 ± 0.373
Liver					
Absolute	2.672 ± 0.144	2.709 ± 0.163	2.936 ± 0.140	2.495 ± 0.134	2.109 ± 0.115
Relative	54.317 ± 2.279	56.573 ± 2.322	60.284 ± 2.831	52.254 ± 1.862	52.939 ± 5.006
Lung					
Absolute	0.355 ± 0.020	0.336 ± 0.013	0.321 ± 0.007	0.375 ± 0.020	0.325 ± 0.021
Relative	7.291 ± 0.445	7.127 ± 0.328	6.597 ± 0.140	8.086 ± 0.693	8.157 ± 0.753
R. Testis					
Absolute	0.128 ± 0.003	0.125 ± 0.003	0.127 ± 0.003	0.128 ± 0.003	0.112 ± 0.002**
Relative	2.626 ± 0.065	2.654 ± 0.067	2.609 ± 0.055	2.715 ± 0.048	2.793 ± 0.134
Thymus					
Absolute	0.051 ± 0.004	0.049 ± 0.003	0.054 ± 0.005	0.049 ± 0.004	0.038 ± 0.003
Relative	1.043 ± 0.084	1.038 ± 0.049	1.117 ± 0.091	1.025 ± 0.089	0.922 ± 0.058
Female					
n	13	14	12	14	9
Necropsy body wt	42.4 ± 2.0	39.8 ± 1.3	36.6 ± 1.6**	35.1 ± 1.2**	31.4 ± 0.9**
Heart					
Absolute	0.184 ± 0.004	0.185 ± 0.007	0.183 ± 0.008	0.169 ± 0.004	0.165 ± 0.009
Relative	4.424 ± 0.150	4.673 ± 0.144	5.047 ± 0.242	4.855 ± 0.148	5.298 ± 0.352**
R. Kidney					
Absolute	0.254 ± 0.007	0.252 ± 0.005	0.261 ± 0.006	0.266 ± 0.007	0.262 ± 0.006
Relative	6.092 ± 0.196	6.413 ± 0.246	7.262 ± 0.293**	7.666 ± 0.297**	8.365 ± 0.175**
Liver					
Absolute	2.051 ± 0.093	1.892 ± 0.068	2.133 ± 0.204	2.201 ± 0.295	1.904 ± 0.295
Relative	48.670 ± 1.112	48.056 ± 2.088	60.141 ± 7.744	64.043 ± 9.728	60.539 ± 8.937
Lung					
Absolute	0.334 ± 0.019	0.378 ± 0.010[b]	0.370 ± 0.019	0.345 ± 0.019	0.340 ± 0.021
Relative	8.363 ± 0.920	9.361 ± 0.277[b]	10.242 ± 0.575	9.986 ± 0.730	10.901 ± 0.748*
Thymus					
Absolute	0.052 ± 0.004	0.049 ± 0.004	0.042 ± 0.005	0.047 ± 0.003	0.035 ± 0.002*
Relative	1.216 ± 0.061	1.211 ± 0.068	1.134 ± 0.108	1.355 ± 0.075	1.108 ± 0.067

* Significantly different ($P \leq 0.05$) from the vehicle control group by William's or Dunnett's test
** $P \leq 0.01$
[a] Organ weights (absolute weights) and body weights are given as grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] n=13

APPENDIX D
REPRODUCTIVE TISSUE EVALUATIONS

TABLE D1
Summary of Reproductive Tissue Evaluations in Male Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
n	13	14	13	14	7
Weights (g)					
Necropsy body weight	48.9 ± 0.9	47.5 ± 1.1	48.7 ± 0.5	47.4 ± 1.2	40.8 ± 2.4**
L. Cauda epididymis	0.0162 ± 0.0005	0.0154 ± 0.0004	0.0152 ± 0.0004	0.0150 ± 0.0004	0.0131 ± 0.0005**
L. Epididymis	0.0506 ± 0.0008	0.0499 ± 0.0009	0.0488 ± 0.0012	0.0490 ± 0.0010	0.0432 ± 0.0023**
L. Testis	0.1250 ± 0.0022	0.1237 ± 0.0025	0.1243 ± 0.0024	0.1250 ± 0.0027	0.1118 ± 0.0045*
Spermatid measurements					
Spermatid heads (10^6/g testis)	170.6 ± 9.8[b]	185.7 ± 6.5[c]	181.9 ± 8.4	185.1 ± 5.8[c]	169.3 ± 6.3
Spermatid heads (10^6/testis)	18.89 ± 0.79[b]	20.27 ± 0.72	20.54 ± 0.88	20.70 ± 0.61	17.29 ± 0.92
Epididymal spermatozoal measurements					
Sperm motility (%)	90.99 ± 0.90	90.18 ± 1.36	91.29 ± 0.68	90.17 ± 0.86	91.27 ± 0.60
Sperm heads (10^6/g cauda epididymis)	838 ± 34	799 ± 35	797 ± 44	840 ± 23	599 ± 105
Sperm heads (10^6/cauda)	13.52 ± 0.59	12.29 ± 0.62	11.97 ± 0.35	12.59 ± 0.40	8.07 ± 1.56**

* Significantly different ($P \le 0.05$) from the vehicle control group by Dunnett's test
** Significantly different ($P \le 0.01$) from the vehicle control group by Dunnett's (body weights), Williams' (left cauda epididymis and epididymis weights), or Dunn's (sperm heads per cauda) test
[a] Data are presented as mean ± standard error. Differences from the vehicle control group for spermatid, epididymal sperm heads per gram cauda, and epididymal sperm motility measurements are not significant by Dunn's test.
[b] n=12
[c] n=13

APPENDIX E
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

Procurement and Characterization of Glycidol

Glycidol was obtained from Aldrich Chemical Co. (Milwaukee, WI) in one lot (01616 BS) and was used in the 40-week study. Identity and purity analyses were conducted by the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC), and the study laboratory, Battelle Columbus Operations (Columbus, OH); stability analyses were also conducted by the analytical chemistry laboratory. Reports on analyses performed in support of the glycidol studies are on file at the National Institute of Environmental Health Sciences.

Lot 01616 BS of the chemical, a viscous, colorless, combustible liquid (NTP, 2004), was identified as glycidol using infrared (IR) and proton nuclear magnetic resonance (NMR) spectroscopy by the analytical chemistry laboratory and IR spectroscopy by the study laboratory. Spectra were consistent with the structure of glycidol, matched reference spectra (*Aldrich*, 1981, 1983, 1985), and matched the spectrum of a reference standard from the same lot. Representative IR and NMR spectra are presented in Figures E1 and E2, respectively.

The purity of lot 01616 BS was determined by the analytical chemistry and study laboratories using gas chromatography (GC). The analytical chemistry laboratory GC system included flame ionization detection (FID; Hewlett-Packard, Palo Alto, CA), a Supelco Nukol column (30 m × 0.25 mm, 0.25-µm film thickness; Supelco, Bellefonte, PA), an oven temperature program of 60° C to 200° C at 10° C/minute, held 26 minutes, and helium as the carrier gas at a flow rate of 1.0 mL/minute. For the impurity identification, the analytical laboratory used an Agilent 6890 GC with a 5973 mass spectrometry detector (Palo Alto, CA) and a Supelco (Bellefonte, PA) Nukol column (30 m × 0.32 mm ID, 0.25-µm film thickness). The oven program was 60° C for 5 minutes, 60° C to 200° C at 10° C/minute, then 200° C for 26 minutes. Helium was the carrier gas at 1.6 mL/minute and electron impact ionization. Quantitation of the impurity peaks was performed with a GC/FID system similar to the system described above. The study laboratory system included a GC/FID (Hewlett-Packard, Palo Alto, CA); a Stabilwax DA column (30 m × 0.25 mm, 0.25-µm film thickness; Restek, Bellefonte, PA); an oven temperature program of 60° C to 200° C at 15° C/minute, held 5 minutes, and helium as the carrier gas at a flow rate of 1 mL/minute.

GC by the analytical chemistry laboratory indicated one major peak, six impurities with peak areas greater than 0.1% of the total peak area, ranging from 0.12% to 1.18%, and seven impurities with peak areas less than 0.1% of the total peak area; the purity of lot 01616 BS was determined to be greater than 96%. Impurities with peak areas exceeding 0.1% were 1-hydroxy-2-propanone (0.6%), diglycidyl ether (0.3%), 3-methoxy-1,2-propanediol (0.5%), 2,5-dimethanol-p-dioxane (0.1%), 2,6-dimethanol-p-dioxane (2.2%), and glycidol-dimethyl ether (0.2%). GC by the study laboratory indicated one major peak and several minor impurities; the purity was determined to be 95.9% by comparison to a reference standard from the same lot. The overall purity of lot 01616 BS was determined to be greater than 95%.

Analyses of the bulk chemical were performed by the study laboratory at approximately 2 and 8 months after the study began and at the end of the study using GC by the system previously described. To ensure stability, the bulk chemical was protected from light in amber glass bottles capped with Teflon®-lined lids and stored at approximately 5° C. No degradation of the bulk chemical was detected.

Preparation and Analysis of Dose Formulations

Dose formulations were prepared at least every month. The dose formulations were prepared by mixing the appropriate amount of glycidol with deionized water to give the required concentrations (Table E1). Formulations were protected from light in amber glass bottles capped with Teflon®-lined lids and stored at approximately 5° C for up to 35 days.

The study laboratory conducted periodic analyses of preadministration dose formulations five times during the study using GC by the system previously described with variations in the oven temperature program (60° C for 1.5 minutes, then 200° C at 20° C/minute, held 7 minutes); postadministration formulations were also analyzed. Of the preadministration dose formulations analyzed, all were within 10% of the target concentrations; of the postadministration formulations analyzed, all 16 were more than 10% less than the target concentrations, ranging from –11% to –17%, probably due to the evaporation of glycidol during administration (Table E2).

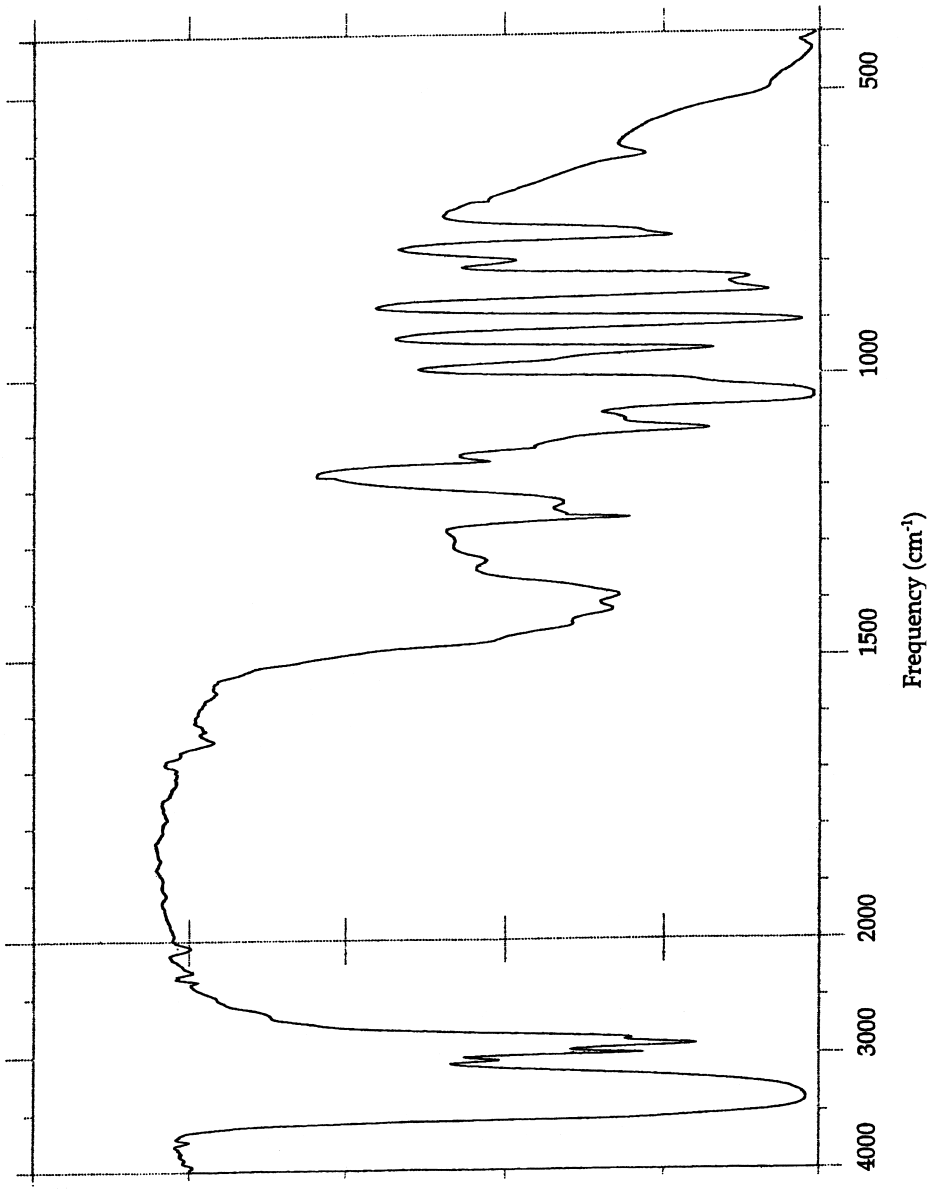

FIGURE E1
Infrared Absorption Spectrum of Glycidol

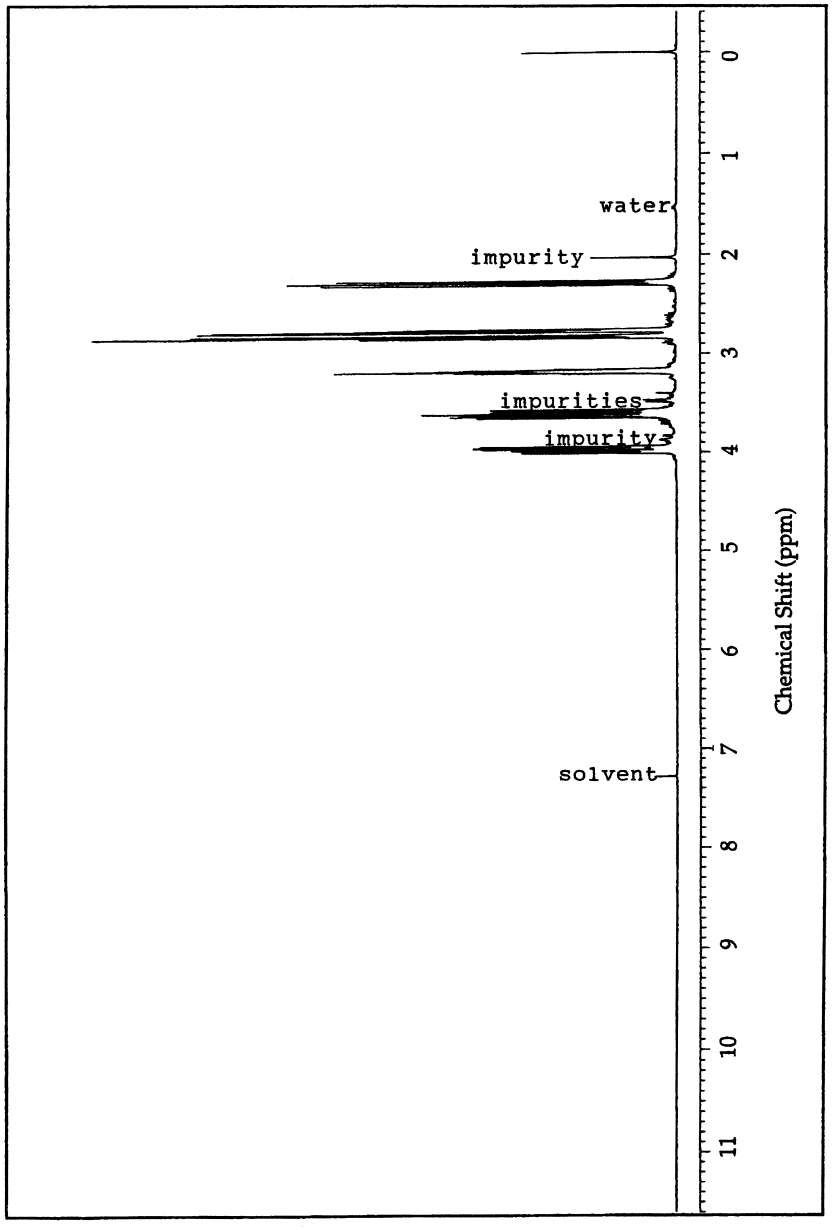

FIGURE E2
Proton Nuclear Magnetic Resonance Spectrum of Glycidol

TABLE E1
Preparation and Storage of Dose Formulations in the 40-Week Gavage Study of Glycidol

Preparation
Dose formulations were prepared at least every 3 months. For each formulation, the appropriate amount of the chemical was pipetted into a calibrated mixing bottle containing deionized water, diluted to volume, and thoroughly mixed.

Chemical Lot Number
01616 BS

Maximum Storage Time
35 days

Storage Conditions
Protected from light in amber glass bottles capped with Teflon® -lined lids and stored at approximately 5° C

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

TABLE E2
Results of Analyses of Dose Formulations Administered to Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice in the 40-Week Gavage Study of Glycidol

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
January 26, 2000	January 27, 2000	2.5	2.409	–4
		5	4.864	–3
		10	9.749	–3
		20	19.79	–1
	March 2-3, 2000[b]	2.5	2.164	–13
		5	4.280	–14
		10	8.619	–14
		20	17.78	–11
April 21, 2000	April 25-26, 2000	2.5	2.374	–5
		5	4.831	–3
		10	10.12	+1
		20	20.09	0
	May 30-31, 2000[b]	2.5	2.066	–17
		5	4.128	–17
		10	8.583	–14
		20	17.42	–13
July 13, 2000	July 14-15, 2000	2.5	2.296	–8
		5	4.649	–7
		10	9.368	–6
		20	19.55	–2
	August 22, 2000[b]	2.5	2.197	–12
		5	4.321	–14
		10	8.276	–17
		20	16.62	–17
October 5, 2000	October 11, 2000	2.5	2.392	–4
		5	4.770	–5
		10	9.318	–7
		20	18.82	–6
	November 14-15, 2000[b]	2.5	2.217	–11
		5	4.252	–15
		10	8.502	–15
		20	17.24	–14

[a] Results of duplicate analysis
[b] Animal room samples

APPENDIX F
HISTORICAL CONTROL INCIDENCES

TABLE F1
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 F2
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 F2
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 F3
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 F4
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 F4
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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