

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY STUDIES OF

BROMODICHLOROACETIC ACID

(CAS NO. 71133-14-7)

IN F344/N RATS AND B6C3F1/N MICE

AND TOXICOLOGY AND CARCINOGENESIS STUDIES

OF BROMODICHLOROACETIC ACID

IN F344/NTac RATS AND B6C3F1/N MICE

(DRINKING WATER STUDIES)

Scheduled Peer Review Date: May 22, 2014

NOTICE

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National Toxicology Program

National Institutes of Health
Public Health Service
U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

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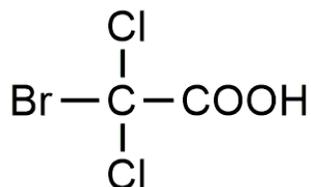
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ABSTRACT



BROMODICHLOROACETIC ACID

CAS No. 71133-14-7

Chemical Formula: $\text{C}_2\text{HBrCl}_2\text{O}_2$ Molecular Weight: 207.84

Synonyms: 2-Bromo-2,2-dichloroacetic acid; bromodichloroethanoic acid

Bromodichloroacetic acid is a haloacetic acid that forms when drinking water supplies containing natural organic matter are disinfected with chlorine-containing oxidizing compounds and when bromide is present in the source water. Bromodichloroacetic acid was nominated for toxicity and carcinogenicity studies by the American Water Works Association Research Foundation and the United States Environmental Protection Agency because of widespread human exposure to this water disinfection by-product and because related dihaloacetic acids were found to be carcinogenic to the liver of rats and mice. Male and female F344/N rats and B6C3F1/N mice were exposed to bromodichloroacetic acid (greater than 97% pure) in drinking water for 2 weeks or 3 months, and male and female F344/NTac rats and B6C3F1/N mice were exposed for 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium*, *Escherichia coli*, and mouse peripheral blood erythrocytes.

2-WEEK STUDY IN F344/N RATS

Groups of five male and five female rats were exposed to 0, 62.5, 125, 250, 500, or 1,000 mg bromodichloroacetic acid/L drinking water for 16 days (equivalent to average daily doses of approximately 7, 15, 31, 56, or 131 mg bromodichloroacetic acid/kg body weight to males and 8, 15, 30, 58, or 113 mg/kg to females). All rats survived to the end of the study. Mean body weights of exposed groups of male and female rats were similar to those of the

controls. Water consumption by exposed and control groups was similar. No organ weight differences were attributed to exposure to bromodichloroacetic acid. No gross or histologic lesions related to bromodichloroacetic acid exposure were observed.

2-WEEK STUDY IN MICE

Groups of five male and five female mice were exposed to 0, 62.5, 125, 250, 500, or 1,000 mg bromodichloroacetic acid/L drinking water for 16 days (equivalent to average daily doses of approximately 10, 20, 41, 84, or 175 mg/kg to males and 11, 16, 40, 78, or 138 mg/kg to females). All mice survived to the end of the study. Mean body weights of exposed groups of male and female mice were similar to those of the controls. Water consumption by exposed and control groups was similar. No gross or histologic lesions related to bromodichloroacetic acid exposure were observed.

3-MONTH STUDY IN F344/N RATS

Groups of 10 male and 10 female rats were exposed to 0, 62.5, 125, 250, 500, or 1,000 mg bromodichloroacetic acid/L drinking water for 14 weeks (equivalent to average daily doses of approximately 5, 9, 19, 37, or 72 mg/kg to males and 5, 10, 20, 43, or 69 mg/kg to females). In addition, groups of 10 male and 10 female clinical pathology study rats were exposed to the same concentrations for 4 weeks. All exposed rats survived until the end of the study. Mean body weights of 1,000 mg/L females were significantly less than those of the controls. Kidney weights of 1,000 mg/L females were significantly greater than those of the controls. Male rats exposed to 1,000 mg/L exhibited decreased left testis weights and sperm motility, suggesting a potential for bromodichloroacetic acid to be a reproductive toxicant in male rats. No chemical-related histologic lesions were noted.

3-MONTH STUDY IN MICE

Groups of 10 male and 10 female mice were exposed to 0, 62.5, 125, 250, 500, or 1,000 mg bromodichloroacetic acid/L drinking water for 14 weeks (equivalent to average daily doses of approximately 7, 15, 30, 59, or 123 mg/kg to males and 9, 17, 36, 70, or 129 mg/kg to females). All mice survived to the end of the study. Mean body weights of exposed groups were similar to those of controls. Water consumption by exposed and control groups was

generally similar. The absolute and relative liver weights of 500 and 1,000 mg/L males were significantly greater than those of controls, and the absolute kidney weight of 1,000 mg/L males was significantly less than that of controls. In the liver of females exposed to 1,000 mg/L, the incidence of glycogen depletion (minimal) was significantly greater than the control incidence.

2-YEAR STUDY IN F344/NTAC RATS

Groups of 66 male and 66 female rats were exposed to 0, 250, 500, or 1,000 mg bromodichloroacetic acid/L drinking water for up to 104 weeks (equivalent to average daily doses of approximately 11, 21, or 43 mg/kg to males and 13, 28, or 57 mg/kg to females). Eight male and eight female rats from each group were removed at 6 and 13 months for interim evaluations.

Survival of 500 and 1,000 mg/L females was significantly less than that of the controls. The mean body weights of 1,000 mg/L males were approximately 10% less than those of controls after week 89, and those of 1,000 mg/L females were less after week 13. Water consumption by 1,000 mg/L males was less than that by controls throughout the study; in 1,000 mg/L females, water consumption was less during the first year of the study but similar to control values during the second year. At the 6-month interim evaluation, relative liver weights of 500 and 1,000 mg/L females were significantly greater than those of the controls.

In females, the incidences of mammary gland fibroadenoma and fibroadenoma or carcinoma (combined) in all exposed groups were significantly greater than the control incidences, as was the incidence of carcinoma at 1,000 mg/L. The incidence of hyperplasia of the mammary gland in 1,000 mg/L females was significantly increased. Two males in the 250 mg/L group, three males in the 500 mg/L group, and one male in the 1,000 mg/L group had mammary gland fibroadenomas. No fibroadenomas were observed in the control males.

In the testis at 13 months, the incidence of malignant mesothelioma in the 1,000 mg/L group was significantly increased. In all organs at 2 years, the incidences of malignant mesothelioma in males occurred with a positive trend and were significantly increased in all exposed groups.

The incidences of keratoacanthoma and of basal cell adenoma or carcinoma (combined) of the skin in males occurred with positive trends, and the 1,000 mg/L group incidences were significantly greater than the control incidences. The combined incidence of squamous cell papilloma, keratoacanthoma, sebaceous gland adenoma, basal cell adenoma, basal cell carcinoma, or squamous cell carcinoma in 1,000 mg/L males was significantly increased. The incidence of subcutaneous fibroma was significantly increased in 1,000 mg/L males.

The incidences of adenoma of the large intestine (cecum, colon, rectum) were slightly increased in 500 and 1,000 mg/L males.

Incidences of glioma, oligodendroglioma, and benign granular cell tumor of the meninges occurred in the brain of male rats in the original evaluation, but the differences between the exposed group incidences and the control group incidences were not statistically significant. An extended evaluation of additional brain sections, up to nine per animal, was conducted; one additional control male had a glioma and one additional 500 mg/L male had an oligodendroglioma. The extended evaluation did not result in statistically significant increases in the incidences of these neoplasms. While there was no statistically significant increase in the incidences of glioma or oligodendroglioma (combined), these neoplasms have not been observed in historical controls.

One male in the control group and three each in the 500 and 1,000 mg/L groups had squamous cell papillomas or squamous cell carcinomas of the oral cavity (oral mucosa or tongue).

In the bone marrow, the incidences of angiectasis in all exposed groups of males and females and hyperplasia in all exposed groups of females and in 1,000 mg/L males were significantly increased when compared to the control group incidences.

At 2 years, the incidence of eosinophilic focus in the liver of 1,000 mg/L males and 500 and 1,000 mg/L females were significantly increased. Also, there were significantly increased incidences of hepatic hematopoietic cell proliferation in 500 and 1,000 mg/L females.

In the spleen of females, significantly increased incidences of hematopoietic cell proliferation were observed in the 500 and 1,000 mg/L groups.

2-YEAR STUDY IN MICE

Groups of 66 male and 66 female mice were exposed to 0, 250, 500, or 1,000 mg bromodichloroacetic acid/L drinking water for up to 105 weeks (equivalent to average daily doses of approximately 23, 52, or 108 mg/kg to males and 17, 34, or 68 mg/kg to females). Seven or eight male and eight female mice from each group were removed at 6 and 14 months for interim evaluations.

Survival of 500 and 1,000 mg/L males was significantly decreased. The most common cause of early deaths in 500 mg/L males and 1,000 mg/L males and females appeared to be liver neoplasms. Compared to those of controls, mean body weights were more than 10% less in 500 mg/L males after week 73, in 1,000 mg/L males after week 57, in 250 and 500 mg/L females after week 89, and in 1,000 mg/L females after week 73. Water consumption by all exposed groups of males and 250 and 500 mg/L females was generally greater than that by controls during the second year of the study. At the 6-month interim evaluation, left and right kidney weights of 1,000 mg/L males were significantly less than those of the controls.

In the liver at 14 months, exposure concentration-related increased incidences of atypical focus of cellular alteration were noted in all exposed groups of male and female mice, and the incidences in 1,000 mg/L males and females were significantly increased. A single case of hepatoblastoma was noted in a 1,000 mg/L male.

At 2 years, the incidences of hepatocellular adenoma in all exposed groups of females, hepatocellular carcinoma in all exposed groups of males and in 500 and 1,000 mg/L females, and hepatoblastoma in all exposed groups of males and in 1,000 mg/L females were significantly increased compared to those in the control groups. The incidences of multiple hepatocellular carcinoma and multiple hepatoblastoma in males and females and multiple hepatocellular adenoma in females were generally increased in an exposure concentration-related manner. When combined, the incidences of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma were significantly increased in 250 and 1,000 mg/L males and in all exposed groups of females.

In the liver, the incidences of eosinophilic focus in exposed females and atypical focus of cellular alteration (i.e., eosinophilic cell focus and mixed cell focus) in exposed groups of males and females (except 250 mg/L) were significantly greater than those in the controls.

In the Harderian gland of males, the incidences of adenoma and adenoma or carcinoma (combined) in the 500 and 1,000 mg/L groups were significantly greater than those in the controls.

The incidences of atrophy of the testis increased with increasing exposure concentration, and the increases in the 500 and 1,000 mg/L groups were significant. In the epididymis, the incidences of atrophy in all exposed groups, hypospermia in the 1,000 mg/L group, and epithelium degeneration in the 500 and 1,000 mg/L groups were significantly increased.

GENETIC TOXICOLOGY

Bromodichloroacetic acid was tested in bacterial gene mutation assays. These *in vitro* assays were conducted with the same well-characterized lot of bromodichloroacetic acid that was used in the 2-year bioassays, positive responses were seen in *S. typhimurium* strains TA97, TA98, and TA100 and the *E. coli* strain WP2 *uvrA*/pkM101 in the absence of S9. With rat S9, equivocal responses were seen with the three *S. typhimurium* tester strains, and a positive response was observed in the *E. coli* strain. *In vivo*, no significant increases in the frequencies of micronucleated normochromatic erythrocytes or percent polychromatic erythrocytes (reticulocytes) were observed in blood samples from male or female B6C3F1/N mice administered bromodichloroacetic acid in drinking water for 3 months.

CONCLUSIONS

Under the conditions of these 2-year studies, there was *clear evidence of carcinogenic activity** of bromodichloroacetic acid in male F344/NTac rats based on increased incidences of malignant mesothelioma and the combined incidences of epithelial tumors of the skin. Increased incidences of glioma or oligodendroglioma (combined) of the brain, squamous cell papilloma or squamous cell carcinoma of the oral cavity (oral mucosa or tongue) and

subcutaneous fibromas were also related to exposure to bromodichloroacetic acid. The occurrences of adenoma of the large intestine and fibroadenoma of the mammary gland may have been related to bromodichloroacetic acid exposure. There was *clear evidence of carcinogenic activity* of bromodichloroacetic acid in female F344/NTac rats based on increased incidences of fibroadenoma and carcinoma of the mammary gland. The increased incidences of glioma or oligodendroglioma (combined) of the brain were also related to bromodichloroacetic acid exposure. There was *clear evidence of carcinogenic activity* of bromodichloroacetic acid in male B6C3F1/N mice based on increased incidences of hepatocellular carcinoma and hepatoblastoma and increased incidences of adenoma or carcinoma (combined) of the Harderian gland. There was *clear evidence of carcinogenic activity* of bromodichloroacetic acid in female B6C3F1/N mice based on increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma.

Exposure to bromodichloroacetic acid for 2 years resulted in increased incidences of nonneoplastic lesions in the bone marrow and liver of male and female rats, spleen of female rats, liver of male and female mice, and testis and epididymis of male mice.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 16.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Bromodichloroacetic Acid

	Male F344/NTac Rats	Female F344/NTac Rats	Male B6C3F1/N Mice	Female B6C3F1/N Mice
Concentrations in drinking water	0, 250, 500, or 1,000 mg/L	0, 250, 500, or 1,000 mg/L	0, 250, 500, or 1,000 mg/L	0, 250, 500, or 1,000 mg/L
Body weights	1,000 mg/L group approximately 10% less than the control group after week 89	1,000 mg/L group at least 10% less than the control group after week 13	500 and 1,000 mg/L groups at least 10% less than the control group after weeks 73 and 57, respectively	250 and 500 mg/L groups at least 10% less than the control group after week 89; 1,000 mg/L group at least 10% less after week 73
Survival rates	19/50, 21/50, 25/50, 19/50	34/50, 26/50, 7/50, 2/50	25/50, 21/50, 12/49, 10/51	30/50, 33/50, 29/50, 27/50
Nonneoplastic effects	<p><u>Bone marrow</u>: angiectasis (4/50, 29/50, 34/50, 40/50); hyperplasia (17/50, 19/50, 20/50, 30/50)</p> <p><u>Liver</u>: eosinophilic focus (6/50, 10/50, 7/50, 14/50)</p>	<p><u>Bone marrow</u>: angiectasis (1/50, 19/50, 32/50, 39/50); hyperplasia (23/50, 35/50, 40/50, 43/50)</p> <p><u>Liver</u>: hematopoietic cell proliferation (3/50, 7/50, 14/50, 9/50); eosinophilic focus (6/50, 13/50, 21/50, 22/50)</p> <p><u>Spleen</u>: hematopoietic cell proliferation (6/50, 13/50, 29/50, 31/50)</p>	<p><u>Liver</u>: focus of cellular alteration, atypical (0/50, 19/50, 42/49, 43/51)</p> <p><u>Testis</u>: atrophy (4/50, 6/50, 13/49, 23/51)</p> <p><u>Epididymis</u>: atrophy (0/50, 7/50, 10/49, 17/51); hypospermia (0/50, 1/50, 0/49, 17/51); epithelium, degeneration (1/50, 1/50, 10/49, 6/51)</p>	<p><u>Liver</u>: eosinophilic focus (22/49, 33/50, 38/49, 40/50); focus of cellular alteration, atypical (0/49, 2/50, 6/49, 16/50)</p>
Neoplastic effects	<p><u>All organs</u>: malignant mesothelioma (1/50, 12/50, 18/50, 37/50)</p> <p><u>Skin</u>: squamous cell papilloma, keratoacanthoma, sebaceous gland adenoma, basal cell adenoma, basal cell carcinoma, or squamous cell carcinoma (9/50, 7/50, 15/50, 21/50)</p> <p><u>Skin (subcutaneous tissue)</u>: fibroma (4/50, 6/50, 10/50, 15/50)</p> <p><u>Brain</u>: glioma or oligodendroglioma (original and extended evaluations combined: 0/50, 1/50, 4/50, 3/50)</p> <p><u>Oral cavity (oral mucosa and tongue)</u>: squamous cell papilloma or squamous cell carcinoma (1/50, 0/50, 3/50, 3/50)</p>	<p><u>Mammary gland</u>: fibroadenoma (28/50, 47/50, 47/50, 39/50); carcinoma (0/50, 1/50, 3/50, 8/50); fibroadenoma, adenoma, or carcinoma (28/50, 47/50, 48/50, 42/50)</p> <p><u>Brain</u>: glioma or oligodendroglioma (original and extended evaluations combined: 0/50, 1/50, 3/50, 1/50)</p>	<p><u>Liver</u>: hepatocellular carcinoma (12/50, 22/50, 27/49, 39/51); hepatoblastoma (4/50, 24/50, 40/49, 34/51)</p> <p><u>Harderian gland</u>: adenoma (6/50, 11/50, 14/49, 19/51); adenoma or carcinoma (6/50, 11/50, 14/49, 20/51)</p>	<p><u>Liver</u>: hepatocellular adenoma (33/49, 42/50, 42/49, 44/50); hepatocellular carcinoma (9/49, 17/50, 22/49, 26/50); hepatoblastoma (0/49, 1/50, 4/49, 6/50); hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma (36/49, 44/50, 43/49, 46/50)</p>

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Bromodichloroacetic Acid

	Male F344/NTac Rats	Female F344/NTac Rats	Male B6C3F1/N Mice	Female B6C3F1/N Mice
Equivocal findings	<u>Large intestine:</u> adenoma (0/50, 0/50, 2/50, 2/50) <u>Mammary gland:</u> fibroadenoma (0/50, 2/50, 3/50, 1/50)	None	None	None
Level of evidence of carcinogenic activity	Clear evidence	Clear evidence	Clear evidence	Clear evidence
Genetic toxicology				
Bacterial gene mutations:		Positive in TA97, TA98, and TA100 in the absence of S9; equivocal in TA97, TA98, and TA100 with S9; positive in <i>E. coli</i> WP2 <i>uvrA</i> /pkM101 with or without S9 (Lot #NJ87-90/9/2005)		
Micronucleated erythrocytes Mouse peripheral blood <i>in vivo</i> :		Negative in males and females (Lot #31542-80)		

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

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

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

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

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

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

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

**NATIONAL TOXICOLOGY PROGRAM TECHNICAL REPORTS
PEER REVIEW PANEL**

The members of the Peer Review Panel who evaluated the draft NTP Technical Report on bromodichloroacetic acid on May 22, 2014, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, panel 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.

SUMMARY OF PEER REVIEW PANEL COMMENTS

NOTE: A summary of the Peer Review Panel's remarks will appear in a future draft of this report.

OVERVIEW OF WATER DISINFECTION BY-PRODUCTS PROGRAM

The disinfection of drinking water by chlorination or chloramination leads to the production of halogenated by-products. The disinfection by-products are produced by interactions with reactive chlorine and humic acids derived from algae and the breakdown of vegetative matter. The composition of the by-products depends on the disinfection process and the source water. There are seasonal variations in concentrations of disinfection by-products due to increased algal blooms in warmer weather. Source water high in bromine will contain higher concentrations of bromo- and mixed chloro/bromo disinfection by-products. Several epidemiologic studies suggest an association between exposure to disinfection by-products and bladder cancer and reproductive toxicity (Richardson *et al.*, 2007; Colman *et al.*, 2011). To better understand the risks associated with exposure to disinfection by-products, the American Water Works Association Research Foundation, the United States Environmental Protection Agency (USEPA), and others have nominated disinfection by-products for study by the NTP. In response to this nomination, the NTP developed a plan to evaluate the potential human health effects of various disinfection by-products. At the start of this program, many of the disinfection by-products in finished drinking water were unidentified. Thus, the initial focus was on the chlorinated and brominated trihalomethanes, haloacetic acids, and the oxyhalides. There are four chloro/bromo trihalomethanes [trichloromethane (chloroform), tribromomethane (bromoform), bromodichloromethane, and chlorodibromomethane], nine chloro/bromo haloacetic acids (mono-, di-, and trichloroacetic acids; mono-, di- and tribromoacetic acids; bromochloroacetic acid, bromodichloroacetic acid, and dibromochloroacetic acid), and three oxyhalides (bromate, chlorate, and chlorite).

Since these chemicals were nominated for toxicity and carcinogenicity studies, mouse and rat cancer bioassays were performed by the NTP on all chemicals except bromoacetic acid, dichloroacetic acid, trichloroacetic acid, tribromoacetic acid, bromate, and chlorite. Bioassay data are available in the peer-reviewed literature for dichloroacetic acid, trichloroacetic acid, bromate, and chlorite (reviewed in Richardson *et al.*, 2007). Dichloroacetic acid induced liver neoplasms in rats (DeAngelo *et al.*, 1996) and mice (Bull *et al.*, 1990; DeAngelo *et al.*, 1996),

while trichloroacetic acid induced liver neoplasms only in mice (Bull *et al.*, 1990; DeAngelo *et al.*, 1997). In mice, bromate increased the incidences of renal neoplasms (DeAngelo *et al.*, 1998); in rats, it increased the incidences of renal and thyroid tumors as well as mesotheliomas (Kurokawa *et al.*, 1983). Sodium chlorite was negative in both rats and mice (Kurokawa *et al.*, 1986; Yokose *et al.*, 1987). Bromoacetic acid and tribromoacetic acid do not have chronic toxicity and carcinogenicity studies.

The NTP has evaluated the toxicity and carcinogenicity of all the trihalomethanes. When administered in corn oil by gavage, all four trihalomethanes were carcinogenic in rats and/or mice (Table 1). Trichloromethane (chloroform) (NCI, 1976) and bromodichloromethane (NTP, 1987, 2006, 2007a) both produced renal tubule adenomas and adenocarcinomas in rats while bromodichloromethane induced these tumors in rats and mice. In mice, liver tumors were observed with trichloromethane (chloroform) (male and female), bromodichloromethane (female) and chlorodibromomethane (male and female) (NTP, 1985). When administered in drinking water, no evidence of carcinogenicity was observed in male rats and female mice receiving bromodichloromethane (NTP, 2006). However, average daily exposures in the drinking water studies of bromodichloromethane are approximately fourfold lower than those of the corn oil gavage studies, and the lower dose levels may explain the discrepancy of the cancer findings between the two studies. Tribromomethane (bromoform) only increased the incidence of neoplasms of the large intestine in male and female rats, and in mice there was no evidence of a carcinogenic effect (NTP, 1989).

Of the nine haloacetic acids, the NTP has evaluated four (monochloroacetic acid, dibromoacetic acid, bromochloroacetic acid and bromodichloroacetic acid) for toxicity and carcinogenicity in rats and mice. Dichloroacetic acid was evaluated using the genetically modified mouse models (NTP, 2007b). Monochloroacetic acid was not carcinogenic in either rats or mice (NTP, 1992). Dibromoacetic acid (NTP, 2007c), bromochloroacetic acid (NTP, 2009), and bromodichloroacetic acid (current study) increased the incidences of malignant mesothelioma in male rats, and all three also increased the incidences of hepatocellular carcinoma and hepatoblastoma in either male or female mice. Bromochloroacetic acid and bromodichloroacetic acid also increased the incidences of mammary gland fibroadenoma in female rats, but dibromoacetic acid did not.

In general, the trihalomethanes and haloacetic acids evaluated by the NTP increased the incidence of mouse liver tumors. Renal tumors in rats and mice may be a common finding for the trihalomethanes, while malignant mesotheliomas in male rats may be a common finding for the brominated haloacetic acids.

The NTP also ran screening-level reproductive and developmental studies and immunotoxicity screens on several of the disinfection by-products. The results from these screens are used to aid in prioritization of disinfection by-products for further testing. The reproductive and developmental screens were typically performed on one rodent species/chemical and examined the reproductive effects on adult males and females. During the past decade, numerous studies of the developmental effects of specific disinfection by-products have become available (reviewed in Colman *et al.*, 2011), and the need for further reproductive and developmental toxicity studies of disinfection by-products conducted by the NTP will be considered in light of the published reports.

The immunotoxicity screens consisted of 28-day repeat dose studies in one gender of rats or mice. These studies evaluated immune parameters such as T-cell dependent antibody response and natural killer cell activity as well as hematology and organ weights. Only five of the 16 disinfection by-products under consideration at the NTP have been evaluated in these immunotoxicity screens, and only dibromoacetic acid produced results consistent with an immunotoxic potential. Since the beginning of the disinfection by-product program, a number of other classes of disinfection by-products have been identified, including the iodinated trihalomethanes and acetic acids, halonitriles, and N-nitrosamines (Richardson *et al.*, 2007).

TABLE 1
NTP Water Disinfection By-Product Program^a

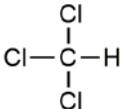
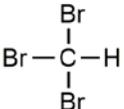
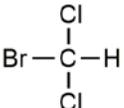
Chemical (CAS #)	Maximum Contaminant Level ^b	Study Conditions	Carcinogenicity (Clear Evidence or Some Evidence)	Genetic Toxicology
Trihalomethanes				
Trichloromethane (Chloroform) (67-66-3) 	Sum of the concentrations of all four trihalomethanes as an annual average: 0.080 mg/L or 80 ppb	By corn oil gavage [NCI, 1976] <u>Rats</u> : males (0, 90 or 180 mg/kg per day); females (0, 100 or 200 mg/kg per day) <u>Mice</u> : males (0, 138 or 277 mg/kg per day); females (0, 238, or 477 mg/kg per day)	<u>Rats</u> : renal tubule adenoma and adenocarcinoma (males) <u>Mice</u> : hepatocellular carcinoma (males and females)	CHO cell sister chromatid exchange: negative CHO cell chromosome aberration: equivocal <i>Salmonella typhimurium</i> : negative Rodent bone marrow cytogenetics chromosome aberrations: negative Rodent bone marrow cytogenetics sister chromosome exchange: equivocal Mouse lymphoma: positive Micronucleus, male mouse: positive
Tribromomethane (Bromoform) (75-25-2) 		By corn oil gavage [NTP, 1989] <u>Rats</u> : males and females (0, 100, or 200 mg/kg per day) <u>Mice</u> : males (0, 50, or 100 mg/kg per day); females (0, 100, or 200 mg/kg per day)	<u>Rats</u> : uncommon neoplasms of the large intestine (males and females) <u>Mice</u> : no evidence	CHO cell sister chromatid exchange: weakly positive CHO cell chromosome aberration: negative <i>Salmonella typhimurium</i> : positive Rodent bone marrow cytogenetics chromosome aberrations: negative Rodent bone marrow cytogenetics sister chromosome exchange: positive Mouse lymphoma: positive Micronucleus, male mouse: negative Drosophila sex-linked recessive lethal: positive Drosophila reciprocal translocations: negative Drosophila sex-linked recessive lethal: positive Drosophila reciprocal translocations: negative
Bromodichloromethane (75-27-4) 		In drinking water [NTP, 2006] <u>Rats</u> : males only (0, 6, 12, or 25 mg/kg per day) <u>Mice</u> : females only (0, 9, 18, or 36 mg/kg per day)	<u>Rats</u> : no evidence <u>Mice</u> : no evidence	CHO cell sister chromatid exchange: equivocal CHO cell chromosome aberration: negative <i>Salmonella typhimurium</i> : negative Rodent bone marrow cytogenetics chromosome aberrations: negative Rodent bone marrow cytogenetics sister chromosome exchange: positive Mouse lymphoma: positive Micronucleus, mouse: negative

TABLE 1
NTP Water Disinfection By-Product Program

Chemical (CAS #)	Maximum Contaminant Level	Study Conditions	Carcinogenicity (Clear Evidence or Some Evidence)	Genetic Toxicology
Trihalomethanes (continued)				
Bromodichloromethane (continued)		<p>By corn oil gavage [NTP, 1987] <u>Rats:</u> males and females (0, 50, or 100 mg/kg per day) <u>Mice:</u> males (0, 25, or 50 mg/kg per day); females (0, 75, or 150 mg/kg per day)</p> <p>By dermal application, in drinking water, or by corn oil gavage [GMM 5] <u>FVB Tg.AC hemizygous mice:</u> Dermal (0, 64, 128, or 256 mg/kg per day) Drinking water (males: 0, 20, 36, or 61 mg/kg per day; females: 0, 31, 61, or 130 mg/kg per day) Gavage (0, 25, 50, or 100 mg/kg per day)</p> <p><u>B6.129-Trp53^{tm1Brd} (N5) haploinsufficient mice:</u> Drinking water (males: 0, 16, 31, or 65 mg/kg per day; females: 0, 26, 50, or 100 mg/kg per day) Gavage (0, 25, 50, or 100 mg/kg per day)</p>	<p><u>Rats:</u> renal tubule cell adenocarcinoma (males and females); large intestine adenocarcinoma (males and females) <u>Mice:</u> renal tubule cell adenoma and adenocarcinoma (males) hepatocellular adenoma and adenocarcinoma (females)</p> <p><u>FVB Tg.AC hemizygous mice:</u> no evidence</p> <p><u>B6.129-Trp53^{tm1Brd} (N5) haploinsufficient mice:</u> no evidence</p>	

TABLE 1
NTP Water Disinfection By-Product Program

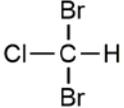
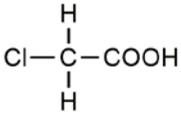
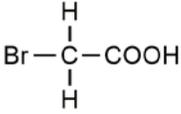
Chemical (CAS #)	Maximum Contaminant Level	Study Conditions	Carcinogenicity (Clear Evidence or Some Evidence)	Genetic Toxicology
Trihalomethanes (continued)				
Chlorodibromomethane (124-48-1) 		By corn oil gavage [NTP, 1985] <u>Rats</u> : males and females (0, 40, or 80 mg/kg per day) <u>Mice</u> : males and females (0, 50, or 100 mg/kg per day)	<u>Rats</u> : no evidence <u>Mice</u> : hepatocellular adenoma or carcinoma (females)	CHO cell sister chromatid exchange: positive CHO cell chromosome aberration: negative <i>Salmonella typhimurium</i> : weakly positive Rodent bone marrow cytogenetics chromosome aberrations: positive Rodent bone marrow cytogenetics sister chromosome exchange: negative Mouse lymphoma: positive Micronucleus, male mouse: negative Drosophila sex-linked recessive lethal: positive Drosophila sex-linked recessive lethal: negative
Regulated Haloacetic Acids				
Monochloroacetic acid (79-11-8) 	Sum of the concentrations of all five regulated haloacetic acids as an annual average: 0.060 mg/L or 60 ppb	By water gavage [NTP, 1992] <u>Rats</u> : 0, 15, or 30 mg/kg per day <u>Mice</u> : 0, 50, or 100 mg/kg per day	<u>Rats</u> : no evidence <u>Mice</u> : no evidence	CHO cell sister chromatid exchange: weakly positive CHO cell chromosome aberration: negative <i>Salmonella typhimurium</i> : negative Mouse lymphoma: positive Micronucleus, male mouse: negative Drosophila sex-linked recessive lethal: positive Drosophila reciprocal translocations: negative Drosophila sex-linked recessive lethal: equivocal
Monobromoacetic acid (79-08-3) 		Not tested by the NTP		<i>Salmonella typhimurium</i> : positive

TABLE 1
NTP Water Disinfection By-Product Program

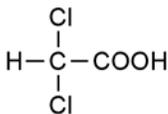
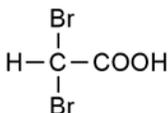
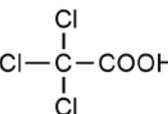
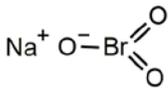
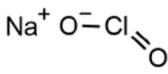
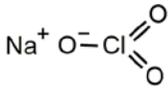
Chemical (CAS #)	Maximum Contaminant Level	Study Conditions	Carcinogenicity (Clear Evidence or Some Evidence)	Genetic Toxicology
Regulated Haloacetic Acids (continued)				
Dichloroacetic acid (79-43-6) 	Sum of the concentrations of all five regulated haloacetic acids as an annual average: 0.060 mg/L or 60 ppb	By dermal application or in drinking water [NTP, 2007b] <u>FVB Tg.AC hemizygous mice:</u> Dermal (0, 31.25, 125, or 500 mg/kg per day) Drinking water (males: 0, 75, 145, or 265 mg/kg per day; females: 0, 100, 185, or 285 mg/kg per day) <u>B6.129-Trp53^{tm1Brd} (N5) haploinsufficient mice:</u> Drinking water (males: 0, 16, 31, or 65 mg/kg per day; females: 0, 26, 50, or 100 mg/kg per day)	<u>FVB Tg.AC hemizygous mice:</u> Dermal: increased incidences of squamous cell papilloma (males and females) Drinking water: marginally increased incidences of alveolar/bronchiolar tumors (males and females) <u>B6.129-Trp53^{tm1Brd} (N5) haploinsufficient mice:</u> no evidence	<i>Salmonella typhimurium</i> : positive Mouse micronuclei: negative
Dibromoacetic acid (631-64-1) 		In drinking water [NTP, 2007c] <u>Rats:</u> males (0, 2, 20, or 40 mg/kg per day); females (0, 2, 25, or 45 mg/kg per day) <u>Mice:</u> males (0, 4, 45, or 87 mg/kg per day); females (0, 4, 35, or 67 mg/kg per day)	<u>Rats:</u> malignant mesothelioma (males) and mononuclear cell leukemia (females) <u>Mice:</u> hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma (males and females); alveolar/bronchiolar adenoma (males)	<i>Salmonella typhimurium</i> : positive Mouse micronuclei: positive
Trichloroacetic acid (76-03-9) 		Not tested by the NTP		<i>Salmonella typhimurium</i> : negative

TABLE 1
NTP Water Disinfection By-Product Program

Chemical (CAS #)	Maximum Contaminant Level	Study Conditions	Carcinogenicity (Clear Evidence or Some Evidence)	Genetic Toxicology
Unregulated Haloacetic Acids				
Bromochloroacetic acid (5589-96-8) $\begin{array}{c} \text{Br} \\ \\ \text{H}-\text{C}-\text{COOH} \\ \\ \text{Cl} \end{array}$	Not regulated	In drinking water [NTP, 2009] <u>Rats</u> : males (0, 10, 20, or 40 mg/kg per day); females (0, 13, 25, or 50 mg/kg per day) <u>Mice</u> : males (0, 20, 50, or 90 mg/kg per day); females (0, 15, 30, or 60 mg/kg per day)	<u>Rats</u> : malignant mesothelioma (males); large intestine adenomas (males and females); mammary gland (females) <u>Mice</u> : hepatocellular adenoma, carcinoma (males and females); hepatoblastoma (males)	<i>Salmonella typhimurium</i> : positive Mouse micronuclei: negative
Tribromoacetic acid (75-96-7) $\begin{array}{c} \text{Br} \\ \\ \text{Br}-\text{C}-\text{COOH} \\ \\ \text{Br} \end{array}$	Not regulated	Not tested by the NTP	Not tested	<i>Salmonella typhimurium</i> : positive
Bromodichloroacetic acid (71133-14-7) $\begin{array}{c} \text{Cl} \\ \\ \text{Br}-\text{C}-\text{COOH} \\ \\ \text{Cl} \end{array}$	Not regulated	In drinking water [current report] <u>Rats (F344/NTac)</u> : males (0, 11, 21, or 43 mg/kg per day); females (0, 13, 28, or 57 mg/kg per day) <u>Mice</u> : males (0, 23, 52, or 108 mg/kg per day); females (0, 17, 34, or 68 mg/kg per day)	<u>Rats</u> : malignant mesothelioma and skin neoplasia (males); mammary gland (females) <u>Mice</u> : hepatocellular carcinoma and hepatoblastoma (males and females); Harderian gland (males)	<i>Salmonella typhimurium</i> : positive Mouse micronuclei: negative
Chlorodibromoacetic acid (5728-95-5) $\begin{array}{c} \text{Br} \\ \\ \text{Cl}-\text{C}-\text{COOH} \\ \\ \text{Br} \end{array}$	Not regulated	Not tested by the NTP		On test

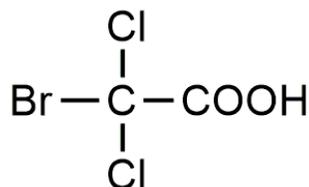
TABLE 1
NTP Water Disinfection By-Product Program

Chemical (CAS #)	Maximum Contaminant Level	Study Conditions	Carcinogenicity (Clear Evidence or Some Evidence)	Genetic Toxicology
Oxyhalides Sodium bromate (7789-38-0) 	0.010 mg/L or 10 ppb as an annual average	By dermal application or in drinking water [NTP, 2007d] <u>FVB Tg.AC hemizygous mice:</u> Dermal (0, 64, 128, or 256 mg/kg per day in ethanol) Drinking water (males: 0, 13, 63, or 129 mg/kg per day; females: 0, 15, 72, or 148 mg/kg per day) <u>B6.129-Trp53^{tm1Brd} (N5)</u> <u>haploinsufficient mice:</u> Drinking Water (males: 0, 16, 31, or 65 mg/kg per day; females: 0, 26, 50, or 100 mg/kg per day)	<u>FVB Tg.AC hemizygous mice:</u> no evidence <u>B6.129-Trp53^{tm1Brd} (N5)</u> <u>haploinsufficient mice:</u> no evidence	Mouse micronuclei: positive in FVB Tg.AC hemizygous and B6.129-Trp53 ^{tm1Brd} (N5) haploinsufficient mice
Sodium chlorite (7758-19-2) 	1.0 mg/L or 1 ppm	Not tested by the NTP	Not tested	Not tested
Sodium chlorate (7775-09-9) 	Not regulated	In drinking water [NTP, 2005] <u>Rats:</u> males (0, 5, 35, or 75 mg/kg per day); females (0, 5, 45, or 95 mg/kg per day) <u>Mice:</u> males (0, 40, 80, or 160 mg/kg per day); females (0, 30, 60, or 120 mg/kg per day)	<u>Rats:</u> thyroid gland follicular cell carcinoma (male) or adenoma (female) <u>Mice:</u> none	<i>Salmonella typhimurium:</i> negative Mouse micronuclei: negative

^a Unless otherwise stated, rats are F344/N and mice are B6C3F1.

^b Maximum concentration levels are set as close to the health goals as possible, considering cost, benefits, and the ability of public water systems to detect and remove contaminants using suitable treatment technologies.

INTRODUCTION



BROMODICHLOROACETIC ACID

CAS No. 71133-14-7

Chemical Formula: $\text{C}_2\text{HBrCl}_2\text{O}_2$ Molecular Weight: 207.84

Synonyms: 2-Bromo-2,2-dichloroacetic acid; bromodichloroethanoic acid

CHEMICAL AND PHYSICAL PROPERTIES

Bromodichloroacetic acid is a crystalline compound with a melting point of 69° to 72° C (Sigma-Aldrich, 2012). It is a moderately strong acid with an estimated pKa of 0.61 (Schultz *et al.*, 1999). The pKa for dihaloacetic acids, including bromochloroacetic acid, dichloroacetic acid, and dibromoacetic acid, is approximately 1.3 (Schultz *et al.*, 1999; Urbansky, 2000). Thus, under most conditions of exposure and in biological tissues, this chemical exists as the carboxylate anion. Bromodichloroacetic acid has an estimated water solubility of 4.9×10^3 mg/L at 25° C and an estimated Henry's Law constant of 7.9×10^{-9} atm-m³/mole at 25° C (HSDB, 2013).

FORMATION, OCCURRENCE, AND HUMAN EXPOSURE

Bromodichloroacetic acid is a member of the haloacetic acid (HAA) family of chemicals. HAAs are formed when drinking water supplies containing natural organic matter (e.g., humic or fulvic acids) are disinfected with chlorine-containing oxidizing compounds such as chlorine gas, hypochlorous acid, and hypochlorite. If bromide is present in the source water, it may be oxidized to hypobromous acid-hypobromite ion, which can react with organic matter to form brominated organic compounds. The reaction of brominated and/or chlorinated oxidizing agents with natural

organic matter produces mixed brominated and chlorinated compounds, including mono-, di-, and trichloroacetic acids; mono-, di-, and tribromoacetic acids; bromochloroacetic acid; bromodichloroacetic acid; and chlorodibromoacetic acid. The relative amount of brominated HAAs produced in chlorinated drinking water is a function of the bromide concentration in the source water and the initial bromine/chlorine ratio. Of the nine chlorinated and/or brominated HAAs, five (mono-, di-, and trichloroacetic acids and mono- and dibromoacetic acids) are regulated by the United States Environmental Protection Agency (USEPA) and four (bromo-, bromodichloro-, and dibromochloroacetic acids and tribromoacetic acid) are unregulated.

Coagulation prior to chlorination removes much of the disinfection by-product precursors from source water and thereby reduces the amount of disinfection by-products formed during disinfection. Although possible reactions of HAAs in water are decarboxylation and nucleophilic substitution (hydrolysis), these processes are very slow in ambient water, and most decreases in concentrations of HAAs in drinking water distribution systems are likely due to biodegradation (Urbansky, 2001).

HAAs are second to trihalomethanes as the most commonly detected class of disinfection by-products in surface drinking water supplies in the United States (Liang and Singer, 2003). The relative amounts of these two families of chemicals as well as other disinfection by-products produced in drinking water supplies are affected by the nature and concentration of the organic precursor materials, water temperature, pH, the type of disinfectant, the disinfectant dose, and contact time (Liang and Singer, 2003; Huang *et al.*, 2004). For example, increasing the pH from 6 to 8 increases trihalomethane formation and decreases trihaloacetic acid formation, but has little effect on mono- and dihaloacetic acid levels.

Levels of HAAs in drinking water are regulated by the USEPA (40 CFR, § 141.64). Under the disinfection by-products rule, the sum of the concentrations of monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, monobromoacetic acid, and dibromoacetic acid is limited to 60 µg/L (60 ppb). This level is believed to reduce risks from cancer as well as reproductive and developmental toxicity. However, bromodichloroacetic acid is not included in the five HAAs regulated by the USEPA under the current disinfection by-products rule. A nationwide study of disinfection by-product occurrence in diverse geographic regions of the United States was conducted between

October 2000 and April 2002 (Weinberg *et al.*, 2002). In this study, 12 water treatment plants that had different source water quality and bromide levels and that employed the major disinfectants chlorine, chloramines, ozone, and chlorine dioxide were sampled quarterly. Concentrations of bromodichloroacetic acid in finished water samples and in the distribution systems ranged from less than 2 to 15 µg/L. Bromodichloroacetic acid's portion of the total HAAs can range from 1% to 20% (Weinberg *et al.*, 2002). The Environmental Working Group (2009) has developed a database of chemical analysis from 47,576 water suppliers, of which 938 have tested for bromodichloroacetic acid from 2004 to 2009. Similar to the study by Weinberg *et al.* (2002), most facilities were below the detection limit of 2 µg/L; the highest yearly average level of bromodichloroacetic acid reported in drinking water from a single facility was 11.12 µg/L with a range of 7.22 to 16.85 µg/L.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

There are few studies of bromodichloroacetic acid in experimental animals, but much can be learned about the absorption, distribution, metabolism, and excretion of bromodichloroacetic acid from the data on other HAAs. Dihaloacetic acids are rapidly absorbed from the gastrointestinal tract of rats after oral exposure (James *et al.*, 1998; Schultz *et al.*, 1999). In male F344 rats, bromodichloroacetic acid bioavailability was estimated at 96% following an oral exposure in an aqueous vehicle (Schultz *et al.*, 1999). Dihaloacetic acids exhibit low binding to rat plasma proteins. For example, in plasma obtained from dosed F344 rats, 93% of the measured bromochloroacetic acid was in the unbound fraction (Schultz *et al.*, 1999). In contrast, bromodichloroacetic acid exhibits higher binding to rat plasma proteins with approximately 49% unbound. Compared to dihaloacetic acids, where 1% to 3% of the dose is eliminated in the urine, trihaloacetic acids are readily excreted (30% to 58%) in the urine of rats following an oral exposure (Schultz *et al.*, 1999). In rats, dihaloacetic acids are minor metabolites of bromodichloroacetic acid or other trihaloacetic acids. Schultz *et al.* (1999) evaluated the toxicokinetics and oral bioavailability of a mixture of haloacetic acids, one of which was bromodichloroacetic acid. In this study, a mixture containing trichloroacetic acid, bromochloroacetic acid, bromodichloroacetic acid, and dibromoacetic acid was administered intravenously and by gavage to naïve rats. In the mixture, the half-life of trichloroacetic acid was approximately 8 hours, while bromodichloroacetic acid had a half-life of approximately 1.85 hours. Bromochloroacetic acid and dibromoacetic acid had half-lives of approximately 44 minutes when given intravenously. In a similar study, Saghir and Schultz

(2005) examined the effects of glutathione depletion on the half-life of a mixture of haloacetic acids. Depletion of glutathione-s-transferase zeta decreased the half-life of bromodichloroacetic acid when given intravenously but had no effect when given by gavage. Oral bioavailability of bromodichloroacetic acid was approximately 80% to 95% in this mixture.

While there are no direct data on bioavailability of bromodichloroacetic acid in mice, following an exposure by oral gavage to bromodichloroacetic acid, over 90% of the radioactivity was recovered in the urine, carcass, and exhaled CO₂, with only 10% of the chemical-derived radioactivity present in the feces (Xu *et al.*, 1995). This study suggests that, following oral exposure, bromodichloroacetic acid is well absorbed in mice. In mice, 24 hours after an oral gavage exposure to [¹⁴C₁]-bromodichloroacetic acid, approximately 10% of the radioactivity remains in the carcass, compared to 20% to 30% of the radioactivity remaining in the carcass of mice treated with [¹⁴C_{1,2}]- trichloroacetic acid or [¹⁴C_{1,2}]-dichloroacetic acid, respectively. Approximately 40% to 50% of bromodichloroacetic acid is eliminated in the urine and 5% to 10% in the feces. A significant amount (25% to 30%) is eliminated as CO₂ through respiration. There does appear to be some dose-dependent metabolism of bromodichloroacetic acid in mice. At a dose of 100 mg/kg body weight, 20 times more, or approximately 4%, bromodichloroacetic acid is eliminated unchanged in the urine compared to a dose of 20 mg/kg. Xu *et al.* (1995) proposed a metabolic scheme for bromodichloroacetic acid in mice (Figure 1). Approximately 30% of bromodichloroacetic acid is eliminated in the urine as oxalate. In contrast, less than 7% of dichloroacetic acid or trichloroacetic acid is eliminated as oxalate. These data suggest that bromodichloroacetic acid metabolism is different than that of di- or trichloroacetic acid and that little of the bromodichloroacetic acid is metabolized to dichloroacetic acid. Based on these metabolic differences, Xu and colleagues postulated that the toxicity of bromodichloroacetic acid may be different than that of trichloroacetic acid. The half-life of bromodichloroacetic acid in mice ranges from 1 to 2 hours and appears dose dependent with shorter half-lives at 5 mg/kg compared to 100 mg/kg (Merdink *et al.*, 2001).

A comparison of the *in vitro* metabolism of bromodichloroacetic acid, chlorodibromoacetic acid, and tribromoacetic acid was conducted by Saghir *et al.* (2011) in mouse, rat, or human liver microsomes. In all three systems, intrinsic metabolic clearance was similar for tribromoacetic acid and chlorodibromoacetic acid, with the intrinsic clearance of

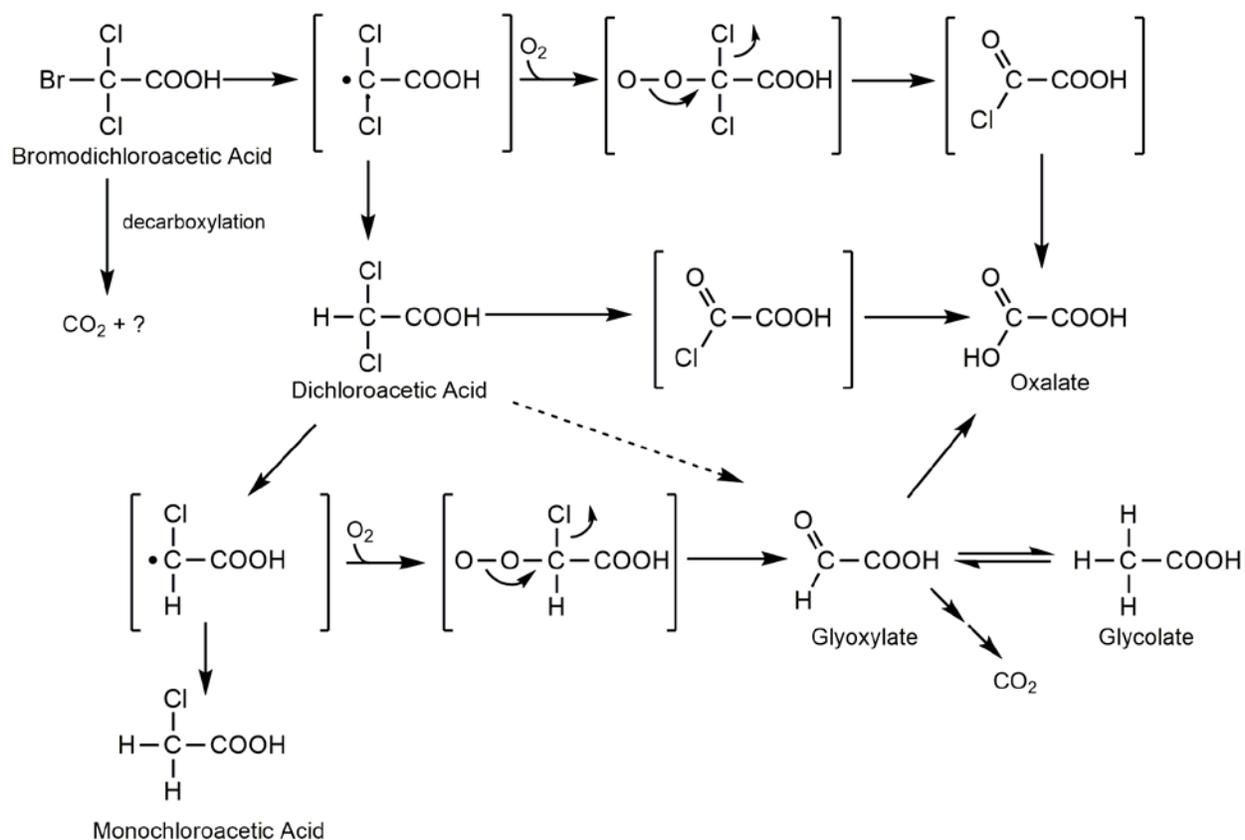


FIGURE 1
Proposed Scheme for the Biotransformation of Bromodichloroacetic Acid in Mice
 (Xu *et al.*, 1995)

bromodichloroacetic acid at about one half of that of the other haloacetic acids. The metabolism appeared to proceed by reductive debromination because the metabolic rates were greatest under nitrogen.

Humans

There are no studies evaluating the absorption, distribution, metabolism, or elimination of bromodichloroacetic acid in humans. One study did evaluate the *in vitro* metabolism of chlorodibromoacetic acid using human microsomes and recombinant enzymes (Saghir *et al.*, 2011). In this study, chlorodibromoacetic acid metabolism was blocked by CO, diphenyleneiodonium (a P450 reductase inhibitor) and inhibitors of CYP 2E1 and 3A4.

TOXICITY

Very few data are available on the toxicity of bromodichloroacetic acid. There are no *in vivo* toxicity studies in which bromodichloroacetic acid is administered alone to experimental animals. Health effects data are available on other HAAs such as dichloroacetic acid, dibromoacetic acid, and bromochloroacetic acid. The results of toxicity studies on other HAAs may provide insight into the potential health effects of bromodichloroacetic acid. However, because of qualitative and quantitative differences in the metabolism of bromodichloroacetic acid compared to other HAAs, the toxic effects of mono- and dihalogenated acetic acids may be different than those produced by bromodichloroacetic acid.

Experimental Animals

Studies comparing the effects of dichloroacetic acid in mouse liver with those of trichloroacetic acid indicate that these agents induce different spectra of hepatotoxicity. Both dichloroacetic acid and trichloroacetic acid increased the incidences of hyperplastic nodules and hepatocellular adenoma and carcinoma in male B6C3F1 mice following 52 weeks of exposure by drinking water (Bull *et al.*, 1990). However, dichloroacetic acid caused hepatomegaly, cytomegaly, focal necrosis, and accumulation of glycogen in hepatocytes, while trichloroacetic acid caused marked accumulation of lipofuscin (indicator of lipid peroxidation), modest accumulation of glycogen, and no evidence of focal necrosis. Similar to dichloroacetic acid, bromochloroacetic acid and dibromoacetic acid caused glycogen accumulation in the liver of B6C3F1 mice (Kato-Weinstein *et al.*, 2001). However, dibromoacetic acid, but not

bromochloroacetic acid, produced transient increases (2 to 4 weeks) in liver cell replication rates and in peroxisomal acyl-CoA oxidase activity. Dibromoacetic acid and dichloroacetic acid also induced peroxisomal palmitoyl-CoA oxidase activity in primary rat hepatocyte cultures (Walgren *et al.*, 2004). Hepatocellular vacuolization was observed in F344/N rats (increased incidence) and B6C3F1 mice (increased severity) administered dibromoacetic acid in drinking water at concentrations ranging from 125 to 2,000 mg/L for 3 months (Melnick *et al.*, 2007; NTP, 2007c). Similarly, bromochloroacetic acid produced hepatocellular vacuolization in female F344/N rats and male and female B6C3F1 mice following exposure in the drinking water for 3 months (NTP, 2009).

Both dibromoacetic acid and dichloroacetic acid induce peripheral neurotoxicity. In adolescent male and female F344/NCR rats exposed to 0.2 to 1.5 g dibromoacetic acid/L drinking water for 6 months, a concentration-related neuromuscular toxicity occurred (Moser *et al.*, 2004). Effects of exposure to dibromoacetic acid included decreased grip strength, mild gait abnormalities, and decreased sensorimotor responsiveness; neuropathologic findings included degeneration of spinal cord nerve fibers and spinal cord cellular vacuolization at the 0.6 and 1.5 g/L concentrations. Dichloroacetic acid exposure induced similar neuromuscular effects in F344/NCR rats (Moser *et al.*, 1999).

The liver is clearly a target for haloacetic acids, but there are some differences in the hepatic effects of these chemicals. The hepatotoxic effects of dibromoacetic and dichloroacetic acid are thought to be mediated, in part by PPAR α activation. In contrast, there was no evidence of PPAR α activation by bromochloroacetic acid. Two haloacetic acids, dibromo- and dichloroacetic acids, induce peripheral neurotoxicity in rats, but others in this class have not been evaluated.

Humans

No studies on the toxicity of bromodichloroacetic acid in humans were found in a review of the literature.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Experimental Animals

No *in vivo* studies examining the effects of bromodichloroacetic acid on reproductive and developmental toxicity are available. Inferences on the potential effects of bromodichloroacetic acid as a reproductive and developmental toxicant may be made from toxicity studies of the dihaloacetic acids. Spermatotoxicity in male rats has been identified as one of the most sensitive toxic endpoints following exposure to dihaloacetic acids. Initial studies evaluated the effects of dibromoacetic acid and dichloroacetic acid, while later studies also examined male reproductive toxicity of bromochloroacetic acid. Fewer studies have examined the effects of HAAs on the female reproductive system. In general, little has been observed in females, with the exception of dibromoacetic acid, which is reported to alter estrus cyclicity (Balchak *et al.*, 2000).

In male Sprague Dawley rats, exposure to bromochloroacetic acid for 14 days decreased epididymal sperm counts, decreased the number of motile sperm, increased the number of epididymal sperm with misshapen heads or tail defects, increased the number of atypical residual bodies in seminiferous tubules, and increased the number of Step 19 spermatids retained in Stages X and XI of the spermatogenic cycle (Klinefelter *et al.*, 2002). Dichloroacetic acid produces similar effects in Sprague Dawley rats (Linder *et al.*, 1997a) as does dibromoacetic acid in Sprague Dawley rats (Linder *et al.*, 1994a,b, 1997b) and in F344/NTac rats and B6C3F1 mice (Melnick *et al.*, 2007; NTP, 2007c). In spite of the changes in sperm quality caused by dibromoacetic acid at daily doses up to approximately 150 mg/kg, the germinal epithelium appeared intact, and there were no obvious changes in sperm production in exposed rats. At higher doses of dibromoacetic acid, germinal epithelial atrophy was induced in exposed rats (Linder *et al.*, 1997b; Melnick *et al.*, 2007).

Juvenile and adult C57BL/6 male mice were exposed daily to 0, 8, 24, 72, or 216 mg/kg bromochloroacetic acid for 14 days and evaluated for reproductive performance in a 40-day continuous breeding assay (Tully *et al.*, 2005). Juvenile mice, exposed from postnatal day 8 through postnatal day 21, were allowed to mature to 14 weeks of age and then entered into the 40-day mating study. Effects on fertility were observed only during the first 10 days of mating in mice exposed as adults (10 weeks old). In the two highest exposure groups, the mean number of litters per male, the percentage of litters per female bred, and the total number of fetuses per male were reduced. In addition,

histopathology evaluations of testes after the final dosing with bromochloroacetic acid revealed spermatids with abnormal head morphology and atypical residual bodies. Thus, diminished male fertility was attributed to disruption of spermatid differentiation by bromochloroacetic acid. Treatment with bromochloroacetic acid did not affect relative testis, epididymal, or seminal vesicle weights.

The fertility of cauda epididymal sperm evaluated by *in utero* insemination was reduced in male rats exposed to 8, 24, or 72 mg bromochloroacetic acid/kg body weight for 14 days (Klinefelter *et al.*, 2002). In male Sprague Dawley rats gavaged daily with up to 50 mg dibromoacetic acid/kg for up to 79 days, fertility was not altered; however, male fertility was compromised in rats treated for 15 days or longer with 250 mg/kg (Linder *et al.*, 1995). The latter evaluation was made through natural breeding and after artificial insemination of female rats with sperm collected from exposed rats. In fertility assessments by intrauterine insemination, the ED₅₀ for decreased fertility of cauda epididymal sperm collected from male Sprague Dawley rats receiving 2.7 mg/kg (15.6 µmol/kg) bromochloroacetic acid by gavage for 14 days was similar to the ED₅₀ for dibromoacetic acid (3.5 mg/kg, 16.1 µmol/kg) (Kaydos *et al.*, 2004).

In a short-term reproductive toxicity screen, male and female rats were exposed to 0, 60, 200, or 600 ppm (mg/L) bromochloroacetic acid in the drinking water during cohabitation and separated after mating (NTP, 1998). Treatment of dams continued until gestation day 20 when they were examined for fertility and pregnancy status. Treatment of males continued until 30 days after mating, at which time they were necropsied and examined for sperm counts, motility, and morphology; organ weights; and histopathologic changes in reproductive organs. In a concomitant developmental toxicity screen, pregnant rats were exposed to the same concentrations of bromochloroacetic acid in drinking water from gestation day 6 until onset of birth. Treatment-related findings included a significant decrease in total implants per litter and a significant decrease in the number of live fetuses per litter at 600 ppm (50 mg/kg per day). At 600 ppm, water consumption was decreased by 21% to 34% and the albumin/globulin ratios were increased, consistent with signs of dehydration. The 600 ppm concentration produced a consistent decrease in water consumption in both sexes, and the decreased water consumption may have affected reproductive function in females. From these data, bromochloroacetic acid is taste-aversive and a general toxicant in both sexes at 600 ppm and also a female reproductive toxicant at 600 ppm. Studies that observed an effect of

bromochloroacetic acid on male reproductive organs (Linder *et al.*, 1995; Klinefelter *et al.*, 2002; Kaydos *et al.*, 2004) used oral gavage doses equal to or higher than those used in the NTP drinking water study (NTP, 1998).

Holtzman rats were administered dibromoacetic acid by gavage at doses ranging from 62.5 to 250 mg/kg per day during the first 8 days of pregnancy to determine if administration during early pregnancy would affect female reproduction (Cummings and Hedge, 1998). No effects were detected on the number of implantation sites, number of pups per litter, number of resorptions, or pup weights on day 20; however, serum levels of estradiol were elevated in dosed dams. In a follow-up study, gavage dosing of 90-day-old female Sprague Dawley rats with 10 to 270 mg dibromoacetic acid/kg for 14 days caused a dose-related alteration in estrous cyclicity, with a tendency toward longer periods of persistent estrus (Balchak *et al.*, 2000). In addition, *in vitro* exposure of preovulatory follicles to dibromoacetic acid (50 µg/mL for 24 hours) caused an elevation in estradiol release and suppression of progesterone secretion stimulated with human chorionic gonadotropin; thus, the disruption of estrous cyclicity by dibromoacetic acid was attributed to alteration of ovarian steroid production. Elevations in circulating estradiol in female rats exposed to dibromoacetic acid were attributed to suppression of estradiol catabolism because serum estradiol levels were elevated in ovariectomized rats implanted with estradiol-containing capsules and then treated with dibromoacetic acid (Goldman and Murr, 2003).

Daily exposure of female Dutch belted rabbits to approximately 1 to 50 mg dibromoacetic acid/kg body weight per day in drinking water beginning on gestation day 15 and continuing through 24 weeks did not produce any gross abnormalities of the reproductive tract or viscera but did reduce the number of primordial follicles in prepubertal and adult rabbits (Bodensteiner *et al.*, 2004). This exposure covers the fetal and neonatal periods when the primordial follicle pool in rabbits is formed. Reduction in the population of primordial follicles could result in early reproductive senescence.

Exposure of Sprague Dawley rats to 4 to 800 ppm dibromoacetic acid in drinking water from gestation day 15 through adulthood induced delays in pubertal development (delayed preputial separation in males and delayed vaginal opening in females at 400 ppm) and decreases in the fertility of cauda epididymal sperm at 600 and 800 ppm (Klinefelter *et al.*, 2004). Altered steroidogenesis was suggested to be a contributor to the pubertal delays.

Dibromoacetic acid was administered to Sprague Dawley rats in drinking water at concentrations ranging from 125 to 1,000 ppm (mg/L) with exposures beginning 14 days before cohabitation and continuing through gestation and lactation (Christian *et al.*, 2001). Exposure to dibromoacetic acid at concentrations of 250, 500, or 1,000 ppm reduced food and water consumption in the dams. The only reported reproductive and developmental effects were decreases in pup body weights at 250 ppm or greater, the severity of which was positively associated with the decreased food and water consumption in the dam.

In a two-generation reproductive toxicity study, Sprague Dawley rats were given dibromoacetic acid in drinking water at concentrations of 0, 250, 500, or 650 ppm (Christian *et al.*, 2002). No effects on estrous cyclicity, mating, fertility, implantation sites, litter sizes, pup viability, or pup sex ratios were observed in the parental or F₁ generation female rats. In parental and F₁ generation male rats, increased incidences of delayed sperm production (retention of Step 19 spermatids in Stage IX and Stage X seminiferous tubules), atypical residual bodies in the testis, abnormal sperm shape, and epididymal abnormalities (atrophy, residual bodies, and hypospermia) were observed in the 250 or 650 ppm groups. Delays in preputial separation and vaginal opening were observed in 650 ppm groups of F₁ generation rats. In contrast to the effect of dibromoacetic acid on follicular development in rabbits (Bodensteiner *et al.*, 2004), no effect on ovarian follicular histology was observed in rats exposed to dibromoacetic acid. For this effect, the rabbit may be a more sensitive species.

Exposure of mouse whole embryo cultures to HAAs for up to 26 hours caused defects in neural tube closure, craniofacial abnormalities, prosencephalic and pharyngeal arch hypoplasia, deficient caudal growth, and eye and heart defects (Hunter *et al.*, 1996, 2006a). Monohaloacetic acids tended to be more potent than dihaloacetic acids, which were more potent than trihaloacetic acids. Brominated acetic acids and mixed bromo/chloroacetic acids were more potent than their chlorinated analogs. In this model, bromodichloroacetic acid disrupted embryogenesis and altered morphogenesis (Hunter *et al.*, 2006b).

In order to study the potential toxic effects of whole disinfection by-product mixtures, the USEPA generated a concentrated chlorinated drinking water. The source water was obtained from East Fork Lake in Ohio, spiked with bromide and iodide, coagulated, chlorinated, and concentrated 136 fold (Richardson *et al.*, 2008). This water was

used as the test article in a multigenerational rat bioassay (Narotsky *et al.*, 2013). Endpoints evaluated were pup weight, prenatal loss, pregnancy rate, gestation length, puberty onset in males, growth, estrous cycle, hormone levels, immunologic, and neurodevelopmental parameters. Significant effects were observed for delayed puberty for F1 females, reduced caput epididymal sperm counts in F1 adult males, and increased incidences of thyroid gland follicular cell hypertrophy in adult females. The role of bromodichloroacetic acid, if any, is uncertain in this study.

A number of haloacetic acids have been evaluated for potential reproductive toxicity in experimental animals. In general, the dihaloacetic acids are male reproductive toxicants in rats and mice. There are fewer studies on the potential for female reproductive toxicity of haloacetic acids and only dibromoacetic acid appears to have any effect on the female reproductive system.

Humans

No studies have been reported on reproductive or developmental effects of bromodichloroacetic acid alone in humans. However, several studies have indicated an association between exposure to disinfection by-products and alterations in reproductive function or fetal development, including spontaneous abortions, stillbirths, low birth weights, and birth defects (Nieuwenhuijsen *et al.*, 2000). Although associations between stillbirth risk and exposure to disinfection by-products have been demonstrated, no association was observed with HAAs after controlling for exposures to trihalomethanes (King *et al.*, 2005; Hoffman *et al.*, 2008). Associations between exposure to the five dihaloacetic acids regulated by the USEPA and impaired fetal growth have been reported (Hinckley *et al.*, 2005), but these studies did not include bromodichloroacetic acid.

CARCINOGENICITY

Experimental Animals

No studies have been reported on the carcinogenicity of bromodichloroacetic acid in animals. However, several studies have shown that dichloroacetic acid administered in drinking water is carcinogenic to the liver of rats and mice. Hepatocellular adenomas and carcinomas were induced in male and female B6C3F1 mice exposed to up to 5 g dichloroacetic acid/L drinking water for at least 61 weeks (Herren-Freund *et al.*, 1987) and up to 104 weeks

(DeAngelo *et al.*, 1991; Daniel *et al.*, 1992). When length of exposure was increased, a lower concentration of dichloroacetic acid was required to induce the hepatocarcinogenic response in mice; the liver tumor response was significantly increased at 0.5 g/L or greater after 104 weeks of exposure (DeAngelo *et al.*, 1991).

Dichloroacetic acid is also carcinogenic to the liver of rats. Male F344/N rats were exposed to 0, 0.05, 0.5, or 1.6 g dichloroacetic acid/L drinking water for 100 weeks (DeAngelo *et al.*, 1996). Mean daily doses of dichloroacetic acid were 3.6, 40, and 139 mg/kg body weight for the three exposed groups. The incidences of hepatocellular adenoma or carcinoma combined in rats that survived more than 78 weeks were 3% (1/33) in controls and 0% (0/26), 24.1% (7/29), and 28.6% (8/28) in the respective exposed groups. The liver tumor response was not associated with peroxisome proliferation, hepatocellular necrosis, or sustained hepatocyte proliferation.

Dibromoacetic acid was administered for 2 years to male and female F344/N rats and B6C3F1 mice in drinking water at concentrations of 0, 50, 500, or 1,000 mg/L (Melnick *et al.*, 2007; NTP, 2007c). Incidences of exposure-related neoplasms were increased at multiple sites; these included mononuclear cell leukemia and abdominal cavity mesothelioma in rats, and liver and lung neoplasms in mice. The increased incidences of hepatocellular neoplasms in male mice were significant even in the 50 mg/L group, an exposure equivalent to an average daily dose of approximately 4 mg/kg.

Bromochloroacetic acid is also carcinogenic in rats and mice. Groups of 50 male and 50 female F344/N and B6C3F1 mice were exposed to drinking water containing 250, 500, or 1,000 mg/L for 2 years (NTP, 2009). Exposed male rats had increased rates of malignant mesothelioma. Adenomas of the large intestine were observed in both male and female rats administered the highest concentration of bromochloroacetic acid. Exposed female rats also had increased incidences of multiple fibroadenoma of the mammary gland. Slightly increased incidences of hepatocellular adenoma in exposed male and female rats and pancreatic islet adenoma in exposed male rats were also observed. Male and female mice administered bromochloroacetic acid had increased incidences of hepatocellular adenoma and carcinoma. Male mice also had an exposure concentration-dependent increase in the incidences of hepatoblastoma.

The results of carcinogenicity studies of trichloroacetic acid are not as consistent as those of the dihaloacetic acids. DeAngelo *et al.* (1997) did not observe increases in the incidences of liver tumors in male F344 rats exposed to up to 5 g/L trichloroacetic acid in drinking water for 104 weeks. In contrast, increased incidences of liver tumors (adenomas and carcinomas) have been observed in male and female B6C3F1 mice following chronic exposure to trichloroacetic acid in the drinking water at concentrations up to 2 g/L (Bull *et al.*, 1990; Periera, 1996). It is possible that these differences represent species specific responses to trichloroacetic acid.

Mechanistic studies examining the potential modes of action of HAAs have focused on the liver. The roles of PPAR- α activation, DNA damage, oxidative stress and cytotoxicity, and regenerative proliferation in liver carcinogenicity of HAAs have been examined in several studies. Single gavage administration of HAAs induced lipid peroxidation and formation of 8-hydroxydeoxyguanosine adducts in nuclear DNA in the liver of male B6C3F1 mice; the relative potencies for these effects were dibromoacetic acid \approx bromochloroacetic acid > dichloroacetic acid > trichloroacetic acid (Austin *et al.*, 1996). These results suggest that DNA damage from oxidative stress induced by these agents may contribute to the hepatocarcinogenic process. In male B6C3F1 mice exposed to 2.0 g dichloroacetic acid/L drinking water, hepatocyte division rates were increased after 14 days of exposure; after 28 or 280 days of exposure, hepatocyte division rates were reduced in livers of dichloroacetic acid-treated mice compared to controls (Stauber and Bull, 1997). Altered hepatic foci and liver tumors in dichloroacetic acid-treated mice showed higher immunoreactivity to the oncoproteins *c-Jun* and *c-Fos* and higher rates of cell division than the surrounding nonlesioned liver tissue. Increased cell replication rates in hepatic foci and tumors and decreased rates in normal hepatocytes of mice exposed to dichloroacetic acid provide a selective growth advantage to initiated cells. In a follow-up study, incubation of primary hepatocyte cultures from untreated male B6C3F1 mice with 0.5 to 2.0 mM dichloroacetic acid enhanced the formation of anchorage-independent colonies in soft agar (Stauber *et al.*, 1998). A fourfold increase in colony formation was measured when hepatocytes were obtained from mice pretreated with 0.5 g dichloroacetic acid/L drinking water for 2 weeks. Although dichloroacetic acid did not induce *c-Jun* expression in hepatocyte monolayers, the colonies promoted by dichloroacetic acid were immunoreactive with *c-Jun* antibody. These results suggest that dichloroacetic acid was selective for *c-Jun*⁺ subpopulations.

Gavage administration of 500 mg dichloroacetic acid/kg body weight to female B6C3F1 mice for 5 days caused decreased DNA methylation and increased mRNA expression of the *c-myc* proto-oncogene (Pereira *et al.*, 2001). Administration of 3.2 g dichloroacetic acid/L drinking water for 36 weeks increased the incidences and multiplicities of liver tumors but not kidney tumors in *N*-methyl-*N*-nitrosourea-initiated mice. Thus, hypomethylation of *c-myc* and increased expression of this gene may be involved in the promotion of liver tumors by dichloroacetic acid. Exposure of female B6C3F1 mice and male F344/N rats to 1,000 or 2,000 mg dibromoacetic acid/L drinking water for 28 days caused a decrease in the 5-methylcytosine content of DNA and increased mRNA expression of the *c-myc* and insulin-like growth factor II genes (Tao *et al.*, 2004). Dibromoacetic acid and dichloroacetic acid also caused hypomethylation of DNA in kidneys of male B6C3F1 mice and male F344 rats exposed to these agents via the drinking water (Tao *et al.*, 2005). Treatment with dibromoacetic acid also caused glycogen accumulation and peroxisome proliferation in the mouse and rat liver. Thus, dibromoacetic acid and dichloroacetic acid appear to induce similar biochemical and molecular effects.

The mutational spectrum at codon 61 of the *H-ras* gene was different in liver tumors obtained from male B6C3F1 mice exposed to 500 mg dichloroacetic acid/L drinking water for 76 weeks compared to liver tumors from control mice (Anna *et al.*, 1994). The frequency of liver tumors with *H-ras* codon 61 dichloroacetic acid-treated mice had increased CAA→CTA and decreased CAA→AAA mutations. Hence, base-substitution mutations may be involved in the hepatocarcinogenicity of dichloroacetic acid.

The di- and trihaloacetic acids that have been tested are carcinogenic in rodents. Target tissues are liver in mice, mesothelium in male rats, and mammary gland in female rats. Other tumors in rats, such as hepatocellular adenomas and pancreatic islet cell adenomas, have been observed, but not consistently, across chemicals.

Humans

No studies have been reported on the carcinogenicity of bromodichloroacetic acid in humans. However, several studies have examined cancer risks associated with exposure to disinfection by-products. A meta-analysis of epidemiology studies published before 1989 on cancer and chlorination by-products in drinking water yielded a relative risk estimate of 1.21 [95% confidence interval (CI): 1.09 to 1.34] for bladder cancer and 1.38 (95% CI:

1.01 to 1.87) for rectal cancer (Morris *et al.*, 1992). A population-based case-control study in Colorado also found an association between prolonged exposures to chlorinated surface water and increased bladder cancer risk in men and women for both smokers and nonsmokers (McGeehin *et al.*, 1993). An elevation in brain cancer risk was also associated with exposure to chlorinated surface water (Cantor *et al.*, 1999).

A more recent meta-analysis of studies on chlorinated drinking water reported long-term drinking of chlorinated water was associated with an increased risk of bladder cancer (Villanueva *et al.*, 2003). This study was followed up by a posterior pooled analysis of six case-control studies of bladder cancer that confirmed an increased risk of bladder cancer associated with exposure to disinfection by-products (Villanueva *et al.*, 2004). In addition to drinking chlorinated water, exposure to disinfection by-products also occurs from activities such as bathing, showering, and swimming pool attendance (Villanueva *et al.*, 2007). In the Spanish bladder cancer study, exposure to disinfection by-products from ingestion or dermal contact resulted in an increased risk of bladder cancer, particularly for the dermal route (Villanueva *et al.*, 2007). One of the challenges to interpreting these studies is the difficulty in attributing the risk to individual chemicals. Disinfection by-products consist of a variety of chemical classes for which one or more representatives have been shown to induce cancer in rodents (Richardson *et al.*, 2007).

GENETIC TOXICITY

There is very little information about the genotoxicity of bromodichloroacetic acid or mixed trihaloacetic acids in general. Only one study was found in the literature that reported on the genotoxicity of bromodichloroacetic acid. In this class study, twelve haloacetic acids were tested for induction of DNA damage using the comet assay in Chinese hamster ovary cells (Plewa *et al.*, 2010). Bromodichloroacetic acid gave negative results at concentrations up to 30 mM, whereas chlorodibromoacetic acid gave positive results at a concentration of 14 mM. However, chlorodibromoacetic acid was the least potent compound among the nine haloacetic acids that were positive in the comet assay and potencies ranged by 1,000-fold.

The genotoxicity of the dihaloacetic acid, dichloroacetic acid, is of interest as dichloroacetic acid is one of the metabolic products of bromodichloroacetic acid in mice, albeit at low levels (Xu *et al.*, 1995), and dichloroacetic

acid is carcinogenic in mice (Herren-Freund *et al.*, 1987; Bull *et al.*, 1990; DeAngelo *et al.*, 1991). Dichloroacetic acid has been tested in bacterial mutagenicity assays, *in vitro* mammalian cell assays, and *in vivo* mutagenesis and micronucleus assays. Dichloroacetic acid was positive in *Salmonella typhimurium* strains TA100, TA98, and RSJ100 (a derivative of TA1535 that expresses the Phase II metabolic enzyme, GST1-1, from rat) in the absence of metabolic activation (DeMarini *et al.*, 1994; Giller *et al.*, 1997; Kargalioglu *et al.*, 2002), whether the exposure was by vapor or in solution. Mixed results were obtained in the presence of rat liver S9 microsomal fraction (S9 mix). Upon further examination of the mutational spectra obtained in TA100 treated with dichloroacetic acid versus background revertants, dichloroacetic acid shifted the mutational spectrum by increasing G:C to A:T transitions (DeMarini *et al.*, 1994). One study found that dichloroacetic acid induced the *Escherichia coli* PQ37 SOS repair system at a concentration of 500 $\mu\text{L/mL}$, but the effect did not occur in the presence of S9 mix (Giller *et al.*, 1997). Conversely, in a prophage induction assay, dichloroacetic acid was a weak positive at a concentration of 2 mg/mL only in the presence of S9 mix (DeMarini *et al.*, 1994).

With regard to *in vitro* mammalian cell assays, dichloroacetic acid has been tested in the comet assay and the mouse lymphoma assay. Dichloroacetic acid did not induce DNA damage, measured by the comet assay, in Chinese hamster ovary cells (Plewa *et al.*, 2002) or in freshly isolated rat or mouse hepatocytes exposed to the chemical for 4 hours at concentrations as high as 10 mM (Chang *et al.*, 1992). However, in the same study, dichloroacetic acid induced DNA damage in a human lymphoblastoid cell line (CCRF-CEM) at a concentration of 1 mM. Dichloroacetic acid was positive in the mouse lymphoma L5178Y/tk^{+/-} assay in the absence of S9 mix at a concentration of 300 $\mu\text{g/mL}$ (Harrington-Brock *et al.*, 1998).

Dichloroacetic acid has been tested for genotoxic effects *in vivo* using the comet assay and the micronucleus assay, and in transgenic mice carrying lambda shuttle vectors for mutational analyses. With regard to the comet assay, Chang *et al.* (1992) obtained negative results in B6C3F1 mice and Fischer 344 rats given single doses of dichloroacetic acid (10 mM/kg) by gavage or multiple exposures in drinking water (up to 5 g/L for mice and up to 2 g/L for rats). The tissues that were assessed for damage after single doses included stomach, duodenum, liver, and spleen. Only liver was assessed after multiple exposures to dichloroacetic acid (Chang *et al.*, 1992). Fuscoe *et al.* (1996) also observed no increase in DNA damage, measured by the comet assay, in blood leukocytes of B6C3F1

mice exposed to dichloroacetic acid in drinking water for 28 days at concentrations up to 3.5 g/L. However, Nelson and Bull (1988) obtained positive results in the comet assay in liver samples from B6C3F1 mice and Sprague Dawley rats exposed to a single dose of dichloroacetic acid (10 mmol/kg) by gavage. Parrish *et al.* (1996) examined whether dichloroacetic acid increased oxidative damage to DNA. Levels of 8-oxo-2'-deoxyguanosine were not increased in DNA from the livers of B6C3F1 mice exposed to dichloroacetic acid in drinking water for 3 or 10 weeks (doses of 3 g/L or 2 g/L, respectively). With regard to the micronucleus assay, micronucleated polychromatic erythrocytes (MN-PCE) in peripheral blood were increased in B6C3F1 mice exposed to dichloroacetic acid for 9 days in drinking water at a concentration of 3.5 g/L (Fusco *et al.*, 1996). Furthermore, the same study showed increases in micronucleated normochromatic erythrocytes (MN-NCE) after 10, 26, or 31 weeks of exposure to 3.5 g/L dichloroacetic acid. However, in studies conducted by the NTP using p53^{+/-} mice or Tg.AC mice (mice carrying one copy of a mutant v-Ha-Ras oncogene), no increases in micronucleated erythrocytes were detected in peripheral blood after 3 months of exposure to dichloroacetic acid (highest concentration of 2 g/L) (NTP, 2007b). Lastly, although dichloroacetic acid did not increase the frequency of mutations at the transgenic *lacI* locus in Big Blue[®] B6C3F1 mice (liver) after 4 or 10 weeks of exposure through drinking water, the mutant frequency was 2.3-fold greater than the control after 60 weeks of exposure at a concentration of 3.5 g/L (Leavitt *et al.*, 1997). Also, the mutation spectrum shifted, with increased mutagenesis at T:A base pairs compared to controls.

The mixed dihaloacetic acid, bromochloroacetic acid was positive in *S. typhimurium* strain TA100, in tests conducted with and without exogenous metabolic activation enzymes (S9); no mutagenicity was detected in strain TA98 or in *E. coli* WP2 *uvrA*/pKM101, with or without S9. No significant increases in the frequencies of micronucleated erythrocytes were observed in blood samples of male or female mice exposed to bromochloroacetic acid for 3 months in drinking water, indicating no induction of chromosomal damage in proerythrocytes under these conditions in mice (NTP, 2009).

Taken together, although there is little published information on the genotoxicity of bromodichloroacetic acid, one of its metabolites, dichloroacetic acid, is consistently positive in bacterial mutagenicity assays in the absence of metabolic activation, gives mixed results in DNA damage (comet) assays, and shows signs of *in vivo* mutagenicity and effects on chromosomal stability in rodents after long-term exposures at high doses.

STUDY RATIONALE

Bromodichloroacetic acid was nominated to the NTP by the American Water Works Association Research Foundation and the USEPA for toxicity and carcinogenicity studies in rats and mice because of widespread human exposure to this water disinfection by-product and because related dihaloacetic acids were found to be carcinogenic in rats and mice. In addition, this study is part of a series of NTP studies to provide data on di- and trihaloacetic acids and to compare the toxicity and carcinogenicity of chlorinated, brominated, and mixed chloro/bromo acetic acids. Drinking water was selected as the route of exposure to mimic human exposure to this chemical.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF BROMODICHLOROACETIC ACID

Bromodichloroacetic acid was obtained from Radian International, LLC (Austin, TX), in one lot (31542-80) and from Chemfinet Services, Inc. (Tarrytown, NY), in one lot (NJ 87-90/9/2005). Lot 31542-80 was used in the 2-week and 3-month studies, and lot NJ 87-90/9/2005 was used in the 2-year studies and in the genetic toxicology studies. Identity and purity analyses were conducted by the analytical chemistry laboratory at Battelle Chemistry Support Services (Columbus, OH) and the study laboratories at Southern Research Institute (SRI) (Birmingham, AL) for the 2-week and 3-month studies and at Battelle Columbus Operations (Columbus, OH) for the 2-year studies. Karl Fischer titration was performed by Galbraith Laboratories (Knoxville, TN), and elemental analyses were performed by Prevalere Life Sciences, Inc. (Whitesboro, NY), and Elemental Analysis Corp. (Lexington, KY) (Appendix J). Reports on analyses performed in support of the bromodichloroacetic acid studies are on file at the National Institute of Environmental Health Sciences.

Both lots of the chemical, an off-white crystalline solid, were identified as bromodichloroacetic acid by the analytical chemistry laboratory using infrared (IR) and proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy and by the study laboratories using IR and NMR (SRI only) spectroscopy. For lot 31542-80, Karl Fischer titration indicated 440 ppm water. High-performance liquid chromatography with ultraviolet detection (HPLC/UV) indicated one major peak and no impurities greater than 0.1% of the total peak area. Ion chromatography indicated one major peak and four impurities, each greater than 0.1% of the total peak area, with a combined area of 1.2%. Titration of the acid functional group indicated a purity of 100%, assuming all titratable acid was present as bromodichloroacetic acid. The overall purity of lot 31542-80 was estimated to be approximately 99%. For lot NJ 87-90/9/2005, Karl Fischer titration indicated approximately 0.4% water; elemental analyses for carbon and hydrogen were in agreement with theoretical values; PIXE analysis indicated the presence of carbon (13%), hydrogen (0.5%), oxygen (17%), chlorine (31%), and bromine (38%), consistent with the theoretical values; no significant impurities were detected. HPLC/UV indicated one major peak with two impurities greater than 0.1%

of the major peak area with a combined area of 0.6%. These two impurities were identified as monochloroacetic acid and dibromochloroacetic acid based on retention times matching to standards. Analysis by gas chromatography (GC) with flame ionization (FID) found two impurities. The larger peak was identified as dibromochloroacetic acid by GC/mass spectroscopy and was quantified at 2.7% by standard addition followed by GC/FID analysis. Functional group titration indicated a purity of approximately 95%, assuming all titratable acid was present as bromodichloroacetic acid. The overall purity of lot NJ87-90/9/2005 was determined to be greater than 97%.

To ensure stability, lot 31542-80 was stored at approximately -20°C in amber glass bottles with Teflon[®]-lined lids protected from light and moisture during the 2-week and 3-month studies. Prior to the 2-year study, a forced degradation study of a reference standard was performed by the study laboratory using HPLC/UV; no degradation occurred. For the 2-year studies, the bulk chemical was stored at -20°C in sealed amber glass bottles with Teflon[®]-lined lids, secured by tape, and placed in a sealed glove bag filled with nitrogen, or repackaged in sealed amber glass bottles under a nitrogen headspace. Periodic reanalyses of the test chemical were performed twice during the 3-month studies and at least every 6 months during the 2-year studies using HPLC/UV; no degradation of the test chemical was observed.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared by mixing bromodichloroacetic acid with tap water and stored in sealed Nalgene[®] containers with a minimal headspace at 5°C for up to 40 (3-month studies) or 42 (2-week and 2-year studies) days (Table J2).

Stability studies of the 62.5 mg/L dose formulation were performed by the analytical chemistry laboratory using HPLC/UV. Stability was confirmed for dose formulations stored in sealed amber glass or Nalgene[®] high density polyethylene containers at 5°C for at least 42 days and for 7 days under simulated animal room conditions.

Periodic analyses of the dose formulations of bromodichloroacetic acid were conducted by the study laboratories using HPLC/UV. During the 2-week studies, the dose formulations were analyzed once; all five dose formulations for rats and mice were within 10% of the target concentrations (Table J3). Animal room samples of these dose

formulations were also analyzed; all five samples for rats and all five for mice were within 10% of the target concentrations. During the 3-month studies, the dose formulations were analyzed at the beginning, midpoint, and end of the studies; all 15 dose formulations for rats and mice were within 10% of the target concentrations (Table J4). Animal room samples of these dose formulations were analyzed, and all 15 for rats and all 14 for mice were within 10% of the target concentrations. During the 2-year studies, the dose formulations were analyzed approximately every 6 weeks, and all 63 dose formulations for rats and all 57 for mice were within 10% of the target concentrations (Table J5). Animal room samples were also analyzed; all 12 for rats and all 12 for mice were within 10% of the target concentrations.

Tap water from the City of Columbus municipal supply was used as the vehicle. Samples of the tap water were taken within 30 days prior to the study start at 3-month intervals throughout the study, and within 30 days of the end of the in-life phase and analyzed by an NIEHS analytical contractor for total chlorates, trihalomethanes, and haloacetic acids. Bromodichloroacetic acid was measured in the tap water and was found below the detection limit (approximately 0.6 µg/mL) in all samples analyzed. This detection limit was 100 times lower than the lowest concentration in the exposed animals in the 2-week and 3-month studies and 400 times lower than the lowest concentrations in the exposed animals in the 2-year studies. Note that in a nationwide study, bromodichloroacetic acid levels ranged from below detection limits to 15 µg/L (Weinberg *et al.*, 2002) and that the highest concentration reported is approximately 4,200 times lower than the lowest concentration used in the 2-week and 3-month studies and 16,666 times lower than the lowest concentration used in the 2-year studies.

ANIMAL SOURCE

Male and female F344/N rats and B6C3F1/N mice were obtained from the NTP colony maintained at Taconic Farms, Inc. (Germantown, NY), for the 2-week and 3-month studies. For the 2-year studies, male and female F344/NTac rats were obtained from the commercial colony at Taconic Farms, Inc., and the B6C3F1/N mice were obtained from the NTP colony maintained at Taconic Farms, Inc. The rationale for change of strain from F344/N to F344/NTac was a programmatic decision. For many years the NTP used the inbred F344/N rat for its toxicity and carcinogenicity studies. Over a period of time, the F344/N exhibited sporadic seizures and idiopathic chylothorax and consistently high rates of mononuclear cell leukemia and testicular neoplasia. Because of these issues in the

F344/N rat and the NTP's desire to find a more fecund rat model that could be used in both reproductive and carcinogenesis studies for comparative purposes, a change in the rat model was explored. Following a workshop in 2005, the F344 rat from the Taconic commercial colony (F344/NTac) was used for a few NTP studies to allow the NTP time to evaluate different rat models. The F344/NTac was used in four subchronic and two chronic studies (cobalt metal and bromodichloroacetic acid) between 2005 and 2006 (King-Herbert *et al.*, 2006).

ANIMAL WELFARE

Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Studies were approved by the Battelle Columbus Operations Animal Care and Use Committee and conducted in accordance with all relevant NIH and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines.

2-WEEK STUDIES

The 2-week study was designed to determine the range of exposure levels for the 3-month study and to evaluate if bromodichloroacetic acid was a PPAR activator. On receipt, the rats and mice were 4 weeks old. Animals were quarantined for 12 (rats) or 14 (mice) days and were 6 weeks old on the first day of the studies. Groups of five male and five female rats and mice were exposed to 0, 62.5, 125, 250, 500, or 1,000 mg bromodichloroacetic acid/L drinking water for 16 days. The exposure concentrations were based on previous studies of dichloroacetic acid (DeAngelo *et al.*, 1991, 1996, 1999; NTP, 2007b), dibromoacetic acid (NTP, 2007c), and bromochloroacetic acid (NTP, 2009). Because bromochloroacetic acid is more potent than dichloroacetic acid based on mass, concentrations of bromodichloroacetic acid equivalent to those used in bromochloroacetic acid studies based on mass were selected. Groups of five male and five female special study rats and mice were exposed to the same concentrations for 17 days. Feed and water were available *ad libitum*. Rats and female mice were housed five per cage, and male mice were housed individually. Clinical findings were recorded daily for core study rats and mice. Water consumption was recorded twice weekly by cage on a 3-day/4-day schedule. The core study animals were

weighed initially, on days 8 and 15, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 2.

Assays of the liver enzyme acetyl-CoA hydrolase were performed using the livers of special study animals at the end of the studies. The liver was perfused with buffer (pH 7.4) to stabilize the enzyme, removed, trimmed, rinsed in ice-cold saline, blotted dry, and weighed. Livers were stored frozen until homogenization, preparation of supernatants, and determination of acetyl-CoA hydrolase activity using the method of Nakanishi *et al.* (1993). Supernatant protein levels were determined using a modification of the Lowry *et al.* (1951) method and used to normalize enzyme activity.

Necropsies were performed on all core study rats and mice, and the heart, right kidney liver, lung, right testis, and thymus were weighed. Histopathology was performed on the colon, small intestine, kidney, liver, and stomach in all core study control and 1,000 mg/L rats and mice. These tissues were examined to a no-effect level in rats. Table 2 lists the tissues and organs examined.

3-MONTH STUDIES

The 3-month studies were conducted to evaluate the cumulative toxic effects of repeated exposure to bromodichloroacetic acid and to determine the appropriate exposure concentrations to be used in the 2-year studies.

On receipt, the rats and mice were 4 to 5 weeks old. Animals were quarantined for 13 or 14 (rats) or 11 or 12 (mice) days and were 6 to 7 weeks old on the first day of the studies. Before the studies began, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix M).

Groups of 10 male and 10 female rats and mice were exposed to 0, 62.5, 125, 250, 500, or 1,000 mg bromodichloroacetic acid/L drinking water for 14 weeks. In addition, groups of 10 male and 10 female clinical pathology study rats were exposed to the same concentrations for 4 weeks. Feed and water were available

ad libitum. Rats and female mice were housed five per cage, and male mice were housed individually. Clinical findings were recorded weekly for core study rats and mice. Water consumption was recorded weekly by cage for all core study animals. The core study animals were weighed initially, weekly, and at the end of the studies; special study animals were weighed initially. Details of the study design and animal maintenance are summarized in Table 2.

Animals were anesthetized with a mixture of CO₂/O₂ prior to collection of blood samples. Blood was collected from the retroorbital plexus of clinical pathology rats on days 3 and 21 and from the retroorbital plexus of core study rats and the retroorbital sinus of mice at the end of the studies for hematology and clinical chemistry (rats only). Blood samples were collected into microcollection serum separator tubes, and serum was obtained by centrifugation for clinical chemistry. Blood was also collected into microcollection tubes containing potassium EDTA as the anticoagulant for hematology. Reagents (except those used for reticulocyte count) were obtained from Bayer, Inc. (Tustin, CA), R&D Systems (Minneapolis, MN), or Fisher Scientific, Inc. (Norcross, GA). Clinical chemistry parameters were measured using a Hitachi 911 chemistry analyzer (Boehringer Mannheim, Indianapolis, IN), and hematology parameters (except reticulocyte count) were measured using the Technicon H•1™ automated hematology analyzer (Bayer HealthCare LLC, Tarrytown, NY). Reticulocytes were counted using a Coulter Model XL flow cytometer (Coulter Corp., Miami, FL); the reagents were manufactured and supplied by Coulter Corp., Molecular Probes (Eugene, OR), or Aldrich Chemical Co. (Milwaukee, WI). Blood smears were prepared within approximately 2 hours of sample collection to evaluate platelet and erythrocyte morphologies by light microscopy. The parameters measured are listed in Table 2.

At the end of the 3-month studies, samples were collected for sperm motility and vaginal cytology evaluations on rats and mice exposed to 0, 250, 500, and 1,000 mg/L. The parameters evaluated are listed in Table 2. For 12 consecutive days prior to scheduled terminal kill, the vaginal vaults of the females were moistened with saline, if necessary, and samples of vaginal fluid and cells were stained with crystal violet. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus). Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was

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

Necropsies were performed on all core study animals; and the heart, right kidney, liver, lung, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (except eyes were fixed in Davidson's solution and testes in modified David's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin (H&E). Complete histopathology examinations were performed by the study laboratory pathologist on 0 and 1,000 mg/L core study rats and mice; tissues were examined to a no-effect level in the remaining exposure groups. Table 2 lists the tissues and organs routinely examined.

After a review of the laboratory reports and selected histopathology slides by a quality assessment (QA) pathologist, the findings and reviewed slides were submitted to a NTP Pathology Working Group (PWG) coordinator for a second independent review. Any inconsistencies in the diagnoses made by the study laboratory and QA pathologists were resolved by the NTP pathology peer review process. Final diagnoses for reviewed lesions represent a consensus of the PWG or a consensus between the study laboratory pathologist, NTP pathologist, QA pathologist(s), and the PWG coordinator. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985).

2-YEAR STUDIES

Study Design

Groups of 66 male and 66 female rats and mice were exposed to 0, 250, 500, or 1,000 mg bromodichloroacetic acid/L drinking water *ad libitum* for up to 104 (rats) or 105 (mice) weeks. Seven or eight male and eight female rats and mice from each group were evaluated at 6 months, 13 months (rats), or 14 months (mice).

Rats were quarantined for 14 (males) or 15 (females) days and mice for 11 (females) or 12 (males) days before the beginning of the studies. Five male and five female rats and mice were randomly selected for parasite evaluation and gross observation of disease. Rats and mice were 5 to 6 weeks old at the beginning of the studies. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix M).

Rats were housed one to three (males) or one to five (females) per cage and mice were housed individually (males) or one to five (females) per cage. Feed and water were available *ad libitum*. Water consumption was measured for a 7-day period every 4 weeks beginning week 4. Cages were changed twice weekly, and racks were rotated once every 2 weeks. Further details of animal maintenance are given in Table 2. Information on feed composition and contaminants is provided in Appendix L.

Clinical Examinations and Pathology

All animals were observed twice daily. Body weights were recorded initially, and clinical findings and body weights were recorded every 4 weeks thereafter and at the end of the studies.

At the 6-month interim evaluation, necropsies were performed on eight male and eight female rats and mice. Organ weights for kidney and liver were collected. The bladder; lungs; kidneys; spleen; a section of the left and median lobes of the liver; and a section of the proximal, medial, and distal colon were collected, fixed in 10 percent neutral buffered formalin, trimmed, and embedded in paraffin blocks. The remainder of the liver was frozen in liquid

nitrogen and the remainder of the colon preserved in 10 percent neutral buffered formalin. Only the liver (rats and mice) and colon (rats only) were examined microscopically.

For the second interim evaluation, eight male and eight female rats (13 months) and seven male and eight female mice (14 months) were shipped live to Dr. Tony DeAngelo at the United States Environmental Protection Agency. One of the 14-month special study mice died prior to shipping, and the data from this mouse was included in the analysis of the 2-year study, resulting in 51 male mice used in the 2-year study. The colon and small intestines from all male and female rats were examined for potential neoplasms. Any neoplasms found were collected and processed to H&E slides and then evaluated microscopically. Additionally, the testes from all male rats were trimmed, processed, sectioned, and stained with H&E for microscopic evaluation. The slides of the rat testes (epididymis not present) were evaluated for possible hyperplasias and neoplasms, particularly of the mesothelium. Only the liver of mice was microscopically examined at this interim evaluation.

Following study termination at the end of the 2-year studies, complete necropsies and microscopic examinations were performed on all core study rats and mice. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin (except eyes which were fixed in Davidson's solution and testes in modified Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μm , and stained with H&E for microscopic examination. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. Tissues examined microscopically are listed in Table 2. An extended evaluation of the male and female rat brains involved the examination of wet tissue for the presence of gross lesions of the brain and the histopathologic examination of an additional three sections of paraffin-embedded brain from the superficial, middle, and deep aspects of the original brain block, resulting in the examination of nine sections of brain not previously examined for most of the animals. In a few cases, the amount of tissue remaining in the block only provided for one or two additional sections.

At the end of the 2-year studies, microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The report, slides, paraffin blocks, residual wet tissues, and pathology data were sent to the NTP Archives for inventory, slide/block match, wet tissue

audit, and storage. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist evaluated slides from all tumors and all potential target organs, which included the liver of rats and mice; the bone marrow, brain, and spleen of rats; the epididymis, prostate gland, seminal vesicle, skin, stomach (forestomach and glandular), and testis of male rats; the mammary gland and pituitary gland of female rats; and the epididymis, Harderian gland, lung, and testis of male mice.

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

Molecular Pathology Studies

A collection of neoplasms for molecular biology analysis was triggered when a potential chemical-related response was noted in moribund animals or at terminal sacrifice. Tumors greater than 5 mm in diameter were sectioned in half; one half was flash frozen in liquid nitrogen, and the other half was placed in 10% neutral buffered formalin for histopathology examination. In this 2-year study, representative mammary gland neoplasm samples from F344/NTac rats and liver neoplasm samples from B6C3F1/N mice were collected in liquid nitrogen for molecular biology studies at study termination.

Targeted qPCR Arrays on Rat Mammary Gland Adenocarcinoma: Four frozen mammary gland adenocarcinomas from exposed female F344/NTac rats were evaluated in this study (Appendix N). No concurrent control normal mammary gland tissues or spontaneous mammary gland adenocarcinomas were collected in this study. However, normal mammary gland tissues from five age-matched female F344/N rats were obtained from the National Institute on Aging. The frozen spontaneous mammary gland adenocarcinomas were sourced from five age-matched F344/N vehicle controls from other NTP chronic studies. RNA was extracted from these laser capture microdissected (LCM) frozen samples. A rat-specific PCR array (PARN-131Z, SABiosciences, Frederick, MD) was used to identify differential expression of genes relevant for mammary gland tumorigenesis (84 genes represented).

Molecular Analysis of Mouse Hepatoblastoma and Hepatocellular Carcinoma: For the microarray studies, six each of frozen hepatoblastoma, hepatocellular carcinoma, and adjacent nontumor liver tissue samples were collected from the exposed mice (Appendix O). In addition, six age-matched normal liver samples from control mice were collected. RNA was extracted from the LCM frozen tissue samples, and microarray was performed using Affymetrix Mouse Genome 430 2.0 GeneChip® arrays. Differentially expressed genes between sample groups were compared to examine the altered molecular pathways.

Mutation analysis was conducted on hotspot regions of the *H-ras* and *Ctnnb1* (β -catenin) genes in the liver neoplasms resulting from chronic exposure to bromodichloroacetic acid. Each of the liver neoplasms selected for mutation analysis contained both the hepatoblastoma and the adjacent hepatocellular carcinoma. Representative hepatoblastoma and hepatocellular carcinoma samples were LCM from formalin fixed, paraffin embedded liver neoplasms (n=30). DNA was extracted from the LCM samples and subjected to PCR amplification and sequencing using previously published methods (Hoenerhoff *et al.*, 2013).

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of Bromodichloroacetic Acid

2-Week Studies	3-Month Studies	2-Year Studies
Study Laboratory Southern Research Institute (Birmingham, AL)	Southern Research Institute (Birmingham, AL)	Battelle Columbus Operations (Columbus, OH)
Strain and Species F344/N rats B6C3F1/N mice	F344/N rats B6C3F1/N mice	F344/NTac rats B6C3F1/N mice
Animal Source Taconic Farms, Inc. (Germantown, NY)	Taconic Farms, Inc. (Germantown, NY)	Taconic Farms, Inc. (Germantown, NY)
Time Held Before Studies Rats: 12 days Mice: 14 days	Rats: 13 (males) or 14 (females) days Mice: 11 (females) or 12 (males) days	Rats: 14 (males) or 15 (females) days Mice: 11 (females) or 12 (males) days
Average Age When Studies Began 6 weeks	6 to 7 weeks	Rats: 5 to 6 (males) or 6 (females) weeks Mice: 5 to 6 (females) or 6 (males) weeks
Date of First Exposure Rats: August 21, 2000 Mice: August 23, 2000	Rats: November 14 (males) or 15 (females), 2000 Mice: November 12 (females) or 13 (males), 2000	Rats: October 26 (males) or 27 (females), 2006 Mice: September 25 (females) or 26 (males), 2006
Duration of Exposure 16 (core study) or 17 (special study) days	4 (clinical pathology) or 14 (core study) weeks	Rats: 104 weeks Mice: 105 weeks
Date of Last Exposure Rats: September 5 (core study) or 6 (special study), 2000 Mice: September 7 (core study) or 8 (special study), 2000	Rats: February 14 (males) or 15 (females), 2001 Mice: February 12 (females) or 13 (males), 2001	Rats: October 21 (males) or 23 (females), 2008 Mice: September 24 (females) or 25 (males), 2008
Necropsy Dates Rats: September 5, 2000 Mice: September 7, 2000	Rats: February 14 (males) or 15 (females), 2001 Mice: February 12 (females) or 13 (males), 2001	Core study rats: October 20 through 21 (males) or 22 through 23 (females), 2008 Core study mice: September 22 through 24 (females) or 24 through 25 (males), 2008
Average Age at Necropsy 8 weeks	19 weeks	Rats: 109 to 110 weeks Mice: 109 to 110 (females) or 110 to 111 (males) weeks

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of Bromodichloroacetic Acid

2-Week Studies	3-Month Studies	2-Year Studies
<p>Size of Study Groups 5 males and 5 females</p>	10 males and 10 females	6-month interim evaluation: 8 males and 8 females 13- (rats) or 14- (mice) month interim evaluation: 7 (1,000 mg/L mice) or 8 males and 8 females Core study: 50 or 51 (1,000 mg/L mice) males and 50 females
<p>Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weights.</p>	Same as 2-week studies	Same as 2-week studies
<p>Animals per Cage Rats: 5 Mice: 1 (males) or 5 (females)</p>	Rats: 5 Mice: 1 (males) or 5 (females)	Rats: 1 to 3 (males) or 1 to 5 (females) Mice: 1 (male) or 5 (study females)
<p>Method of Animal Identification Tail tattoo</p>	Tail tattoo	Tail tattoo
<p>Diet Irradiated NTP-2000 wafer feed (Zeigler Brothers, Inc., Gardners, PA), available <i>ad libitum</i>, changed weekly</p>	Same as 2-week studies	Same as 2-week studies
<p>Water Tap water (Birmingham, AL, municipal supply) via amber glass water bottles with plastic screw caps with Teflon[®] liners (Wheaton, Millville, NJ) and stainless steel double-ball sipper tubes (Allentown Caging, Allentown, NJ), available <i>ad libitum</i>; sipper tubes changed twice weekly</p>	Same as 2-week studies	Tap water (Columbus, OH, municipal supply) via glass bottles (Allentown Caging, Allentown, NJ; VWR, Westchester, PA; or Qorpak, Bridgeville, PA) with bottle caps lined with Teflon [®] -coated septa (Qorpak or Supelco, Bellefonte, PA) and with stainless steel double-ball bearing sipper tubes (Ancare Corp., Bellmore, NY), available <i>ad libitum</i> and changed twice weekly
<p>Cages Solid bottom polycarbonate cages (Lab Products, Inc., Maywood, NJ), changed once (male mice) or twice weekly</p>	Same as 2-week studies	Polycarbonate cages (Lab Products, Inc., Seaford, DE), changed twice weekly and rotated in racks every 2 weeks
<p>Bedding Irradiated hardwood chip bedding (P.J. Murphy Forest Products, Inc., Montville, NJ), changed once (male mice) or twice weekly</p>	Same as 2-week studies	Irradiated Sani-Chips hardwood chip bedding (P.J. Murphy Forest Products, Inc., Montville, NJ), changed twice weekly
<p>Rack Filters Disposable Reemay[®] spun-bonded polyester filter (Andico, Birmingham, AL), changed every 2 weeks</p>	Same as 2-week studies	Spun-bonded polyester filter sheets (Snow Filtration Co. (Cincinnati, OH), changed every 2 weeks

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of Bromodichloroacetic Acid

2-Week Studies	3-Month Studies	2-Year Studies
<p>Racks Stainless steel (Lab Products, Inc., Maywood, NJ), changed every 2 weeks</p>	<p>Same as 2-week studies</p>	<p>Stainless steel (Lab Products, Inc., Seaford, DE), changed and rotated every 2 weeks</p>
<p>Animal Room Environment Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour</p>	<p>Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour</p>	<p>Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour</p>
<p>Exposure Concentrations 0, 62.5, 125, 250, 500, or 1,000 mg/L drinking water, available <i>ad libitum</i></p>	<p>0, 62.5, 125, 250, 500, or 1,000 mg/L drinking water, available <i>ad libitum</i></p>	<p>0, 250, 500, or 1,000 mg/L drinking water, available <i>ad libitum</i></p>
<p>Type and Frequency of Observation Observed twice daily; core study animals were weighed initially, on days 8 and 15, and at the end of the studies; and clinical findings were recorded daily. Special study animals were weighed initially and at the end of the studies. Water consumption was recorded twice weekly by cage, on a 3-day/4-day schedule.</p>	<p>Observed twice daily. Core study animals were weighed initially, weekly, and at the end of the studies; special study animals were weighed initially. Clinical findings and water consumption were recorded weekly for core study animals.</p>	<p>Observed twice daily; animals were weighed initially; and animals were weighed and clinical findings were recorded every four weeks thereafter, and at the end of the studies. Water consumption was recorded one week per month beginning week 4.</p>
<p>Method of Kill Carbon dioxide asphyxiation</p>	<p>Same as 2-week studies</p>	<p>Same as 2-week studies</p>
<p>Necropsy Necropsies were performed on all core study animals; and the heart, right kidney, liver, lung, right testis, and thymus were weighed.</p>	<p>Necropsies were performed on all core study animals. Organs weighed were heart, right kidney, liver, lung, right testis, and thymus.</p>	<p>Necropsies were performed on all animals. Organs weighed for 6-month interim evaluation rats and mice were kidneys (both left and right) and liver.</p>
<p>Clinical Pathology None</p>	<p>Blood was collected from the retroorbital plexus of clinical pathology rats on days 3 and 21 and from retroorbital plexus of core study rats and the retroorbital sinus in mice at the end of the studies for hematology and clinical chemistry (rats only). Hematology: hematocrit, hemoglobin concentration; erythrocyte, reticulocyte, platelet, and nucleated erythrocyte counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials Clinical chemistry: urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and bile acids</p>	

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of Bromodichloroacetic Acid

2-Week Studies	3-Month Studies	2-Year Studies
<p>Histopathology In addition to gross lesions and tissue masses, the following tissues were examined in controls, to a no-effect level in rats, and in 1,000 mg/L mice: colon, small intestine (duodenum, jejunum, ileum), kidney, liver, and stomach (forestomach and glandular).</p>	<p>Complete histopathology was performed on 0 and 1,000 mg/L core study animals. In addition to gross lesions and tissue masses, the following tissues were examined to a no-effect level: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eye, gallbladder (mice), Harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, preputial gland, prostate gland, salivary gland, seminal vesicle, spleen, stomach (forestomach and glandular), testis (with epididymis and vaginal tunics), thymus, thyroid gland, trachea, urinary bladder, and uterus.</p>	<p>Complete histopathology was performed on all core study rats and mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eye, gallbladder (mice), Harderian gland, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, seminal vesicle, skin, spleen, stomach (forestomach and glandular), testis with epididymis and vaginal tunics, thymus, thyroid gland, tongue (rats), trachea, urinary bladder, and uterus. For the 6-month interim evaluation, the large intestine (rats only) and liver were examined. For the 13-month (rats) or 14-month (mice) interim evaluation, the large intestine (rats only), small intestine (rats only), testes (rats only), and liver (mice only) were examined.</p>
<p>Sperm Motility and Vaginal Cytology None</p>	<p>At the end of the studies, spermatid and sperm samples were collected from male animals in the 0, 250, 500, and 1,000 mg/L groups. The following parameters were evaluated: spermatid heads per testis and per gram testis, sperm motility, and sperm per cauda epididymis and per gram cauda epididymis. The left cauda, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from females exposed to 0, 250, 500, or 1,000 mg/L.</p>	<p>None</p>
<p>Acetyl-CoA Hydrolase Activity The livers of all special study animals were weighed, and acetyl-CoA hydrolase activity was measured.</p>	<p>None</p>	<p>None</p>

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of Bromodichloroacetic Acid

2-Week Studies	3-Month Studies	2-Year Studies
Molecular Pathology Studies		
Targeted qPCR Arrays on Rat Mammary Gland Adenocarcinoma None	None	Four frozen mammary gland adenocarcinomas from female F344/NTac rats in the current study, five spontaneous mammary gland adenocarcinomas from unexposed F344/N rats from five other 2-year NTP studies, and laser capture microdissected mammary gland epithelium from five age- and sex-matched F344/N rats (obtained from the National Institute on Aging) were used for pathway specific breast cancer qPCR arrays.
Molecular Analysis of Mouse Hepatoblastoma and Hepatocellular Carcinoma None	None	Frozen hepatoblastoma, associated hepatocellular carcinoma, and adjacent normal liver samples were obtained from B6C3F1/N mice using laser capture microdissection, and Affymetrix microarray analysis was performed to identify differentially expressed genes between sample groups. To study the comparative mutation profile in hepatoblastoma and associated hepatocellular carcinoma, <i>H-ras</i> codon 61 and exon 2 & 3 <i>Ctnnb1</i> mutation spectra were analyzed in 30 formalin-fixed paraffin embedded hepatoblastoma and associated hepatocellular carcinoma tissues from mice.

STATISTICAL METHODS

Survival Analyses

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

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions are presented in Tables A1, A3, B1, B3, C1, C4, D1, and D4 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A2, B2, C2, and D2) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., mesentery, pleura, peripheral nerve, skeletal muscle, tongue, tooth, and Zymbal's gland) before microscopic evaluation, the denominators consist of the number of animals that had a gross abnormality. When neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed. Tables A2, B2, C2, and D2 also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm. This survival-adjusted rate (based on the Poly-3 method described below) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, only to site-specific, lesion-free animals that do not reach terminal kill.

Analysis of Neoplasm and Nonneoplastic Lesion Incidences

The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997) was used to assess neoplasm and nonneoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to take survival differences into account. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total

number of animal years at risk. For analysis of a given site, each animal is assigned a risk weight. This value is one if the animal had a lesion at that site or if it survived until terminal kill; if the animal died prior to terminal kill and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the k th power.

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

Tests of significance included pairwise comparisons of each dosed group with controls and a test for an overall dose-related trend. Continuity-corrected Poly-3 tests were used in the analysis of lesion incidence, and reported P values are one sided. The significance of lower incidences or decreasing trends in lesions is represented as 1-P with the letter N added (e.g., $P=0.99$ is presented as $P=0.01N$). For neoplasms and nonneoplastic lesions detected at the interim evaluation, the Fisher exact test (Gart *et al.*, 1979), a procedure based on the overall proportion of affected animals, was used.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology, clinical chemistry, acetyl-CoA hydrolase activity, 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. Proportions of regular cycling females in each dosed group were compared to the control group using the Fisher exact test (Gart *et al.*, 1979). Tests for extended periods of estrus, diestrus, metestrus, and proestrus, as well as skipped estrus and skipped diestrus, were constructed based on a Markov chain model proposed by Girard and Sager (1987). For each dose group, a transition probability matrix was estimated for transitions among the proestrus, estrus, metestrus, and diestrus stages, with provision for extended stays within each stage as well as for skipping estrus or diestrus within a cycle. Equality of transition matrices among dose groups and between the control group and each dosed group was tested using chi-square statistics.

Historical Control Data

The concurrent control group represents the most valid comparison to the treated groups and is the only control group analyzed statistically in NTP bioassays. However, historical control data are often helpful in interpreting potential treatment-related effects, particularly for uncommon or rare neoplasm types. For meaningful comparisons, the conditions for studies in the historical control database must be generally similar. Significant factors affecting the background incidences of neoplasms at a variety of sites are diet, sex, strain/stock, and route of exposure. The NTP historical control database contains all 2-year studies for each species, sex, and strain/stock with histopathology findings in control animals completed within the most recent 5-year period (Haseman, 1992, 1995; Haseman and Rao, 1992). In general, the historical control database for a given study includes studies using the same route of administration, and the overall incidences of neoplasms in controls for all routes of administration are included for comparison, including the current mouse study. There are only two studies that used the F344/NTac rats in the historical control database, the current study and a cobalt metal study. The current study is a drinking water study in F344/NTac rats; the cobalt metal study was by inhalation. Therefore, only historical control incidences for all routes and all vehicles are presented for F344/NTac rats in this Technical Report.

QUALITY ASSURANCE METHODS

The 2-week, 3-month, and 2-year studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 3-month and 2-year studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assessment contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

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

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

DNA reactivity combined with *Salmonella* mutagenicity is highly correlated with induction of carcinogenicity in multiple species/sexes of rodents and at multiple tissue sites (Ashby and Tennant, 1991). A positive response in the

Salmonella test was shown to be the most predictive *in vitro* indicator for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens) (Tennant *et al.*, 1987; Zeiger *et al.*, 1990). Additionally, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. However, these other tests can provide useful information on the types of DNA and chromosomal damage induced by the chemical under investigation.

The predictivity for carcinogenicity of a positive response in acute *in vivo* bone marrow chromosome aberration or micronucleus tests appears to be less than that in the *Salmonella* test (Shelby *et al.*, 1993; Shelby and Witt, 1995). However, clearly positive results in long-term peripheral blood micronucleus tests have high predictivity for rodent carcinogenicity; a weak response in one sex only or negative results in both sexes in this assay do not correlate well with either negative or positive results in rodent carcinogenicity studies (Witt *et al.*, 2000). Because of the theoretical and observed associations between induced genetic damage and adverse effects in somatic and germ cells, the determination of *in vivo* genetic effects is important to the overall understanding of the risks associated with exposure to a particular chemical.

RESULTS

2-WEEK STUDY IN F344/N RATS

All rats survived to the end of the study (Table 3). Final mean body weights and body weight gains of exposed groups of male and female rats were similar to those of the controls. Water consumption by exposed and control groups was similar. Drinking water concentrations of 62.5, 125, 250, 500, and 1,000 mg/L resulted in average daily doses of approximately 7, 15, 31, 56, and 131 mg bromodichloroacetic acid/kg body weight to males and 8, 15, 30, 58, and 113 mg/kg to females. There were no clinical findings related to bromodichloroacetic acid exposure.

TABLE 3
Survival, Body Weights, and Water Consumption of F344/N Rats
in the 2-Week Drinking Water Study of Bromodichloroacetic Acid^a

Concentration (mg/L)	Survival ^b	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)	Water Consumption Week 1	Water Consumption Week 3
Male							
0	5/5	102 ± 2	163 ± 5	61 ± 4		13	16
62.5	5/5	105 ± 2	170 ± 4	66 ± 3	104	15	17
125	5/5	101 ± 2	165 ± 4	64 ± 3	102	14	17
250	5/5	103 ± 2	170 ± 7	68 ± 5	105	16	17
500	5/5	102 ± 2	158 ± 5	55 ± 6	97	14	15
1,000	5/5	100 ± 2	164 ± 3	64 ± 3	101	15	— ^c
Female							
0	5/5	90 ± 1	124 ± 2	34 ± 1		13	12
62.5	5/5	91 ± 2	125 ± 3	35 ± 1	101	13	14
125	5/5	88 ± 2	124 ± 2	36 ± 3	100	13	13
250	5/5	88 ± 1	123 ± 2	35 ± 1	99	12	13
500	5/5	90 ± 2	118 ± 3	29 ± 1	96	12	12
1,000	5/5	88 ± 3	120 ± 3	32 ± 2	97	11	12

^a Weights and weight changes are given as mean ± standard error. Water consumption is expressed as grams per animal per day. Differences in weights and weight changes from the control group are not significant by Dunnett's test.

^b Number of animals surviving at 16 days/number initially in group

^c Used bottle was spilled; no water consumption data available for week 3.

At the end of the 2-week study, no increases in acetyl-CoA hydrolase activities were observed in either male or female rats (Table G1), suggesting that bromodichloroacetic acid does not activate PPAR α , and these assays were not performed in the 3-month and 2-year studies.

No organ weight differences were attributed to exposure to bromodichloroacetic acid (Table H1). No gross or microscopic lesions related to chemical exposure were observed.

Exposure Concentration Rationale: Because no mortality or toxicologic effects were seen in the 2-week study, exposure concentrations selected for the 3-month drinking water study in F344/N rats were 62.5, 125, 250, 500, and 1,000 mg/L.

3-MONTH STUDY IN F344/N RATS

All rats survived until the end of the study except one female in the control group that died during week 7 (Table 4). Final mean body weights and body weight gains of 1,000 mg/L females were significantly less than those of the controls by approximately 5% and 12%, respectively (Table 4 and Figure 2). No treatment-related effects on body weights or body weight gains were observed in male rats. Drinking water concentrations of 62.5, 125, 250, 500, and 1,000 mg/L resulted in average daily doses of approximately 5, 9, 19, 37, and 72 mg bromodichloroacetic acid/kg body weight to males and 5, 10, 20, 43, and 69 mg/kg to females. No chemical-related clinical findings were observed.

TABLE 4
Survival, Body Weights, and Water Consumption of F344/N Rats
in the 3-Month Drinking Water Study of Bromodichloroacetic Acid^a

Concentration (mg/L)	Survival ^b	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)	Water Consumption Week 1 (g)	Water Consumption Week 13 (g)
Male							
0	10/10	115 ± 1	341 ± 5	226 ± 5		16	16
62.5	10/10	115 ± 1	349 ± 8	234 ± 7	102	16	16
125	10/10	114 ± 1	349 ± 6	235 ± 7	102	16	17
250	10/10	116 ± 2	356 ± 5	240 ± 6	104	16	17
500	10/10	115 ± 2	350 ± 6	235 ± 6	103	16	16
1,000	10/10	113 ± 2	336 ± 6	223 ± 6	99	14	15
Female							
0	9/10 ^c	103 ± 1	194 ± 3	91 ± 2		14	11
62.5	10/10	103 ± 1	206 ± 3	103 ± 3	106	14	15
125	10/10	104 ± 1	203 ± 2	99 ± 2	105	13	12
250	10/10	102 ± 1	193 ± 3	90 ± 3	100	14	11
500	10/10	104 ± 1	197 ± 2	93 ± 2	101	14	12
1,000	10/10	103 ± 1	184 ± 2**	80 ± 2**	95	11	10

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

^a Weights and weight changes are given as mean ± standard error. Water consumption is expressed as grams per animal per day.

^b Number of animals surviving at 14 weeks/number initially in group

^c Week of death: 7

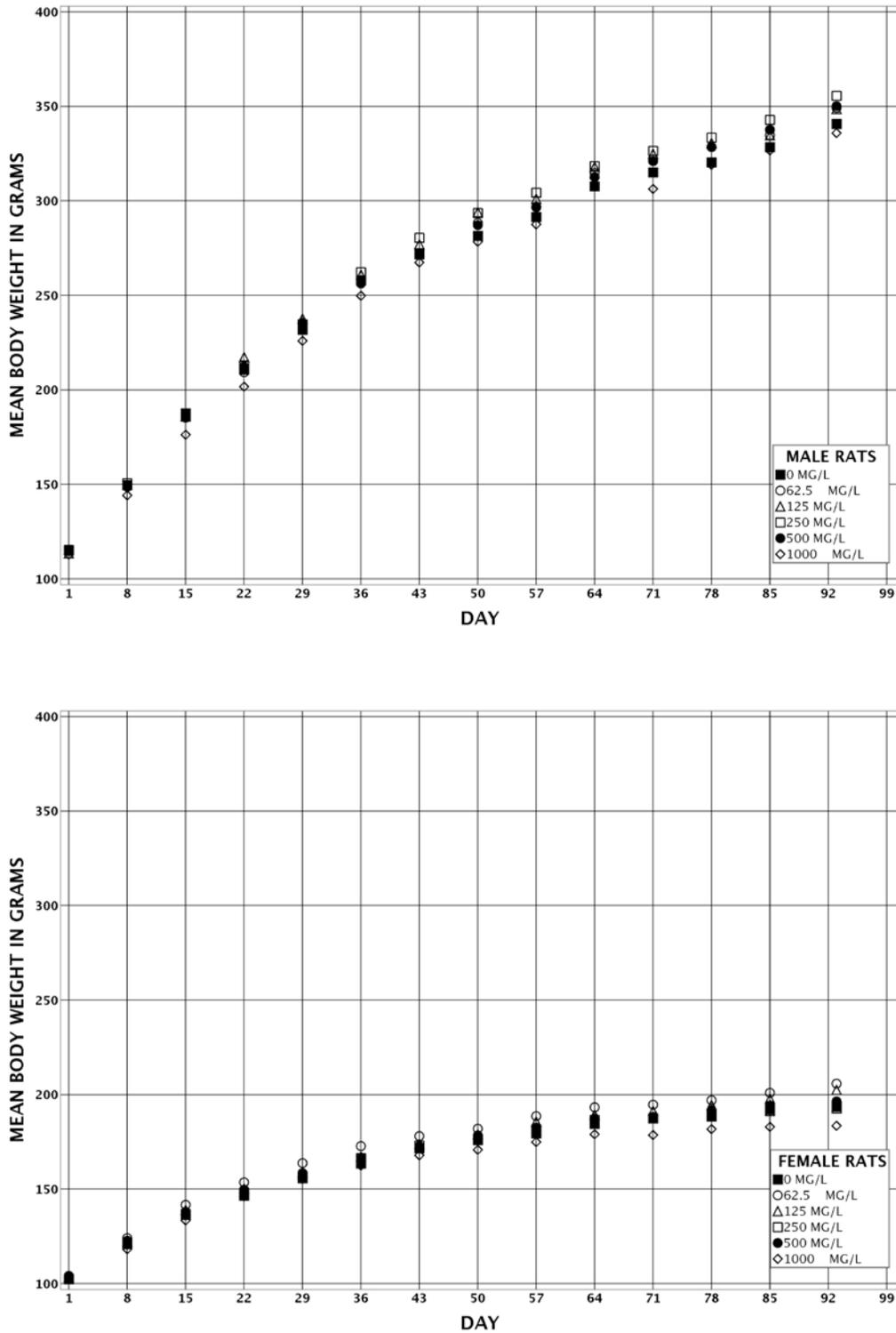


FIGURE 2
Growth Curves for F344/N Rats Exposed to Bromodichloroacetic Acid
in Drinking Water for 3 Months

Compared to the control group, on day 3, there were mild increases ($\leq 11\%$) in hematocrit values, hemoglobin concentrations, and red blood cell counts in several exposed groups of female rats, but most consistently at 1,000 mg/L (Table F1). These increases were not seen on day 23 and were consistent with mild transient hemoconcentration, as evidenced by the decrease in water consumption by the 1,000 mg/L females. Urea nitrogen concentrations were also mildly increased in females, consistent with mild dehydration as the rats acclimated to the study; this parameter was mildly elevated in all the exposed groups on day 3 and in the 250 mg/L or greater groups on day 23. This change in urea nitrogen concentration resolved by week 14.

At week 14, the 500 and 1,000 mg/L females had mild decreases in serum total protein concentrations (Table F1). In addition, serum albumin concentrations were mildly decreased in all exposed female groups. The magnitude of the decrease was greatest in the 1,000 mg/L females ($\geq 11\%$). The decreases in the total protein concentration were driven by the decreases in albumin concentration, as albumin is a major component of the total protein concentration. The mechanism for the decrease in albumin concentration was not clear.

The absolute kidney weights of 1,000 mg/L females and relative kidney weights of 250, 500, and 1,000 mg/L females were significantly greater than those of the controls by approximately 5% to 15% (Table H2). Relative liver weights were increased by approximately 9% in males exposed to 1,000 mg/L. These changes in organ weights, though statistically significant, were considered minor effects.

Male rats exposed to 1,000 mg/L bromodichloroacetic acid in drinking water exhibited decreased left testis weights and sperm motility (approximately 5% less than concurrent controls) (Table I1). A similar decrease in weight was not observed in the right testis (Table H2) or epididymis measurement (Table I1). No correlating histopathology findings were noted. Given the small magnitude of change, inconsistent response in testis weight, and absence of correlating histopathology, the relationship of the observed changes to bromodichloroacetic acid exposure is uncertain. Female rats exposed to 250 mg/L exhibited a higher probability of extended estrus (Table I2). Given that this was only observed at the lowest exposure concentration tested and occurred in the absence of histopathology findings, its relationship to bromodichloroacetic acid exposure is uncertain.

No chemical-related gross or histopathology findings were observed in either male or female rats.

Exposure Concentration Selection Rationale: Based on the limited effects of bromodichloroacetic acid exposure in the 3-month study, exposure concentrations selected for the 2-year drinking water study in F344/NTac rats were 250, 500, and 1,000 mg/L. Higher concentrations of bromodichloroacetic acid were not considered based on the 2-year NTP study of bromochloroacetic acid (NTP, 2009), which used these exposure concentrations and resulted in clear evidence of carcinogenicity in rats.

2-YEAR STUDY IN F344/NTAC RATS

Survival

Estimates of 2-year survival probabilities for male and female rats are shown in Table 5 and in the Kaplan-Meier survival curves (Figure 3). Survival of 500 and 1,000 mg/L females was significantly less than that of the controls. Early deaths in the female rats were due to moribund sacrifice of animals with mammary gland fibroadenomas and carcinomas. There was no significant effect on survivability in male rats.

TABLE 5
Survival of F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male				
Animals initially in study	66	66	66	66
6-Month interim evaluation ^a	8	8	8	8
13-Month interim evaluation ^a	8	8	8	8
Moribund	26	23	20	27
Natural deaths	5	6	5	4
Animals surviving to study termination	19	21	25	19
Percent probability of survival at end of study ^b	38	42	50	38
Mean survival (days) ^c	658	655	676	654
Survival analysis ^d	P=1.000	P=0.648N	P=0.186N	P=1.000
Female				
Animals initially in study	66	66	66	66
6-Month interim evaluation ^a	8	8	8	8
13-Month interim evaluation ^a	8	8	8	8
Moribund	10	22	42	44
Natural deaths	6	2	1	4
Animals surviving to study termination	34	26	7	2
Percent probability of survival at end of study	68	52	14	4
Mean survival (days)	694	683	616	589
Survival analysis	P<0.001	P=0.141	P<0.001	P<0.001

^a Censored from survival analyses

^b Kaplan-Meier determinations

^c Mean of all deaths (uncensored and terminal kill)

^d The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed group columns. A lower mortality in an exposure group is indicated by N.

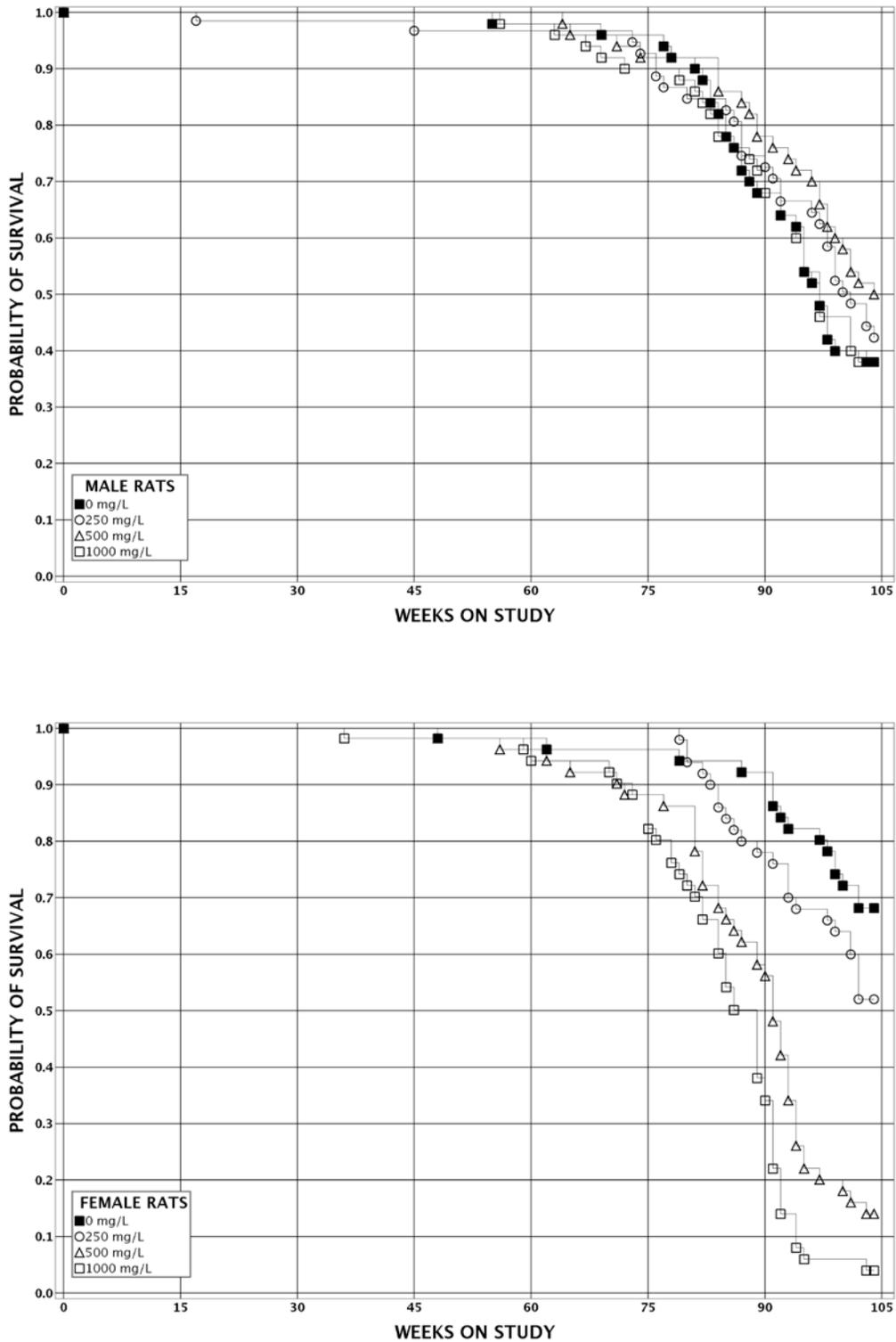


FIGURE 3
Kaplan-Meier Survival Curves for F344/NTac Rats Exposed to Bromodichloroacetic Acid in Drinking Water for 2 Years

Body Weights, Water and Compound Consumption, Clinical Findings, and Organ Weights

The mean body weights of 1,000 mg/L males were approximately 10% less than those of controls after week 89 (Table 6; Figure 4). This was associated with an approximately 10% decrease in water consumption (Table K1). Body weights of the 1,000 mg/L females were 10% less than those of the controls after week 13 and dropped to about 80% of controls by 52 weeks (Table 7; Figure 4). In 1,000 mg/L females, water consumption was less during the first year of the study but similar to control values during the second year (Table K2). The decreased body weight and moribundity and mortality appears unrelated to the decreased water intake in the females. Drinking water concentrations of 250, 500, and 1,000 mg/L resulted in average daily doses of approximately 11, 21, and 43 mg bromodichloroacetic acid/kg body weight to males and approximately 13, 28, and 57 mg/kg to females. No clinical findings related to chemical exposure were observed. At the 6-month interim evaluation, absolute and relative liver weights of 500 mg/L females were significantly increased, as was the relative live weight in 1,000 mg/L females (Table H3).

TABLE 6
Mean Body Weights and Survival of Male F344/NTac Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

Day	0 mg/L		250 mg/L			500 mg/L			1,000 mg/L		
	Av. Wt. (g)	No. of Survivors	Av. Wt (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt (g)	Wt. (% of Controls)	No. of Survivors
1	94	66	93	99	66	93	99	66	94	100	66
29	211	66	206	98	66	205	97	66	201	95	66
57	282	66	276	98	66	270	96	66	267	95	66
85	330	66	321	97	66	312	95	66	309	94	66
113	365	66	356	98	66	345	95	66	342	94	66
141	395	66	382	97	65	370	94	66	367	93	66
169	420	66	405	97	65	393	94	66	387	92	66
197 ^a	431	58	420	97	57	407	95	58	400	93	58
225	449	58	438	97	57	425	95	58	417	93	58
253	464	58	449	97	57	438	94	58	430	93	58
281	478	58	464	97	57	452	95	58	440	92	58
309	490	58	474	97	56	462	94	58	451	92	58
337	500	58	486	97	56	474	95	58	457	91	58
365	510	58	491	96	56	479	94	58	463	91	58
393 ^a	517	49	498	96	48	484	94	50	470	91	49
421	520	49	502	97	48	486	94	50	472	91	49
449	524	49	505	96	48	490	94	49	472	90	48
477	527	48	506	96	48	492	94	48	473	90	47
505	526	48	511	97	48	497	95	47	473	90	45
533	528	48	512	97	43	500	95	46	470	89	45
561	519	45	507	98	42	496	95	46	472	91	43
589	509	40	504	99	42	496	97	43	470	92	39
617	509	34	503	99	37	491	96	41	468	92	37
645	512	32	498	97	33	482	94	38	457	89	32
673	494	26	479	97	32	474	96	35	445	90	27
701	485	20	465	96	25	468	96	29	434	89	23
Mean for Weeks											
1-13	229		224	98		220	96		218	95	
14-52	444		430	97		418	94		410	92	
53-101	514		499	97		487	95		465	90	

^a Animals removed for interim evaluations during weeks 27 and 54

TABLE 7
Mean Body Weights and Survival of Female F344/NTac Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

Day	0 mg/L		250 mg/L			500 mg/L			1,000 mg/L		
	Av. Wt. (g)	No. of Survivors	Av. Wt (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt (g)	Wt. (% of Controls)	No. of Survivors
1	87	66	87	100	66	88	100	66	88	100	66
29	147	66	145	99	66	143	97	66	138	94	66
57	175	66	171	98	66	167	95	66	159	91	66
85	189	66	184	98	66	180	95	66	171	90	66
113	202	66	195	96	66	191	94	66	178	88	66
141	213	66	204	96	66	198	93	66	186	87	66
169	223	66	212	95	66	206	92	66	191	86	66
197 ^a	231	58	220	95	58	212	92	58	197	85	58
225	241	58	229	95	58	221	92	58	204	85	58
253	250	58	238	95	58	227	91	58	210	84	57
281	258	58	246	95	58	235	91	58	214	83	57
309	266	58	255	96	58	241	91	58	218	82	57
337	277	57	263	95	58	252	91	57	224	81	57
365	285	57	272	95	58	258	91	57	229	80	57
393 ^a	295	49	282	96	50	269	91	48	238	81	49
421	304	49	293	96	50	276	91	48	244	80	47
449	313	48	300	96	50	283	91	46	247	79	47
477	321	48	306	95	50	290	90	46	251	78	47
505	327	48	314	96	50	294	90	44	256	78	45
533	334	48	320	96	50	300	90	43	260	78	40
561	340	47	327	96	47	306	90	40	266	78	35
589	344	47	328	95	43	307	89	34	272	79	30
617	349	46	335	96	40	313	90	31	266	76	25
645	353	41	338	96	35	312	88	18	268	76	7
673	351	40	340	97	34	306	87	10	266	76	3
701	348	36	342	98	32	312	90	9	265	76	3
Mean for Weeks											
1-13	150		147	98		145	97		139	94	
14-52	240		229	95		220	92		202	85	
53-101	328		315	96		294	90		256	78	

^a Animals removed for interim evaluations during weeks 27 and 54

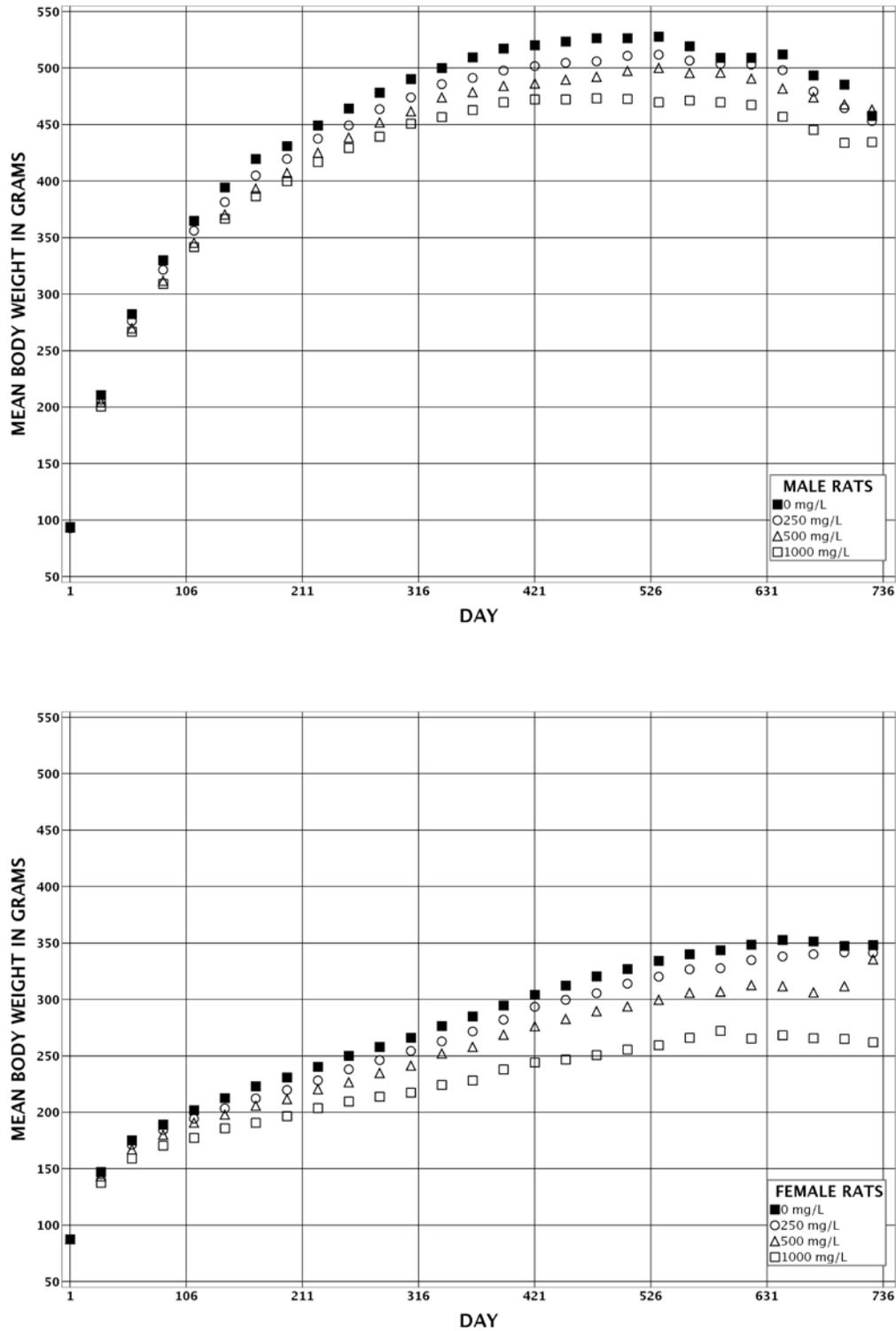


FIGURE 4
Growth Curves for F344/NTac Rats Exposed to Bromodichloroacetic Acid
in Drinking Water for 2 Years

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of mesothelioma and neoplasms and/or nonneoplastic lesions of the mammary gland, skin, large intestine, brain, bone marrow, liver, oral cavity (oral mucosa and tongue), and spleen. Summaries of the incidences of neoplasms and nonneoplastic lesions and statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group are presented in Appendix A for male rats and Appendix B for female rats.

Mammary Gland: Fibroadenoma was noted in all exposed groups of males (Tables 8, A1, and A2). In females, there were positive trends in the incidences of fibroadenoma; carcinoma; and fibroadenoma, adenoma, or carcinoma (combined) (Tables 8, B1, and B2). The incidences of fibroadenoma and of the combined neoplasms in all exposed groups were significantly greater than the control incidences, as was the incidence of carcinoma at 1,000 mg/L. In both male and female rats, mammary gland fibroadenomas were characterized by multiple, discrete, variably sized islands of well differentiated glandular alveoli that frequently contained secretory material within abundant fibrous connective tissue stroma. In the case of female mammary gland adenoma, the neoplasm was well demarcated from the adjacent mammary tissue. The regular architecture of the gland was distorted, and there was an increase in size and variability in diameter of glandular lobules. Mammary gland carcinomas occasionally arose within fibroadenomas and consisted of epithelium arranged in alveolar, papillary, or solid patterns. Neoplastic alveolar epithelium often piled up to 10 layers thick and the nuclear/cytoplasmic ratio was increased in epithelial cells. Mammary gland carcinomas sometimes contained areas of squamous differentiation and/or central areas of necrosis.

Increased incidences of hyperplasia of the mammary gland were noted in all female exposed groups; the incidence in the 1,000 mg/L group was significantly increased. In most cases, the severity assigned was minimal. Mammary gland hyperplasia is characterized by enlarged lobules with increased numbers of alveoli of variable sizes lined usually by a single layer of well differentiated epithelium with minimal cellular atypia. The interlobular ducts and the alveoli are separated by scant collagenous connective tissue stroma.

TABLE 8
Incidences of Neoplasms and Nonneoplastic Lesions of the Mammary Gland in F344/NTac Rats
in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male				
Number Necropsied	50	50	50	50
Hyperplasia ^a	6 (1.2) ^b	1* (1.0)	2 (1.0)	1 (1.0)
Fibroadenoma ^c	0	2	3	1
Female				
Number Necropsied	50	50	50	50
Hyperplasia	0	4 (1.3)	2 (1.5)	10** (1.2)
Fibroadenoma, Multiple	6	34**	37**	27**
Fibroadenoma (includes multiple) ^d				
Overall rate ^e	28/50 (56%)	47/50 (94%)	47/50 (94%)	39/50 (78%)
Adjusted rate ^f	60.1%	96.6%	99.1%	89.6%
Terminal rate ^g	21/34 (62%)	26/26 (100%)	7/7 (100%)	2/2 (100%)
First incidence (days)	551	558	449	414
Poly-3 test ^h	P<0.001	P<0.001	P<0.001	P<0.001
Adenoma ⁱ	1	2	3	1
Carcinoma ⁱ				
Overall rate	0/50 (0%)	1/50 (2%)	3/50 (6%)	8/50 (16%)
Adjusted rate	0.0%	2.3%	9.1%	25.8%
Terminal rate	0/34 (0%)	0/26 (0%)	0/7 (0%)	0/2 (0%)
First incidence (days)	— ^j	558	533	509
Poly-3 test	P<0.001	P=0.492	P=0.074	P<0.001
Adenoma or Carcinoma ^k	1	3	6*	9**
Fibroadenoma, Adenoma, or Carcinoma ^l				
Overall rate	28/50 (56%)	47/50 (94%)	48/50 (96%)	42/50 (84%)
Adjusted rate	60.1%	96.6%	99.4%	92.5%
Terminal rate	21/34 (62%)	26/26 (100%)	7/7 (100%)	2/2 (100%)
First incidence (days)	551	558	386	414
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001

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

** P≤0.01

^a Number of animals with lesion

^b Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^c Historical control incidence for 2-year studies (all routes): 1/100

^d Historical control incidence: 46/100

^e Number of animals with neoplasm per number of animals necropsied

^f Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^g Observed incidence at terminal kill

^h Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill.

ⁱ Historical control incidence: 2/100

^j Not applicable; no neoplasms in animal group

^k Historical control incidence: 4/100

^l Historical control incidence: 48/100

Malignant Mesothelioma: In the testis at 13 months, one 500 mg/L male and five 1,000 mg/L males had malignant mesothelioma, and the incidence in the 1,000 mg/L group was significantly increased (Table 9). Minimal to moderate hyperplasia of the mesothelial layer was also observed in these groups [0 mg/L, 0/8; 250 mg/L, 0/8; 500 mg/L, 1/8 (1.0); 1,000 mg/L, 3/8 (2.0)]. Malignant mesothelioma was characterized by discrete proliferative single or multiple layers of plump mesothelial cells often overlying fibrovascular cores, and these neoplasms occasionally invaded the subjacent tissues.

In multiple organs at 2 years, the incidences of malignant mesothelioma occurred with a positive trend and were significantly increased in all exposed groups (Tables 9 and A2). Mesotheliomas were seen primarily in the tunica vaginalis of the testis and epididymis and in some cases extended to the seminal vesicle and prostate gland of male rats.

TABLE 9
Incidences of Malignant Mesothelioma in Male F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
13-Month Interim Evaluation				
Number with Testis Examined Microscopically	8	8	8	8
Malignant Mesothelioma, Bilateral ^a	0	0	1	1
Malignant Mesothelioma (includes bilateral) ^b	0	0	1	5*
2-Year Study				
Multiple Organs, Malignant Mesothelioma ^c				
Overall rate ^d	1/50 (2%)	12/50 (24%)	18/50 (36%)	37/50 (74%)
Adjusted rate ^e	2.6%	28.0%	40.6%	77.5%
Terminal rate ^f	0/19 (0%)	3/21 (14%)	10/25 (40%)	12/19 (63%)
First incidence (days)	683	309	442	389
Poly-3 test ^g	P<0.001	P<0.001	P<0.001	P<0.001

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

^a Number of animals with neoplasm

^b Historical control data not available

^c Historical control incidence for 2-year studies (all routes): 3/100

^d Number of animals with neoplasm per number of animals necropsied

^e Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^f Observed incidence at terminal kill

^g Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill.

Histologically, malignant mesotheliomas were characterized by areas of mesothelial proliferation along the peritoneal surface of the tunica vaginalis of male reproductive tissues and comprised plump papillary projections of neoplastic mesothelial cells overlying fibrovascular cores (Plate 1). The most prominent proliferations were generally located along the epididymides with smaller areas associated with the testes, seminal vesicles, and prostate gland.

Skin: The incidences of basal cell adenoma or carcinoma (combined) in males occurred with a positive trend, and the 1,000 mg/L group incidence was significantly greater than the control incidence (Tables 10, A1, and A2). Microscopically, basal cell adenomas were present as elevated nodules that extended into the dermis and were composed of ribbons and cords of basal cells separated by a thin fibrovascular stroma. In some of these cases, there was multifocal sebaceous differentiation. The superficial surface of some basal cell adenomas was ulcerated and contained small areas of hemorrhage. Basal cell carcinoma consisted of an epithelial mass that extended far into the dermis and was composed of basal cells arranged in cords, nests, and solid sheets. Basal cell carcinomas displayed moderate nuclear pleomorphism and numerous mitotic figures. Frequently, areas of squamous and sebaceous differentiation were present, as well as small areas of necrosis and associated inflammation.

Two cases each of sebaceous gland adenoma were noted in the exposed male groups. Histologically, sebaceous gland adenomas were characterized by well circumscribed lobular dermal masses composed of irregularly shaped acini of sebaceous epithelium containing abundant foamy cytoplasm lined by a thin layer of basal epithelium.

The incidences of keratoacanthoma in males exposed to 500 and 1,000 mg/L were increased (significantly for the 1,000 mg/L group) and occurred with a positive trend. Keratoacanthoma is a well demarcated cup-shaped mass arising in the epithelium and extending into the dermis. The central portion of these masses was filled with abundant layers of desquamated keratin debris and lined by a thickened layer of squamous epithelium.

The combined incidence of squamous cell papilloma, keratoacanthoma, sebaceous gland adenoma, basal cell adenoma, basal cell carcinoma, or squamous cell carcinoma in 1,000 mg/L males was significantly increased.

TABLE 10
Incidences of Neoplasms of the Skin in Male F344/NTac Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Number Necropsied	50	50	50	50
Squamous Cell Papilloma ^{a, b}	3	1	0	1
Keratoacanthoma, Multiple	0	0	0	3
Keratoacanthoma (includes multiple) ^c				
Overall rate ^d	7/50 (14%)	3/50 (6%)	10/50 (20%)	15/50 (30%)
Adjusted rate ^e	17.3%	7.6%	23.2%	37.1%
Terminal rate ^f	1/19 (5%)	2/21 (10%)	5/25 (20%)	7/19 (37%)
First incidence (days)	544	716	493	621
Poly-3 test ^g	P=0.003	P=0.162N	P=0.346	P=0.035
Basal Cell Adenoma ^h	0	0	4	4
Basal Cell Carcinoma ⁱ	0	0	0	1
Basal Cell Adenoma or Carcinoma ^h				
Overall rate	0/50 (0%)	0/50 (0%)	4/50 (8%)	5/50 (10%)
Adjusted rate	0.0%	0.0%	9.3%	12.8%
Terminal rate	0/19 (0%)	0/21 (0%)	1/25 (4%)	3/19 (16%)
First incidence (days)	— ^j	—	513	629
Poly-3 test	P=0.004	— ^k	P=0.074	P=0.030
Squamous Cell Carcinoma ^l	0	1	1	0
Squamous Cell Papilloma, Keratoacanthoma, Basal Cell Adenoma, Basal Cell Carcinoma, or Squamous Cell Carcinoma ^l				
Overall rate	9/50 (18%)	5/50 (10%)	14/50 (28%)	19/50 (38%)
Adjusted rate	22.2%	12.7%	31.8%	46.8%
Terminal rate	2/19 (11%)	3/21 (14%)	7/25 (28%)	10/19 (53%)
First incidence (days)	544	716	493	621
Poly-3 test	P<0.001	P=0.201N	P=0.226	P=0.014
Sebaceous Gland, Adenoma ^m	0	2	2	2
Squamous Cell Papilloma, Keratoacanthoma, Sebaceous Gland Adenoma, Basal Cell Adenoma, Basal Cell Carcinoma, or Squamous Cell Carcinoma ^m				
Overall rate	9/50 (18%)	7/50 (14%)	15/50 (30%)	21/50 (42%)
Adjusted rate	22.2%	17.4%	33.7%	50.4%
Terminal rate	2/19 (11%)	4/21 (19%)	7/25 (28%)	10/19 (53%)
First incidence (days)	544	509	493	436
Poly-3 test	P<0.001	P=0.397N	P=0.171	P=0.005
Subcutaneous Tissue, Fibroma, Multiple	0	1	0	2

TABLE 10
Incidences of Neoplasms of the Skin in Male F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Subcutaneous Tissue, Fibroma (includes multiple) ^m				
Overall rate	4/50 (8%)	6/50 (12%)	10/50 (20%)	15/50 (30%)
Adjusted rate	10.2%	14.8%	23.3%	36.0%
Terminal rate	2/19 (11%)	2/21 (10%)	7/25 (28%)	7/19 (37%)
First incidence (days)	657	595	442	467
Poly-3 test	P<0.001	P=0.391	P=0.098	P=0.005

^a Number of animals with neoplasm

^b Historical control incidence for 2-year studies (all routes): 3/100

^c Historical control incidence: 11/100

^d Number of animals with neoplasm per number of animals necropsied

^e Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^f Observed incidence at terminal kill

^g Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill. A lower incidence in an exposure group is indicated by N.

^h Historical control incidence: 2/100

ⁱ Historical control incidence: 0/100

^j Not applicable; no neoplasms in animal group

^k Value of statistic cannot be computed.

^l Historical control incidence: 1/100

^m No historical control data available

Also in males, exposure concentration-related increased incidences of fibroma in the subcutaneous tissue were noted in the exposed groups, and the increase was significant in the 1,000 mg/L group. Fibroma is a noninvasive expansile circumscribed neoplasm consisting of proliferative fibrocytes in an abundant collagenous stroma. Mitoses and cellular atypia are minimal to absent.

Large Intestine: At 6 and 13 months, no significant microscopic findings were present in the colon. At 2 years, the incidences of adenoma of the large intestine (cecum, colon, or rectum) in both sexes were greater at 1,000 mg/L than those in controls (Tables 11, A1, and B1).

The adenomas consisted of well circumscribed solitary exophytic masses composed of single to multiple layered neoplastic epithelial cells arranged in variably differentiated acinar structures and overlying a broad fibromuscular stalk. The neoplastic cells were tall, columnar with indistinct borders, moderate amphophilic cytoplasm, ovoid nuclei with finely stippled chromatin and up to three nucleoli. Epithelial cells often piled up to six layers thick and

TABLE 11
Incidences of Adenoma of the Large Intestine in F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male				
Cecum ^a	50	50	49	50
Adenoma ^{b, c}	0	0	1	0
Colon	50	50	50	50
Adenoma ^d	0	0	1	1
Rectum	50	50	50	50
Adenoma ^d	0	0	0	1
Colon or Rectum: Adenoma ^d				
Overall rate ^e	0/50 (0%)	0/50 (0%)	1/50 (2%)	2/50 (4%)
Adjusted rate ^f	0.0%	0.0%	2.4%	5.2%
Terminal rate ^g	0/19 (0%)	0/21 (0%)	1/25 (4%)	2/19 (11%)
First incidence (days)	— ⁱ	—	726 (T)	726 (T)
Poly-3 test ^h	P=0.068	— ^j	P=0.516	P=0.235
Cecum, Colon, or Rectum: Adenoma ^c				
Overall rate	0/50 (0%)	0/50 (0%)	2/50 (4%)	2/50 (4%)
Adjusted rate	0.0%	0.0%	4.8%	5.2%
Terminal rate	0/19 (0%)	0/21 (0%)	2/25 (8%)	2/19 (11%)
First incidence (days)	—	—	726 (T)	726 (T)
Poly-3 test	P=0.082	—	P=0.254	P=0.235
Female				
Colon	50	50	50	50
Adenoma ^d	0	0	0	2
Rectum	50	50	50	50
Adenoma ^k	1	0	1	0
Colon or Rectum: Adenoma ^k				
Overall rate	1/50 (0%)	0/50 (0%)	1/50 (2%)	2/50 (4%)
Adjusted rate	2.2%	0.0%	3.1%	7.0%
Terminal rate	1/34 (3%)	0/26 (0%)	0/7 (0%)	0/2 (0%)
First incidence (days)	727 (T)	—	643	631
Poly-3 test	P=0.165	P=0.511N	P=0.684	P=0.353

(T) Terminal kill

^a Number of animals with tissue examined microscopically

^b Number of animals with neoplasm

^c No historical control data available

^d Historical control incidence for 2-year studies (all routes): 0/100

^e Number of animals with neoplasm per number of animals necropsied

^f Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^g Observed incidence at terminal kill

^h Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill. A lower incidence in an exposure group is indicated by N.

ⁱ Not applicable; no neoplasms in animal group

^j Value of statistic cannot be computed.

^k Historical control incidence: 1/100

the mitotic index averaged up to six per high power field among all cases. The neoplastic cells had increased basophilia when compared to the adjacent unaffected intestinal epithelium due to loss of goblet cells and a decreased amount of intervening fibrous connective tissue.

Brain: Sporadic incidences of brain neoplasms were seen in control and exposed groups of males and females in the original, routine three sections of the brain (Tables 12, A1, and B1). These included glioma, oligodendroglioma, and benign granular cell tumors of the meninges. Due to the microscopic and focal nature of these lesions, an extended evaluation of additional brain sections, up to nine per animal, was conducted. No additional neoplasms were found in females. One control male had a glioma, and one additional 500 mg/L male had an oligodendroglioma; benign granular cell tumors of the meninges were found in one male each in the 250 and 1,000 mg/L groups. Based on the combined original and extended evaluations, there were no statistically significant differences between exposed and control incidences of glioma or oligodendroglioma (combined) or the benign granular cell tumors of the meninges based on the Poly-3 test in either males or females.

Gliomas were invasive and were not clearly demarcated from the surrounding normal neuropil. The neoplastic cells occasionally demonstrated invasion of meninges and cuffing of blood vessels. Neoplastic cells had indistinct cell borders, moderate to abundant eosinophilic granular cytoplasm, and elongated nuclei. Oligodendrogliomas consisted of moderately well-demarcated tumors with small, uniformly sized neoplastic cells with small round hyperchromatic nuclei and perinuclear halos that imparted a classic honey-comb appearance. Characteristic endothelial hyperplasia and hypertrophy (vascular garlands/glomeruloid vascular proliferations) were often noted in oligodendrogliomas. Benign granular cell tumors of the meninges are usually circumscribed, noninvasive, and slightly compressive and are confined to the meninges. These tumors are characterized by a relatively homogenous neoplastic polygonal cell population within a fine vascular stroma. The neoplastic cells have a fine granular eosinophilic cytoplasm and central to eccentric round nuclei with little atypia.

TABLE 12
Incidences of Neoplasms of the Brain and Meninges in F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male				
Original Evaluation				
Number Examined Microscopically	50	50	50	50
Brain, Glioma ^{a, b}	0	1	2	2
Brain, Oligodendroglioma NOS ^b	0	0	1	1
Brain, Glioma or Oligodendroglioma ^b				
Overall rate ^c	0/50 (0%)	1/50 (2%)	3/50 (6%)	3/50 (6%)
Adjusted rate ^d	0.0%	2.5%	7.1%	7.7%
Terminal rate ^e	0/19 (0%)	1/21 (5%)	0/25 (0%)	0/19 (0%)
First incidence (days)	— ^g	726 (T)	685	644
Poly-3 test ^f	P=0.066	P=0.504	P=0.134	P=0.119
Meninges, Granular Cell Tumor Benign ^h	2	0	1	0
Extended Evaluation				
Number Examined Microscopically	50	50	50	50
Brain, Glioma	1	0	0	0
Brain, Oligodendroglioma NOS	0	0	1	0
Meninges, Granular Cell Tumor Benign	0	1	0	1
Original and Extended Evaluations (Combined)				
Number Examined Microscopically	50	50	50	50
Brain, Glioma	1	1	2	2
Brain, Oligodendroglioma NOS	0	0	2	1
Brain, Glioma or Oligodendroglioma				
Overall rate	1/50 (2%)	1/50 (2%)	4/50 (8%)	3/50 (6%)
Adjusted rate	2.6%	2.5%	9.5%	7.7%
Terminal rate	0/19 (0%)	1/21 (5%)	1/25 (4%)	0/19 (0%)
First incidence (days)	719	726 (T)	685	644
Poly-3 test	P=0.162	P=0.757N	P=0.204	P=0.306
Meninges, Granular Cell Tumor Benign	2	1	1	1

TABLE 12
Incidences of Neoplasms of the Brain and Meninges in F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Femaleⁱ				
Number Examined Microscopically	50	50	50	50
Brain, Glioma ^l	1	0	2	0
Brain, Oligodendroglioma NOS ^b	0	0	1	1
Brain, Glioma or Oligodendroglioma ^l				
Overall rate	1/50 (2%)	0/50 (0%)	3/50 (6%)	1/50 (2%)
Adjusted rate	2.2%	0.0%	9.0%	3.5%
Terminal rate	0/34 (0%)	0/26 (0%)	1/7 (14%)	0/2 (0%)
First incidence (days)	645	—	561	631
Poly-3 test	P=0.266	P=0.512N	P=0.207	P=0.647

(T) Terminal kill

^a Number of animals with neoplasm

^b Historical control incidence for 2-year studies (all routes) (mean ± standard deviation): 0/100

^c Number of animals with neoplasm per number of animals with brain examined microscopically

^d Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^e Observed incidence at terminal kill

^f Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill. A lower incidence in an exposure group is indicated by N.

^g Not applicable; no neoplasms in animal group

^h Historical control incidence: 2/100

ⁱ While an extended evaluation of the female brain was conducted, no additional neoplasms were found.

^j Historical control incidence: 1/100

Oral cavity (oral mucosa and tongue): Hyperplasia of the epithelium was seen in one 500 and two 1,000 mg/L males, and one male each in these groups had squamous cell carcinoma (Tables 13, A1, and A3). One 1,000 mg/L male had a squamous cell papilloma. One female control rat had hyperplasia of the epithelium, two females each in the 250 and 1,000 mg/L groups had squamous cell papilloma, and one 500 mg/L female had squamous cell carcinoma (Tables 13, B1, and B3).

All proliferative lesions in the tongue arose from the dorsal center or slightly dorsolateral surface of the tongue with no involvement of the ventral aspect of the tongue. The microscopically examined oral mucosa consisted of the dorsal palate. Squamous hyperplasia was characterized by a focal epithelial thickening comprised of 20 cell layers of squamous epithelium in contrast to about a five cell layer thick normal squamous epithelium. These areas were occasionally covered by a moderate amount of laminated keratin. Rete pegs in hyperplastic regions were elongate, branching, and anastomosing, in comparison with more normal regions in which the pegs were all of similar size with a flattened ventral surface. In contrast to papillomas and carcinomas, areas of squamous hyperplasia did not form exophytic projections or significantly distort the surface of the oral mucosa or tongue. Squamous cell papillomas were characterized by focally extensive, fairly well demarcated exophytic masses projecting from the oral mucosa or the central dorsal surface of the tongue. These neoplasms were composed of squamous epithelial cells that formed multiple, elongated papillary projections thickened by orthokeratotic hyperkeratosis. Squamous cell carcinoma consisted of an unencapsulated, invasive neoplastic mass that originated from and effaced the oral mucosa or the dorsal surface of the tongue. The mass was composed of polygonal cells arranged in nests, cords, and anastomosing trabeculae supported on a dense fibrous stroma with elongate fronds that projected from the oral mucosa or the dorsal surface of the tongue.

TABLE 13
Incidences of Neoplasms and Nonneoplastic Lesions of the Oral Cavity (Oral Mucosa or Tongue)
in F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male				
Number Necropsied	50	50	50	50
Epithelium, Hyperplasia ^a	0	0	1 (2.0) ^b	2 (2.5)
Squamous Cell Papilloma ^c	0	0	2	2
Squamous Cell Carcinoma ^d	1	0	1	1
Squamous Cell Papilloma or Squamous Cell Carcinoma ^d				
Overall rate ^e	1/50 (2%)	0/50 (0%)	3/50 (6%)	3/50 (6%)
Adjusted rate ^f	2.6%	0.0%	7.0%	7.7%
Terminal rate ^g	0/19 (0%)	0/21 (0%)	1/25 (4%)	1/19 (5%)
First incidence (days)	593	— ⁱ	442	642
Poly-3 test ^h	P=0.105	P=0.498N	P=0.338	P=0.302
Female				
Number Necropsied	50	50	50	50
Epithelium, Hyperplasia	1 (1.0)	0	0	0
Squamous Cell Papilloma ^d	0	2	0	2
Squamous Cell Carcinoma ^c	0	0	1	0
Squamous Cell Papilloma or Squamous Cell Carcinoma ^d				
Overall rate	0/50 (0%)	2/50 (4%)	1/50 (2%)	2/50 (4%)
Adjusted rate	0.0%	4.6%	3.1%	6.9%
Terminal rate	0/34 (0%)	0/26 (0%)	0/7 (0%)	0/2 (0%)
First incidence (days)	—	588	533	529
Poly-3 test	P=0.121	P=0.230	P=0.439	P=0.157

^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 control incidence for 2-year studies (all routes): 0/100

^d Historical control incidence: 1/100

^e Number of animals with neoplasm per number of animals necropsied

^f Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^g Observed incidence at terminal kill

^h Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill. A lower incidence in an exposure group is indicated by N.

ⁱ Not applicable; no neoplasms in animal group

Bone Marrow: The incidences of angiectasis in exposed groups of males and females increased with increasing exposure concentration and were significantly increased when compared to the control group incidences (Tables 14, A3, and B3). Angiectasis consisted of a larger blood-filled area that exceeded the normal vascular boundary

TABLE 14
Incidences of Nonneoplastic Lesions of the Bone Marrow in F344/NTac Rats
in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male				
Number Examined Microscopically	50	50	50	50
Angiectasis ^a	4 (1.3) ^b	29** (1.7)	34** (2.1)	40** (2.1)
Hyperplasia	17 (3.1)	19 (2.7)	20 (2.9)	30* (2.7)
Female				
Number Examined Microscopically	50	50	50	50
Angiectasis	1 (1.0)	19** (1.5)	32** (1.7)	39** (1.7)
Hyperplasia	23 (2.5)	35** (2.7)	40** (2.7)	43** (2.7)

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

** $P \leq 0.01$

^a Number of animals with lesion

^b Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

(Plate 2). The criteria for assigning severity grades to areas of angiectasis were as follows: Minimal severity was represented by one or two small focal areas of angiectasis, mild severity was used when one to three areas were present that were a little larger and encompassed up to 30% of the marrow cavity, moderate severity consisted of multiple areas that encompassed approximately 30% to 70% of the marrow cavity, and marked severity was assigned when multiple areas of angiectasis were present that filled over 70% of the marrow cavity. Sections of both femur bones were evaluated in determining severity grade. Angiectasis was occasionally surrounded by clusters of histiocytes containing intracytoplasmic pale golden yellow pigment with numerous clear acicular clefts, (Plate 3). These aggregates were considered to be a response to areas of angiectasis and therefore did not warrant a separate diagnosis.

Increased incidences of hyperplasia of the bone marrow were noted in all exposed groups when compared to controls; the increases were significant in all exposed groups of females and in the 1,000 mg/L males. Bone marrow hyperplasia was composed of large numbers of hematopoietic cells that completely filled the marrow cavity and reduced the number of adipocytes. The proportion of myeloid to erythroid precursors was equivalent with no single population predominating.

Liver: The incidences of eosinophilic focus were significantly increased in 1,000 mg/L males (6/50, 10/50, 7/50, 14/50, Table A3) and 500 and 1,000 mg/L females (6/50, 13/50, 21/50, 22/50, Table B3). Eosinophilic focus is a slightly hypereosinophilic aggregate of hepatocytes that are well demarcated, noncompressive, and blend imperceptibly with the adjacent normal hepatocytes. The hepatocytes within the focus are usually enlarged with finely granular pale or intensely eosinophilic cytoplasm and enlarged nuclei with prominent nucleoli. Significantly increased incidences of hematopoietic cell proliferation were noted in 500 and 1,000 mg/L females [3/50 (1.0), 7/50 (1.1), 14/50 (1.0), 9/50 (1.1), Table B3]. Hematopoietic cell proliferation was characterized by multifocal aggregates of multiple hematopoietic cell lineage precursors.

Spleen: Exposure concentration-related increased incidences of hematopoietic cell proliferation in females were observed, and the increases were significant at 500 and 1,000 mg/L (6/50 (1.8), 13/50 (2.4), 29/50 (2.1), 31/50 (2.1), Table B3). Hematopoietic cell proliferation was characterized by multifocal poorly demarcated foci of erythroid and myeloid precursors associated with megakaryocytes within the red pulp.

Rat Mammary Gland Adenocarcinoma qPCR Arrays

Within the mammary gland adenocarcinomas from the bromodichloroacetic acid exposed female F344/NTac rats, several genes involved in the *Tgfb* pathway were overrepresented. Pairwise comparison analysis identified eight genes that were significantly upregulated in mammary gland adenocarcinomas from bromodichloroacetic acid-exposed female rats compared to the spontaneous mammary gland adenocarcinomas from unexposed female rats. Five of these eight genes are associated with *Tgfb* pathway signaling, including its effects on matrix remodeling, mammary gland cancer progression, tumor invasion, and metastasis (*Mmp9*, *Mmp2*, *Id1*, *Vegfa*, and *Thbs1*; Table N2).

MICE

2-WEEK STUDY

All mice survived to the end of the study (Table 15). Final mean body weights and body weight gains of exposed groups of male and female mice were similar to those of the controls. Water consumption by exposed and control groups was similar. Drinking water concentrations of 62.5, 125, 250, 500, and 1,000 mg/L resulted in average daily doses of approximately 10, 20, 41, 84, and 175 mg bromodichloroacetic acid/kg body weight to males and 11, 16, 40, 78, and 138 mg/kg to females. There were no clinical findings related to bromodichloroacetic acid exposure.

TABLE 15
Survival, Body Weights, and Water Consumption of Mice
in the 2-Week Drinking Water Study of Bromodichloroacetic Acid^a

Concentration (mg/L)	Survival ^b	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)	Water Consumption Week 1	Water Consumption Week 3
Male							
0	5/5	23.4 ± 0.4	26.2 ± 0.4	2.9 ± 0.2		4	5
62.5	5/5	23.2 ± 0.4	26.4 ± 0.5	3.3 ± 0.3	101	4	4
125	5/5	23.6 ± 0.3	25.9 ± 0.3	2.3 ± 0.3	99	4	4
250	5/5	23.1 ± 0.6	25.9 ± 0.9	2.8 ± 0.8	99	4	4
500	5/5	23.6 ± 0.5	26.9 ± 0.7	3.3 ± 0.5	102	4	4
1,000	5/5	22.6 ± 0.7	26.2 ± 0.5	3.6 ± 0.4	100	4	5
Female							
0	5/5	18.9 ± 0.3	21.5 ± 0.4	2.6 ± 0.4		3	3
62.5	5/5	18.4 ± 0.2	21.9 ± 0.4	3.4 ± 0.3	101	3	3
125	5/5	18.7 ± 0.6	20.9 ± 0.5	2.2 ± 0.4	97	3	3
250	5/5	18.7 ± 0.4	21.3 ± 0.4	2.6 ± 0.3	99	3	3
500	5/5	19.0 ± 0.3	21.3 ± 0.2	2.3 ± 0.3	99	3	3
1,000	5/5	18.7 ± 0.1	21.3 ± 0.2	2.6 ± 0.2	99	2	3

^a Weights and weight changes are given as mean ± standard error. Water consumption is expressed as grams per animal per day. Differences in weights and weight changes from the control group are not significant by Dunnett's test.

^b Number of animals surviving at 16 days/number initially in group

Significantly decreased acetyl-CoA hydrolase activities occurred in males exposed to 125, 250, or 500 mg/L, and significantly increased activities occurred in females administered 250 or 500 mg/L (Table G2); these assays were not performed in the 3-month and 2-year studies.

Organ weights were unaffected by exposure to bromodichloroacetic acid (Table H4). No chemical-related gross or microscopic lesions were observed.

Exposure Concentration Selection Rationale: Due to the lack of effects of bromodichloroacetic acid exposure in the 2-week study, exposure concentrations selected for the 3-month drinking water study in mice were 62.5, 125, 250, 500, and 1,000 mg/L.

3-MONTH STUDY

All mice survived to the end of the study (Table 16). The final mean body weight of 1,000 mg/L females was somewhat less than that of controls, but the difference was not statistically significant (Table 16 and Figure 5). Water consumption by exposed groups was similar. Drinking water concentrations of 62.5, 125, 250, 500, and 1,000 mg/L resulted in average daily doses of approximately 7, 15, 30, 59, and 123 mg bromodichloroacetic acid/kg body weight per day to males and 9, 17, 36, 70, and 129 mg/kg to females. No chemical-related clinical findings were noted.

TABLE 16
Survival, Body Weights, and Water Consumption of Mice
in the 3-Month Drinking Water Study of Bromodichloroacetic Acid^a

Concentration (mg/L)	Survival ^b	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)	Water Consumption Week 1	Water Consumption Week 13
Male							
0	10/10	22.3 ± 0.3	39.3 ± 1.0	17.1 ± 0.8		4	4
62.5	10/10	22.3 ± 0.4	40.0 ± 0.6	17.6 ± 0.6	102	4	3
125	10/10	22.2 ± 0.3	39.6 ± 0.8	17.4 ± 0.7	101	3	4
250	10/10	22.6 ± 0.2	40.1 ± 1.0	17.6 ± 0.9	102	4	4
500	10/10	22.4 ± 0.3	39.8 ± 0.8	17.4 ± 0.9	101	4	3
1,000	10/10	22.0 ± 0.4	36.8 ± 0.7	14.8 ± 0.9	94	4	3
Female							
0	10/10	18.6 ± 0.2	33.8 ± 0.6	15.2 ± 0.6		3	3
62.5	10/10	18.6 ± 0.3	32.8 ± 1.3	14.2 ± 1.2	97	3	3
125	10/10	18.5 ± 0.2	35.4 ± 0.7	16.9 ± 0.8	105	3	4
250	10/10	18.9 ± 0.2	32.6 ± 0.7	13.7 ± 0.8	96	4	4
500	10/10	18.8 ± 0.2	33.2 ± 0.9	14.4 ± 1.0	98	3	3
1,000	10/10	18.5 ± 0.3	30.7 ± 0.7	12.2 ± 0.7	91	4	3

^a Weights and weight changes are given as mean ± standard error. Water consumption is expressed as grams per animal per day. Differences in weights and weight changes from the control group are not significant by Dunnett's test.

^b Number of animals surviving at 14 weeks/number initially in group

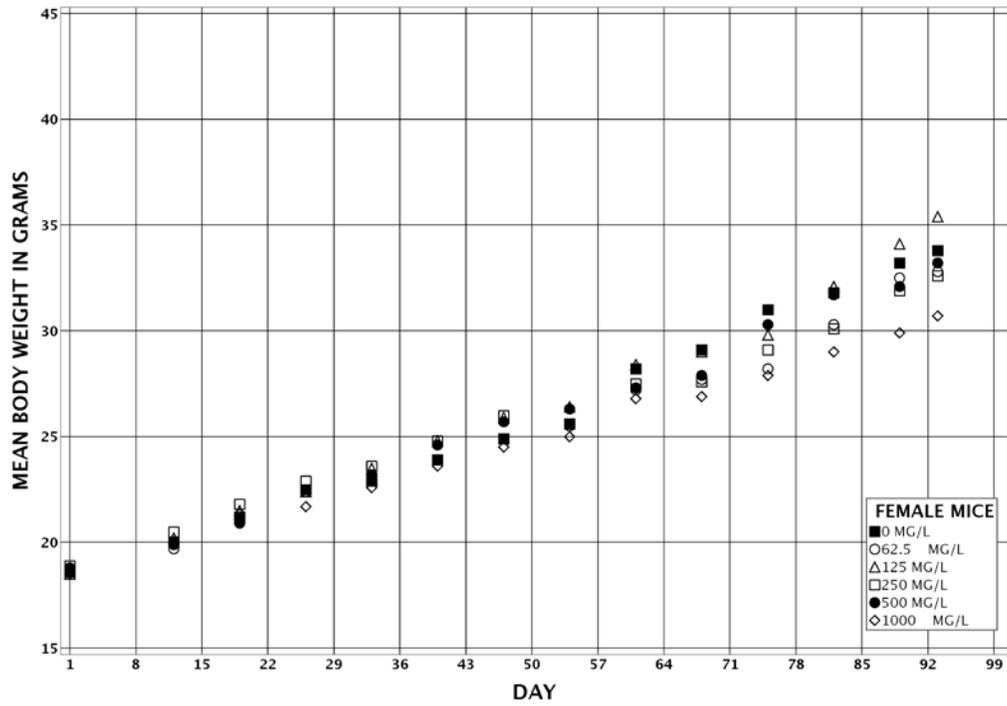
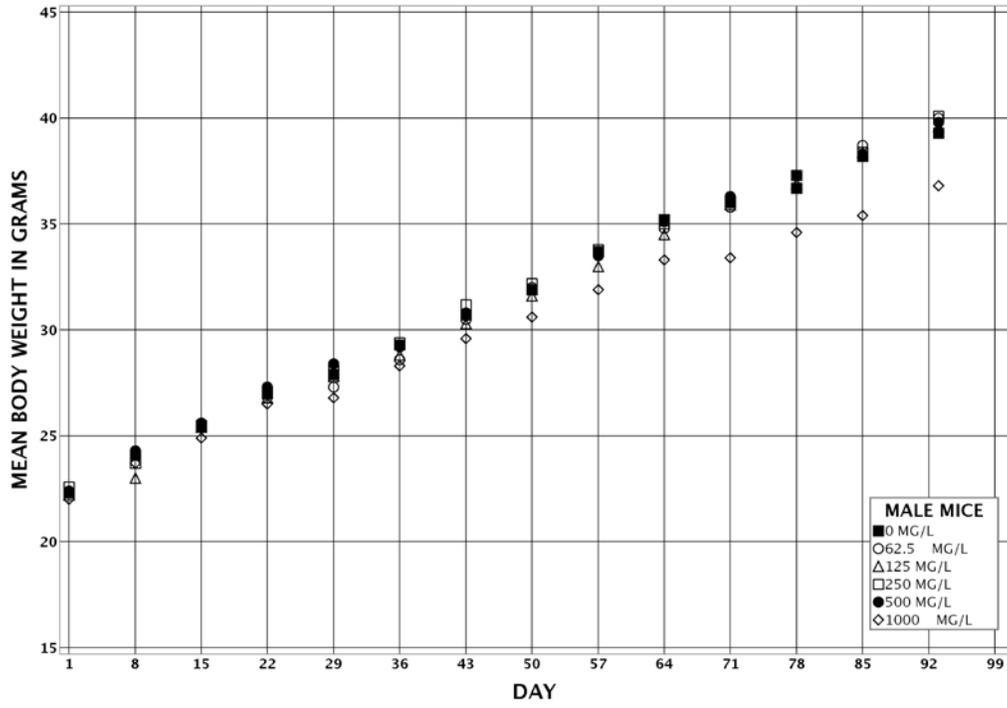


FIGURE 5
Growth Curves for Mice Exposed to Bromodichloroacetic Acid
in Drinking Water for 3 Months

There were no changes in hematology parameters attributable to bromodichloroacetic acid exposure (Table F2).

The absolute and relative liver weights of 500 and 1,000 mg/L males were significantly greater than those of the concurrent controls (Table H5). The absolute kidney weight of 1,000 mg/L males was significantly less than that of the controls.

B6C3F1/N mice did not display any findings suggestive of an effect of bromodichloroacetic acid on the male or female reproductive system (Tables I3 and I4).

Exposure Concentration Selection Rationale: Based on the limited effects of bromodichloroacetic acid exposure observed in the 3-month study, the exposure concentrations selected for the 2-year drinking water study in mice were 250, 500, and 1,000 mg/L. Higher concentrations of bromodichloroacetic acid were not considered, based on the 2-year NTP study of bromochloroacetic acid (NTP, 2009), which used these exposure concentrations and resulted in clear evidence of carcinogenicity in mice.

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 17 and in the Kaplan-Meier survival curves (Figure 6). Survival of the 500 and 1,000 mg/L males was significantly decreased, but survival was unaffected by exposure in females. The most common cause of early deaths in 500 mg/L males and 1,000 mg/L males and females appeared to be liver neoplasms.

TABLE 17
Survival of Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male				
Animals initially in study	66	66	66	66
6-Month interim evaluation ^a	8	8	8	8
14-Month interim evaluation ^a	8	8	8	7
Missing ^a	0	0	1	0
Moribund	15	14	27	27
Natural deaths	10	15	10	14
Animals surviving to study termination	25	21	12	10
Percent probability of survival at end of study ^b	50	42	25	20
Mean survival (days) ^c	660	668	618	605
Survival analysis ^d	P<0.001	P=0.824	P=0.016	P=0.002
Female				
Animals initially in study	66	66	66	66
6-Month interim evaluation ^a	8	8	8	8
14-Month interim evaluation ^a	8	8	8	8
Moribund	9	9	8	11
Natural deaths	11	8	13	12
Animals surviving to study termination	30	33	29	27 ^e
Percent probability of survival at end of study	60	66	58	54
Mean survival (days)	680	683	681	680
Survival analysis	P=0.463	P=0.674N	P=1.000	P=0.722

^a Censored from survival analyses

^b Kaplan-Meier determinations

^c Mean of all deaths (uncensored and terminal kill)

^d The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed group columns. A lower mortality in an exposure group is indicated by N.

^e Includes one animal that died during the last week of the study

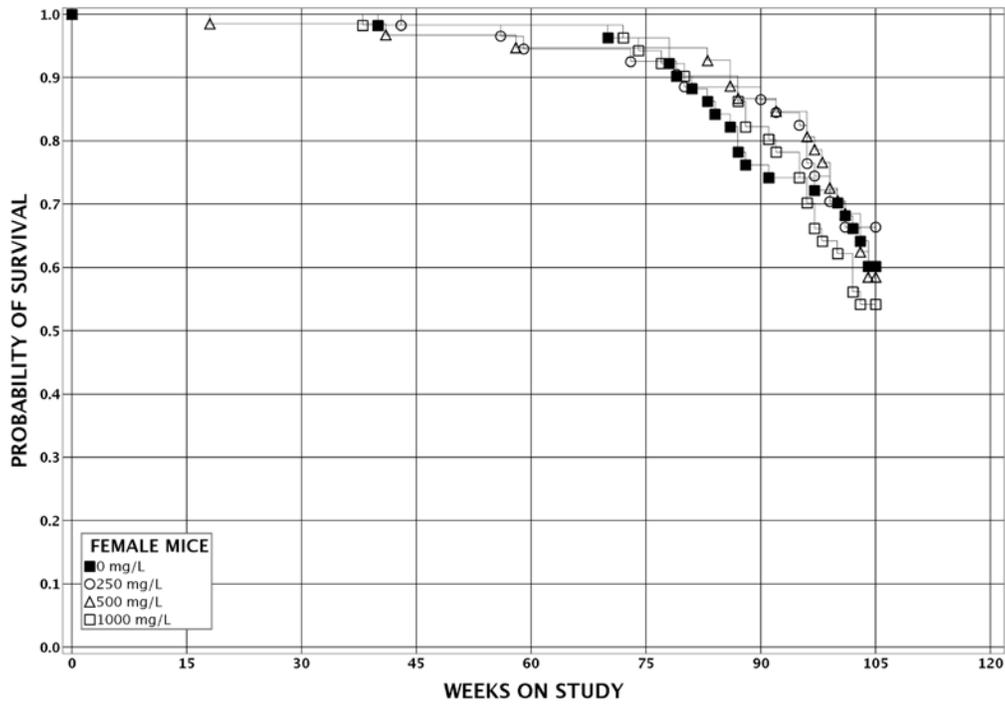
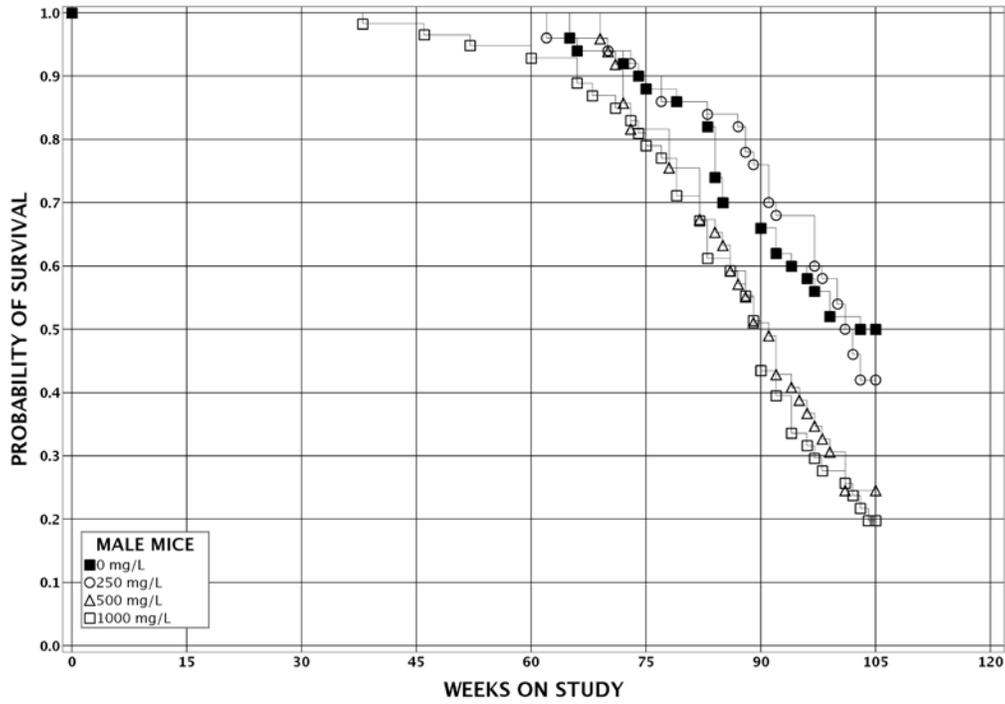


FIGURE 6
Kaplan-Meier Survival Curves for Mice Exposed to Bromodichloroacetic Acid in Drinking Water for 2 Years

Body Weights, Water and Compound Consumption, Clinical Findings, and Organ Weights

Compared to those of controls, mean body weights were more than 10% less in 500 mg/L males after week 73, in 1,000 mg/L males after week 57, in 250 and 500 mg/L females after week 89, and in 1,000 mg/L females after week 73 (Figure 7; Tables 18 and 19). Water consumption by all exposed groups of males and 250 and 500 mg/L females was generally greater than that by controls during the second year of the study (Tables K3 and K4). Drinking water concentrations of 250, 500, and 1,000 mg/L resulted in average daily doses of approximately 23, 52, and 108 mg bromodichloroacetic acid/kg body weight to males and approximately 17, 34, and 68 mg/kg to females. No clinical findings related to chemical exposure were observed. At the 6-month interim evaluation, absolute and relative left and right kidney weights of 1,000 mg/L males were significantly less than those of the controls (Table H6).

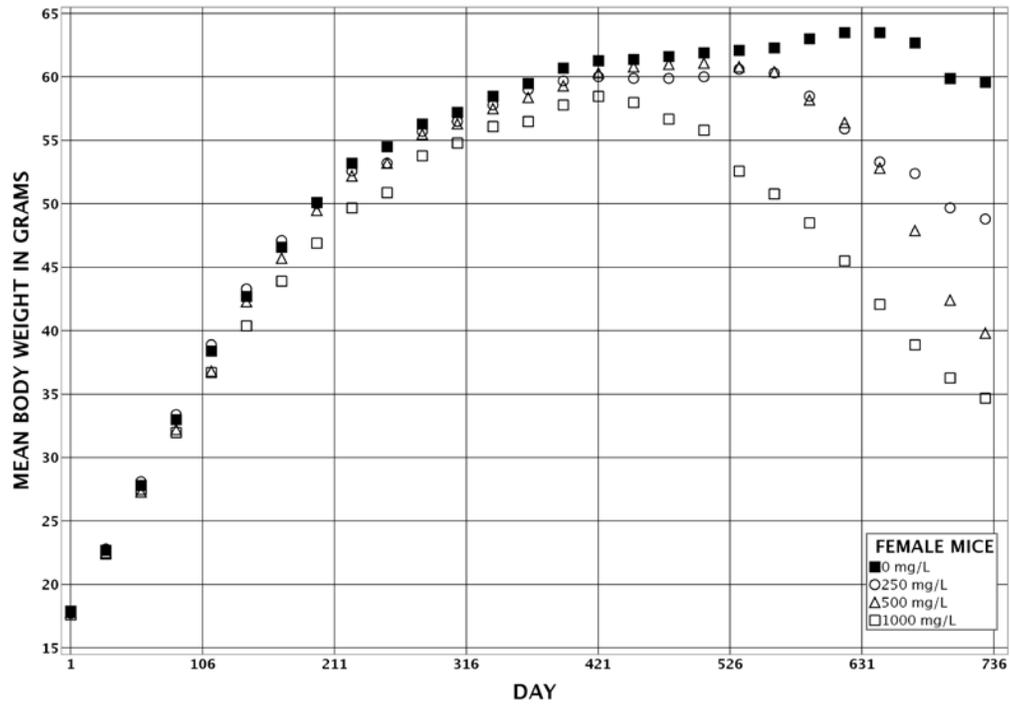
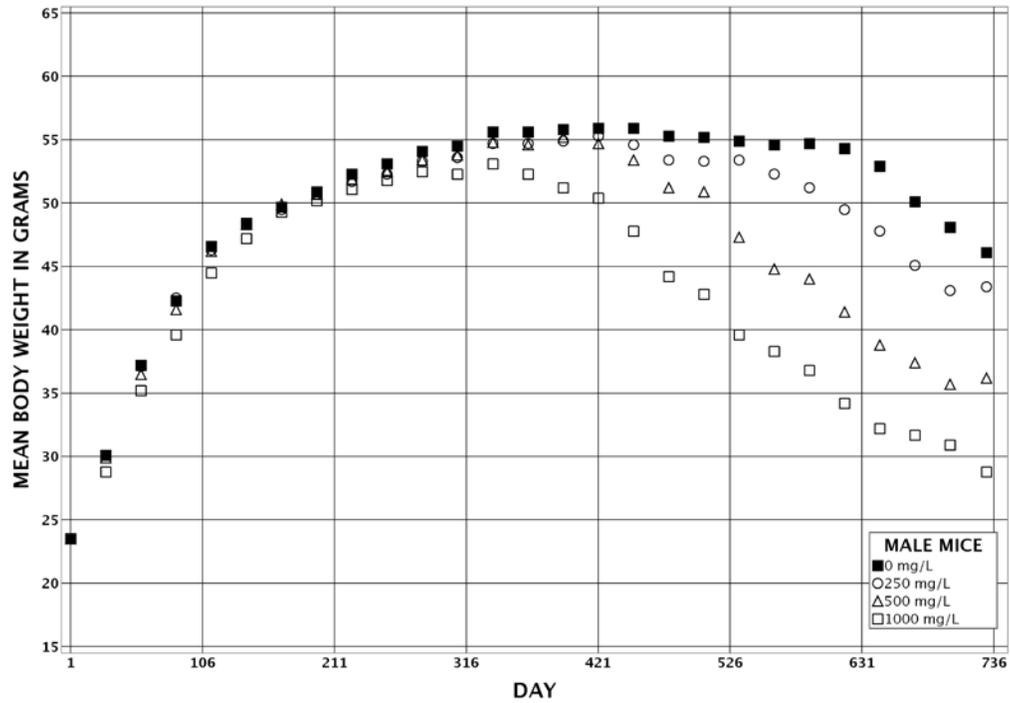


FIGURE 7
Growth Curves for Mice Exposed to Bromodichloroacetic Acid
in Drinking Water for 2 Years

TABLE 18
Mean Body Weights and Survival of Male Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

Day	0 mg/L		250 mg/L			500 mg/L			1,000 mg/L		
	Av. Wt. (g)	No. of Survivors	Av. Wt (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt (g)	Wt. (% of Controls)	No. of Survivors
1	23.5	66	23.5	100	66	23.5	100	66	23.5	100	66
29	30.1	66	30.1	100	66	29.9	99	66	28.8	96	66
57	37.2	66	37.1	100	66	36.5	98	66	35.2	95	66
85	42.3	66	42.5	100	66	41.6	98	66	39.6	94	66
113	46.6	66	46.3	99	66	46.2	99	66	44.5	96	66
141	48.4	66	48.3	100	66	48.3	100	66	47.2	97	66
169	49.7	66	49.5	100	66	49.9	100	66	49.3	99	66
197 ^a	50.9	58	50.7	100	58	50.7	100	58	50.2	99	58
225	52.3	58	51.7	99	58	51.9	99	58	51.1	98	58
253	53.1	58	52.3	99	58	52.5	99	57	51.8	98	58
281	54.1	58	53.3	99	58	53.4	99	57	52.5	97	57
309	54.5	58	53.6	98	58	53.8	99	57	52.3	96	57
337	55.6	58	54.7	98	58	54.8	99	57	53.1	96	56
365	55.6	58	54.7	99	58	54.6	98	57	52.3	94	55
393	55.8	58	54.9	98	58	55.2	99	57	51.2	92	55
421 ^a	55.9	50	55.3	99	50	54.7	98	49	50.4	90	47
449	55.9	48	54.6	98	48	53.4	96	49	47.8	86	47
477	55.3	47	53.4	97	48	51.2	93	48	44.2	80	44
505	55.2	46	53.3	97	47	50.9	92	41	42.8	78	43
533	54.9	44	53.4	97	43	47.3	86	40	39.6	72	40
561	54.6	43	52.3	96	43	44.8	82	37	38.3	70	36
589	54.7	37	51.2	94	42	44.0	80	32	36.8	67	31
617	54.3	35	49.5	91	39	41.4	76	27	34.2	63	28
645	52.9	31	47.8	91	34	38.8	73	21	32.2	61	20
673	50.1	29	45.1	90	34	37.4	75	17	31.7	63	16
701	48.1	26	43.1	90	27	35.7	74	14	30.9	64	13
Mean for Weeks											
1-13	33.3		33.3	100		32.9	99		31.8	96	
14-52	51.7		51.2	99		51.3	99		50.2	97	
53-101	54.1		51.4	95		46.9	86		41.0	75	

^a Animals removed for interim evaluation during weeks 27 and 57

TABLE 19
Mean Body Weights and Survival of Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

Day	0 mg/L		250 mg/L			500 mg/L			1,000 mg/L		
	Av. Wt. (g)	No. of Survivors	Av. Wt (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt (g)	Wt. (% of Controls)	No. of Survivors
1	17.9	66	17.7	99	66	17.8	99	66	17.6	98	66
29	22.7	66	22.8	100	66	22.5	99	66	22.4	99	66
57	27.8	66	28.1	101	66	27.3	98	66	27.5	99	66
85	33.0	66	33.4	101	66	32.2	98	66	32.0	97	66
113	38.4	66	38.9	101	66	36.8	96	66	36.7	96	66
141	42.7	66	43.3	102	66	42.3	99	65	40.4	95	66
169	46.6	66	47.1	101	66	45.7	98	65	43.9	94	66
197 ^a	50.1	58	50.0	100	58	49.5	99	57	46.9	94	58
225	53.2	58	52.6	99	58	52.2	98	57	49.7	93	58
253	54.5	58	53.2	98	58	53.2	98	57	50.9	93	58
281	56.3	57	55.7	99	58	55.5	99	57	53.8	96	57
309	57.2	57	56.5	99	57	56.3	98	56	54.8	96	57
337	58.5	57	57.8	99	57	57.5	98	56	56.1	96	57
365	59.5	57	59.0	99	57	58.4	98	56	56.5	95	57
393	60.7	57	59.7	98	56	59.3	98	56	57.8	95	57
421 ^a	61.3	49	60.0	98	47	60.3	98	47	58.5	95	49
449	61.4	49	59.9	97	47	60.8	99	47	58.0	94	49
477	61.6	49	59.9	97	47	61.0	99	47	56.7	92	49
505	61.9	48	60.0	97	47	61.1	99	47	55.8	90	48
533	62.1	48	60.6	98	46	60.8	98	47	52.6	85	47
561	62.3	44	60.3	97	44	60.4	97	47	50.8	82	45
589	63.0	42	58.5	93	44	58.2	92	46	48.5	77	45
617	63.5	38	55.9	88	44	56.4	89	43	45.5	72	41
645	63.5	37	53.3	84	42	52.8	83	42	42.1	66	39
673	62.7	36	52.4	84	37	47.9	76	39	38.9	62	34
701	59.9	34	49.7	83	34	42.4	71	34	36.3	61	31
Mean for Weeks											
1-13	25.4		25.5	100		25.0	99		24.9	98	
14-52	50.8		50.6	100		49.9	98		48.1	95	
53-101	61.8		57.6	93		56.9	92		50.6	82	

^a Animals removed for interim evaluation during weeks 27 and 58

Pathology and Statistical Analyses

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

Liver: At necropsy, larger numbers of liver nodules and masses were observed macroscopically in exposed mice than in control mice. These most often corresponded microscopically to hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma. At 6 months, the average severity of hepatic glycogen depletion in exposed groups was greater than that of the controls in male and female mice and increased with increasing exposure concentration in males (Table 20). The lesion was characterized by a majority of hepatocytes containing lacy diaphanous cytoplasmic vacuoles, consistent with glycogen accumulation. The decrease in cytoplasmic vacuolization is consistent with glycogen depletion which can occur with stress and may be interpreted as the minimal change seen in the 1,000 mg/L groups.

At 14 months, hepatocellular adenomas were noted in exposed groups of males and in 500 and 1,000 mg/L females; none were seen in the controls (Table 20). Hepatocellular adenomas were characterized by well circumscribed nodules that compressed the adjacent parenchyma and often lacked the normal lobular architecture. Cells within adenomas usually consisted of a mixture of eosinophilic, basophilic, clear, or vacuolated cells, and hepatic cords in adenomas often intersected the cords of the adjacent normal parenchyma at an angle indicating autonomous growth.

Exposure concentration-related increased incidences of atypical focus of cellular alteration (i.e., eosinophilic cell focus and mixed cell focus) were noted in all exposed groups of male and female mice at 14 months, and the incidences in 1,000 mg/L males and females were significantly increased (Table 20). This change occurred as either single or multifocal nodules. The cells within the foci displayed marked cytomegaly and karyomegaly with intranuclear invaginations and multiple nucleoli in some cases.

TABLE 20
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male				
6-Month Interim Evaluation				
Number Examined Microscopically	8	8	8	8
Glycogen Depletion ^a	8 (1.0) ^b	8 (1.4)	8 (2.0)	8 (2.4)
14-Month Interim Evaluation				
Number Examined Microscopically	7	8	8	7
Focus of Cellular Alteration, Atypical	1	2	4	6*
Hepatocellular Adenoma	0	2	1	1
Hepatoblastoma	0	0	0	1
2-Year Study				
Number Examined Microscopically	50	50	49	51
Focus of Cellular Alteration, Atypical	0	19**	42**	43**
Hepatocellular Adenoma, Multiple	28	29	27	23
Hepatocellular Adenoma (includes multiple) ^c	39	41	42	40
Hepatocellular Carcinoma, Multiple	4	12*	10	17**
Hepatocellular Carcinoma (includes multiple) ^d				
Overall rate ^e	12/50 (24%)	22/50 (44%)	27/49 (55%)	39/51 (76%)
Adjusted rate ^f	29.3%	49.5%	66.1%	87.1%
Terminal rate ^g	7/25 (28%)	8/21 (38%)	7/12 (58%)	8/10 (80%)
First incidence (days)	548	511	480	260
Poly-3 test ^h	P<0.001	P=0.041	P<0.001	P<0.001
Hepatoblastoma, Multiple	1	6	12**	12**
Hepatoblastoma (includes multiple) ⁱ				
Overall rate	4/50 (8%)	24/50 (48%)	40/49 (82%)	34/51 (67%)
Adjusted rate	10.1%	53.6%	87.1%	77.5%
Terminal rate	1/25 (4%)	8/21 (38%)	9/12 (75%)	5/10 (50%)
First incidence (days)	590	433	477	360
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma ^j				
Overall rate	42/50 (84%)	50/50 (100%)	48/49 (98%)	49/51 (96%)
Adjusted rate	91.2%	100.0%	98.0%	99.1%
Terminal rate	25/25 (100%)	21/21 (100%)	11/12 (92%)	10/10 (100%)
First incidence (days)	449	433	477	260
Poly-3 test	P=0.036	P=0.015	P=0.110	P=0.034
Hepatocholangiocarcinoma ^k	0	0	2	2

TABLE 20
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Female				
6-Month Interim Evaluation				
Number Examined Microscopically	8	8	8	8
Glycogen Depletion	8 (1.7)	8 (2.1)	8 (2.9)	8 (2.5)
14-Month Interim Evaluation				
Number Examined Microscopically	8	8	8	8
Focus of Cellular Alteration, Atypical	0	2	3	4*
Hepatocellular Adenoma	0	0	1	1
2-Year Study				
Number Examined Microscopically	49	50	49	50
Eosinophilic Focus	22	33**	38**	40**
Focus of Cellular Alteration, Atypical	0	2	6*	16**
Hepatocellular Adenoma, Multiple	25	38*	37*	37*
Hepatocellular Adenoma (includes multiple) ^l				
Overall rate	33/49 (67%)	42/50 (84%)	42/49 (86%)	44/50 (88%)
Adjusted rate	75.3%	90.7%	92.5%	93.0%
Terminal rate	26/30 (87%)	32/33 (97%)	28/29 (97%)	27/27 (100%)
First incidence (days)	540	386	597	503
Poly-3 test	P=0.009	P=0.030	P=0.015	P=0.010
Hepatocellular Carcinoma, Multiple	0	5	6*	13**
Hepatocellular Carcinoma (includes multiple) ^m				
Overall rate	9/49 (18%)	17/50 (34%)	22/49 (45%)	26/50 (52%)
Adjusted rate	21.1%	37.9%	49.8%	59.1%
Terminal rate	5/30 (17%)	13/33 (39%)	15/29 (52%)	20/27 (74%)
First incidence (days)	604	509	603	607
Poly-3 test	P<0.001	P=0.065	P=0.004	P<0.001
Hepatoblastoma, Multiple	0	0	1	2
Hepatoblastoma (includes multiple) ⁿ				
Overall rate	0/49 (0%)	1/50 (2%)	4/49 (8%)	6/50 (12%)
Adjusted rate	0.0%	2.3%	9.3%	13.9%
Terminal rate	0/30 (0%)	0/33 (0%)	3/29 (10%)	3/27 (11%)
First incidence (days)	— ^o	688	716	636
Poly-3 test	P=0.003	P=0.506	P=0.062	P=0.016

TABLE 20
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Female (continued)				
Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma ^P				
Overall rate	36/49 (73%)	44/50 (88%)	43/49 (88%)	46/50 (92%)
Adjusted rate	81.1%	93.7%	94.7%	95.5%
Terminal rate	26/30 (87%)	33/33 (100%)	29/29 (100%)	27/27 (100%)
First incidence (days)	540	386	597	503
Poly-3 test	P=0.013	P=0.043	P=0.030	P=0.017
Hepatocholangiocarcinoma ^Q				
	0	0	0	1

* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test (interim evaluations) or the Poly-3 test (2-year study)

** $P \leq 0.01$

^a Number of animals with lesion

^b Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^c Historical incidence for 2-year drinking water studies with control groups (mean \pm standard deviation): 72/100 (72.0% \pm 8.5%), range 66%-78%; all routes: 594/949 (62.6% \pm 9.1%), range 48%-78%

^d Historical incidence for drinking water studies: 38/100 (38.0% \pm 19.8%), range 24%-52%; all routes: 348/949 (36.7% \pm 11.4%), range 22%-56%

^e Number of animals with neoplasm per number of animals with liver examined microscopically

^f Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^g Observed incidence at terminal kill

^h Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill.

ⁱ Historical incidence for drinking water studies: 10/100 (10.0% \pm 2.8%), range 8%-12%; all routes: 40/949 (4.2% \pm 3.5%), range 0%-12%

^j Historical incidence for drinking water studies: 87/100 (87.0% \pm 4.2%), range 84%-90%; all routes: 746/949 (78.6% \pm 7.2%), range 64%-90%

^k Historical incidence for drinking water studies: 2/100 (2.0% \pm 2.8%), range 0%-4%; all routes: 10/949 (1.1% \pm 2.2%), range 0%-8%

^l Historical incidence for drinking water studies: 71/98 (72.5% \pm 7.2%), range 67%-78%; all routes: 378/948 (39.9% \pm 18.7%), range 14%-78%

^m Historical incidence for drinking water studies: 20/98 (20.4% \pm 2.9%), range 18%-22%; all routes: 152/948 (16.0% \pm 10.6%), range 4%-46%

ⁿ Historical incidence for drinking water studies: 1/98 (1.0% \pm 1.44%), range 0%-2%; all routes: 4/948 (0.4% \pm 0.8%), range 0%-2%

^o Not applicable; no neoplasms in animal group

^p Historical incidence for drinking water studies: 76/98 (77.6% \pm 5.8%), range 73%-82%; all routes: 448/948 (47.3% \pm 19.3%), range 20%-82%

^q Historical incidence for drinking water studies: 0/98; all routes: 0/948

A single case of hepatoblastoma at 14 months was noted in males exposed to 1,000 mg/L (Table 20).

Histologically, the hepatoblastoma consisted of a variably demarcated mass composed of sheets of small, elongate, hyperchromatic cells that often formed rosettes or palisaded around vessels. The center portion of the mass was necrotic and contained focal areas of mineralization.

Compared to those of the control groups at 2 years, the incidences of hepatocellular adenoma in all exposed groups of females, hepatocellular carcinoma in all exposed groups of males and 500 and 1,000 mg/L females, and hepatoblastoma in all exposed groups of males and 1,000 mg/L females were significantly increased (Tables 20, C1, C2, D1, and D2). The incidences of multiple hepatocellular carcinoma and multiple hepatoblastoma in males and females and multiple hepatocellular adenoma in females were generally increased in an exposure concentration-related manner. When combined, the incidences of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma were significantly increased in 250 and 1,000 mg/L males and all exposed groups of females.

There were sporadic cases of hepatocholangiocarcinoma in males exposed to 500 mg/L and males and females exposed to 1,000 mg/L.

The incidences of eosinophilic focus in exposed females and atypical focus of cellular alteration (i.e., eosinophilic cell focus and mixed cell focus) in exposed groups of males and females (except 250 mg/L) were significantly greater than controls (Tables 20, C4, and D4).

Hepatocellular adenomas were characterized as well-circumscribed nodules that compressed the adjacent parenchyma and lacked the normal lobular architecture and portal triads. Cells within adenomas usually consisted of a mixture of eosinophilic, basophilic, clear, or vacuolated cells, and hepatic cords in adenomas often intersected the cords of the adjacent normal parenchyma at an angle indicating autonomous growth. Hepatocellular carcinomas were characterized by a fairly well-demarcated mass composed of broad trabeculae (greater than three hepatocytes thick) that often rounded up or formed sheets of solid growth. These masses often compressed and/or invaded the adjacent hepatic parenchyma. Neoplastic hepatocytes exhibited features of cellular atypia and increased mitotic figures and the central portion of the masses often contained hemorrhage and necrosis. Hepatoblastomas were variably demarcated masses composed of sheets of small, elongated, hyperchromatic cells that often formed rosettes or palisaded around vessels (Plate 4). These masses were often necrotic in the center and/or consisted of a blood-filled spaces surrounded by neoplastic cells. Metastasis to different organs, such as the lungs (most commonly), mesentery, mesenteric lymph nodes, and skeletal muscle, were noted (Plate 5).

Eosinophilic focus consisted of a well-demarcated collection of hepatocytes with finely granular eosinophilic cytoplasm that blended imperceptibly into the adjacent hepatic parenchyma, while larger foci caused slight compression of surrounding hepatocytes. Atypical focus of cellular alteration occurred as single or multifocal nodular aggregates of enlarged hepatocytes that merged with the surrounding parenchyma, occasionally caused slight compression in other areas, and occasionally had irregular borders. Hepatocytes within the foci had either solid eosinophilic or mixed contents (i.e., distended by multiple small discrete lipid vacuoles – microvesicular vacuolation). The lesions resembled an eosinophilic focus of altered hepatocytes, but there was cellular atypia and hypertrophy. The cells within the foci displayed marked cytomegaly and karyomegaly with intracytoplasmic accumulation of pale globular eosinophilic material in some cases. Affected hepatocytes also contained numerous intranuclear invaginations and multiple prominent nucleoli (Plates 6 and 7). There was no evidence for hyperplasia within the lesions.

Harderian Gland: In males, the incidences of epithelium hyperplasia, adenoma, and carcinoma occurred with positive trends, and the incidences of adenoma and adenoma or carcinoma (combined) in the 500 and 1,000 mg/L groups were significantly greater than those in the controls (Tables 21, C1, C2, and C4). Hyperplasia of the epithelium was seen as focal areas of cellular proliferation that did not distort the architecture of the gland and did not compress the surrounding parenchyma. The epithelial cells within hyperplastic lesions maintained one cell layer of thickness and did not display cellular atypia (Plate 8). Adenoma was a well-demarcated proliferative lesion that compressed the adjacent parenchyma and altered the tissue architecture. Neoplastic cells generally maintained one cell layer thickness, but occasionally piled up to project into the acinar lumen (Plate 9). Carcinoma was seen as a large, poorly demarcated mass that distorted the glandular architecture (Plate 10). Neoplastic epithelial cells displayed cellular and nuclear pleomorphism and often piled up to three or four cell layers thick. The nuclear to cytoplasmic ratio was generally increased.

TABLE 21
Incidences of Neoplasms and Nonneoplastic Lesions of the Harderian Gland in Male Mice
in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Number Necropsied	50	50	49	51
Epithelium, Hyperplasia ^a	1 (2.0) ^b	1 (1.0)	3 (2.3)	4 (2.0)
Adenoma ^c				
Overall rate ^d	6/50 (12%)	11/50 (22%)	14/49 (29%)	19/51 (37%)
Adjusted rate ^e	14.7%	26.1%	38.0%	48.8%
Terminal rate ^f	2/25 (8%)	4/21 (19%)	5/12 (42%)	3/10 (30%)
First incidence (days)	548	533	491	458
Poly-3 test ^g	P<0.001	P=0.155	P=0.015	P<0.001
Carcinoma ^h	0	0	0	3
Adenoma or Carcinoma ⁱ				
Overall rate	6/50 (12%)	11/50 (22%)	14/49 (29%)	20/51 (39%)
Adjusted rate	14.7%	26.1%	38.0%	51.4%
Terminal rate	2/25 (8%)	4/21 (19%)	5/12 (42%)	4/10 (40%)
First incidence (days)	548	533	491	458
Poly-3 test	P<0.001	P=0.155	P=0.015	P<0.001

^a Number of animals with lesion

^b Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^c Historical incidence for 2-year drinking water studies with control groups (mean ± standard deviation): 11/100 (11.0% ± 1.4%), range 10%-12%; all routes: 119/950 (12.5% ± 4.8%), range 4%-22%

^d Number of animals with neoplasm per number of animals necropsied

^e Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^f Observed incidence at terminal kill

^g Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill.

^h Historical incidence for drinking water studies: 2/100 (2.0% ± 2.8%), range 0%-4%; all routes: 34/950 (3.6% ± 2.9%), range 0%-10%

ⁱ Historical incidence for drinking water studies: 13/100 (13.0% ± 1.4%), range 12%-14%; all routes: 153/950 (16.1% ± 4.9%), range 6%-24%

Testis and Epididymis: The incidences and severities of atrophy of the testis increased with increasing exposure concentration, and the increases at 500 and 1,000 mg/L were significant (Tables 22 and C4). Atrophy was characterized by testicular tubular degeneration seen as reduced numbers of germinal epithelial cells within seminiferous tubules in comparison to normal tubules. Spermatozoa were often fewer or absent in affected tubular lumens.

In the epididymis, the incidences of atrophy in all exposed groups, hypospermia in the 1,000 mg/L group, and epithelium degeneration in the 500 and 1,000 mg/L groups were significantly increased compared to the control

TABLE 22
Incidences of Nonneoplastic Lesions of the Testis and Epididymis in Male Mice
in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Testes ^a	(50)	(50)	(49)	(51)
Atrophy ^b	4 (1.3) ^c	6 (2.2)	13** (2.4)	23** (2.9)
Epididymis	(50)	(50)	(49)	(51)
Atrophy	0	7* (1.9)	10** (2.0)	17** (2.1)
Hypospermia	0	1 (2.0)	0	17** (2.7)
Epithelium, Degeneration	1 (3.0)	1 (2.0)	10** (1.6)	6* (1.3)

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

** $P \leq 0.01$

^a Number of animals with tissue examined microscopically.

^b Number of animals with lesion.

^c Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

group incidences (Tables 22 and C4). Atrophy was characterized by a constellation of histologic changes including decreased size of tubules; condensation of the connective tissues surrounding the tubules, imparting a thicker appearance; and a reduction in the size of the epithelial cells lining the tubules. Hypospermia was characterized by reduced numbers of spermatozoa within tubular lumens when compared with normal epididymides. In some cases, although devoid of spermatozoa, tubular lumens were filled with sloughed epithelial cells, macrophages, or other cellular debris (Plates 11 and 12). Epithelium degeneration was characterized by cellular changes affecting the epithelium lining the tubules of the epididymis including individual cell necrosis, sloughing of clusters of eosinophilic degenerative cells, excessive karyomegaly, disorganization of the epithelial layer, and flattening of abnormal cells (Plate 13).

Molecular Analysis of Mouse Hepatoblastoma and Hepatocellular Carcinoma

In bromodichloroacetic acid-exposed adjacent nontumor liver tissue, major oncogenic, metabolic, and hepatic function related pathways were altered compared to normal liver of control mice (Appendix O). In terms of oncogenic signaling, Ingenuity Pathway Analysis™ (IPA) showed that top biological functions altered in adjacent nontumor liver compared to normal liver of control mice included genes involved in cancer, cellular function and

maintenance, and cell morphology. Top toxicology functions perturbed in adjacent nontumor liver tissue samples included genes associated with liver injury, including liver necrosis/cell death, regeneration, and proliferation.

In bromodichloroacetic acid-exposed hepatocellular carcinoma, IPA indicated dysregulation of a variety of metabolic and cancer related pathways. Overrepresented pathways altered in bromodichloroacetic acid-exposed hepatocellular carcinoma included biological functions such as organismal function, cell movement, cancer signaling, cellular development, and cell growth and proliferation. Top overrepresented canonical pathways in hepatocellular carcinoma included fatty acid metabolism, xenobiotic signaling, cell cycle: G2/M DNA damage checkpoint regulation, aryl hydrocarbon receptor signaling, and apoptosis signaling. In bromodichloroacetic acid-exposed hepatocellular carcinoma, many of the top dysregulated cancer pathways included upregulation of oncogenes (*Gpc3*, *Akr1c3*, *Plat*, *Itih5*, *Areg*, *Tff3*, *Afp*) and downregulation of tumor suppressor genes (*Dct*, *Gas1*, *Prlr*, *Socs2*, *Wnt5b*) similar to that seen in human hepatocellular carcinoma.

Principal Component Analysis showed that hepatoblastoma samples clustered distinctly from hepatocellular carcinoma, adjacent nontumor liver tissue, and normal liver of control mice. Compared to adjacent nontumor liver tissue, there were 10,346 differentially expressed genes in hepatoblastoma. Genes involved in *Wnt/Ctnnb1* pathway signaling were dysregulated in hepatoblastoma, including upregulation of various *Wnt* signaling genes (*Wnt9a*, *Wnt10a*, *Wnt7a*), genes involved in *Wnt* feedback-regulation (*Axin2*, *Nkd1*), positive effectors (*Lef1*, *Dvl3*), and *Wnt* antagonists (*Dkk2*, *Dkk3*, *Wif1*). Secondly, there was significant upregulation of a number of genes involved in genomic imprinting (*Igf2*, *Peg1*, *Peg10*, *Bex1*, *Meg3*, *H19*, *Ndn*), which are typically expressed in fetal liver. Finally, genes involved in embryonic stem cell pluripotency (*T*, *Bmp4*, *Fzd6*, *Fzd10*, *Nog*) and stem cell-related target genes (*Sox9*, *Tbx3*, *Suz12*) were upregulated, while *Zfp42*, *Gata4*, *Fzd4*, and *Fzd8* were downregulated, indicating distinctive expression of hepatic stem/progenitor markers. Meta-analysis of hepatoblastoma using NextBio also revealed high concordance with embryonic mouse liver (E10.5 to 12.5). Gene network and pathway analysis using IPA revealed significant alterations in pathways involved in hepatic development, metabolic pathways, embryonic stem cell regulation, and genomic imprinting in hepatoblastoma compared to hepatocellular carcinoma.

Further, bromodichloroacetic acid-exposed hepatoblastoma and hepatocellular carcinoma showed relatively low incidence of *H-ras* mutation and high incidence of *β-catenin* mutation, compared to historical spontaneous hepatocellular carcinoma. Between hepatoblastoma and hepatocellular carcinoma from exposed mice, there was not much difference in the incidences of *H-ras* (7% and 13%, respectively) and *β-catenin* mutations (23% and 10%, respectively). Interestingly, for hepatoblastoma and its adjacent hepatocellular carcinoma, the mutation spectra were different and there was no mutation overlap for *H-ras* and *β-catenin* genes.

GENETIC TOXICOLOGY

Bromodichloroacetic acid was tested in bacterial gene mutation assays. These *in vitro* assays were conducted with the same lot of bromodichloroacetic acid that was used in the 2-year bioassays, positive responses were seen in *S. typhimurium* strains, TA97, TA98, and TA100 and the *Escherichia coli* strain WP2 *uvrA*/pkM101 over a concentration range of 500 to 6,000 µg/plate in the absence of S9 (Table E1). With S9, equivocal responses were seen with the three *S. typhimurium* strains and a positive response was observed in the *E. coli* strain.

In vivo, no significant increases in the frequencies of micronucleated normochromatic erythrocytes were observed in blood samples from male or female B6C3F1/N mice administered bromodichloroacetic acid (concentration range of 62.5 to 1,000 mg/L) in drinking water for 3 months (Table E2). Small increases were seen in the percentage of polychromatic erythrocytes (reticulocytes) among total red blood cells in the highest exposure concentration groups of male and female mice, but these were not statistically significant.

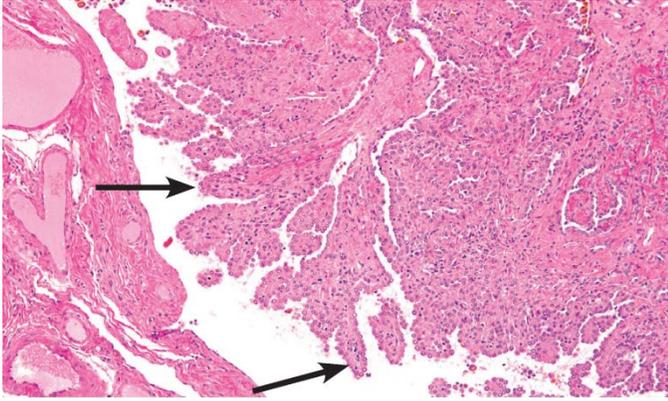


PLATE 1

Malignant mesothelioma growing on the epididymis of a male F344/NTac rat exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note the plump papillary projections of mesothelial cells (arrows) covering a fibrovascular core. H&E



PLATE 2

Bone marrow angiectasis in a male F344/NTac rat exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note the presence of multiple larger blood-filled areas that exceeded the normal vascular boundary (arrows). H&E

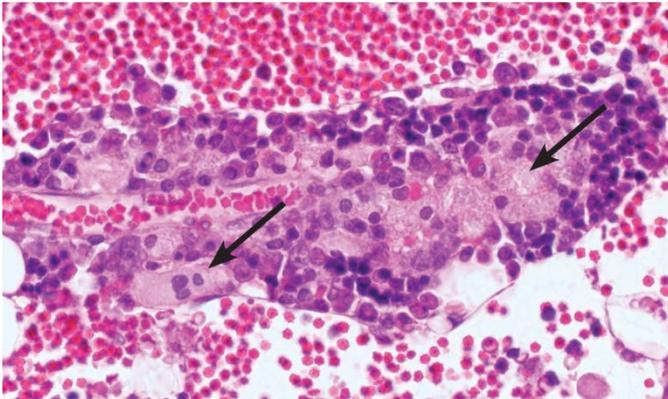


PLATE 3

Histiocytic cell infiltration in the bone marrow of a male F344/NTac rat exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note the collections of histiocytic cells (arrows) close to angiectatic cavities. H&E

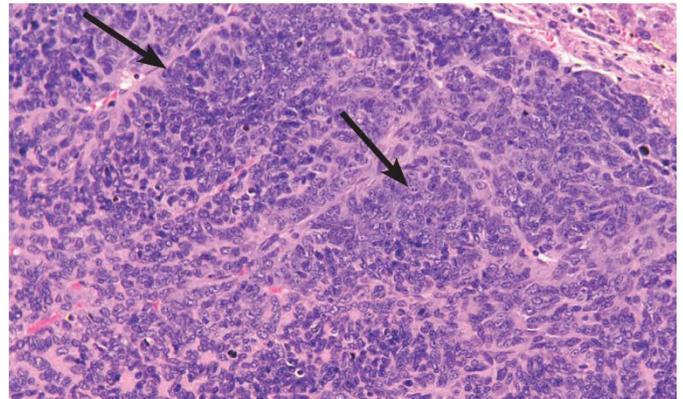


PLATE 4

Hepatoblastoma in the liver of a male B6C3F1/N mouse exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note hepatoblastoma composed of sheets of small, elongate, hyperchromatic cells (arrows) that often formed rosettes or palisaded around vessels. H&E

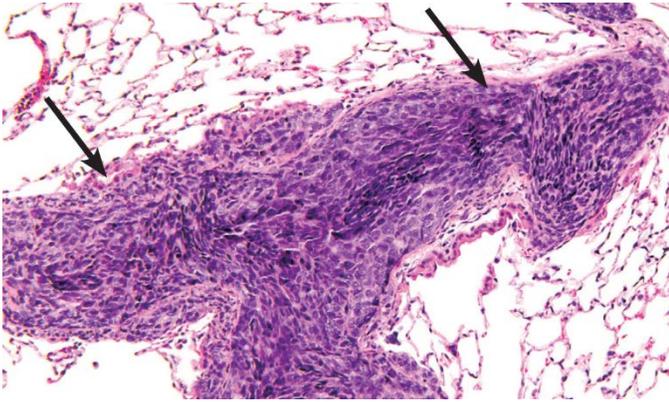


PLATE 5

Metastatic hepatoblastoma in the lung of the same male B6C3F1/N mouse shown in Plate 4. Note the neoplastic hepatoblastoma cells within a pulmonary blood vessel (arrows). H&E

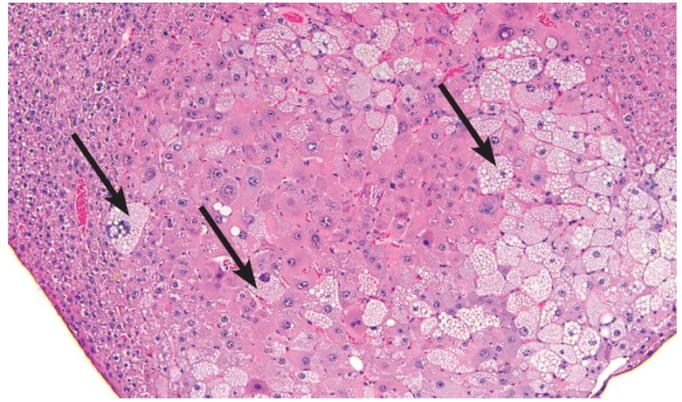


PLATE 6

Focus of cellular alteration, atypical, in the liver of a male B6C3F1/N mouse exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note nodular aggregates of enlarged hepatocytes that merge with the surrounding parenchyma and cause slight compression. The hepatocytes within the focus have solid eosinophilic and mixed contents (i.e., distended by multiple small discrete lipid vacuoles – microvesicular vacuolation - arrows), marked cytomegaly, and intranuclear invaginations and multiple prominent nucleoli. H&E

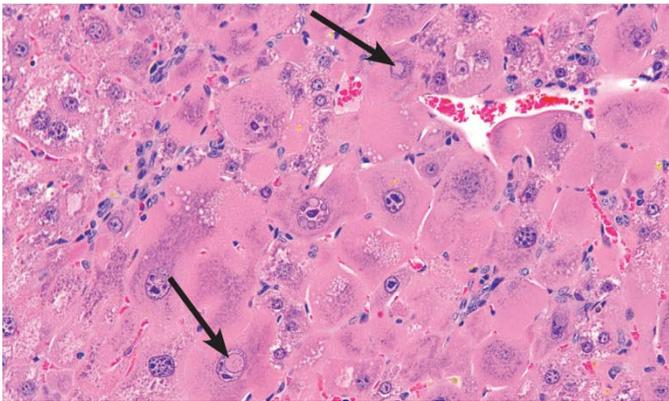


PLATE 7

Focus of cellular alteration, atypical, in the liver of a male B6C3F1/N mouse exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note nodular aggregates of enlarged hepatocytes that merge with the surrounding parenchyma and cause slight compression. The hepatocytes within the focus have solid eosinophilic contents, marked cytomegaly, intranuclear invaginations (arrows), and multiple prominent nucleoli. H&E

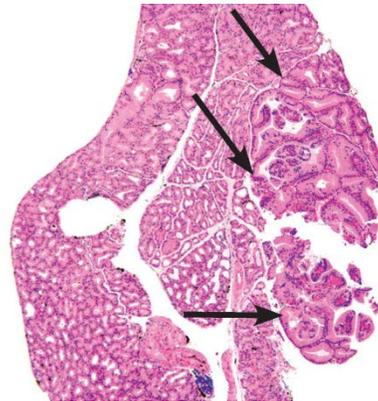


PLATE 8

Hyperplasia in the Harderian gland of a male B6C3F1/N mouse exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note an area of cellular proliferation that did not distort the architecture of the gland and did not compress the surrounding parenchyma (arrows). The epithelial cells within hyperplastic lesions maintained one cell layer of thickness and did not display cellular atypia. H&E

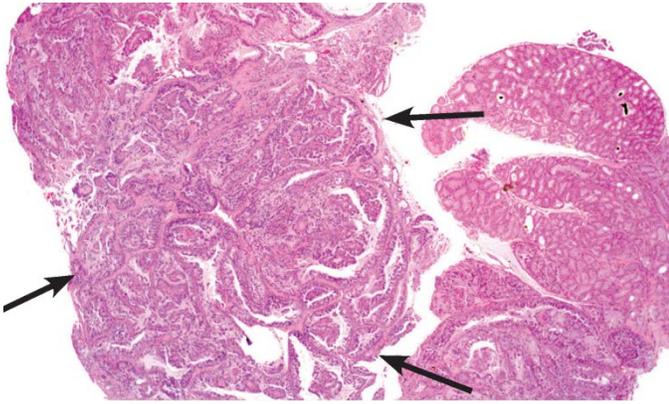


PLATE 9

Adenoma in the Harderian gland of a male B6C3F1/N mouse exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note a well-demarcated proliferative lesion (arrows) that compresses the adjacent parenchyma and does not maintain tissue architecture. H&E

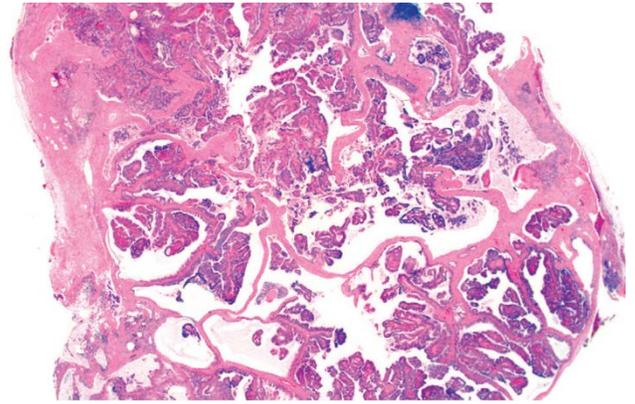


PLATE 10

Carcinoma in the Harderian gland of a male B6C3F1/N mouse exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note large, poorly demarcated, locally invading mass that distorted the glandular architecture. H&E

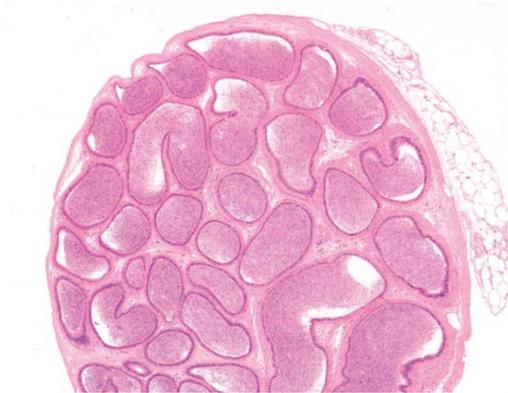


PLATE 11

Normal contents of spermatozoa in a male control B6C3F1/N mouse from the 2-year drinking water study of bromodichloroacetic acid. H&E

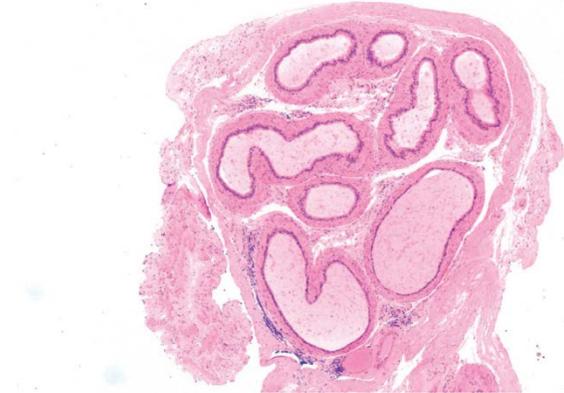


PLATE 12

Hypospermia in the epididymis of a male B6C3F1/N mouse exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note the reduced numbers of spermatozoa within tubular lumens when compared with a normal epididymis from a control mouse (Plate 11). H&E

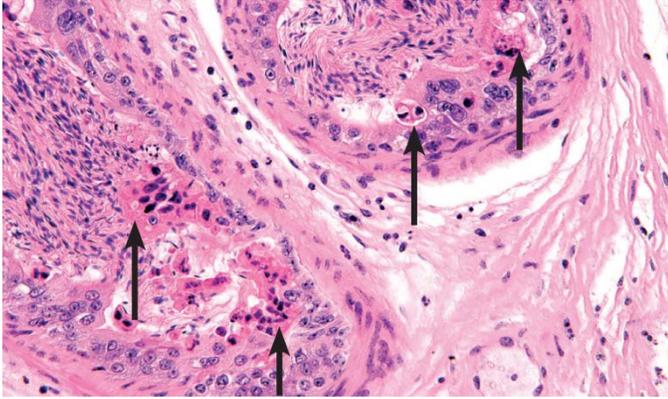


PLATE 13

Epithelium degeneration in the epididymis of a male B6C3F1/N mouse exposed to 1,000 mg/L bromodichloroacetic acid in drinking water for 2 years. Note the cellular changes affecting the epithelium lining the tubules of the epididymis (arrows) including individual cell necrosis, sloughing of clusters of eosinophilic degenerative cells, excessive karyomegaly, disorganization of the epithelial layer, and flattening of abnormal cells. H&E

DISCUSSION AND CONCLUSIONS

Bromodichloroacetic acid is a haloacetic acid that is produced by the interaction of naturally occurring bromides in water reacting with humic acids and halogenated oxidants used in water disinfection. There are nine chlorinated, brominated, or mixed chloro/bromo haloacetic acids. The United States Environmental Protection Agency (USEPA) regulates five haloacetic acids in drinking water at a level of 60 µg/L. Bromodichloroacetic acid is not included in this standard, and no drinking water standard has been developed for this chemical. In a nationwide survey, bromodichloroacetic acid was found in water distribution systems as well as finished water at concentrations up to 15 µg/L (Weinberg *et al.*, 2002). Bromodichloroacetic acid typically makes up 1% to 20% of the haloacetic acids present in water distribution systems and finished water (Weinberg *et al.*, 2002). The disinfection by-products were nominated to the NTP by the USEPA and the American Water Works Association Research Foundation for evaluation of their carcinogenic potential. Bromodichloroacetic acid is the latest in this class to be evaluated. There were no previous *in vivo* toxicity studies available for bromodichloroacetic acid.

Including the present study, cancer bioassays in rats and/or mice are available for six of the nine haloacetic acids. No cancer data for bromoacetic acid, dibromochloroacetic acid, or tribromoacetic acid are available in the published literature. The carcinogenic potential of monochloroacetic acid in drinking water was evaluated in rats and mice by the NTP and no evidence of carcinogenicity was observed (NTP, 1992). Dichloroacetic acid in drinking water increased the incidences of liver neoplasms in male mice (Bull *et al.*, 1990; DeAngelo *et al.*, 1999) and rats (DeAngelo *et al.*, 1996). Dibromoacetic acid exposure in drinking water was carcinogenic in male (liver and lung) and female (liver) mice and male (mesothelioma and leukemia) and female (mesothelioma) rats (NTP, 2007c). The potential carcinogenic effects of bromochloroacetic acid in drinking water were evaluated by the NTP and clear evidence of carcinogenicity was observed in male (hepatocellular carcinoma and hepatocellular adenoma or carcinoma) and female (hepatocellular adenoma or carcinoma) mice and male (malignant mesothelioma and large intestine adenoma) and female (large intestine adenoma and mammary gland fibroadenoma) rats (NTP, 2009). Trichloroacetic acid induced liver neoplasms in male mice but not in male rats. Given some of the limitations of

these data, it appears that the monohalogenated chemicals are less likely to be carcinogenic and that the addition of bromine increases the potency and carcinogenicity of the haloacetic acid. A common neoplasm site for haloacetic acids is the liver in male and female mice, and mesotheliomas are common neoplasms in male rats.

Drinking water concentrations of bromodichloroacetic acid ranged from 62.5 to 1,000 mg/L in the current 2-week and 3-month studies in F344/N rats. These concentrations were based on earlier studies of haloacetic acids. In the current 2-week and 3-month rat studies, exposure to bromodichloroacetic acid in drinking water produced minimal effects and no chemical-related gross or histopathology findings were observed. In the 3-month study, there were minor changes (less than 10% from control) in organ weights and/or organ weight to body weight ratios in the liver of male rats and the kidney of female rats, which were not considered biologically significant. Based on the minimal effects of bromodichloroacetic acid in the subchronic studies, the drinking water concentrations of bromodichloroacetic acid were set at 0, 250, 500 and 1,000 mg/L for the 2-year study in F344/NTac rats. These concentrations resulted in average daily doses in the 2-year study of approximately 11, 21, and 43 mg bromodichloroacetic acid/kg body weight to males and approximately 13, 28, and 57 mg/kg to females.

In female F344/NTac rats exposed to 1,000 mg/L for 2 years, body weights were 90% of controls starting on day 85 and decreased to about 80% of controls by day 365. Exposure to bromodichloroacetic acid in the drinking water significantly decreased survival at the two highest exposure concentrations in female rats. Sixty-eight percent of the control females survived to the end of the study, while only 14% and 4% of the females exposed to 500 and 1,000 mg/L, respectively, survived to the end of the study. The morbidity associated with bromodichloroacetic acid exposure was due to increased incidences of mammary gland fibroadenoma and carcinoma.

Bromodichloroacetic acid exposure caused clear exposure concentration-dependent increases in incidences of mammary gland fibroadenoma (including multiples) and carcinoma in female F344/NTac rats. The incidences of fibroadenoma in females were significantly increased at all exposure concentrations tested, and there was a statistically significant increase in the incidence of carcinoma in the 1,000 mg/L group. Bromodichloroacetic acid also caused an exposure concentration-dependent increase in the incidences of mammary gland hyperplasia. The

determination of clear evidence is supported by the exposure concentration-dependent increase in the incidences of fibroadenomas that are associated with possible progression to mammary gland carcinomas.

In males, the incidences of mammary gland fibroadenoma were greater in the exposed animals compared to the controls, but these differences did not reach statistical significance. Due to the low background incidence of these tumors in male rats and the clear evidence of mammary gland neoplasms in the females, these neoplasms may also have been related to bromodichloroacetic acid exposure.

Examination of gene expression using PCR arrays (Appendix N) indicated that the female mammary gland adenocarcinomas from bromodichloroacetic acid-treated animals were enriched for *Mmp2*, *Mmp9*, *Id1*, *Thbs1*, *Hic1*, *Adam23*, *Vegfa*, and *Cdh13* compared to spontaneous mammary gland adenocarcinomas from control female F344/N and Sprague Dawley rats from other NTP studies. Five of these eight genes are associated with *Tgfb* pathway signaling, including its effects on matrix remodeling, mammary cancer progression, tumor invasion, and metastasis (*Mmp2*, *Mmp9*, *Id1*, *Vegfa*, *Thbs1*). The overrepresentation of *Tgfb* mediators that were significantly upregulated in mammary gland adenocarcinomas from bromodichloroacetic acid-exposed female F344/N rats compared to spontaneous mammary gland adenocarcinomas may suggest a correlation between this pathway and the increased incidences of proliferative mammary gland lesions observed in bromodichloroacetic acid-exposed animals. These data suggest that mammary gland carcinogenesis in bromodichloroacetic acid-exposed animals may be influenced in part by *Tgfb*-dependent mechanisms.

In the NTP study of bromochloroacetic acid, the incidences of multiple mammary gland fibroadenoma in female rats were significantly increased at the two highest exposure concentrations; these lesions were considered exposure-related (NTP, 2009). Bromochloroacetic acid and bromodichloroacetic acid are mixed bromo/chloro substituted haloacetic acids. While the mechanism for these increased incidences of fibroadenoma is uncertain, the structural and toxicologic similarity of these two chemicals further supports the determination of clear evidence for increased mammary gland neoplasms caused by bromodichloroacetic acid exposure. The remaining mixed bromo/chloroacetic acids and dibromochloroacetic acid have not been evaluated for carcinogenicity in rodents.

Exposure to bromodichloroacetic acid in the drinking water was clearly related to increased incidences of malignant mesotheliomas in male rats. Support for the determination of clear evidence of carcinogenicity is the exposure concentration-dependent increase in the incidences of malignant mesothelioma that were found primarily in the epididymis, testis, seminal vesicle, and prostate gland of male F344/NTac rats. In addition, malignant mesotheliomas were found in the male interim evaluation rats at 13 months. Despite group sizes of only eight at the 13-month necropsy, one 500 mg/L male and five 1,000 mg/L males had malignant mesothelioma, and the incidence in the 1,000 mg/L group was significantly greater than the control incidence. These findings were similar to those observed with dibromoacetic acid (NTP, 2007c) and bromochloroacetic acid (NTP, 2009). The similarity of the structures and the carcinogenic effect increases confidence that exposure to haloacetic acids causes an increase in the incidences of malignant mesothelioma in rats and provides further support for clear evidence of carcinogenicity.

Chemically induced mesotheliomas in rats have been observed with 19 test chemicals out of the 580 tested at the NTP. This neoplasm has been found predominantly in male rats compared to female rats or male or female mice. Positive male rat findings were reported in 17 of the 19 studies with an increase in mesotheliomas. These chemicals are structurally diverse, ranging from persistent organic pollutants such as pentachlorophenol to rapidly metabolized chemicals such as glycidol. Of these 17 studies, including the current study of bromodichloroacetic acid, male rat mesotheliomas were chemically induced; 13 of these chemicals also induced clear or some evidence of female rat mammary gland carcinogenic activity. The exceptions to this list are pentachlorophenol, phenoxybenzamine hydrochloride, and dibromoacetic acid. Phenoxybenzamine hydrochloride was administered by intraperitoneal injection and is the only chemical that increased the incidences of mesothelioma in both male and female rats and mice, and it is also the only chemical that induced only mesotheliomas (NTP, 1978); all other chemicals that induced mesothelioma also induced tumors at multiple sites. In the pentachlorophenol study, male rat mesotheliomas were only observed in animals from the stop-exposure study and not in animals exposed for the full 2 years (NTP, 1999). Conversely, there are 31 chemicals that resulted in clear or some evidence of mammary gland carcinogenic activity in female rats with 13 of 31 inducing male rat mesothelioma. If a chemical induced male rat mesothelioma and female rat mammary gland adenomas or carcinomas, it was also positive for carcinogenicity in mice, although there was no consistent target tissue or tumor finding. The mechanistic relationship, if any, between male rat mesotheliomas and female rat mammary gland carcinogenesis is undetermined.

In male F344/NTac rats, there were clear exposure concentration-dependent increases in the combined incidences of all cutaneous epithelial neoplasms including squamous cell papilloma, keratoacanthoma, basal cell adenoma, basal cell carcinoma, or squamous cell carcinoma, and the increase was statistically significant at the highest exposure concentration. The incidence of keratoacanthoma (including multiples) was significantly increased at the highest exposure concentration tested as was the incidence of the combination of basal cell adenoma or carcinoma. Because the squamous cell papillomas, keratoacanthomas, basal cell adenomas, basal cell carcinomas, and squamous cell carcinomas are all epithelial neoplasms in the skin, the increased incidences of the combined neoplasms increases confidence that the neoplasms are indeed treatment related.

The incidences of fibroma (including multiples) in subcutaneous tissue also demonstrated an exposure concentration-dependent effect that was statistically significant in 1,000 mg/L male rats. These neoplasms appear to be related to systemic exposure because they did not occur around the mouth or snout, which may be considered sites of contact for a drinking water study. While bromodichloroacetic acid is the only haloacetic acid to increase incidences of neoplasms of the skin in rats, the statistically significant exposure concentration-dependent trends in these neoplasms support the determination of clear evidence of carcinogenicity.

Exposure to bromodichloroacetic acid also caused small increases in neoplasm incidences at several sites that have very low background rates. Because of the low background incidence, it is difficult to discriminate between spontaneous and chemically induced tumors. In order to confirm the low tumor incidence, extended histological evaluation was done on a case by case basis. In the present rat study, the brain was chosen for extended evaluation, and all tongues, not just those with macroscopic lesions, were examined microscopically.

In the original evaluation of the brain, sporadic neoplasms were observed in F344/NTac rats, and an extended evaluation was performed on this tissue to rule out any missed microscopic lesions. In the males, there was an exposure concentration-dependent increase in the incidences of glioma and oligodendroglioma. The incidences for the combined neoplasms from both evaluations were 1/50, 1/50, 4/50, and 3/50 in male rats and 1/50, 0/50, 3/50, and 1/50 in female rats. While exposed groups of animals had increased incidences of these neoplasms, the rates were very low. However, the 2013 historical control database indicates that the occurrence of oligodendrogliomas or

gliomas was 0/699 male F344/N rats and 3/700 female F344/N rats. In the 2009 historical control database, the occurrence of oligodendrogliomas or gliomas was 7/1,397 male F344/N rats and 8/1,350 female F344/N rats. There were no studies in males that had an occurrence of oligodendrogliomas or gliomas of more than 1/50. Occurrences of oligodendrogliomas or gliomas in male F344/NTac rats of 4/50 and 3/50 were observed at the 500 mg/L and 1,000 mg/L concentrations, respectively, in the current study. While these neoplasms were not significantly increased, they are greater than any historical controls in the past 10 years; and bromodichloroacetic acid is genotoxic. This was considered some evidence of carcinogenicity at this site.

Similarly, three males in the 500 and 1,000 mg/L groups had a squamous cell papilloma or squamous cell carcinoma of the oral cavity (oral mucosa or tongue). The carcinomas were associated with hyperplasia of the lingual epithelium. Because bromodichloroacetic acid is genotoxic, the oral cavity is the one of the portals of entry, and these are rare neoplasms (in F344/N male rats, the 2013 historical control range is 0% to 2% with an incidence of 5/699 animals and in the 2009 historical control range is 0% to 2% with an incidence of 11/1,398), this was considered some evidence of carcinogenicity at this site.

The large intestine was a target site for bromochloroacetic acid in male and female F344/N rats with adenoma incidences of 4/50 and 7/50 in 1,000 mg/L males and females, respectively (NTP, 2009) with no adenomas present in the control male or female rats. In the current study in F344/NTac rats, the background rates of large intestine adenoma were 0/50 for males and 1/50 for females. At 500 and 1,000 mg/L in male rats and 1,000 mg/L in female rats, the incidences of large intestine adenoma were 2/50 each. This was considered equivocal evidence of carcinogenicity because, in F344/N male rats, the 2013 historical control range is 0% with an incidence of 0/699 animals, the 2009 historical control range was 0% to 2% with an incidence of 3/1,398, and neither database ever had a study with more than 1/50 large intestine adenomas. These neoplasms may have been related to chemical exposure; however, while these incidences of large intestine adenoma are greater than those in the concurrent controls, the overall incidences are relatively small.

Exposure to bromodichloroacetic acid for 2 years was associated with additional nonneoplastic effects in the liver, bone marrow, and spleen of F344/NTac rats. Significantly increased incidences of hematopoietic cell proliferation

were noted in the liver of 500 and 1,000 mg/L females; the lesion was characterized by multifocal aggregations of erythroid and myeloid precursors. Significantly increased incidences of hematopoietic cell proliferation were also noted in the spleen of female rats exposed to 500 and 1,000 mg/L. In the bone marrow, exposure concentration-dependent increased incidences of angiectasis, a dilation of the blood vessels, and hematopoietic hyperplasia were observed in male and female rats.

Similar to F344/N rats, drinking water concentrations of bromodichloroacetic acid ranged from 62.5 to 1,000 mg/L in the 2-week and 3-month studies in mice. In the 2-week and 3-month studies, exposure to bromodichloroacetic acid did not induce chemical-related gross or clinical findings. While histopathology findings were observed in the liver of females and kidney of males in the 3-month study, these effects were considered minor. Based on the minimal effects of bromodichloroacetic acid in the subchronic studies, the drinking water concentrations of bromodichloroacetic acid were set at 0, 250, 500, and 1,000 mg/L for the 2-year study in mice. In mice, these concentrations resulted in average daily doses in the 2-year study of approximately 23, 52, and 108 mg bromodichloroacetic acid/kg body weight to males and approximately 17, 34, and 68 mg/kg to females.

In the 2-year study, exposure to bromodichloroacetic acid in the drinking water resulted in significantly decreased survival in male mice exposed to 500 or 1,000 mg/L. The most common cause of early death appeared to be liver neoplasms. In male mice, there was clear evidence of a carcinogenic effect based on increased incidences of hepatocellular carcinoma (including multiples) and hepatoblastoma (including multiples); the incidences of these neoplasms were significantly increased at all exposure concentrations. The exposure concentration-dependent increased incidences of hepatocellular carcinoma and hepatoblastoma support the determination of clear evidence of hepatocarcinogenicity of bromodichloroacetic acid in male mice. In females, there were exposure concentration-dependent increases in incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma. Statistically significant increases were observed in the incidences of adenoma in all exposed groups, carcinoma in the 500 and 1,000 mg/L groups, and hepatoblastoma in the 1,000 mg/L group. For the combination of all three neoplasms, statistically significant increases were observed in all exposed groups. The exposure concentration-dependent increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma support the determination of clear evidence of hepatocarcinogenicity of bromodichloroacetic acid in female mice.

The mouse liver is a common target site for the carcinogenic effects of the trihalomethanes and haloacetic acids that occur in drinking water as disinfection by-products. The proposed mechanism of hepatocarcinogenicity of these chemicals involves both genotoxic and non-genotoxic mechanisms. *In vitro*, bromodichloroacetic acid was mutagenic in *Salmonella typhimurium* strains TA97, TA98, and TA100 and the *Escherichia coli* strain WP2 *uvrA*/pkM101 over a concentration range of 500 to 6,000 µg/plate in the absence of S9. With S9, equivocal responses were seen with the three *S. typhimurium* tester strains, and a positive response was observed in the *E. coli* strain. This data supports the role of a genotoxic mechanism for the mouse liver neoplasms due to bromodichloroacetic acid.

Non-genotoxic mechanisms for the haloacetic acids may involve activation of PPAR α . Trichloroacetic acid is thought to be carcinogenic, in part due to activation of PPAR α (Corton, 2008). In the 2-week studies, activation of PPAR α by bromodichloroacetic acid was evaluated by determining hepatic acetyl-CoA hydrolase activity in F344/N rats and mice. Previous studies have used induction of hepatic acetyl-CoA hydrolase as a marker for PPAR α activation (Nakanishi *et al.*, 1993). Acetyl-CoA hydrolase activity was not consistently induced by bromodichloroacetic acid in either rats or mice. If bromodichloroacetic acid activated PPAR α , induction of hepatic peroxisome proliferation would have been evident in either the 3-month or 2-year studies. Because neither the induction of an enzymatic marker of PPAR α activation nor peroxisome proliferation was observed in either rats or mice, it is unlikely that bromodichloroacetic acid is a PPAR α activator. These findings are consistent with the literature reports that bromodichloroacetic acid does not induce PPAR α activation even at high doses (Xu *et al.*, 1995).

The increased incidences of hepatic neoplasms were associated with increased incidences of atypical foci of cellular alteration in mice. This lesion was observed at necropsy at the 14-month interim evaluation and consisted of eosinophilic cell focus and mixed cell focus, which occurred as either single or multifocal nodular aggregates of abnormally enlarged hepatocytes that merged with the surrounding parenchyma and occasionally caused slight compression. In males at 2 years, this lesion occurred more frequently and the incidences were significantly increased in all exposed groups, while in females, the incidences of this lesion were significantly increased in the

500 and 1,000 mg/L groups. At 2 years, this lesion did not occur in the control mice. The relationship between this lesion and the hepatic neoplasms is undetermined.

Key differences in gene expression were observed between bromodichloroacetic acid-exposed adjacent nontumor liver tissue compared to control liver involving genes mostly involved in metabolic pathways, cell death, cell growth and proliferation, and neoplasia, suggesting that bromodichloroacetic acid causes specific toxic and carcinogenic effects in the liver of exposed B6C3F1/N mice. Gene changes in bromodichloroacetic acid-treated adjacent nontumor liver tissue are consistent with neoplastic signaling and may suggest microenvironmental changes preceding neoplastic transformation due to chemical exposure or a type of “field cancerization.” However, given the fact that these bromodichloroacetic acid-exposed nontumor liver tissues were isolated from sections adjacent to hepatocellular carcinoma, these gene expression changes may represent microenvironmental effects of the adjacent hepatocellular carcinoma on the histologically normal adjacent tissue.

In bromodichloroacetic acid-exposed hepatocellular carcinoma, a number of genes were altered that were either classic oncogenes involved in hepatocarcinogenesis or alterations in genes that were suggestive of loss of normal hepatic function. In terms of dysregulated cancer pathways, well known pathways involved in mouse hepatic carcinogenesis were altered including cell cycle G2/M checkpoint regulation and apoptosis regulation.

Further, bromodichloroacetic acid-treated mouse hepatoblastomas are markedly different from adjacent hepatocellular carcinoma in terms of their morphology, global gene expression, and *Hras/Ctnnb1* mutation profiles. Mouse hepatoblastoma and human hepatoblastoma share significant similarities in global gene expression, including dysregulation of genes involved in *Wnt*/ β -catenin signaling, embryonic/stem cell pluripotency pathways, metabolic dysregulation, and expression of genomic imprinting genes. Furthermore, meta-analysis using Nextbio shows that mouse hepatoblastoma is very similar to mouse early embryonic liver in terms of gene expression profile. These findings suggest that hepatoblastoma and hepatocellular carcinoma are very different entities, likely arising from the same hepatic lineage, but hepatoblastoma arising as a result of transformation of a hepatic stem or multipotential progenitor cell. However, further studies are required to further understand the molecular tumorigenesis of hepatoblastoma and hepatocellular carcinoma.

Exposure to bromodichloroacetic acid increased the incidences of glandular epithelial hyperplasia, adenoma, and the combination of adenoma or carcinoma in the Harderian gland of male mice. The associated increased incidences of Harderian gland adenoma and the combination of adenoma or carcinoma were considered clear evidence of carcinogenicity of bromodichloroacetic acid. The determination of clear evidence is supported by the exposure concentration-dependent increase in the incidences of adenoma, at 500 and 1,000 mg/L, and the exposure concentration-dependent increased incidences of glandular epithelial hyperplasia.

Bromodichloroacetic acid exposure increased the incidences and severities of testicular and epididymal atrophy in male mice; the incidences were significantly increased in the 500 and 1,000 mg/L groups for the testes and in all exposed groups for the epididymis. Incidences of hypospermia (1,000 mg/L) and epithelium degeneration (500 and 1,000 mg/L) were also significantly increased in the epididymis of males treated with bromodichloroacetic acid.

The testis was not a target in the 3-month study in mice. There were minimal changes in left testis weight, but not right testis weight, in the 3-month study in F344/N rats, and no testicular lesions were observed in the 2-year study in F344/NTac rats. Bromochloroacetic acid was evaluated in a reproductive and developmental screen by the NTP and was negative in males but caused a 16% decrease in total implants per litter and a 50% decrease in the number of live fetuses per litter in female Sprague Dawley rats following peri-conception exposure (NTP, 1998).

Dibromoacetic acid has been shown to decrease the number of sperm in the caput epididymis in male rats, resulting in decreased epididymal weight (Linder *et al.*, 1994b). Bromochloroacetic acid also decreased the number and progressive motility of cauda epididymal sperm in male rats (Klinefelter *et al.*, 2002). Similar findings were also reported for dichloroacetic acid (Linder *et al.*, 1997a).

Bromodichloroacetic acid is not routinely monitored by water suppliers. However, the Environmental Working Group (2009) has developed a database of chemical analysis of 47,576 water suppliers, of which 938 have tested for bromodichloroacetic acid from 2004 to 2009. The highest average level of bromodichloroacetic acid reported in drinking water was 11.12 µg/L with a range of 7.22 to 16.85 µg/L. These levels are similar to those reported by Weinberg *et al.* (2002), in which bromodichloroacetic acid was reported up to 15 µg/L in 12 water supplies. At the highest end of the range, 16.85 µg/L, the concentration is approximately 15,000 times lower than the concentration used as the lowest exposure in the present study. In addition, if an adult male ingests 2 L of water/day and weighs

70 kg, then the average daily exposure for adult males is 0.48 µg/kg and is approximately 23,000 times lower than the lowest exposure of male rats from the present study. While these margins of exposure may be high, it should be noted that the lowest exposure used in the current study had statistically significant increases in a number of neoplasms, and bromodichloroacetic acid is genotoxic.

There are nine haloacetic acids of which carcinogenicity data are available for seven either from the NTP or in the published literature. For the di- and trihaloacetic acids, the mouse liver is one of the target sites for dibromoacetic acid, bromochloroacetic acid, bromodichloroacetic acid, dichloroacetic acid, and trichloroacetic acid. Of interest is the similarity in neoplasias between bromochloroacetic acid and bromodichloroacetic acid. Both chemicals are associated with malignant mesotheliomas in male rats; mammary gland fibroadenomas in female rats; and hepatocellular adenomas, hepatocellular carcinomas, and hepatoblastomas in male and female mice. The finding of consistent neoplasias for these chemicals further supports the determination of clear evidence of carcinogenicity for bromodichloroacetic acid. Bromodichloroacetic acid exposure also increased the incidences of cutaneous and subcutaneous epithelial skin neoplasms in male rats and Harderian gland tumors in male mice, which were not increased with other haloacetic acids. The remaining mixed bromo/chloro acetic acid, dibromochloroacetic acid has not been evaluated for carcinogenicity. The NTP has evaluated a number of the trihalomethanes and haloacetic acids that are drinking water disinfection by-products. In general, the higher the halogenation of the haloacetic acid, the greater its carcinogenic potential. In addition, it would appear that the mixed chloro/bromo haloacetic acids are associated with a greater number of neoplasias than the chlorinated- or brominated-only analogues.

CONCLUSIONS

Under the conditions of these 2-year studies, there was *clear evidence of carcinogenic activity** of bromodichloroacetic acid in male F344/NTac rats based on increased incidences of malignant mesothelioma and the combined incidences of epithelial tumors of the skin. Increased incidences of glioma or oligodendroglioma (combined) of the brain, squamous cell papilloma or squamous cell carcinoma of the oral cavity (oral mucosa or tongue) and subcutaneous fibromas were also related to bromodichloroacetic acid exposure. The occurrences of adenoma of the large intestine and fibroadenoma of the mammary gland may have been related to bromodichloroacetic acid exposure. There was *clear evidence of carcinogenic activity* of bromodichloroacetic acid in female

F344/NTac rats based on increased incidences of fibroadenoma and carcinoma of the mammary gland.

The increased incidences of glioma or oligodendroglioma (combined) of the brain were also

related to bromodichloroacetic acid exposure. There was *clear evidence of carcinogenic activity*

of bromodichloroacetic acid in male B6C3F1/N mice based on increased incidences of hepatocellular carcinoma and hepatoblastoma and increased incidences of adenoma or carcinoma (combined) of the Harderian gland. There was *clear evidence of carcinogenic activity* of bromodichloroacetic acid in female B6C3F1/N mice based on increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma.

Exposure to bromodichloroacetic acid for 2 years resulted in increased incidences of nonneoplastic lesions in the bone marrow and liver of male and female rats, spleen of female rats, liver of male and female mice, and testis and epididymis of male mice.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 16.

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APPENDIX A
SUMMARY OF LESIONS IN MALE RATS
IN THE 2-YEAR DRINKING WATER STUDY
OF BROMODICHLOROACETIC ACID

TABLE A1	Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid	A-2
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TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Disposition Summary				
Animals initially in study	66	66	66	66
<i>6-Month interim evaluation^b</i>	8	8	8	8
<i>13-Month interim evaluation^b</i>	8	8	8	8
Early deaths				
Moribund	26	23	20	27
Natural deaths	5	6	5	4
Survivors				
Terminal kill	19	21	25	19
Animals examined microscopically	66	66	66	66
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Intestine large, cecum	(50)	(50)	(49)	(50)
Adenoma			1 (2%)	
Intestine large, colon	(50)	(50)	(50)	(50)
Adenoma			1 (2%)	1 (2%)
Intestine large, rectum	(50)	(50)	(50)	(50)
Adenoma				1 (2%)
Intestine small, duodenum	(50)	(50)	(49)	(50)
Intestine small, ileum	(50)	(50)	(49)	(50)
Intestine small, jejunum	(50)	(50)	(49)	(50)
Liver	(50)	(50)	(50)	(50)
Cholangiocarcinoma			1 (2%)	
Fibrous histiocytoma	1 (2%)			
Hemangioma				1 (2%)
Hepatocellular adenoma		1 (2%)	2 (4%)	
Osteosarcoma, metastatic, bone				1 (2%)
Mesentery	(7)	(4)	(9)	(10)
Oral mucosa	(50)	(50)	(50)	(50)
Squamous cell carcinoma	1 (2%)			
Squamous cell papilloma			2 (4%)	1 (2%)
Pancreas	(50)	(50)	(49)	(50)
Adenoma, mixed cell				1 (2%)
Acinus, adenoma	1 (2%)		2 (4%)	1 (2%)
Salivary glands	(50)	(50)	(50)	(50)
Stomach, forestomach	(50)	(50)	(50)	(50)
Squamous cell papilloma	1 (2%)		1 (2%)	
Stomach, glandular	(50)	(50)	(50)	(50)
Tongue	(50)	(50)	(50)	(50)
Squamous cell carcinoma			1 (2%)	1 (2%)
Squamous cell papilloma				1 (2%)
Tooth	(1)	(0)	(0)	(0)
Cardiovascular System				
Blood vessel	(50)	(50)	(50)	(50)
Heart	(50)	(50)	(50)	(50)
Atrium, endocardium, schwannoma malignant				1 (2%)

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Adenoma	2 (4%)		4 (8%)	
Carcinoma		1 (2%)		
Osteosarcoma, metastatic, bone				1 (2%)
Adrenal medulla	(50)	(50)	(50)	(50)
Pheochromocytoma benign	8 (16%)	7 (14%)	8 (16%)	11 (22%)
Pheochromocytoma malignant		1 (2%)	2 (4%)	1 (2%)
Bilateral, carcinoma, metastatic, thyroid gland		1 (2%)		
Bilateral, pheochromocytoma benign	2 (4%)	1 (2%)	1 (2%)	
Islets, pancreatic	(50)	(50)	(49)	(50)
Adenoma		3 (6%)	2 (4%)	
Parathyroid gland	(49)	(48)	(46)	(44)
Pituitary gland	(50)	(49)	(49)	(48)
Pars distalis, adenoma	26 (52%)	29 (59%)	26 (53%)	20 (42%)
Pars distalis, carcinoma		1 (2%)		
Thyroid gland	(50)	(50)	(50)	(50)
Bilateral, C-cell, adenoma	2 (4%)		1 (2%)	
C-cell, adenoma	5 (10%)	9 (18%)	8 (16%)	8 (16%)
C-cell, carcinoma	1 (2%)	1 (2%)		
Follicular cell, adenoma	1 (2%)	3 (6%)		2 (4%)
Follicular cell, carcinoma				2 (4%)
General Body System				
None				
Genital System				
Epididymis	(50)	(50)	(50)	(50)
Preputial gland	(50)	(50)	(50)	(49)
Adenoma	2 (4%)	1 (2%)	5 (10%)	
Carcinoma	2 (4%)			1 (2%)
Bilateral, carcinoma		1 (2%)		
Prostate	(50)	(50)	(50)	(50)
Adenoma	1 (2%)	1 (2%)		
Osteosarcoma		1 (2%)		
Seminal vesicle	(50)	(50)	(50)	(50)
Testes	(50)	(50)	(50)	(50)
Bilateral, interstitial cell, adenoma	25 (50%)	20 (40%)	19 (38%)	20 (40%)
Interstitial cell, adenoma	9 (18%)	11 (22%)	12 (24%)	19 (38%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Lymph node	(5)	(2)	(8)	(7)
Mediastinal, fibrous histiocytoma	1 (20%)			
Renal, hemangiosarcoma			1 (13%)	
Lymph node, mandibular	(50)	(50)	(50)	(50)
Lymph node, mesenteric	(50)	(50)	(49)	(50)
Spleen	(50)	(50)	(49)	(50)
Hemangiosarcoma			1 (2%)	1 (2%)
Osteosarcoma, metastatic, bone				1 (2%)
Thymus	(48)	(47)	(48)	(49)
Thymoma benign				2 (4%)

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Integumentary System				
Mammary gland	(49)	(50)	(49)	(49)
Fibroadenoma		2 (4%)	3 (6%)	1 (2%)
Skin	(50)	(50)	(50)	(50)
Basal cell adenoma			4 (8%)	4 (8%)
Basal cell carcinoma				1 (2%)
Keratoacanthoma	7 (14%)	3 (6%)	10 (20%)	12 (24%)
Keratoacanthoma, multiple				3 (6%)
Squamous cell carcinoma		1 (2%)	1 (2%)	
Squamous cell papilloma	2 (4%)	1 (2%)		1 (2%)
Pinna, squamous cell papilloma	1 (2%)			
Sebaceous gland, adenoma		2 (4%)	2 (4%)	2 (4%)
Subcutaneous tissue, fibroma	4 (8%)	5 (10%)	10 (20%)	13 (26%)
Subcutaneous tissue, fibroma, multiple		1 (2%)		2 (4%)
Subcutaneous tissue, fibrosarcoma	1 (2%)		2 (4%)	1 (2%)
Subcutaneous tissue, fibrosarcoma, multiple				1 (2%)
Subcutaneous tissue, fibrous histiocytoma	1 (2%)			
Subcutaneous tissue, lipoma	1 (2%)		1 (2%)	
Subcutaneous tissue, schwannoma malignant		1 (2%)		
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Osteoma	1 (2%)			1 (2%)
Osteosarcoma				2 (4%)
Tendon, fibrosarcoma				1 (2%)
Skeletal muscle	(4)	(0)	(2)	(0)
Fibroma			1 (50%)	
Fibrous histiocytoma	1 (25%)			
Nervous System				
Brain	(50)	(50)	(50)	(50)
Fibrous histiocytoma	1 (2%)			
Glioma		1 (2%)	2 (4%)	2 (4%)
Oligodendroglioma NOS			1 (2%)	1 (2%)
Meninges, granular cell tumor benign	2 (4%)		1 (2%)	
Peripheral nerve	(0)	(2)	(0)	(0)
Spinal cord	(0)	(2)	(0)	(0)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	3 (6%)	2 (4%)		1 (2%)
Alveolar/bronchiolar carcinoma				1 (2%)
Carcinoma, metastatic, Harderian gland	1 (2%)		1 (2%)	
Carcinoma, metastatic, thyroid gland		1 (2%)		
Fibrous histiocytoma	1 (2%)			
Osteosarcoma, metastatic, bone				1 (2%)
Squamous cell carcinoma	1 (2%)			1 (2%)
Nose	(50)	(50)	(50)	(50)
Trachea	(50)	(50)	(50)	(50)

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Special Senses System				
Ear	(1)	(0)	(0)	(0)
Neural crest tumor	1 (100%)			
Eye	(50)	(50)	(50)	(50)
Iris, melanoma malignant				1 (2%)
Harderian gland	(50)	(50)	(50)	(50)
Adenoma		1 (2%)		
Carcinoma	1 (2%)		1 (2%)	
Zymbal's gland	(1)	(2)	(0)	(2)
Carcinoma	1 (100%)	2 (100%)		2 (100%)
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Lipoma	1 (2%)			
Mesenchymal tumor malignant			1 (2%)	
Renal tubule, adenoma	1 (2%)		1 (2%)	1 (2%)
Urinary bladder	(50)	(50)	(50)	(50)
Systemic Lesions				
Multiple organs ^c	(50)	(50)	(50)	(50)
Leukemia mononuclear	15 (30%)	11 (22%)	15 (30%)	11 (22%)
Mesothelioma malignant	1 (2%)	12 (24%)	18 (36%)	37 (74%)
Neoplasm Summary				
Total animals with primary neoplasms ^d	49	48	48	50
Total primary neoplasms	139	137	174	199
Total animals with benign neoplasms	46	44	47	49
Total benign neoplasms	108	103	127	130
Total animals with malignant neoplasms	24	25	33	46
Total malignant neoplasms	30	33	44	66
Total animals with metastatic neoplasms	1	1	1	1
Total metastatic neoplasms	1	2	1	4
Total animals with uncertain neoplasms- benign or malignant	1	1	3	3
Total uncertain neoplasms	1	1	3	3

^a Number of core study animals examined microscopically at the site and the number of animals with neoplasm

^b Pathology data for interim evaluation animals are not presented in this table.

^c Number of animals with any tissue examined microscopically

^d Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Adrenal Cortex: Adenoma				
Overall rate ^a	2/50 (4%)	0/50 (0%)	4/50 (8%)	0/50 (0%)
Adjusted rate ^b	5.2%	0.0%	9.4%	0.0%
Terminal rate ^c	2/19 (11%)	0/21 (0%)	2/25 (8%)	0/19 (0%)
First incidence (days)	726 (T)	— ^e	611	—
Poly-3 test ^d	P=0.345N	P=0.232N	P=0.380	P=0.238N
Adrenal Medulla: Pheochromocytoma Benign				
Overall rate	10/50 (20%)	8/50 (16%)	9/50 (18%)	11/50 (22%)
Adjusted rate	24.9%	20.1%	21.2%	27.7%
Terminal rate	5/19 (26%)	5/21 (24%)	5/25 (20%)	6/19 (32%)
First incidence (days)	561	685	657	561
Poly-3 test	P=0.377	P=0.403N	P=0.444N	P=0.489
Adrenal Medulla: Benign or Malignant Pheochromocytoma				
Overall rate	10/50 (20%)	9/50 (18%)	11/50 (22%)	12/50 (24%)
Adjusted rate	24.9%	22.6%	25.9%	30.2%
Terminal rate	5/19 (26%)	6/21 (29%)	6/25 (24%)	7/19 (37%)
First incidence (days)	561	685	657	561
Poly-3 test	P=0.290	P=0.510N	P=0.560	P=0.388
Bone: Osteoma or Osteosarcoma				
Overall rate	1/50 (2%)	0/50 (0%)	0/50 (0%)	3/50 (6%)
Adjusted rate	2.6%	0.0%	0.0%	7.8%
Terminal rate	0/19 (0%)	0/21 (0%)	0/25 (0%)	2/19 (11%)
First incidence (days)	671	—	—	678
Poly-3 test	P=0.084	P=0.497N	P=0.485N	P=0.301
Brain, Glioma or Oligodendroglioma (Original Evaluation)				
Overall rate	0/50 (0%)	1/50 (2%)	3/50 (6%)	3/50 (6%)
Adjusted rate	0.0%	2.5%	7.1%	7.7%
Terminal rate	0/19 (0%)	1/21 (5%)	0/25 (0%)	0/19 (0%)
First incidence (days)	—	726 (T)	685	644
Poly-3 test	P=0.066	P=0.504	P=0.134	P=0.119
Brain, Glioma or Oligodendroglioma (Original and Extended Evaluations)				
Overall rate	1/50 (0%)	1/50 (2%)	4/50 (8%)	3/50 (6%)
Adjusted rate	2.6%	2.5%	9.5%	7.7%
Terminal rate	0/19 (0%)	1/21 (5%)	1/25 (4%)	0/19 (0%)
First incidence (days)	719	726 (T)	685	644
Poly-3 test	P=0.162	P=0.504	P=0.071	P=0.119
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	3/50 (6%)	2/50 (4%)	0/50 (0%)	1/50 (2%)
Adjusted rate	7.7%	5.1%	0.0%	2.6%
Terminal rate	2/19 (11%)	2/21 (10%)	0/25 (0%)	1/19 (5%)
First incidence (days)	664	726 (T)	—	726 (T)
Poly-3 test	P=0.159N	P=0.493N	P=0.105N	P=0.309N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	3/50 (6%)	2/50 (4%)	0/50 (0%)	2/50 (4%)
Adjusted rate	7.7%	5.1%	0.0%	5.2%
Terminal rate	2/19 (11%)	2/21 (10%)	0/25 (0%)	2/19 (11%)
First incidence (days)	664	726 (T)	—	726 (T)
Poly-3 test	P=0.368N	P=0.493N	P=0.105N	P=0.506N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Mammary Gland: Fibroadenoma				
Overall rate	0/50 (0%)	2/50 (4%)	3/50 (6%)	1/50 (2%)
Adjusted rate	0.0%	5.1%	7.2%	2.6%
Terminal rate	0/19 (0%)	2/21 (10%)	2/25 (8%)	1/19 (5%)
First incidence (days)	—	726 (T)	685	726 (T)
Poly-3 test	P=0.437	P=0.241	P=0.133	P=0.499
Oral Cavity (Oral Mucosa or Tongue): Squamous Cell Papilloma or Squamous Cell Carcinoma				
Overall rate	1/50 (2%)	0/50 (0%)	3/50 (6%)	3/50 (6%)
Adjusted rate	2.6%	0.0%	7.0%	7.7%
Terminal rate	0/19 (0%)	0/21 (0%)	1/25 (4%)	1/19 (5%)
First incidence (days)	593	—	442	642
Poly-3 test	P=0.105	P=0.498N	P=0.338	P=0.302
Pancreatic Islets: Adenoma				
Overall rate	0/50 (0%)	3/50 (6%)	2/49 (4%)	0/50 (0%)
Adjusted rate	0.0%	7.6%	4.9%	0.0%
Terminal rate	0/19 (0%)	2/21 (10%)	2/25 (8%)	0/19 (0%)
First incidence (days)	—	719	726 (T)	—
Poly-3 test	P=0.409N	P=0.121	P=0.251	— ^f
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	26/50 (52%)	29/49 (59%)	26/49 (53%)	20/48 (42%)
Adjusted rate	59.5%	66.8%	58.1%	49.3%
Terminal rate	11/19 (58%)	13/21 (62%)	14/24 (58%)	8/18 (44%)
First incidence (days)	477	516	442	499
Poly-3 test	P=0.118N	P=0.306	P=0.537N	P=0.228N
Pituitary Gland (Pars Distalis): Adenoma or Carcinoma				
Overall rate	26/50 (52%)	30/49 (61%)	26/49 (53%)	20/48 (42%)
Adjusted rate	59.5%	69.1%	58.1%	49.3%
Terminal rate	11/19 (58%)	14/21 (67%)	14/24 (58%)	8/18 (44%)
First incidence (days)	477	516	442	499
Poly-3 test	P=0.102N	P=0.226	P=0.537N	P=0.228N
Preputial Gland: Adenoma				
Overall rate	2/50 (4%)	1/50 (2%)	5/50 (10%)	0/49 (0%)
Adjusted rate	5.1%	2.5%	11.7%	0.0%
Terminal rate	1/19 (5%)	0/21 (0%)	2/25 (8%)	0/19 (0%)
First incidence (days)	657	644	442	—
Poly-3 test	P=0.328N	P=0.493N	P=0.254	P=0.243N
Preputial Gland: Adenoma or Carcinoma				
Overall rate	4/50 (8%)	2/50 (4%)	5/50 (10%)	1/49 (2%)
Adjusted rate	10.2%	5.0%	11.7%	2.6%
Terminal rate	1/19 (5%)	1/21 (5%)	2/25 (8%)	0/19 (0%)
First incidence (days)	657	644	442	610
Poly-3 test	P=0.219N	P=0.331N	P=0.551	P=0.185N
Skin: Squamous Cell Papilloma				
Overall rate	3/50 (6%)	1/50 (2%)	0/50 (0%)	1/50 (2%)
Adjusted rate	7.7%	2.5%	0.0%	2.6%
Terminal rate	2/19 (11%)	1/21 (5%)	0/25 (0%)	0/19 (0%)
First incidence (days)	680	726 (T)	—	674
Poly-3 test	P=0.196N	P=0.298N	P=0.105N	P=0.306N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Skin: Keratoacanthoma				
Overall rate	7/50 (14%)	3/50 (6%)	10/50 (20%)	15/50 (30%)
Adjusted rate	17.3%	7.6%	23.2%	37.1%
Terminal rate	1/19 (5%)	2/21 (10%)	5/25 (20%)	7/19 (37%)
First incidence (days)	544	716	493	621
Poly-3 test	P=0.003	P=0.162N	P=0.346	P=0.035
Skin: Squamous Cell Papilloma or Keratoacanthoma				
Overall rate	9/50 (18%)	4/50 (8%)	10/50 (20%)	16/50 (32%)
Adjusted rate	22.2%	10.1%	23.2%	39.4%
Terminal rate	2/19 (11%)	3/21 (14%)	5/25 (20%)	7/19 (37%)
First incidence (days)	544	716	493	621
Poly-3 test	P=0.010	P=0.120N	P=0.559	P=0.069
Skin: Squamous Cell Papilloma, Keratoacanthoma, or Squamous Cell Carcinoma				
Overall rate	9/50 (18%)	5/50 (10%)	11/50 (22%)	16/50 (32%)
Adjusted rate	22.2%	12.7%	25.6%	39.4%
Terminal rate	2/19 (11%)	3/21 (14%)	6/25 (24%)	7/19 (37%)
First incidence (days)	544	716	493	621
Poly-3 test	P=0.013	P=0.201N	P=0.459	P=0.069
Skin: Basal Cell Adenoma				
Overall rate	0/50 (0%)	0/50 (0%)	4/50 (8%)	4/50 (8%)
Adjusted rate	0.0%	0.0%	9.3%	10.4%
Terminal rate	0/19 (0%)	0/21 (0%)	1/25 (4%)	3/19 (16%)
First incidence (days)	—	—	513	653
Poly-3 test	P=0.012	—	P=0.074	P=0.059
Skin: Basal Cell Adenoma or Basal Cell Carcinoma				
Overall rate	0/50 (0%)	0/50 (0%)	4/50 (8%)	5/50 (10%)
Adjusted rate	0.0%	0.0%	9.3%	12.8%
Terminal rate	0/19 (0%)	0/21 (0%)	1/25 (4%)	3/19 (16%)
First incidence (days)	—	—	513	629
Poly-3 test	P=0.004	—	P=0.074	P=0.030
Skin: Squamous Cell Papilloma, Keratoacanthoma, Basal Cell Adenoma, Basal Cell Carcinoma, or Squamous Cell Carcinoma				
Overall rate	9/50 (18%)	5/50 (10%)	14/50 (28%)	19/50 (38%)
Adjusted rate	22.2%	12.7%	31.8%	46.8%
Terminal rate	2/19 (11%)	3/21 (14%)	7/25 (28%)	10/19 (53%)
First incidence (days)	544	716	493	621
Poly-3 test	P<0.001	P=0.201N	P=0.226	P=0.014
Squamous Cell Papilloma, Keratoacanthoma, Sebaceous Gland Adenoma, Basal Cell Adenoma, Basal Cell Carcinoma, or Squamous Cell Carcinoma				
Overall rate	9/50 (18%)	7/50 (14%)	15/50 (30%)	21/50 (42%)
Adjusted rate	22.2%	17.4%	33.7%	50.4%
Terminal rate	2/19 (11%)	4/21 (19%)	7/25 (28%)	10/19 (53%)
First incidence (days)	544	509	493	436
Poly-3 test	P<0.001	P=0.397N	P=0.171	P=0.005
Skin (Subcutaneous Tissue): Fibroma				
Overall rate	4/50 (8%)	6/50 (12%)	10/50 (20%)	15/50 (30%)
Adjusted rate	10.2%	14.8%	23.3%	36.0%
Terminal rate	2/19 (11%)	2/21 (10%)	7/25 (28%)	7/19 (37%)
First incidence (days)	657	595	442	467
Poly-3 test	P<0.001	P=0.391	P=0.098	P=0.005

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Skin (Subcutaneous Tissue): Fibroma, Fibrous Histiocytoma, or Fibrosarcoma				
Overall rate	6/50 (12%)	6/50 (12%)	12/50 (24%)	17/50 (34%)
Adjusted rate	15.2%	14.8%	27.9%	40.4%
Terminal rate	2/19 (11%)	2/21 (10%)	8/25 (32%)	7/19 (37%)
First incidence (days)	657	595	442	467
Poly-3 test	P<0.001	P=0.601N	P=0.128	P=0.009
Testes: Adenoma				
Overall rate	34/50 (68%)	31/50 (62%)	31/50 (62%)	39/50 (78%)
Adjusted rate	76.1%	73.9%	69.1%	86.3%
Terminal rate	15/19 (79%)	19/21 (91%)	19/25 (76%)	19/19 (100%)
First incidence (days)	537	526	513	467
Poly-3 test	P=0.107	P=0.503N	P=0.292N	P=0.136
Thyroid Gland (C-Cell): Adenoma				
Overall rate	7/50 (14%)	9/50 (18%)	9/50 (18%)	8/50 (16%)
Adjusted rate	17.4%	22.4%	20.9%	20.4%
Terminal rate	3/19 (16%)	7/21 (33%)	5/25 (20%)	4/19 (21%)
First incidence (days)	544	533	585	642
Poly-3 test	P=0.468	P=0.390	P=0.451	P=0.478
Thyroid Gland (C-Cell): Adenoma or Carcinoma				
Overall rate	8/50 (16%)	10/50 (20%)	9/50 (18%)	8/50 (16%)
Adjusted rate	19.8%	24.6%	20.9%	20.4%
Terminal rate	3/19 (16%)	7/21 (33%)	5/25 (20%)	4/19 (21%)
First incidence (days)	544	533	585	642
Poly-3 test	P=0.513N	P=0.397	P=0.557	P=0.582
Thyroid Gland (Follicular Cell): Adenoma				
Overall rate	1/50 (2%)	3/50 (6%)	0/50 (0%)	2/50 (4%)
Adjusted rate	2.6%	7.6%	0.0%	5.1%
Terminal rate	1/19 (5%)	1/21 (5%)	0/25 (0%)	0/19 (0%)
First incidence (days)	726 (T)	674	—	610
Poly-3 test	P=0.566	P=0.314	P=0.484N	P=0.504
Thyroid Gland (Follicular Cell): Adenoma or Carcinoma				
Overall rate	1/50 (2%)	3/50 (6%)	0/50 (0%)	4/50 (8%)
Adjusted rate	2.6%	7.6%	0.0%	10.2%
Terminal rate	1/19 (5%)	1/21 (5%)	0/25 (0%)	1/19 (5%)
First incidence (days)	726 (T)	674	—	610
Poly-3 test	P=0.168	P=0.314	P=0.484N	P=0.181
All Organs: Mononuclear Cell Leukemia				
Overall rate	15/50 (30%)	11/50 (22%)	15/50 (30%)	11/50 (22%)
Adjusted rate	36.1%	27.1%	34.3%	27.0%
Terminal rate	6/19 (32%)	6/21 (29%)	6/25 (24%)	5/19 (26%)
First incidence (days)	544	629	587	481
Poly-3 test	P=0.289N	P=0.258N	P=0.524N	P=0.253N
All Organs: Malignant Mesothelioma				
Overall rate	1/50 (2%)	12/50 (24%)	18/50 (36%)	37/50 (74%)
Adjusted rate	2.6%	28.0%	40.6%	77.5%
Terminal rate	0/19 (0%)	3/21 (14%)	10/25 (40%)	12/19 (63%)
First incidence (days)	683	309	442	389
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
All Organs: Osteoma or Osteosarcoma				
Overall rate	1/50 (2%)	1/50 (2%)	0/50 (0%)	3/50 (6%)
Adjusted rate	2.6%	2.5%	0.0%	7.8%
Terminal rate	0/19 (0%)	0/21 (0%)	0/25 (0%)	2/19 (11%)
First incidence (days)	671	516	—	678
Poly-3 test	P=0.158	P=0.754N	P=0.485N	P=0.301
All Organs: Benign Neoplasms				
Overall rate	46/50 (92%)	44/50 (88%)	47/50 (94%)	49/50 (98%)
Adjusted rate	96.3%	93.7%	96.0%	99.7%
Terminal rate	19/19 (100%)	20/21 (95%)	24/25 (96%)	19/19 (100%)
First incidence (days)	477	509	442	436
Poly-3 test	P=0.129	P=0.445N	P=0.708N	P=0.253
All Organs: Malignant Neoplasms				
Overall rate	24/50 (48%)	25/50 (50%)	33/50 (66%)	46/50 (92%)
Adjusted rate	54.7%	56.7%	71.5%	93.4%
Terminal rate	7/19 (37%)	11/21 (52%)	16/25 (64%)	17/19 (90%)
First incidence (days)	544	309	442	389
Poly-3 test	P<0.001	P=0.512	P=0.066	P<0.001
All Organs: Benign or Malignant Neoplasms				
Overall rate	49/50 (98%)	48/50 (96%)	48/50 (96%)	50/50 (100%)
Adjusted rate	99.7%	98.8%	97.5%	100.0%
Terminal rate	19/19 (100%)	21/21 (100%)	24/25 (96%)	19/19 (100%)
First incidence (days)	477	309	442	389
Poly-3 test	P=0.630	P=0.819N	P=0.464N	P=1.000

(T) Terminal kill

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

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Disposition Summary				
Animals initially in study	66	66	66	66
<i>6-Month interim evaluation^b</i>	8	8	8	8
<i>13-Month interim evaluation^b</i>	8	8	8	8
Early deaths				
Moribund	26	23	20	27
Natural deaths	5	6	5	4
Survivors				
Terminal kill	19	21	25	19
Animals examined microscopically	66	66	66	66
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Intestine large, cecum	(50)	(50)	(49)	(50)
Erosion			1 (2%)	
Inflammation			2 (4%)	
Intestine large, colon	(50)	(50)	(50)	(50)
Amyloid deposition		1 (2%)		
Inflammation	1 (2%)	1 (2%)		
Parasite metazoan	6 (12%)	8 (16%)	10 (20%)	8 (16%)
Thrombosis		1 (2%)		
Intestine large, rectum	(50)	(50)	(50)	(50)
Intestine small, duodenum	(50)	(50)	(49)	(50)
Intestine small, ileum	(50)	(50)	(49)	(50)
Intestine small, jejunum	(50)	(50)	(49)	(50)
Liver	(50)	(50)	(50)	(50)
Angiectasis		1 (2%)	3 (6%)	4 (8%)
Basophilic focus	13 (26%)	23 (46%)	17 (34%)	20 (40%)
Clear cell focus	19 (38%)	20 (40%)	21 (42%)	16 (32%)
Congestion				1 (2%)
Degeneration, cystic	12 (24%)	12 (24%)	11 (22%)	14 (28%)
Eosinophilic focus	6 (12%)	10 (20%)	7 (14%)	14 (28%)
Fatty change	8 (16%)	7 (14%)	4 (8%)	1 (2%)
Fatty change, focal			1 (2%)	4 (8%)
Fibrosis			1 (2%)	
Hematopoietic cell proliferation	1 (2%)			3 (6%)
Hemorrhage		1 (2%)	1 (2%)	
Hepatodiaphragmatic nodule	9 (18%)	4 (8%)	3 (6%)	2 (4%)
Inflammation, chronic active	32 (64%)	36 (72%)	36 (72%)	36 (72%)
Mixed cell focus	3 (6%)	2 (4%)	6 (12%)	1 (2%)
Necrosis	2 (4%)	1 (2%)	4 (8%)	5 (10%)
Pigmentation, hemosiderin	1 (2%)		2 (4%)	1 (2%)
Regeneration			1 (2%)	
Tension lipidosis			1 (2%)	2 (4%)
Thrombosis				1 (2%)
Bile duct, cyst	1 (2%)			1 (2%)
Bile duct, hyperplasia	49 (98%)	48 (96%)	49 (98%)	48 (96%)
Hepatocyte, degeneration			1 (2%)	3 (6%)
Oval cell, hyperplasia			1 (2%)	
Mesentery	(7)	(4)	(9)	(10)
Fat, necrosis	7 (100%)	3 (75%)	3 (33%)	3 (30%)
Oral mucosa	(50)	(50)	(50)	(50)

^a Number of core study animals examined microscopically at the site and the number of animals with lesion

^a Pathology data for interim evaluation animals are not presented in this table.

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Alimentary System (continued)				
Pancreas	(50)	(50)	(49)	(50)
Atrophy	18 (36%)	19 (38%)	15 (31%)	21 (42%)
Cytoplasmic alteration	1 (2%)			
Acinus, hyperplasia	4 (8%)	1 (2%)	3 (6%)	4 (8%)
Salivary glands	(50)	(50)	(50)	(50)
Atrophy			1 (2%)	
Metaplasia, serous	1 (2%)			
Stomach, forestomach	(50)	(50)	(50)	(50)
Erosion				1 (2%)
Inflammation	13 (26%)	8 (16%)	4 (8%)	
Perforation	1 (2%)			
Ulcer	6 (12%)	3 (6%)	1 (2%)	
Epithelium, dysplasia	1 (2%)			
Epithelium, hyperplasia	12 (24%)	8 (16%)	4 (8%)	1 (2%)
Stomach, glandular	(50)	(50)	(50)	(50)
Erosion	1 (2%)		1 (2%)	
Hyperplasia, focal	1 (2%)			
Inflammation	7 (14%)	1 (2%)	2 (4%)	
Mineralization	1 (2%)		1 (2%)	1 (2%)
Ulcer	2 (4%)			
Epithelium, hyperplasia			1 (2%)	
Tongue	(50)	(50)	(50)	(50)
Inflammation	1 (2%)			
Epithelium, hyperplasia			1 (2%)	2 (4%)
Tooth	(1)	(0)	(0)	(0)
Degeneration	1 (100%)			
Cardiovascular System				
Blood vessel	(50)	(50)	(50)	(50)
Aneurysm			1 (2%)	
Inflammation	5 (10%)		3 (6%)	
Aorta, mineralization		1 (2%)		
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	47 (94%)	48 (96%)	48 (96%)	48 (96%)
Mineralization		3 (6%)		
Atrium, inflammation		1 (2%)		
Atrium, thrombosis	5 (10%)	6 (12%)	7 (14%)	5 (10%)
Pericardium, inflammation, chronic active	1 (2%)			
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Atrophy	1 (2%)			
Degeneration, cystic	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Hyperplasia	5 (10%)	2 (4%)	2 (4%)	
Hypertrophy	1 (2%)	3 (6%)		
Mineralization			1 (2%)	
Vacuolization cytoplasmic	14 (28%)	9 (18%)	17 (34%)	13 (26%)
Adrenal medulla	(50)	(50)	(50)	(50)
Hyperplasia	15 (30%)	18 (36%)	15 (30%)	9 (18%)
Islets, pancreatic	(50)	(50)	(49)	(50)
Hyperplasia		2 (4%)		1 (2%)
Parathyroid gland	(49)	(48)	(46)	(44)
Hyperplasia	6 (12%)	1 (2%)	1 (2%)	1 (2%)

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Endocrine System (continued)				
Pituitary gland	(50)	(49)	(49)	(48)
Atrophy	2 (4%)			
Pars distalis, atrophy	1 (2%)			
Pars distalis, hyperplasia	12 (24%)	14 (29%)	18 (37%)	21 (44%)
Thyroid gland	(50)	(50)	(50)	(50)
C-cell, hyperplasia	22 (44%)	20 (40%)	21 (42%)	24 (48%)
Follicle, cyst				2 (4%)
Follicular cell, hyperplasia	2 (4%)			1 (2%)
General Body System				
None				
Genital System				
Epididymis	(50)	(50)	(50)	(50)
Atrophy	8 (16%)	7 (14%)	5 (10%)	4 (8%)
Inflammation	1 (2%)			1 (2%)
Mesothelium, hyperplasia			1 (2%)	
Preputial gland	(50)	(50)	(50)	(49)
Hyperplasia	1 (2%)	2 (4%)	2 (4%)	
Inflammation	2 (4%)			
Duct, hyperplasia, squamous			1 (2%)	
Duct, inflammation			1 (2%)	
Prostate	(50)	(50)	(50)	(50)
Atrophy	1 (2%)			
Cyst			1 (2%)	
Inflammation	42 (84%)	41 (82%)	45 (90%)	35 (70%)
Epithelium, hyperplasia	11 (22%)	17 (34%)	12 (24%)	14 (28%)
Seminal vesicle	(50)	(50)	(50)	(50)
Atrophy	1 (2%)			
Hyperplasia			1 (2%)	1 (2%)
Inflammation	3 (6%)	3 (6%)	2 (4%)	1 (2%)
Testes	(50)	(50)	(50)	(50)
Atrophy	15 (30%)	16 (32%)	16 (32%)	17 (34%)
Inflammation	1 (2%)			
Germinal epithelium, degeneration	8 (16%)	10 (20%)	8 (16%)	9 (18%)
Interstitial cell, hyperplasia	23 (46%)	26 (52%)	24 (48%)	19 (38%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Angiectasis	4 (8%)	29 (58%)	34 (68%)	40 (80%)
Hemorrhage		5 (10%)	1 (2%)	
Hyperplasia	17 (34%)	19 (38%)	20 (40%)	30 (60%)
Myelofibrosis	1 (2%)			2 (4%)
Necrosis		1 (2%)		
Thrombosis		4 (8%)		1 (2%)
Lymph node	(5)	(2)	(8)	(7)
Iliac, ectasia				1 (14%)
Iliac, hyperplasia, lymphoid				1 (14%)
Iliac, necrosis, lymphoid				1 (14%)
Inguinal, ectasia				1 (14%)
Lumbar, ectasia			1 (13%)	
Lumbar, infiltration cellular, histiocyte			1 (13%)	
Mediastinal, hyperplasia			1 (13%)	
Mediastinal, hyperplasia, lymphoid		1 (50%)		

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Hematopoietic System (continued)				
Lymph node (continued)	(5)	(2)	(8)	(7)
Mediastinal, infiltration cellular, histiocyte				1 (14%)
Mediastinal, infiltration cellular, mast cell		1 (50%)		
Mediastinal, infiltration cellular, plasma cell				1 (14%)
Pancreatic, inflammation, histiocytic				1 (14%)
Renal, hyperplasia, lymphoid			1 (13%)	
Renal, infiltration cellular, histiocyte				1 (14%)
Renal, infiltration cellular, plasma cell	2 (40%)			
Lymph node, mandibular	(50)	(50)	(50)	(50)
Ectasia	4 (8%)	1 (2%)	2 (4%)	
Lymph node, mesenteric	(50)	(50)	(49)	(50)
Atrophy	2 (4%)			4 (8%)
Ectasia			1 (2%)	1 (2%)
Hemorrhage				2 (4%)
Necrosis				1 (2%)
Spleen	(50)	(50)	(49)	(50)
Hematopoietic cell proliferation	32 (64%)	31 (62%)	31 (63%)	32 (64%)
Hemorrhage				1 (2%)
Hyperplasia, lymphoid		1 (2%)		
Infarct	2 (4%)	1 (2%)		1 (2%)
Pigmentation, hemosiderin	35 (70%)	36 (72%)	32 (65%)	28 (56%)
Stromal hyperplasia	1 (2%)		1 (2%)	2 (4%)
Thrombosis				1 (2%)
Capsule, fibrosis				1 (2%)
Lymphoid follicle, atrophy			3 (6%)	
Red pulp, atrophy		1 (2%)	1 (2%)	
Thymus	(48)	(47)	(48)	(49)
Atrophy	47 (98%)	46 (98%)	46 (96%)	49 (100%)
Integumentary System				
Mammary gland	(49)	(50)	(49)	(49)
Hyperplasia	6 (12%)	1 (2%)	2 (4%)	1 (2%)
Inflammation, chronic	1 (2%)			
Inflammation, chronic active	1 (2%)			
Skin	(50)	(50)	(50)	(50)
Cyst epithelial inclusion	1 (2%)		2 (4%)	
Hyperplasia, squamous		1 (2%)		
Inflammation	3 (6%)	5 (10%)	5 (10%)	5 (10%)
Ulcer	2 (4%)	3 (6%)	1 (2%)	3 (6%)
Dermis, fibrosis				1 (2%)
Epidermis, hyperkeratosis	2 (4%)	1 (2%)	2 (4%)	2 (4%)
Epidermis, hyperplasia	3 (6%)	5 (10%)	5 (10%)	2 (4%)
Epidermis, hyperplasia, basal cell				1 (2%)
Epidermis, hyperplasia, squamous	1 (2%)			
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Fibrous osteodystrophy	1 (2%)			
Osteopetrosis	3 (6%)	1 (2%)		4 (8%)
Cartilage, sternum, degeneration				1 (2%)
Tibia, fracture				1 (2%)
Skeletal muscle	(4)	(0)	(2)	(0)

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Nervous System				
Brain	(50)	(50)	(50)	(50)
Gliosis		1 (2%)		
Hemorrhage		2 (4%)		
Infiltration cellular, lymphocyte		1 (2%)		
Meninges, hyperplasia				1 (2%)
Peripheral nerve	(0)	(2)	(0)	(0)
Degeneration		1 (50%)		
Spinal cord	(0)	(2)	(0)	(0)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Fibrosis				1 (2%)
Inflammation, chronic active	15 (30%)	11 (22%)	11 (22%)	19 (38%)
Metaplasia, osseous			1 (2%)	
Mineralization	1 (2%)			
Thrombosis	1 (2%)			
Alveolar epithelium, hyperplasia	5 (10%)	7 (14%)	12 (24%)	10 (20%)
Alveolar epithelium, hypertrophy				2 (4%)
Alveolus, infiltration cellular, histiocyte	20 (40%)	22 (44%)	25 (50%)	22 (44%)
Bronchiole, hyperplasia	1 (2%)			
Nose	(50)	(50)	(50)	(50)
Foreign body	12 (24%)	16 (32%)	10 (20%)	17 (34%)
Inflammation	15 (30%)	21 (42%)	14 (28%)	22 (44%)
Glands, olfactory epithelium, hyperplasia		1 (2%)		
Glands, respiratory epithelium, hyperplasia	1 (2%)			
Olfactory epithelium, metaplasia				1 (2%)
Respiratory epithelium, hyperplasia	4 (8%)			
Trachea	(50)	(50)	(50)	(50)
Special Senses System				
Ear	(1)	(0)	(0)	(0)
Eye	(50)	(50)	(50)	(50)
Anterior chamber, inflammation			1 (2%)	1 (2%)
Cornea, inflammation				1 (2%)
Iris, inflammation	1 (2%)	1 (2%)		
Lens, cataract	2 (4%)	5 (10%)	1 (2%)	6 (12%)
Retina, degeneration	4 (8%)	2 (4%)	3 (6%)	5 (10%)
Retina, dysplasia				1 (2%)
Retina, gliosis		2 (4%)		
Harderian gland	(50)	(50)	(50)	(50)
Hyperplasia, squamous				1 (2%)
Inflammation	1 (2%)		1 (2%)	
Metaplasia, squamous				1 (2%)
Zymbal's gland	(1)	(2)	(0)	(2)

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Accumulation, hyaline droplet	3 (6%)		1 (2%)	
Mineralization	16 (32%)	21 (42%)	25 (50%)	23 (46%)
Nephropathy	48 (96%)	49 (98%)	49 (98%)	49 (98%)
Cortex, cyst	1 (2%)	1 (2%)		
Cortex, inflammation, focal	1 (2%)			
Pelvis, dilatation		2 (4%)		
Renal tubule, dilatation		2 (4%)		
Renal tubule, hyperplasia, atypical				1 (2%)
Renal tubule, necrosis				1 (2%)
Urinary bladder	(50)	(50)	(50)	(50)
Inflammation	1 (2%)	2 (4%)		

APPENDIX B
SUMMARY OF LESIONS IN FEMALE RATS
IN THE 2-YEAR DRINKING WATER STUDY
OF BROMODICHLOROACETIC ACID

TABLE B1	Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid	B-2
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TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Disposition Summary				
Animals initially in study	66	66	66	66
<i>6-Month interim evaluation^b</i>	8	8	8	8
<i>13-Month interim evaluation^b</i>	8	8	8	8
Early deaths				
Moribund	10	22	42	44
Natural deaths	6	2	1	4
Survivors				
Terminal kill	34	26	7	2
Animals examined microscopically	66	66	66	66
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Intestine large, cecum	(50)	(50)	(50)	(50)
Intestine large, colon	(50)	(50)	(50)	(50)
Adenoma				2 (4%)
Intestine large, rectum	(50)	(50)	(50)	(50)
Adenoma	1 (2%)		1 (2%)	
Intestine small, duodenum	(50)	(50)	(50)	(50)
Intestine small, ileum	(50)	(50)	(50)	(50)
Intestine small, jejunum	(50)	(50)	(50)	(50)
Sarcoma		1 (2%)		
Liver	(50)	(50)	(50)	(50)
Hemangioma	1 (2%)			
Hemangiosarcoma				1 (2%)
Hepatocellular adenoma			2 (4%)	1 (2%)
Mesentery	(3)	(4)	(3)	(2)
Oral mucosa	(0)	(0)	(0)	(1)
Pharyngeal, squamous cell papilloma				1 (100%)
Pancreas	(50)	(50)	(50)	(50)
Salivary glands	(50)	(50)	(50)	(50)
Stomach, forestomach	(50)	(50)	(50)	(50)
Stomach, glandular	(50)	(50)	(50)	(50)
Tongue	(50)	(50)	(50)	(50)
Squamous cell carcinoma			1 (2%)	
Squamous cell papilloma		2 (4%)		1 (2%)
Cardiovascular System				
Blood vessel	(50)	(50)	(50)	(50)
Heart	(50)	(50)	(50)	(50)
Schwannoma malignant		1 (2%)		
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Adenoma			1 (2%)	
Adrenal medulla	(50)	(50)	(50)	(50)
Pheochromocytoma benign	1 (2%)	1 (2%)	2 (4%)	1 (2%)
Pheochromocytoma benign, multiple				1 (2%)
Pheochromocytoma malignant	1 (2%)	1 (2%)		1 (2%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Adenoma	1 (2%)			
Carcinoma		1 (2%)		
Parathyroid gland	(47)	(46)	(49)	(49)

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Endocrine System (continued)				
Pituitary gland	(50)	(50)	(49)	(50)
Pars distalis, adenoma	23 (46%)	20 (40%)	11 (22%)	11 (22%)
Thyroid gland	(50)	(50)	(50)	(50)
C-cell, adenoma	11 (22%)	10 (20%)	6 (12%)	7 (14%)
C-cell, carcinoma			1 (2%)	
Follicular cell, adenoma			1 (2%)	1 (2%)
General Body System				
None				
Genital System				
Clitoral gland	(50)	(49)	(49)	(48)
Adenoma	7 (14%)	11 (22%)	8 (16%)	7 (15%)
Carcinoma	1 (2%)			
Bilateral, adenoma	1 (2%)			
Ovary	(50)	(50)	(50)	(50)
Granulosa cell tumor malignant			1 (2%)	
Hemangiosarcoma			1 (2%)	
Pheochromocytoma malignant, metastatic, adrenal medulla				1 (2%)
Uterus	(50)	(50)	(50)	(50)
Deciduoma NOS	1 (2%)			
Fibrous histiocytoma	1 (2%)			
Leiomyosarcoma	1 (2%)			
Polyp stromal	3 (6%)	6 (12%)	8 (16%)	5 (10%)
Polyp stromal, multiple	1 (2%)			
Sarcoma stromal	1 (2%)			1 (2%)
Cervix, granular cell tumor NOS			1 (2%)	
Cervix, hemangiosarcoma			1 (2%)	
Endometrium, adenoma		1 (2%)		
Endometrium, carcinoma				1 (2%)
Vagina	(1)	(0)	(0)	(0)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Lymph node	(4)	(4)	(2)	(2)
Lymph node, mandibular	(50)	(50)	(50)	(50)
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Spleen	(50)	(50)	(50)	(50)
Hemangiosarcoma				1 (2%)
Thymus	(48)	(49)	(47)	(48)
Thymoma malignant	1 (2%)			
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Adenoma	1 (2%)	2 (4%)	3 (6%)	1 (2%)
Carcinoma		1 (2%)	3 (6%)	8 (16%)
Fibroadenoma	22 (44%)	13 (26%)	10 (20%)	12 (24%)
Fibroadenoma, multiple	6 (12%)	34 (68%)	37 (74%)	27 (54%)
Skin	(50)	(50)	(50)	(50)
Basal cell adenoma				1 (2%)
Subcutaneous tissue, fibroma	2 (4%)		3 (6%)	2 (4%)
Subcutaneous tissue, lipoma	2 (4%)			

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Skeletal muscle	(0)	(1)	(0)	(0)
Rhabdomyosarcoma		1 (100%)		
Nervous System				
Brain	(50)	(50)	(50)	(50)
Glioma	1 (2%)		2 (4%)	
Oligodendroglioma NOS			1 (2%)	1 (2%)
Spinal cord	(0)	(0)	(1)	(0)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma		1 (2%)		
Carcinoma, metastatic, mammary gland			1 (2%)	
Pheochromocytoma malignant, metastatic, adrenal medulla	1 (2%)	1 (2%)		1 (2%)
Nose	(50)	(50)	(50)	(50)
Trachea	(50)	(50)	(50)	(50)
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Harderian gland	(50)	(50)	(50)	(50)
Lacrimal gland	(0)	(2)	(0)	(0)
Zymbal's gland	(0)	(0)	(0)	(2)
Carcinoma				2 (100%)
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Urinary bladder	(50)	(50)	(50)	(50)
Systemic Lesions				
Multiple organs ^c	(50)	(50)	(50)	(50)
Histiocytic sarcoma		1 (2%)		
Leukemia mononuclear	19 (38%)	17 (34%)	8 (16%)	10 (20%)

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Neoplasm Summary				
Total animals with primary neoplasms ^d	47	50	49	48
Total primary neoplasms	110	125	113	107
Total animals with benign neoplasms	41	48	48	46
Total benign neoplasms	83	101	93	81
Total animals with malignant neoplasms	23	21	13	22
Total malignant neoplasms	25	24	16	25
Total animals with metastatic neoplasms	1	2	1	1
Total metastatic neoplasms	1	2	1	2
Total animals with uncertain neoplasms- benign or malignant	2		4	1
Total uncertain neoplasms	2		4	1

^a Number of core study animals examined microscopically at the site and the number of animals with neoplasm

^b Pathology data for interim evaluation animals are not presented in this table.

^c Number of animals with any tissue examined microscopically

^d Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Brain, Glioma or Oligodendrogloma				
Overall rate ^a	1/50 (2%)	0/50 (0%)	3/50 (6%)	1/50 (2%)
Adjusted rate ^b	2.2%	0.0%	9.0%	3.5%
Terminal rate ^c	0/34 (0%)	0/26 (0%)	1/7 (14%)	0/2 (0%)
First incidence (days)	645	—	561	631
Poly-3 test ^d	P=0.266	P=0.512N	P=0.207	P=0.647
Clitoral Gland: Adenoma				
Overall rate	8/50 (16%) ^e	11/49 (22%)	8/49 (16%)	7/48 (15%)
Adjusted rate	17.8%	25.7%	23.9%	23.2%
Terminal rate	7/34 (21%)	7/25 (28%)	2/6 (33%)	0/2 (0%)
First incidence (days)	690	558	564	509
Poly-3 test	P=0.348	P=0.262	P=0.354	P=0.392
Mammary Gland: Fibroadenoma				
Overall rate	28/50 (56%)	47/50 (94%)	47/50 (94%)	39/50 (78%)
Adjusted rate	60.1%	96.6%	99.1%	89.6%
Terminal rate	21/34 (62%)	26/26 (100%)	7/7 (100%)	2/2 (100%)
First incidence (days)	551	558	449	414
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
Mammary Gland: Adenoma				
Overall rate	1/50 (2%)	2/50 (4%)	3/50 (6%)	1/50 (2%)
Adjusted rate	2.2%	4.7%	8.9%	3.5%
Terminal rate	0/34 (0%)	2/26 (8%)	0/7 (0%)	0/2 (0%)
First incidence (days)	635	727 (T)	386	488
Poly-3 test	P=0.365	P=0.479	P=0.213	P=0.650
Mammary Gland: Fibroadenoma or Adenoma				
Overall rate	28/50 (56%)	47/50 (94%)	48/50 (96%)	40/50 (80%)
Adjusted rate	60.1%	96.6%	99.4%	90.5%
Terminal rate	21/34 (62%)	26/26 (100%)	7/7 (100%)	2/2 (100%)
First incidence (days)	551	558	386	414
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
Mammary Gland: Carcinoma				
Overall rate	0/50 (0%)	1/50 (2%)	3/50 (6%)	8/50 (16%)
Adjusted rate	0.0%	2.3%	9.1%	25.8%
Terminal rate	0/34 (0%)	0/26 (0%)	0/7 (0%)	0/2 (0%)
First incidence (days)	— ^f	558	533	509
Poly-3 test	P<0.001	P=0.492	P=0.074	P<0.001
Mammary Gland: Adenoma or Carcinoma				
Overall rate	1/50 (2%)	3/50 (6%)	6/50 (12%)	9/50 (18%)
Adjusted rate	2.2%	7.0%	17.2%	28.4%
Terminal rate	0/34 (0%)	2/26 (8%)	0/7 (0%)	0/2 (0%)
First incidence (days)	635	558	386	488
Poly-3 test	P<0.001	P=0.288	P=0.024	P<0.001
Mammary Gland: Fibroadenoma, Adenoma, or Carcinoma				
Overall rate	28/50 (56%)	47/50 (94%)	48/50 (96%)	42/50 (84%)
Adjusted rate	60.1%	96.6%	99.4%	92.5%
Terminal rate	21/34 (62%)	26/26 (100%)	7/7 (100%)	2/2 (100%)
First incidence (days)	551	558	386	414
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	23/50 (46%)	20/50 (40%)	11/49 (22%)	11/50 (22%)
Adjusted rate	48.7%	45.2%	31.3%	34.5%
Terminal rate	13/34 (38%)	12/26 (46%)	1/7 (14%)	0/2 (0%)
First incidence (days)	551	551	449	551
Poly-3 test	P=0.070N	P=0.449N	P=0.084N	P=0.153N
Skin: Fibroma				
Overall rate	2/50 (4%)	0/50 (0%)	3/50 (6%)	2/50 (4%)
Adjusted rate	4.4%	0.0%	8.9%	6.9%
Terminal rate	1/34 (3%)	0/26 (0%)	0/7 (0%)	0/2 (0%)
First incidence (days)	608	—	574	585
Poly-3 test	P=0.249	P=0.251N	P=0.369	P=0.524
Thyroid Gland (C-Cell): Adenoma				
Overall rate	11/50 (22%)	10/50 (20%)	6/50 (12%)	7/50 (14%)
Adjusted rate	24.2%	22.3%	18.1%	23.1%
Terminal rate	9/34 (27%)	4/26 (15%)	2/7 (29%)	1/2 (50%)
First incidence (days)	635	558	601	586
Poly-3 test	P=0.446N	P=0.514N	P=0.354N	P=0.562N
Thyroid Gland (C-Cell): Adenoma or Carcinoma				
Overall rate	11/50 (22%)	10/50 (20%)	7/50 (14%)	7/50 (14%)
Adjusted rate	24.2%	22.3%	21.1%	23.1%
Terminal rate	9/34 (27%)	4/26 (15%)	3/7 (43%)	1/2 (50%)
First incidence (days)	635	558	601	586
Poly-3 test	P=0.482N	P=0.514N	P=0.477N	P=0.562N
Uterus: Stromal Polyp				
Overall rate	4/50 (8%)	6/50 (12%)	8/50 (16%)	5/50 (10%)
Adjusted rate	8.9%	13.6%	22.4%	16.7%
Terminal rate	4/34 (12%)	2/26 (8%)	0/7 (0%)	0/2 (0%)
First incidence (days)	727 (T)	551	498	544
Poly-3 test	P=0.138	P=0.359	P=0.085	P=0.265
Uterus: Stromal Polyp or Stromal Sarcoma				
Overall rate	5/50 (10%)	6/50 (12%)	8/50 (16%)	6/50 (12%)
Adjusted rate	11.1%	13.6%	22.4%	19.6%
Terminal rate	4/34 (12%)	2/26 (8%)	0/7 (0%)	0/2 (0%)
First incidence (days)	641	551	498	519
Poly-3 test	P=0.133	P=0.484	P=0.143	P=0.247
All Organs: Mononuclear Cell Leukemia				
Overall rate	19/50 (38%)	17/50 (34%)	8/50 (16%)	10/50 (20%)
Adjusted rate	41.4%	39.1%	23.6%	31.6%
Terminal rate	14/34 (41%)	11/26 (42%)	1/7 (14%)	1/2 (50%)
First incidence (days)	551	594	609	554
Poly-3 test	P=0.126N	P=0.497N	P=0.076N	P=0.261N
All Organs: Benign Neoplasms				
Overall rate	41/50 (82%)	48/50 (96%)	48/50 (96%)	46/50 (92%)
Adjusted rate	85.4%	97.5%	99.4%	97.3%
Terminal rate	29/34 (85%)	26/26 (100%)	7/7 (100%)	2/2 (100%)
First incidence (days)	551	551	386	414
Poly-3 test	P=0.011	P=0.028	P=0.009	P=0.034

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
All Organs: Malignant Neoplasms				
Overall rate	23/50 (46%)	21/50 (42%)	14/50 (28%)	22/50 (44%)
Adjusted rate	48.5%	46.8%	39.1%	60.5%
Terminal rate	14/34 (41%)	11/26 (42%)	2/7 (29%)	1/2 (50%)
First incidence (days)	432	558	429	509
Poly-3 test	P=0.189	P=0.518N	P=0.261N	P=0.183
All Organs: Benign or Malignant Neoplasms				
Overall rate	47/50 (94%)	50/50 (100%)	49/50 (98%)	48/50 (96%)
Adjusted rate	95.7%	100.0%	99.8%	99.5%
Terminal rate	32/34 (94%)	26/26 (100%)	7/7 (100%)	2/2 (100%)
First incidence (days)	432	551	386	414
Poly-3 test	P=0.155	P=0.214	P=0.239	P=0.274

(T) Terminal kill

- ^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for brain, clitoral gland, pituitary gland, and thyroid gland; for other tissues, denominator is number of animals necropsied.
- ^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
- ^c Observed incidence at terminal kill
- ^d Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill. A negative trend or a lower incidence in an exposure group is indicated by N.
- ^e A single incidence of carcinoma occurred in an animal that also had an adenoma.
- ^f Not applicable; no neoplasms in animal group

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Disposition Summary				
Animals initially in study	66	66	66	66
<i>6-Month interim evaluation^b</i>	8	8	8	8
<i>13-Month interim evaluation^b</i>	8	8	8	8
Early deaths				
Moribund	10	22	42	44
Natural deaths	6	2	1	4
Survivors				
Terminal kill	34	26	7	2
Animals examined microscopically	66	66	66	66
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Intestine large, cecum	(50)	(50)	(50)	(50)
Inflammation		1 (2%)		
Intestine large, colon	(50)	(50)	(50)	(50)
Parasite metazoan	6 (12%)	9 (18%)	5 (10%)	4 (8%)
Intestine large, rectum	(50)	(50)	(50)	(50)
Intestine small, duodenum	(50)	(50)	(50)	(50)
Intestine small, ileum	(50)	(50)	(50)	(50)
Intestine small, jejunum	(50)	(50)	(50)	(50)
Liver	(50)	(50)	(50)	(50)
Angiectasis	3 (6%)		1 (2%)	2 (4%)
Basophilic focus	42 (84%)	46 (92%)	46 (92%)	46 (92%)
Clear cell focus	14 (28%)	10 (20%)	5 (10%)	8 (16%)
Degeneration, cystic	1 (2%)	1 (2%)		
Eosinophilic focus	6 (12%)	13 (26%)	21 (42%)	22 (44%)
Fatty change	10 (20%)	7 (14%)	5 (10%)	5 (10%)
Fatty change, focal	1 (2%)	2 (4%)		
Hematopoietic cell proliferation	3 (6%)	7 (14%)	14 (28%)	9 (18%)
Hepatodiaphragmatic nodule	5 (10%)	9 (18%)	12 (24%)	15 (30%)
Inflammation, chronic active	36 (72%)	33 (66%)	40 (80%)	35 (70%)
Mixed cell focus	2 (4%)	3 (6%)	2 (4%)	3 (6%)
Necrosis	2 (4%)	1 (2%)	1 (2%)	2 (4%)
Necrosis, multifocal	1 (2%)			
Tension lipidosis	1 (2%)			
Bile duct, hyperplasia	28 (56%)	27 (54%)	28 (56%)	22 (44%)
Oval cell, hyperplasia	2 (4%)			
Serosa, fibrosis				1 (2%)
Mesentery	(3)	(4)	(3)	(2)
Fat, necrosis	3 (100%)	4 (100%)	3 (100%)	2 (100%)
Oral mucosa	(0)	(0)	(0)	(1)
Pancreas	(50)	(50)	(50)	(50)
Atrophy	9 (18%)	13 (26%)	11 (22%)	14 (28%)
Acinus, hyperplasia	6 (12%)	1 (2%)		
Salivary glands	(50)	(50)	(50)	(50)
Degeneration		1 (2%)		
Inflammation		1 (2%)		
Stomach, forestomach	(50)	(50)	(50)	(50)
Inflammation	2 (4%)	1 (2%)	1 (2%)	
Ulcer	1 (2%)	1 (2%)	1 (2%)	
Epithelium, dysplasia				1 (2%)
Epithelium, hyperplasia	2 (4%)	1 (2%)	4 (8%)	

^a Number of core study animals examined microscopically at the site and the number of animals with lesion

^b Pathology data for interim evaluation animals are not presented in this table.

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Alimentary System (continued)				
Stomach, glandular	(50)	(50)	(50)	(50)
Erosion				1 (2%)
Mineralization	1 (2%)			
Tongue	(50)	(50)	(50)	(50)
Foreign body	1 (2%)			
Inflammation	1 (2%)			
Ulcer	1 (2%)			
Epithelium, hyperplasia	1 (2%)			
Cardiovascular System				
Blood vessel	(50)	(50)	(50)	(50)
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	45 (90%)	42 (84%)	39 (78%)	35 (70%)
Atrium, thrombosis		1 (2%)		
Epicardium, inflammation				1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Degeneration, cystic	4 (8%)	2 (4%)	2 (4%)	6 (12%)
Hematopoietic cell proliferation	1 (2%)			
Hyperplasia		1 (2%)		
Hypertrophy	5 (10%)		1 (2%)	5 (10%)
Necrosis				2 (4%)
Vacuolization cytoplasmic	3 (6%)	6 (12%)	2 (4%)	
Zona fasciculata, hyperplasia		4 (8%)	1 (2%)	1 (2%)
Adrenal medulla	(50)	(50)	(50)	(50)
Hyperplasia	2 (4%)		5 (10%)	1 (2%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Parathyroid gland	(47)	(46)	(49)	(49)
Pituitary gland	(50)	(50)	(49)	(50)
Cyst		1 (2%)		
Inflammation		1 (2%)		
Pars distalis, angiectasis			1 (2%)	1 (2%)
Pars distalis, cyst			1 (2%)	
Pars distalis, hyperplasia	15 (30%)	18 (36%)	22 (45%)	17 (34%)
Pars distalis, vacuolization cytoplasmic			1 (2%)	
Thyroid gland	(50)	(50)	(50)	(50)
Atrophy			1 (2%)	
Cyst		1 (2%)		
C-cell, hyperplasia	33 (66%)	31 (62%)	26 (52%)	26 (52%)
Follicular cell, hyperplasia	1 (2%)	2 (4%)		1 (2%)
General Body System				
None				
Genital System				
Clitoral gland	(50)	(49)	(49)	(48)
Cyst	1 (2%)	1 (2%)		
Hyperplasia	1 (2%)	5 (10%)	3 (6%)	
Inflammation	2 (4%)			

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Genital System (continued)				
Ovary	(50)	(50)	(50)	(50)
Atrophy	9 (18%)	9 (18%)	6 (12%)	3 (6%)
Cyst	10 (20%)	12 (24%)	10 (20%)	6 (12%)
Infiltration cellular, histiocyte	1 (2%)			
Uterus	(50)	(50)	(50)	(50)
Atrophy		1 (2%)		
Cervix, hypertrophy		1 (2%)	1 (2%)	
Cervix, inflammation	2 (4%)	3 (6%)		1 (2%)
Cervix, metaplasia, squamous	2 (4%)	1 (2%)		1 (2%)
Cervix, epithelium, hyperplasia	1 (2%)			
Endometrium, hyperplasia, adenomatous			1 (2%)	
Endometrium, hyperplasia, cystic	10 (20%)	9 (18%)	12 (24%)	9 (18%)
Vagina	(1)	(0)	(0)	(0)
Epithelium, hyperplasia	1 (100%)			
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Angiectasis	1 (2%)	19 (38%)	32 (64%)	39 (78%)
Atrophy				1 (2%)
Hemorrhage	1 (2%)	1 (2%)		
Hyperplasia	23 (46%)	35 (70%)	40 (80%)	43 (86%)
Infiltration cellular, histiocyte		1 (2%)	1 (2%)	1 (2%)
Myelofibrosis				1 (2%)
Necrosis	1 (2%)			1 (2%)
Lymph node	(4)	(4)	(2)	(2)
Inguinal, infiltration cellular, plasma cell	1 (25%)			
Lumbar, ectasia		1 (25%)		
Mediastinal, hyperplasia, lymphoid		1 (25%)		
Mediastinal, infiltration cellular, histiocyte	1 (25%)			
Mediastinal, infiltration cellular, plasma cell				1 (50%)
Pancreatic, infiltration cellular, histiocyte	1 (25%)			
Lymph node, mandibular	(50)	(50)	(50)	(50)
Atrophy			1 (2%)	
Infiltration cellular, plasma cell	1 (2%)		1 (2%)	
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Atrophy	4 (8%)		2 (4%)	
Ectasia			1 (2%)	
Infiltration cellular, histiocyte				1 (2%)
Spleen	(50)	(50)	(50)	(50)
Hematopoietic cell proliferation	6 (12%)	13 (26%)	29 (58%)	31 (62%)
Hyperplasia, lymphoid		1 (2%)		
Pigmentation, hemosiderin	39 (78%)	43 (86%)	41 (82%)	39 (78%)
Thrombosis				2 (4%)
Capsule, mineralization	1 (2%)			
Lymphoid follicle, atrophy	5 (10%)	1 (2%)	1 (2%)	3 (6%)
Thymus	(48)	(49)	(47)	(48)
Atrophy	45 (94%)	48 (98%)	44 (94%)	45 (94%)
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Galactocele		1 (2%)		
Hyperplasia		4 (8%)	2 (4%)	10 (20%)
Inflammation	1 (2%)			

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Integumentary System (continued)				
Skin	(50)	(50)	(50)	(50)
Cyst epithelial inclusion	1 (2%)	1 (2%)		
Inflammation	3 (6%)			2 (4%)
Ulcer	2 (4%)			
Epidermis, hyperkeratosis				1 (2%)
Epidermis, hyperplasia, squamous	2 (4%)			1 (2%)
Sebaceous gland, hyperplasia			1 (2%)	
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Fibrous osteodystrophy	1 (2%)	1 (2%)		
Hyperplasia			1 (2%)	
Osteopetrosis	2 (4%)	2 (4%)	8 (16%)	5 (10%)
Tibia, fracture		1 (2%)		
Vertebra, fracture				1 (2%)
Skeletal muscle	(0)	(1)	(0)	(0)
Nervous System				
Brain	(50)	(50)	(50)	(50)
Hemorrhage		2 (4%)		
Necrosis		1 (2%)		
Spinal cord	(0)	(0)	(1)	(0)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Inflammation, chronic active	20 (40%)	26 (52%)	26 (52%)	28 (56%)
Alveolar epithelium, hyperplasia	11 (22%)	4 (8%)	8 (16%)	8 (16%)
Alveolar epithelium, hypertrophy	1 (2%)			
Alveolus, hyperplasia			1 (2%)	
Alveolus, infiltration cellular, histiocyte	38 (76%)	46 (92%)	43 (86%)	43 (86%)
Bronchiole, hyperplasia		1 (2%)		1 (2%)
Mediastinum, inflammation	1 (2%)			
Nose	(50)	(50)	(50)	(50)
Foreign body	2 (4%)	1 (2%)	2 (4%)	1 (2%)
Inflammation	5 (10%)	5 (10%)	9 (18%)	5 (10%)
Respiratory epithelium, hyperplasia			1 (2%)	
Trachea	(50)	(50)	(50)	(50)
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Fibrosis			1 (2%)	
Anterior chamber, inflammation			1 (2%)	
Ciliary body, fibrosis	1 (2%)			
Cornea, inflammation		1 (2%)		1 (2%)
Iris, inflammation	1 (2%)			
Lens, cataract	5 (10%)	1 (2%)	6 (12%)	3 (6%)
Posterior chamber, inflammation	1 (2%)			
Retina, degeneration	6 (12%)	2 (4%)	5 (10%)	3 (6%)
Retina, gliosis	1 (2%)	1 (2%)	1 (2%)	
Harderian gland	(50)	(50)	(50)	(50)
Inflammation			1 (2%)	1 (2%)
Lacrimal gland	(0)	(2)	(0)	(0)
Zymbal's gland	(0)	(0)	(0)	(2)

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Infarct	1 (2%)	1 (2%)		1 (2%)
Infiltration cellular, lymphoid		1 (2%)		
Inflammation	1 (2%)			
Mineralization	28 (56%)	21 (42%)	20 (40%)	27 (54%)
Nephropathy	43 (86%)	40 (80%)	44 (88%)	46 (92%)
Cortex, cyst	1 (2%)		1 (2%)	
Papilla, necrosis	1 (2%)			1 (2%)
Pelvis, calculus microscopic observation only		3 (6%)	4 (8%)	
Pelvis, inflammation	1 (2%)		1 (2%)	
Renal tubule, dilatation				1 (2%)
Renal tubule, hyperplasia, atypical		1 (2%)	1 (2%)	
Renal tubule, necrosis				1 (2%)
Urinary bladder	(50)	(50)	(50)	(50)
Transitional epithelium, hyperplasia		1 (2%)		

APPENDIX C
SUMMARY OF LESIONS IN MALE MICE
IN THE 2-YEAR DRINKING WATER STUDY
OF BROMODICHLOROACETIC ACID

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TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Disposition Summary				
Animals initially in study	66	66	66	66
<i>6-Month interim evaluation^b</i>	8	8	8	8
<i>14-Month interim evaluation^b</i>	8	8	8	7
Early deaths				
Moribund	15	14	27	27
Natural deaths	10	15	10	14
Survivors				
Terminal kill	25	21	12	10
Missing			1	
Animals examined microscopically	66	66	65	66
Alimentary System				
Esophagus	(50)	(50)	(49)	(51)
Gallbladder	(48)	(49)	(48)	(47)
Intestine large, cecum	(50)	(50)	(49)	(51)
Carcinoma	1 (2%)			
Intestine large, colon	(50)	(50)	(49)	(51)
Intestine large, rectum	(50)	(50)	(49)	(51)
Serosa, hemangiosarcoma, metastatic, liver		1 (2%)		
Intestine small, duodenum	(50)	(50)	(49)	(51)
Adenoma	1 (2%)		1 (2%)	
Carcinoma	1 (2%)	1 (2%)		
Intestine small, ileum	(50)	(50)	(49)	(51)
Intestine small, jejunum	(50)	(50)	(49)	(51)
Carcinoma	1 (2%)	3 (6%)		2 (4%)
Liver	(50)	(50)	(49)	(51)
Cholangiocarcinoma				1 (2%)
Hemangioma			1 (2%)	
Hemangiosarcoma	1 (2%)	4 (8%)	2 (4%)	4 (8%)
Hemangiosarcoma, metastatic, spleen	1 (2%)		1 (2%)	
Hepatoblastoma	3 (6%)	18 (36%)	28 (57%)	22 (43%)
Hepatoblastoma, multiple	1 (2%)	6 (12%)	12 (24%)	12 (24%)
Hepatocellular adenoma	11 (22%)	12 (24%)	15 (31%)	17 (33%)
Hepatocellular adenoma, multiple	28 (56%)	29 (58%)	27 (55%)	23 (45%)
Hepatocellular carcinoma	8 (16%)	10 (20%)	17 (35%)	22 (43%)
Hepatocellular carcinoma, multiple	4 (8%)	12 (24%)	10 (20%)	17 (33%)
Hepatocholangiocarcinoma			2 (4%)	2 (4%)
Schwannoma malignant, metastatic, heart		1 (2%)		
Mesentery	(7)	(1)	(3)	(6)
Carcinoma, metastatic, uncertain primary site				1 (17%)
Fibrous histiocytoma				1 (17%)
Hepatoblastoma, metastatic, liver			1 (33%)	
Hepatocholangiocarcinoma, metastatic, liver			1 (33%)	
Lipoma	1 (14%)			
Pancreas	(50)	(50)	(49)	(51)
Sarcoma			1 (2%)	
Salivary glands	(50)	(50)	(49)	(51)
Stomach, forestomach	(50)	(50)	(49)	(51)
Squamous cell papilloma		1 (2%)		1 (2%)
Stomach, glandular	(50)	(50)	(49)	(51)
Tooth	(35)	(22)	(13)	(0)

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Cardiovascular System				
Blood vessel	(50)	(50)	(49)	(51)
Heart	(50)	(50)	(49)	(51)
Alveolar/bronchiolar carcinoma, metastatic, lung			2 (4%)	
Schwannoma malignant		1 (2%)		
Pericardium, alveolar/bronchiolar carcinoma, metastatic, lung			1 (2%)	
Endocrine System				
Adrenal cortex	(50)	(50)	(49)	(50)
Subcapsular, adenoma	2 (4%)		1 (2%)	
Adrenal medulla	(50)	(50)	(49)	(50)
Islets, pancreatic	(50)	(50)	(49)	(51)
Parathyroid gland	(44)	(45)	(44)	(44)
Pituitary gland	(50)	(49)	(49)	(51)
Thyroid gland	(50)	(50)	(49)	(51)
Follicular cell, adenoma	1 (2%)		1 (2%)	
General Body System				
Peritoneum	(0)	(0)	(0)	(1)
Genital System				
Epididymis	(50)	(50)	(49)	(51)
Fibrous histiocytoma, metastatic, mesentery				1 (2%)
Penis	(0)	(1)	(0)	(0)
Preputial gland	(50)	(50)	(49)	(51)
Prostate	(50)	(50)	(49)	(51)
Seminal vesicle	(50)	(50)	(49)	(51)
Fibrous histiocytoma, metastatic, mesentery				1 (2%)
Testes	(50)	(50)	(49)	(51)
Interstitial cell, adenoma	1 (2%)			
Hematopoietic System				
Bone marrow	(50)	(50)	(49)	(51)
Hemangiosarcoma				1 (2%)
Hemangiosarcoma, metastatic, liver		1 (2%)		
Hemangiosarcoma, metastatic, spleen	1 (2%)		2 (4%)	
Lymph node	(0)	(1)	(2)	(5)
Mediastinal, alveolar/bronchiolar carcinoma, metastatic, lung			1 (50%)	
Pancreatic, carcinoma, metastatic, uncertain primary site				1 (20%)
Pancreatic, hepatoblastoma, metastatic, liver				1 (20%)
Pancreatic, hepatocellular carcinoma, metastatic, liver				1 (20%)
Lymph node, mandibular	(50)	(50)	(49)	(51)
Lymph node, mesenteric	(50)	(48)	(48)	(48)
Carcinoma, metastatic, uncertain primary site				1 (2%)
Hepatoblastoma, metastatic, liver		1 (2%)		

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Hematopoietic System (continued)				
Spleen	(50)	(50)	(49)	(50)
Hemangiosarcoma	2 (4%)		2 (4%)	
Thymus	(45)	(45)	(45)	(44)
Fibrous histiocytoma, metastatic, mesentery				1 (2%)
Osteosarcoma, metastatic, bone		1 (2%)		
Schwannoma malignant, metastatic, heart		1 (2%)		
Thymoma benign		1 (2%)		
Integumentary System				
Mammary gland	(5)	(3)	(4)	(7)
Skin	(50)	(50)	(49)	(51)
Fibrous histiocytoma	1 (2%)	1 (2%)		1 (2%)
Fibrous histiocytoma, metastatic, mesentery				1 (2%)
Hemangiosarcoma	1 (2%)	1 (2%)		
Hemangiosarcoma, metastatic, spleen	1 (2%)			
Lipoma	1 (2%)			
Squamous cell papilloma		1 (2%)		1 (2%)
Musculoskeletal System				
Bone	(50)	(50)	(49)	(51)
Alveolar/bronchiolar carcinoma, metastatic, lung			1 (2%)	
Osteoma			1 (2%)	
Osteosarcoma		1 (2%)		
Skeletal muscle	(0)	(2)	(2)	(2)
Alveolar/bronchiolar carcinoma, metastatic, lung			1 (50%)	
Carcinoma, metastatic, uncertain primary site				1 (50%)
Hepatoblastoma				1 (50%)
Hepatoblastoma, metastatic, liver		1 (50%)		1 (50%)
Hepatocholangiocarcinoma, metastatic, liver			1 (50%)	
Osteosarcoma, metastatic, bone		1 (50%)		
Nervous System				
Brain	(50)	(50)	(49)	(51)
Meningioma malignant	1 (2%)			
Peripheral nerve	(2)	(1)	(0)	(0)
Spinal cord	(2)	(1)	(0)	(0)
Respiratory System				
Lung	(50)	(50)	(49)	(51)
Alveolar/bronchiolar adenoma	11 (22%)	14 (28%)	8 (16%)	2 (4%)
Alveolar/bronchiolar adenoma, multiple	2 (4%)		1 (2%)	
Alveolar/bronchiolar carcinoma	7 (14%)	10 (20%)	4 (8%)	2 (4%)
Alveolar/bronchiolar carcinoma, multiple		1 (2%)	1 (2%)	
Hepatoblastoma, metastatic, liver		3 (6%)	12 (24%)	5 (10%)
Hepatocellular carcinoma, metastatic, liver	8 (16%)	11 (22%)	5 (10%)	15 (29%)

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Respiratory System (continued)				
Lung (continued)	(50)	(50)	(49)	(51)
Hepatocolangiocarcinoma, metastatic, liver			1 (2%)	
Osteosarcoma, metastatic, bone		1 (2%)		
Schwannoma malignant, metastatic, heart		1 (2%)		
Nose	(50)	(50)	(49)	(51)
Glands, olfactory epithelium, adenoma		1 (2%)		
Trachea	(50)	(50)	(49)	(51)
Special Senses System				
Eye	(50)	(50)	(49)	(51)
Iris, melanoma malignant	1 (2%)			
Harderian gland	(50)	(50)	(49)	(51)
Adenoma	6 (12%)	11 (22%)	14 (29%)	19 (37%)
Carcinoma				3 (6%)
Urinary System				
Kidney	(50)	(50)	(49)	(51)
Adenoma	2 (4%)	2 (4%)		2 (4%)
Alveolar/bronchiolar carcinoma, metastatic, lung		1 (2%)		
Carcinoma, metastatic, uncertain primary site				1 (2%)
Ureter	(1)	(0)	(0)	(0)
Urethra	(1)	(0)	(0)	(0)
Hemangiosarcoma	1 (100%)			
Urinary bladder	(50)	(50)	(49)	(51)
Systemic Lesions				
Multiple organs ^c	(50)	(50)	(49)	(51)
Histiocytic sarcoma	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Leukemia lymphocytic			1 (2%)	
Lymphoma malignant			1 (2%)	1 (2%)
Mesothelioma malignant	1 (2%)			3 (6%)
Neoplasm Summary				
Total animals with primary neoplasms ^d	48	50	49	51
Total primary neoplasms	103	142	152	161
Total animals with benign neoplasms	43	44	46	44
Total benign neoplasms	67	72	70	65
Total animals with malignant neoplasms	30	42	49	47
Total malignant neoplasms	36	70	82	96
Total animals with metastatic neoplasms	10	17	20	21
Total metastatic neoplasms	12	25	30	38
Total animals with malignant neoplasms of uncertain primary site	1			1

^a Number of core study animals examined microscopically at the site and the number of animals with neoplasm

^b Pathology data for interim evaluation animals are not presented in this table.

^c Number of animals with any tissue examined microscopically

^d Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Harderian Gland: Adenoma				
Overall rate ^a	6/50 (12%)	11/50 (22%)	14/49 (29%)	19/51 (37%)
Adjusted rate ^b	14.7%	26.1%	38.0%	48.8%
Terminal rate ^c	2/25 (8%)	4/21 (19%)	5/12 (42%)	3/10 (30%)
First incidence (days)	548	533	491	458
Poly-3 test ^d	P<0.001	P=0.155	P=0.015	P<0.001
Harderian Gland: Carcinoma				
Overall rate	0/50 (0%)	0/50 (0%)	0/49 (0%)	3/51 (6%)
Adjusted rate	0.0%	0.0%	0.0%	9.3%
Terminal rate	0/25 (0%)	0/21 (0%)	0/12 (0%)	2/10 (20%)
First incidence (days)	— ^e	—	—	624
Poly-3 test	P=0.008	— ^f	—	P=0.087
Harderian Gland: Adenoma or Carcinoma				
Overall rate	6/50 (12%)	11/50 (22%)	14/49 (29%)	20/51 (39%)
Adjusted rate	14.7%	26.1%	38.0%	51.4%
Terminal rate	2/25 (8%)	4/21 (19%)	5/12 (42%)	4/10 (40%)
First incidence (days)	548	533	491	458
Poly-3 test	P<0.001	P=0.155	P=0.015	P<0.001
Small Intestine (Jejunum): Carcinoma				
Overall rate	1/50 (2%)	3/50 (6%)	0/49 (0%)	2/51 (4%)
Adjusted rate	2.5%	7.3%	0.0%	6.2%
Terminal rate	0/25 (0%)	1/21 (5%)	0/12 (0%)	0/10 (0%)
First incidence (days)	548	581	—	618
Poly-3 test	P=0.470	P=0.317	P=0.538N	P=0.431
Small Intestine (Duodenum or Jejunum): Carcinoma				
Overall rate	2/50 (4%)	4/50 (8%)	0/49 (0%)	2/51 (4%)
Adjusted rate	5.1%	9.7%	0.0%	6.2%
Terminal rate	0/25 (0%)	1/21 (5%)	0/12 (0%)	0/10 (0%)
First incidence (days)	548	581	—	618
Poly-3 test	P=0.484N	P=0.355	P=0.283N	P=0.619
Small Intestine (Duodenum or Jejunum): Adenoma or Carcinoma				
Overall rate	3/50 (6%)	4/50 (8%)	1/49 (2%)	2/51 (4%)
Adjusted rate	7.6%	9.7%	3.1%	6.2%
Terminal rate	1/25 (4%)	1/21 (5%)	1/12 (8%)	0/10 (0%)
First incidence (days)	548	581	730 (T)	618
Poly-3 test	P=0.382N	P=0.522	P=0.378N	P=0.592N
Liver: Hemangiosarcoma				
Overall rate	1/50 (2%)	4/50 (8%)	2/49 (4%)	4/51 (8%)
Adjusted rate	2.6%	9.7%	6.1%	12.3%
Terminal rate	1/25 (4%)	1/21 (5%)	1/12 (8%)	1/10 (10%)
First incidence (days)	730 (T)	609	603	600
Poly-3 test	P=0.139	P=0.196	P=0.443	P=0.128
Liver: Hepatocellular Adenoma				
Overall rate	39/50 (78%)	41/50 (82%)	42/49 (86%)	40/51 (78%)
Adjusted rate	87.2%	89.8%	90.9%	91.3%
Terminal rate	25/25 (100%)	20/21 (95%)	10/12 (83%)	10/10 (100%)
First incidence (days)	449	434	477	322
Poly-3 test	P=0.288	P=0.471	P=0.388	P=0.355

TABLE C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Liver: Hepatocellular Carcinoma				
Overall rate	12/50 (24%)	22/50 (44%)	27/49 (55%)	39/51 (76%)
Adjusted rate	29.3%	49.5%	66.1%	87.1%
Terminal rate	7/25 (28%)	8/21 (38%)	7/12 (58%)	8/10 (80%)
First incidence (days)	548	511	480	260
Poly-3 test	P<0.001	P=0.041	P<0.001	P<0.001
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	42/50 (84%)	47/50 (94%)	46/49 (94%)	48/51 (94%)
Adjusted rate	91.2%	97.7%	96.5%	98.1%
Terminal rate	25/25 (100%)	21/21 (100%)	11/12 (92%)	10/10 (100%)
First incidence (days)	449	434	477	260
Poly-3 test	P=0.071	P=0.109	P=0.211	P=0.081
Liver: Hepatoblastoma				
Overall rate	4/50 (8%)	24/50 (48%)	40/49 (82%)	34/51 (67%)
Adjusted rate	10.1%	53.6%	87.1%	77.5%
Terminal rate	1/25 (4%)	8/21 (38%)	9/12 (75%)	5/10 (50%)
First incidence (days)	590	433	477	360
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
Liver: Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	15/50 (30%)	34/50 (68%)	48/49 (98%)	45/51 (88%)
Adjusted rate	36.1%	71.9%	98.0%	95.5%
Terminal rate	8/25 (32%)	11/21 (52%)	11/12 (92%)	9/10 (90%)
First incidence (days)	548	433	477	260
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
Liver: Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma				
Overall rate	42/50 (84%)	50/50 (100%)	48/49 (98%)	49/51 (96%)
Adjusted rate	91.2%	100.0%	98.0%	99.1%
Terminal rate	25/25 (100%)	21/21 (100%)	11/12 (92%)	10/10 (100%)
First incidence (days)	449	433	477	260
Poly-3 test	P=0.036	P=0.015	P=0.110	P=0.034
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	13/50 (26%)	14/50 (28%)	9/49 (18%)	2/51 (4%)
Adjusted rate	31.5%	32.7%	24.9%	6.1%
Terminal rate	7/25 (28%)	6/21 (29%)	1/12 (8%)	0/10 (0%)
First incidence (days)	578	434	498	458
Poly-3 test	P=0.005N	P=0.547	P=0.344N	P=0.006N
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	7/50 (14%)	11/50 (22%)	5/49 (10%)	2/51 (4%)
Adjusted rate	17.9%	26.6%	14.6%	6.2%
Terminal rate	5/25 (20%)	6/21 (29%)	2/12 (17%)	1/10 (10%)
First incidence (days)	688	511	480	625
Poly-3 test	P=0.058N	P=0.253	P=0.474N	P=0.130N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	19/50 (38%)	25/50 (50%)	13/49 (27%)	4/51 (8%)
Adjusted rate	45.8%	56.5%	34.3%	12.0%
Terminal rate	11/25 (44%)	12/21 (57%)	2/12 (17%)	1/10 (10%)
First incidence (days)	578	434	480	458
Poly-3 test	P<0.001N	P=0.212	P=0.199N	P<0.001N

TABLE C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
All Organs: Hemangiosarcoma				
Overall rate	5/50 (10%)	5/50 (10%)	4/49 (8%)	5/51 (10%)
Adjusted rate	12.6%	12.1%	11.7%	15.3%
Terminal rate	2/25 (8%)	1/21 (5%)	1/12 (8%)	2/10 (20%)
First incidence (days)	626	499	499	600
Poly-3 test	P=0.428	P=0.604N	P=0.591N	P=0.504
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	5/50 (10%)	5/50 (10%)	5/49 (10%)	5/51 (10%)
Adjusted rate	12.6%	12.1%	14.6%	15.3%
Terminal rate	2/25 (8%)	1/21 (5%)	2/12 (17%)	2/10 (20%)
First incidence (days)	626	609	499	600
Poly-3 test	P=0.398	P=0.604N	P=0.538	P=0.504
All Organs: Malignant Mesothelioma				
Overall rate	1/50 (2%)	0/50 (0%)	0/49 (0%)	3/51 (6%)
Adjusted rate	2.6%	0.0%	0.0%	9.1%
Terminal rate	1/25 (4%)	0/21 (0%)	0/12 (0%)	0/10 (0%)
First incidence (days)	730 (T)	—	—	521
Poly-3 test	P=0.071	P=0.494N	P=0.535N	P=0.249
All Organs: Benign Neoplasms				
Overall rate	43/50 (86%)	44/50 (88%)	46/49 (94%)	44/51 (86%)
Adjusted rate	92.8%	93.8%	96.0%	94.3%
Terminal rate	25/25 (100%)	20/21 (95%)	11/12 (92%)	10/10 (100%)
First incidence (days)	449	434	477	322
Poly-3 test	P=0.435	P=0.612	P=0.398	P=0.570
All Organs: Malignant Neoplasms				
Overall rate	30/50 (60%)	42/50 (84%)	49/49 (100%)	47/51 (92%)
Adjusted rate	66.7%	85.6%	100.0%	98.2%
Terminal rate	14/25 (56%)	15/21 (71%)	12/12 (100%)	10/10 (100%)
First incidence (days)	449	433	477	260
Poly-3 test	P<0.001	P=0.021	P<0.001	P<0.001
All Organs: Benign or Malignant Neoplasms				
Overall rate	48/50 (96%)	50/50 (100%)	49/49 (100%)	51/51 (100%)
Adjusted rate	98.3%	100.0%	100.0%	100.0%
Terminal rate	25/25 (100%)	21/21 (100%)	12/12 (100%)	10/10 (100%)
First incidence (days)	449	433	477	260
Poly-3 test	P=0.307	P=0.604	P=0.609	P=0.599

(T) Terminal kill

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for liver and lung; for other tissues, denominator is number of animals necropsied.

^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill. A negative trend or a lower incidence in an exposure group is indicated by N.

^e Not applicable; no neoplasms in animal group

^f Value of statistic cannot be computed.

TABLE C3a
Historical Incidence of Liver Neoplasms in Control Male B6C3F1 Mice^a

Study (Study Start)	Hepatocellular Carcinoma	Hepatoblastoma
Historical Incidence: Drinking Water Studies		
β-Picoline (November 2004)	26/50	6/50
Bromodichloroacetic acid (September 2006)	12/50	4/50
Total (%)	38/100 (38.0%)	10/100 (10.0%)
Mean ± standard deviation	38.0% ± 19.8%	10.0% ± 2.8%
Range	24%-52%	8%-12%
Overall Historical Incidence: All Routes		
Total (%)	348/949 (36.7%)	40/949 (4.2%)
Mean ± standard deviation	36.7% ± 11.4%	4.2%±3.5%
Range	22%-56%	0%-12%

^a Data as of June 2013

TABLE C3b
Historical Incidence of Harderian Gland Neoplasms in Control Male B6C3F1 Mice^a

Study (Study Start)	Adenoma or Carcinoma
Historical Incidence: Drinking Water Studies	
β-Picoline (November 2004)	7/50
Bromodichloroacetic acid (September 2006)	6/50
Total (%)	13/100 (13.0%)
Mean ± standard deviation	13.0% ± 1.4%
Range	12%-14%
Overall Historical Incidence: All Routes	
Total (%)	153/950 (16.1%)
Mean ± standard deviation	16.1% ± 4.9%
Range	6%-24%

^a Data as of June 2013

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Disposition Summary				
Animals initially in study	66	66	66	66
<i>6-Month interim evaluation^b</i>	8	8	8	8
<i>14-Month interim evaluation^b</i>	8	8	8	7
Early deaths				
Moribund	15	14	27	27
Natural deaths	10	15	10	14
Survivors				
Terminal kill	25	21	12	10
Missing			1	
Animals examined microscopically	566	66	65	66
Alimentary System				
Esophagus	(50)	(50)	(49)	(51)
Gallbladder	(48)	(49)	(48)	(47)
Intestine large, cecum	(50)	(50)	(49)	(51)
Lymphoid tissue, hyperplasia		1 (2%)		
Intestine large, colon	(50)	(50)	(49)	(51)
Epithelium, hyperplasia		1 (2%)		
Intestine large, rectum	(50)	(50)	(49)	(51)
Intestine small, duodenum	(50)	(50)	(49)	(51)
Intestine small, ileum	(50)	(50)	(49)	(51)
Inflammation	3 (6%)	1 (2%)		
Epithelium, hyperplasia	2 (4%)			
Muscularis, hyperplasia	1 (2%)			
Intestine small, jejunum	(50)	(50)	(49)	(51)
Inflammation	1 (2%)			2 (4%)
Peyer's patch, hyperplasia, lymphoid		2 (4%)		1 (2%)
Liver	(50)	(50)	(49)	(51)
Amyloid deposition			1 (2%)	
Angiectasis	1 (2%)		1 (2%)	1 (2%)
Basophilic focus	1 (2%)	2 (4%)	6 (12%)	3 (6%)
Clear cell focus	18 (36%)	14 (28%)	4 (8%)	14 (27%)
Congestion				1 (2%)
Eosinophilic focus	30 (60%)	33 (66%)	30 (61%)	16 (31%)
Fatty change	11 (22%)	1 (2%)		4 (8%)
Fatty change, focal		1 (2%)		
Focus of cellular alteration, atypical		19 (38%)	42 (86%)	43 (84%)
Hematopoietic cell proliferation	1 (2%)		1 (2%)	
Infarct		1 (2%)	1 (2%)	1 (2%)
Infiltration cellular, mixed cell				1 (2%)
Inflammation	7 (14%)	6 (12%)	3 (6%)	4 (8%)
Mineralization			1 (2%)	
Mixed cell focus	13 (26%)	6 (12%)	4 (8%)	5 (10%)
Tension lipidosis	3 (6%)	1 (2%)		
Bile duct, cyst		1 (2%)		1 (2%)
Capsule, fibrosis				1 (2%)
Centrilobular, fatty change			1 (2%)	1 (2%)
Hepatocyte, necrosis	5 (10%)	9 (18%)	10 (20%)	8 (16%)
Oval cell, hyperplasia	1 (2%)		3 (6%)	
Mesentery	(7)	(1)	(3)	(6)
Inflammation				1 (17%)
Necrosis	5 (71%)	1 (100%)	1 (33%)	2 (33%)

^a Number of core study animals examined microscopically at the site and the number of animals with lesion

^b Pathology data for interim evaluation animals are not presented in this table.

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Alimentary System (continued)				
Pancreas	(50)	(50)	(49)	(51)
Amyloid deposition	1 (2%)			
Fibrosis				1 (2%)
Acinus, atrophy	2 (4%)			
Acinus, hyperplasia		1 (2%)	1 (2%)	
Duct, cyst				1 (2%)
Salivary glands	(50)	(50)	(49)	(51)
Hyperplasia		1 (2%)		
Stomach, forestomach	(50)	(50)	(49)	(51)
Inflammation	1 (2%)	1 (2%)		1 (2%)
Ulcer	3 (6%)	1 (2%)	1 (2%)	2 (4%)
Epithelium, hyperplasia	2 (4%)		1 (2%)	1 (2%)
Stomach, glandular	(50)	(50)	(49)	(51)
Amyloid deposition			3 (6%)	1 (2%)
Erosion				1 (2%)
Inflammation	1 (2%)			
Mineralization	1 (2%)			
Ulcer			2 (4%)	
Epithelium, hyperplasia	2 (4%)			
Glands, ectasia		1 (2%)		1 (2%)
Tooth	(35)	(22)	(13)	(0)
Dysplasia	1 (3%)			
Malformation	34 (97%)	20 (91%)	13 (100%)	
Peridental tissue, fibrosis		2 (9%)		
Peridental tissue, inflammation		1 (5%)		
Cardiovascular System				
Blood vessel	(50)	(50)	(49)	(51)
Aorta, inflammation				1 (2%)
Aorta, mineralization			1 (2%)	1 (2%)
Heart	(50)	(50)	(49)	(51)
Cardiomyopathy	31 (62%)	41 (82%)	30 (61%)	33 (65%)
Arteriole, hyperplasia	1 (2%)			
Arteriole, inflammation	1 (2%)	1 (2%)		
Artery, inflammation				1 (2%)
Artery, mineralization			1 (2%)	
Atrium, thrombosis	2 (4%)		1 (2%)	
Endocardium, inflammation				1 (2%)
Epicardium, fibrosis	1 (2%)			
Myocardium, mineralization	1 (2%)		1 (2%)	2 (4%)
Valve, inflammation	1 (2%)			
Endocrine System				
Adrenal cortex	(50)	(50)	(49)	(50)
Angiectasis		1 (2%)		1 (2%)
Hyperplasia		2 (4%)		1 (2%)
Hypertrophy	14 (28%)	21 (42%)	9 (18%)	9 (18%)
Necrosis				1 (2%)
Subcapsular, hyperplasia			1 (2%)	
Adrenal medulla	(50)	(50)	(49)	(50)
Angiectasis	1 (2%)			
Hyperplasia		1 (2%)	1 (2%)	1 (2%)
Mineralization				1 (2%)
Necrosis				1 (2%)
Islets, pancreatic	(50)	(50)	(49)	(51)

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Endocrine System (continued)				
Parathyroid gland	(44)	(45)	(44)	(44)
Pituitary gland	(50)	(49)	(49)	(51)
Cyst		5 (10%)		2 (4%)
Pars distalis, hyperplasia	1 (2%)	2 (4%)	4 (8%)	
Thyroid gland	(50)	(50)	(49)	(51)
Cyst		1 (2%)		
C-cell, hyperplasia		1 (2%)		
Follicle, cyst	1 (2%)	3 (6%)	1 (2%)	1 (2%)
Follicular cell, hyperplasia			1 (2%)	
General Body System				
Peritoneum	(0)	(0)	(0)	(1)
Genital System				
Epididymis	(50)	(50)	(49)	(51)
Atrophy		7 (14%)	10 (20%)	17 (33%)
Granuloma sperm				1 (2%)
Hypospermia		1 (2%)		17 (33%)
Inflammation	2 (4%)	1 (2%)	1 (2%)	
Epithelium, degeneration	1 (2%)	1 (2%)	10 (20%)	6 (12%)
Mesothelium, hyperplasia			1 (2%)	1 (2%)
Penis	(0)	(1)	(0)	(0)
Hyperplasia, squamous		1 (100%)		
Preputial gland	(50)	(50)	(49)	(51)
Atrophy			1 (2%)	
Inflammation	7 (14%)	4 (8%)	3 (6%)	8 (16%)
Duct, ectasia	6 (12%)	6 (12%)	5 (10%)	10 (20%)
Prostate	(50)	(50)	(49)	(51)
Atrophy		1 (2%)		
Inflammation	3 (6%)	3 (6%)	1 (2%)	2 (4%)
Arteriole, inflammation				1 (2%)
Seminal vesicle	(50)	(50)	(49)	(51)
Atrophy		1 (2%)		1 (2%)
Dilatation	1 (2%)	1 (2%)	1 (2%)	
Inflammation	2 (4%)	2 (4%)	3 (6%)	1 (2%)
Testes	(50)	(50)	(49)	(51)
Atrophy	4 (8%)	6 (12%)	13 (27%)	23 (45%)
Fibrosis		1 (2%)		
Hematopoietic System				
Bone marrow	(50)	(50)	(49)	(51)
Angiectasis				2 (4%)
Hyperplasia		2 (4%)		
Necrosis		1 (2%)		
Lymph node	(0)	(1)	(2)	(5)
Axillary, hyperplasia, plasma cell				1 (20%)
Lumbar, hyperplasia, lymphoid		1 (100%)		
Lumbar, hyperplasia, plasma cell		1 (100%)		1 (20%)
Lumbar, inflammation, suppurative				1 (20%)
Renal, hyperplasia, plasma cell				1 (20%)
Lymph node, mandibular	(50)	(50)	(49)	(51)
Degeneration, cystic				1 (2%)
Hyperplasia, lymphoid		1 (2%)		
Hyperplasia, plasma cell	3 (6%)	1 (2%)		1 (2%)

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Hematopoietic System (continued)				
Lymph node, mesenteric	(50)	(48)	(48)	(48)
Hematopoietic cell proliferation		1 (2%)		2 (4%)
Hemorrhage				1 (2%)
Hyperplasia, lymphoid				1 (2%)
Hyperplasia, plasma cell	2 (4%)	4 (8%)		1 (2%)
Necrosis, lymphoid				1 (2%)
Spleen	(50)	(50)	(49)	(50)
Atrophy			3 (6%)	1 (2%)
Hematopoietic cell proliferation	1 (2%)	3 (6%)	5 (10%)	4 (8%)
Hyperplasia, lymphoid	4 (8%)	7 (14%)	5 (10%)	5 (10%)
Infarct			1 (2%)	
Thymus	(45)	(45)	(45)	(44)
Atrophy		1 (2%)		
Mineralization	1 (2%)			
Integumentary System				
Mammary gland	(5)	(3)	(4)	(7)
Skin	(50)	(50)	(49)	(51)
Inflammation, suppurative			1 (2%)	
Ulcer	1 (2%)	2 (4%)	1 (2%)	1 (2%)
Dermis, fibrosis		1 (2%)		
Epidermis, hyperplasia		1 (2%)		
Musculoskeletal System				
Bone	(50)	(50)	(49)	(51)
Cranium, periosteum, fibrosis				1 (2%)
Femur, osteopetrosis		1 (2%)		
Skeletal muscle	(0)	(2)	(2)	(2)
Nervous System				
Brain	(50)	(50)	(49)	(51)
Cerebrum, demyelination			1 (2%)	
Peripheral nerve	(2)	(1)	(0)	(0)
Degeneration	1 (50%)	1 (100%)		
Spinal cord	(2)	(1)	(0)	(0)
Demyelination		1 (100%)		
Respiratory System				
Lung	(50)	(50)	(49)	(51)
Inflammation	1 (2%)		3 (6%)	4 (8%)
Thrombosis	1 (2%)	2 (4%)		2 (4%)
Alveolar epithelium, hyperplasia	2 (4%)	2 (4%)	4 (8%)	4 (8%)
Alveolus, infiltration cellular, histiocyte		1 (2%)	4 (8%)	1 (2%)
Capillary, thrombosis				1 (2%)
Perivascular, infiltration cellular, mononuclear cell	1 (2%)			
Vein, mineralization			1 (2%)	

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Respiratory System (continued)				
Nose	(50)	(50)	(49)	(51)
Inflammation	5 (10%)	9 (18%)	7 (14%)	9 (18%)
Glands, dilatation			1 (2%)	
Glands, fibrosis			1 (2%)	
Glands, hyperplasia		3 (6%)	2 (4%)	
Respiratory epithelium, hyperplasia		1 (2%)		
Respiratory epithelium, metaplasia		1 (2%)		
Turbinate, fibrosis, focal				1 (2%)
Trachea	(50)	(50)	(49)	(51)
Inflammation				1 (2%)
Peritracheal tissue, cyst	1 (2%)			
Special Senses System				
Eye	(50)	(50)	(49)	(51)
Cataract	1 (2%)		1 (2%)	1 (2%)
Phthisis bulbi				2 (4%)
Synechia	1 (2%)			1 (2%)
Cornea, inflammation		2 (4%)	2 (4%)	1 (2%)
Optic nerve, degeneration	1 (2%)	1 (2%)		
Retina, dysplasia	1 (2%)	1 (2%)	1 (2%)	
Harderian gland	(50)	(50)	(49)	(51)
Inflammation				1 (2%)
Epithelium, hyperplasia	1 (2%)	1 (2%)	3 (6%)	4 (8%)
Urinary System				
Kidney	(50)	(50)	(49)	(51)
Atrophy, diffuse				1 (2%)
Casts protein				1 (2%)
Hydronephrosis	2 (4%)		1 (2%)	1 (2%)
Infarct	3 (6%)	3 (6%)		5 (10%)
Infiltration cellular, mononuclear cell		1 (2%)		2 (4%)
Inflammation	3 (6%)	4 (8%)	2 (4%)	3 (6%)
Metaplasia, osseous		3 (6%)		
Mineralization	2 (4%)	1 (2%)	1 (2%)	
Nephropathy	49 (98%)	47 (94%)	45 (92%)	42 (82%)
Papilla, necrosis	1 (2%)		1 (2%)	
Renal tubule, cyst	4 (8%)	3 (6%)	1 (2%)	
Renal tubule, hyperplasia		2 (4%)		
Ureter	(1)	(0)	(0)	(0)
Urethra	(1)	(0)	(0)	(0)
Urinary bladder	(50)	(50)	(49)	(51)
Infiltration cellular, mononuclear cell				1 (2%)
Inflammation	2 (4%)			1 (2%)
Metaplasia, squamous	1 (2%)			
Transitional epithelium, hyperplasia				1 (2%)

APPENDIX D
SUMMARY OF LESIONS IN FEMALE MICE
IN THE 2-YEAR DRINKING WATER STUDY
OF BROMODICHLOROACETIC ACID

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TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Disposition Summary				
Animals initially in study	66	66	66	66
6-Month interim evaluation^b	8	8	8	8
14-Month interim evaluation^b	8	8	8	8
Early deaths				
Moribund	9	9	8	11
Natural deaths	11	8	13	12
Survivors				
Died last week of study				1
Terminal kill	30	33	29	26
Animals examined microscopically	66	66	66	66
Alimentary System				
Esophagus	(50)	(50)	(47)	(48)
Sarcoma				1 (2%)
Gallbladder	(47)	(48)	(50)	(44)
Leiomyosarcoma, metastatic, mesentery		1 (2%)		
Intestine large, cecum	(50)	(50)	(50)	(50)
Hemangiosarcoma			1 (2%)	
Intestine large, colon	(50)	(50)	(50)	(50)
Fibrous histiocytoma, metastatic, skin	1 (2%)			
Intestine large, rectum	(49)	(50)	(50)	(50)
Intestine small, duodenum	(49)	(50)	(50)	(49)
Adenoma			1 (2%)	
Leiomyosarcoma		1 (2%)		
Intestine small, ileum	(50)	(50)	(50)	(49)
Carcinoma		1 (2%)		
Leiomyosarcoma, metastatic, urinary bladder			1 (2%)	
Intestine small, jejunum	(50)	(50)	(50)	(50)
Leiomyosarcoma		1 (2%)		
Liver	(49)	(50)	(49)	(50)
Hemangiosarcoma	2 (4%)	4 (8%)	4 (8%)	8 (16%)
Hemangiosarcoma, metastatic, skin			1 (2%)	
Hemangiosarcoma, metastatic, spleen		1 (2%)		
Hepatoblastoma		1 (2%)	3 (6%)	4 (8%)
Hepatoblastoma, multiple			1 (2%)	2 (4%)
Hepatocellular adenoma	8 (16%)	4 (8%)	5 (10%)	7 (14%)
Hepatocellular adenoma, multiple	25 (51%)	38 (76%)	37 (76%)	37 (74%)
Hepatocellular carcinoma	9 (18%)	12 (24%)	16 (33%)	13 (26%)
Hepatocellular carcinoma, multiple		5 (10%)	6 (12%)	13 (26%)
Hepatocholangiocarcinoma				1 (2%)
Sarcoma, metastatic, uncertain primary site			1 (2%)	
Mesentery	(10)	(13)	(13)	(10)
Fibrosarcoma, metastatic, skin			1 (8%)	
Fibrous histiocytoma		1 (8%)	1 (8%)	
Hemangiosarcoma	1 (10%)			
Leiomyosarcoma		1 (8%)		
Leiomyosarcoma, metastatic, urinary bladder			1 (8%)	
Oral mucosa	(2)	(2)	(0)	(0)
Pancreas	(49)	(50)	(50)	(48)
Cystadenoma		1 (2%)		
Leiomyosarcoma		1 (2%)		
Salivary glands	(48)	(49)	(44)	(45)

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Alimentary System (continued)				
Stomach, forestomach	(50)	(50)	(50)	(50)
Hemangiosarcoma				1 (2%)
Squamous cell papilloma	1 (2%)			2 (4%)
Stomach, glandular	(50)	(50)	(50)	(50)
Tongue	(0)	(0)	(0)	(1)
Squamous cell carcinoma				1 (100%)
Tooth	(0)	(2)	(0)	(1)
Cardiovascular System				
Blood vessel	(50)	(50)	(48)	(49)
Heart	(50)	(50)	(48)	(48)
Endocrine System				
Adrenal cortex	(49)	(50)	(47)	(49)
Leiomyosarcoma		1 (2%)		
Subcapsular, adenoma		1 (2%)		
Adrenal medulla	(50)	(50)	(48)	(49)
Leiomyosarcoma		1 (2%)		
Pheochromocytoma benign			1 (2%)	
Islets, pancreatic	(49)	(50)	(50)	(48)
Parathyroid gland	(45)	(46)	(41)	(38)
Pituitary gland	(49)	(49)	(46)	(48)
Pars distalis, adenoma	2 (4%)	1 (2%)	3 (7%)	
Thyroid gland	(49)	(50)	(46)	(45)
C-cell, carcinoma				1 (2%)
Follicular cell, adenoma		1 (2%)		
General Body System				
None				
Genital System				
Clitoral gland	(47)	(50)	(50)	(48)
Carcinoma				1 (2%)
Ovary	(50)	(50)	(50)	(50)
Cystadenoma	1 (2%)	3 (6%)	2 (4%)	2 (4%)
Granulosa cell tumor benign			1 (2%)	
Granulosa cell tumor malignant				1 (2%)
Hemangiosarcoma		1 (2%)	2 (4%)	
Uterus	(50)	(50)	(50)	(50)
Hemangiosarcoma	1 (2%)		2 (4%)	
Polyp stromal	4 (8%)	3 (6%)	1 (2%)	1 (2%)
Sarcoma stromal			1 (2%)	
Vagina	(0)	(0)	(0)	(1)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hemangiosarcoma			1 (2%)	
Hemangiosarcoma, metastatic, liver	1 (2%)			

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Hematopoietic System (continued)				
Lymph node	(2)	(3)	(3)	(5)
Axillary, schwannoma malignant, metastatic, skin				1 (20%)
Lumbar, plasma cell tumor malignant			1 (33%)	
Lumbar, schwannoma malignant, metastatic, skin				1 (20%)
Renal, plasma cell tumor malignant			1 (33%)	
Lymph node, mandibular	(48)	(49)	(44)	(45)
Plasma cell tumor malignant			1 (2%)	
Lymph node, mesenteric	(48)	(50)	(47)	(47)
Plasma cell tumor malignant			1 (2%)	
Spleen	(49)	(50)	(50)	(48)
Hemangiosarcoma		2 (4%)	1 (2%)	
Hemangiosarcoma, metastatic, skin			1 (2%)	
Plasma cell tumor malignant			1 (2%)	
Thymus	(50)	(50)	(46)	(46)
Alveolar/bronchiolar carcinoma, metastatic, lung		1 (2%)		
Plasma cell tumor malignant			1 (2%)	
Schwannoma malignant, metastatic, skin	1 (2%)			
Thymoma malignant				1 (2%)
Integumentary System				
Mammary gland	(50)	(50)	(49)	(50)
Carcinoma	1 (2%)			
Skin	(50)	(50)	(50)	(50)
Basal cell carcinoma				1 (2%)
Fibrosarcoma			1 (2%)	
Fibrous histiocytoma	3 (6%)	1 (2%)	2 (4%)	4 (8%)
Hemangioma				1 (2%)
Hemangiosarcoma	1 (2%)	1 (2%)	1 (2%)	
Osteosarcoma		1 (2%)		
Sarcoma	1 (2%)		1 (2%)	
Schwannoma malignant	3 (6%)		1 (2%)	5 (10%)
Squamous cell carcinoma				1 (2%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Hemangiosarcoma, metastatic, spleen			1 (2%)	
Osteoma	1 (2%)			1 (2%)
Osteosarcoma		1 (2%)		
Skeletal muscle	(1)	(2)	(2)	(1)
Fibrosarcoma, metastatic, skin			1 (50%)	
Fibrous histiocytoma, metastatic, mesentery		1 (50%)		
Hemangiosarcoma	1 (100%)			
Leiomyosarcoma		1 (50%)		
Sarcoma			1 (50%)	
Nervous System				
Brain	(49)	(50)	(47)	(49)

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Respiratory System				
Lung	(50)	(50)	(48)	(48)
Alveolar/bronchiolar adenoma	3 (6%)	2 (4%)	1 (2%)	2 (4%)
Alveolar/bronchiolar carcinoma	2 (4%)	2 (4%)	1 (2%)	5 (10%)
Alveolar/bronchiolar carcinoma, multiple		1 (2%)		
Fibrous histiocytoma, metastatic, skin				1 (2%)
Hemangiosarcoma, metastatic, liver	1 (2%)			
Hepatoblastoma, metastatic, liver			2 (4%)	
Hepatocellular carcinoma, metastatic, liver	1 (2%)	6 (12%)	4 (8%)	7 (15%)
Schwannoma malignant, metastatic, skin	1 (2%)			1 (2%)
Nose	(50)	(50)	(50)	(50)
Trachea	(50)	(50)	(46)	(46)
Special Senses System				
Eye	(48)	(50)	(46)	(46)
Harderian gland	(48)	(50)	(46)	(46)
Adenoma	5 (10%)	4 (8%)	6 (13%)	5 (11%)
Carcinoma		1 (2%)	1 (2%)	1 (2%)
Urinary System				
Kidney	(50)	(50)	(50)	(49)
Urinary bladder	(50)	(50)	(50)	(50)
Fibrous histiocytoma, metastatic, skin	1 (2%)			
Leiomyosarcoma			1 (2%)	
Systemic Lesions				
Multiple organs ^c	(50)	(50)	(50)	(50)
Histiocytic sarcoma	2 (4%)	3 (6%)	1 (2%)	3 (6%)
Lymphoma malignant	6 (12%)	3 (6%)	6 (12%)	10 (20%)
Neoplasm Summary				
Total animals with primary neoplasms ^d	45	47	46	49
Total primary neoplasms	83	106	120	136
Total animals with benign neoplasms	36	43	43	44
Total benign neoplasms	50	58	58	58
Total animals with malignant neoplasms	31	33	38	45
Total malignant neoplasms	33	48	62	78
Total animals with metastatic neoplasms	4	10	10	8
Total metastatic neoplasms	7	10	14	11
Total animals with malignant neoplasms of uncertain primary site			1	

^a Number of core study animals examined microscopically at the site and the number of animals with neoplasm

^b Pathology data for interim evaluation animals are not presented in this table.

^c Number of animals with any tissue examined microscopically

^d Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Harderian Gland: Adenoma				
Overall rate ^a	5/50 (10%)	4/50 (8%)	6/50 (12%)	5/50 (10%)
Adjusted rate ^b	11.5%	9.1%	13.5%	11.7%
Terminal rate ^c	3/30 (10%)	3/33 (9%)	3/29 (10%)	4/27 (15%)
First incidence (days)	540	509	575	614
Poly-3 test ^d	P=0.484	P=0.494N	P=0.513	P=0.619
Harderian Gland: Adenoma or Carcinoma				
Overall rate	5/50 (10%)	4/50 (8%)	7/50 (14%)	6/50 (12%)
Adjusted rate	11.5%	9.1%	15.8%	14.0%
Terminal rate	3/30 (10%)	3/33 (9%)	4/29 (14%)	4/27 (15%)
First incidence (days)	540	509	575	614
Poly-3 test	P=0.336	P=0.494N	P=0.392	P=0.488
Liver: Hemangiosarcoma				
Overall rate	2/49 (4%)	4/50 (8%)	4/49 (8%)	8/50 (16%)
Adjusted rate	4.7%	9.2%	9.2%	18.6%
Terminal rate	1/30 (3%)	3/33 (9%)	3/29 (10%)	5/27 (19%)
First incidence (days)	596	659	575	660
Poly-3 test	P=0.026	P=0.347	P=0.346	P=0.045
Liver: Hepatocellular Adenoma				
Overall rate	33/49 (67%)	42/50 (84%)	42/49 (86%)	44/50 (88%)
Adjusted rate	75.3%	90.7%	92.5%	93.0%
Terminal rate	26/30 (87%)	32/33 (97%)	28/29 (97%)	27/27 (100%)
First incidence (days)	540	386	597	503
Poly-3 test	P=0.009	P=0.030	P=0.015	P=0.010
Liver: Hepatocellular Carcinoma				
Overall rate	9/49 (18%)	17/50 (34%)	22/49 (45%)	26/50 (52%)
Adjusted rate	21.1%	37.9%	49.8%	59.1%
Terminal rate	5/30 (17%)	13/33 (39%)	15/29 (52%)	20/27 (74%)
First incidence (days)	604	509	603	607
Poly-3 test	P<0.001	P=0.065	P=0.004	P<0.001
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	36/49 (73%)	44/50 (88%)	43/49 (88%)	46/50 (92%)
Adjusted rate	81.1%	93.7%	94.7%	95.5%
Terminal rate	26/30 (87%)	33/33 (100%)	29/29 (100%)	27/27 (100%)
First incidence (days)	540	386	597	503
Poly-3 test	P=0.013	P=0.043	P=0.030	P=0.017
Liver: Hepatoblastoma				
Overall rate	0/49 (0%)	1/50 (2%)	4/49 (8%)	6/50 (12%)
Adjusted rate	0.0%	2.3%	9.3%	13.9%
Terminal rate	0/30 (0%)	0/33 (0%)	3/29 (10%)	3/27 (11%)
First incidence (days)	— ^e	688	716	636
Poly-3 test	P=0.003	P=0.506	P=0.062	P=0.016
Liver: Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	9/49 (18%)	18/50 (36%)	24/49 (49%)	28/50 (56%)
Adjusted rate	21.1%	40.0%	54.4%	63.0%
Terminal rate	5/30 (17%)	13/33 (39%)	17/29 (59%)	20/27 (74%)
First incidence (days)	604	509	603	607
Poly-3 test	P<0.001	P=0.043	P<0.001	P<0.001

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Liver: Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma				
Overall rate	36/49 (73%)	44/50 (88%)	43/49 (88%)	46/50 (92%)
Adjusted rate	81.1%	93.7%	94.7%	95.5%
Terminal rate	26/30 (87%)	33/33 (100%)	29/29 (100%)	27/27 (100%)
First incidence (days)	540	386	597	503
Poly-3 test	P=0.013	P=0.043	P=0.030	P=0.017
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	3/50 (6%)	2/50 (4%)	1/48 (2%)	2/48 (4%)
Adjusted rate	7.1%	4.6%	2.4%	4.8%
Terminal rate	3/30 (10%)	2/33 (6%)	0/29 (0%)	1/27 (4%)
First incidence (days)	729 (T)	729 (T)	687	614
Poly-3 test	P=0.418N	P=0.491N	P=0.306N	P=0.509N
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	2/50 (4%)	3/50 (6%)	1/48 (2%)	5/48 (10%)
Adjusted rate	4.7%	6.8%	2.4%	12.0%
Terminal rate	1/30 (3%)	0/33 (0%)	0/29 (0%)	4/27 (15%)
First incidence (days)	584	549	698	503
Poly-3 test	P=0.146	P=0.514	P=0.506N	P=0.205
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	5/50 (10%)	5/50 (10%)	2/48 (4%)	7/48 (15%)
Adjusted rate	11.7%	11.3%	4.7%	16.6%
Terminal rate	4/30 (13%)	2/33 (6%)	0/29 (0%)	5/27 (19%)
First incidence (days)	584	549	687	503
Poly-3 test	P=0.305	P=0.611N	P=0.220N	P=0.367
Ovary: Cystadenoma				
Overall rate	1/50 (2%)	3/50 (6%)	2/50 (4%)	2/50 (4%)
Adjusted rate	2.4%	6.9%	4.6%	4.7%
Terminal rate	0/30 (0%)	3/33 (9%)	1/29 (3%)	1/27 (4%)
First incidence (days)	694	729 (T)	721	710
Poly-3 test	P=0.498	P=0.311	P=0.508	P=0.499
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	2/49 (4%)	1/49 (2%)	3/46 (7%)	0/48 (0%)
Adjusted rate	4.8%	2.3%	7.5%	0.0%
Terminal rate	2/30 (7%)	1/33 (3%)	3/29 (10%)	0/27 (0%)
First incidence (days)	729 (T)	729 (T)	729 (T)	—
Poly-3 test	P=0.251N	P=0.488N	P=0.483	P=0.239N
Skin: Fibrous Histiocytoma				
Overall rate	3/50 (6%)	1/50 (2%)	2/50 (4%)	4/50 (8%)
Adjusted rate	7.0%	2.3%	4.6%	9.4%
Terminal rate	0/30 (0%)	0/33 (0%)	0/29 (0%)	1/27 (4%)
First incidence (days)	604	666	666	662
Poly-3 test	P=0.287	P=0.303N	P=0.492N	P=0.495
Skin: Fibrous Histiocytoma, Fibrosarcoma, or Sarcoma				
Overall rate	4/50 (8%)	1/50 (2%)	4/50 (8%)	4/50 (8%)
Adjusted rate	9.2%	2.3%	9.0%	9.4%
Terminal rate	0/30 (0%)	0/33 (0%)	0/29 (0%)	1/27 (4%)
First incidence (days)	576	666	597	662
Poly-3 test	P=0.394	P=0.180N	P=0.633N	P=0.632

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Skin: Malignant Schwannoma				
Overall rate	3/50 (6%)	0/50 (0%)	1/50 (2%)	5/50 (10%)
Adjusted rate	7.1%	0.0%	2.3%	11.4%
Terminal rate	1/30 (3%)	0/33 (0%)	0/29 (0%)	1/27 (4%)
First incidence (days)	701	—	669	534
Poly-3 test	P=0.113	P=0.115N	P=0.295N	P=0.372
Uterus: Stromal Polyp				
Overall rate	4/50 (8%)	3/50 (6%)	1/50 (2%)	1/50 (2%)
Adjusted rate	9.4%	6.9%	2.3%	2.3%
Terminal rate	2/30 (7%)	3/33 (9%)	1/29 (3%)	0/27 (0%)
First incidence (days)	712	729 (T)	729 (T)	614
Poly-3 test	P=0.091N	P=0.490N	P=0.171N	P=0.176N
Uterus: Stromal Polyp or Stromal Sarcoma				
Overall rate	4/50 (8%)	3/50 (6%)	2/50 (4%)	1/50 (2%)
Adjusted rate	9.4%	6.9%	4.6%	2.3%
Terminal rate	2/30 (7%)	3/33 (9%)	2/29 (7%)	0/27 (0%)
First incidence (days)	712	729 (T)	729 (T)	614
Poly-3 test	P=0.113N	P=0.490N	P=0.326N	P=0.176N
All Organs: Hemangiosarcoma				
Overall rate	6/50 (12%)	8/50 (16%)	12/50 (24%)	9/50 (18%)
Adjusted rate	13.8%	18.4%	26.9%	20.9%
Terminal rate	3/30 (10%)	6/33 (18%)	7/29 (24%)	6/27 (22%)
First incidence (days)	596	659	575	660
Poly-3 test	P=0.219	P=0.386	P=0.101	P=0.275
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	6/50 (12%)	8/50 (16%)	12/50 (24%)	10/50 (20%)
Adjusted rate	13.8%	18.4%	26.9%	23.3%
Terminal rate	3/30 (10%)	6/33 (18%)	7/29 (24%)	7/27 (26%)
First incidence (days)	596	659	575	660
Poly-3 test	P=0.146	P=0.386	P=0.101	P=0.193
All Organs: Histiocytic Sarcoma				
Overall rate	2/50 (4%)	3/50 (6%)	1/50 (2%)	3/50 (6%)
Adjusted rate	4.7%	6.9%	2.3%	7.0%
Terminal rate	1/30 (3%)	1/33 (3%)	0/29 (0%)	0/27 (0%)
First incidence (days)	549	666	682	636
Poly-3 test	P=0.476	P=0.508	P=0.494N	P=0.500
All Organs: Malignant Lymphoma				
Overall rate	6/50 (12%)	3/50 (6%)	6/50 (12%)	10/50 (20%)
Adjusted rate	13.8%	6.9%	13.8%	22.6%
Terminal rate	4/30 (13%)	3/33 (9%)	3/29 (10%)	5/27 (19%)
First incidence (days)	544	729 (T)	701	265
Poly-3 test	P=0.071	P=0.244N	P=0.619N	P=0.213
All Organs: Benign Neoplasms				
Overall rate	36/50 (72%)	43/50 (86%)	43/50 (86%)	44/50 (88%)
Adjusted rate	81.4%	91.5%	92.5%	93.0%
Terminal rate	27/30 (90%)	32/33 (97%)	28/29 (97%)	27/27 (100%)
First incidence (days)	540	386	575	503
Poly-3 test	P=0.058	P=0.101	P=0.074	P=0.057

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
All Organs: Malignant Neoplasms				
Overall rate	31/50 (62%)	33/50 (66%)	38/50 (76%)	45/50 (90%)
Adjusted rate	64.9%	70.6%	81.2%	92.9%
Terminal rate	14/30 (47%)	22/33 (67%)	21/29 (72%)	25/27 (93%)
First incidence (days)	544	509	575	265
Poly-3 test	P<0.001	P=0.353	P=0.056	P<0.001
All Organs: Benign or Malignant Neoplasms				
Overall rate	45/50 (90%)	47/50 (94%)	46/50 (92%)	49/50 (98%)
Adjusted rate	93.1%	97.9%	98.3%	99.1%
Terminal rate	27/30 (90%)	33/33 (100%)	29/29 (100%)	27/27 (100%)
First incidence (days)	540	386	575	265
Poly-3 test	P=0.070	P=0.247	P=0.205	P=0.137

(T) Terminal kill

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for liver, lung, ovary, and pituitary gland; for other tissues, denominator is number of animals necropsied.

^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal kill. A negative trend or a lower incidence in an exposure group is indicated by N.

^e Not applicable; no neoplasms in animal group

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

Study (Study Start)	Hepatocellular Adenoma	Hepatocellular Carcinoma	Hepatoblastoma
Historical Incidence: Drinking Water Studies			
β-Picoline (November 2004)	38/49	11/49	1/49
Bromodichloroacetic acid (September 2006)	33/49	9/49	0/49
Total (%)	71/98 (72.5%)	20/98 (20.4%)	10/98 (10.0%)
Mean ± standard deviation	72.5% ± 7.2%	20.4% ± 2.9%	10.0% ± 2.8%
Range	67%-78%	18%-22%	8%-12%
Overall Historical Incidence: All Routes			
Total (%)	378/948 (39.9%)	152/948 (16.0%)	40/948 (4.2%)
Mean ± standard deviation	39.9% ± 18.7%	16.0% ± 10.6%	4.2% ± 3.5%
Range	14%-78%	4%-46%	0%-12%

^a Data as of June 2013

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Disposition Summary				
Animals initially in study	66	66	66	66
<i>6-Month interim evaluation^b</i>	8	8	8	8
<i>14-Month interim evaluation^b</i>	8	8	8	8
Early deaths				
Moribund	9	9	8	11
Natural deaths	11	8	13	12
Survivors				
Died last week of study				1
Terminal kill	30	33	29	26
Animals examined microscopically	66	66	66	66
Alimentary System				
Esophagus	(50)	(50)	(47)	(48)
Gallbladder	(47)	(48)	(50)	(44)
Intestine large, cecum	(50)	(50)	(50)	(50)
Diverticulum		1 (2%)		
Epithelium, hyperplasia		1 (2%)		
Lymphoid tissue, hyperplasia		1 (2%)		
Intestine large, colon	(50)	(50)	(50)	(50)
Serosa, inflammation			1 (2%)	
Intestine large, rectum	(49)	(50)	(50)	(50)
Serosa, inflammation			1 (2%)	
Intestine small, duodenum	(49)	(50)	(50)	(49)
Intestine small, ileum	(50)	(50)	(50)	(49)
Inflammation	1 (2%)	1 (2%)	1 (2%)	
Intestine small, jejunum	(50)	(50)	(50)	(50)
Inflammation				1 (2%)
Epithelium, hyperplasia		1 (2%)		
Peyer's patch, hyperplasia, lymphoid				1 (2%)
Peyer's patch, mineralization		1 (2%)		
Liver	(49)	(50)	(49)	(50)
Angiectasis		1 (2%)	6 (12%)	3 (6%)
Basophilic focus	3 (6%)	1 (2%)	1 (2%)	3 (6%)
Clear cell focus	5 (10%)	5 (10%)	8 (16%)	5 (10%)
Eosinophilic focus	22 (45%)	33 (66%)	38 (78%)	40 (80%)
Fatty change	4 (8%)	2 (4%)	2 (4%)	1 (2%)
Fatty change, focal				2 (4%)
Focus of cellular alteration, atypical		2 (4%)	6 (12%)	16 (32%)
Hematopoietic cell proliferation	1 (2%)	2 (4%)	2 (4%)	
Hemorrhage	1 (2%)			
Infarct		1 (2%)		
Inflammation	27 (55%)	18 (36%)	9 (18%)	15 (30%)
Mineralization		1 (2%)	1 (2%)	
Mixed cell focus	4 (8%)	10 (20%)	4 (8%)	4 (8%)
Pigmentation				1 (2%)
Tension lipidosis	4 (8%)	1 (2%)	3 (6%)	
Thrombosis			1 (2%)	1 (2%)
Bile duct, cyst	1 (2%)			
Bile duct, hyperplasia	2 (4%)	4 (8%)	3 (6%)	3 (6%)
Centrilobular, fatty change		2 (4%)		
Hepatocyte, hypertrophy		1 (2%)		
Hepatocyte, necrosis	2 (4%)	4 (8%)	2 (4%)	6 (12%)

^a Number of core study animals examined microscopically at the site and the number of animals with lesion

^b Pathology data for interim evaluation animals are not presented in this table.

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Alimentary System (continued)				
Liver (continued)	(49)	(50)	(49)	(50)
Oval cell, hyperplasia			1 (2%)	1 (2%)
Portal, fibrosis			1 (2%)	
Mesentery	(10)	(13)	(13)	(10)
Inflammation	1 (10%)			
Necrosis	7 (70%)	11 (85%)	11 (85%)	9 (90%)
Oral mucosa	(2)	(2)	(0)	(0)
Gingival, hyperplasia	1 (50%)			
Pharyngeal, hyperplasia		2 (100%)		
Pharyngeal, inflammation	1 (50%)			
Pancreas	(49)	(50)	(50)	(48)
Inflammation		1 (2%)		
Mineralization, focal		1 (2%)		
Acinus, atrophy		3 (6%)	1 (2%)	
Acinus, hyperplasia			1 (2%)	
Duct, cyst				1 (2%)
Salivary glands	(48)	(49)	(44)	(45)
Stomach, forestomach	(50)	(50)	(50)	(50)
Inflammation			1 (2%)	1 (2%)
Ulcer	1 (2%)			1 (2%)
Epithelium, hyperplasia				2 (4%)
Stomach, glandular	(50)	(50)	(50)	(50)
Inflammation	1 (2%)			
Mineralization	1 (2%)	3 (6%)		
Epithelium, hyperplasia				2 (4%)
Glands, ectasia	2 (4%)			2 (4%)
Tongue	(0)	(0)	(0)	(1)
Tooth	(0)	(2)	(0)	(1)
Malformation				1 (100%)
Pulp, inflammation		2 (100%)		
Cardiovascular System				
Blood vessel	(50)	(50)	(48)	(49)
Heart	(50)	(50)	(48)	(48)
Cardiomyopathy	38 (76%)	34 (68%)	33 (69%)	35 (73%)
Atrium, thrombosis				1 (2%)
Endocardium, inflammation	1 (2%)			1 (2%)
Myocardium, inflammation	1 (2%)			
Myocardium, mineralization	1 (2%)			
Valve, thrombosis				1 (2%)
Ventricle, thrombosis	1 (2%)	1 (2%)		
Endocrine System				
Adrenal cortex	(49)	(50)	(47)	(49)
Degeneration, cystic		2 (4%)		
Degeneration, fatty			1 (2%)	
Hematopoietic cell proliferation		3 (6%)	1 (2%)	1 (2%)
Hyperplasia	7 (14%)	12 (24%)	5 (11%)	1 (2%)
Hypertrophy	5 (10%)	10 (20%)	3 (6%)	5 (10%)
Infiltration cellular, mononuclear cell	1 (2%)			1 (2%)
Necrosis	1 (2%)			
Vacuolization cytoplasmic		2 (4%)		

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Endocrine System (continued)				
Adrenal medulla	(50)	(50)	(48)	(49)
Amyloid deposition		1 (2%)		
Hyperplasia	2 (4%)	1 (2%)		
Infiltration cellular, mononuclear cell			1 (2%)	
Islets, pancreatic	(49)	(50)	(50)	(48)
Parathyroid gland	(45)	(46)	(41)	(38)
Cyst	2 (4%)			1 (3%)
Pituitary gland	(49)	(49)	(46)	(48)
Angiectasis	3 (6%)			
Cyst	2 (4%)	1 (2%)		2 (4%)
Mineralization	1 (2%)			
Pars distalis, hyperplasia	14 (29%)	12 (24%)	8 (17%)	11 (23%)
Thyroid gland	(49)	(50)	(46)	(45)
Infiltration cellular, mononuclear cell	1 (2%)		1 (2%)	
Inflammation		2 (4%)		1 (2%)
C-cell, hyperplasia		2 (4%)		1 (2%)
Follicle, cyst	1 (2%)	6 (12%)	4 (9%)	3 (7%)
Follicular cell, hyperplasia			1 (2%)	
General Body System				
None				
Genital System				
Clitoral gland	(47)	(50)	(50)	(48)
Ovary	(50)	(50)	(50)	(50)
Angiectasis		1 (2%)		
Atrophy				2 (4%)
Cyst	4 (8%)	3 (6%)	5 (10%)	4 (8%)
Infiltration cellular, mononuclear cell	1 (2%)			1 (2%)
Mineralization			1 (2%)	1 (2%)
Pigmentation, hemosiderin			1 (2%)	
Periovarian tissue, inflammation			1 (2%)	
Uterus	(50)	(50)	(50)	(50)
Angiectasis	2 (4%)			
Infiltration cellular, mononuclear cell				1 (2%)
Inflammation	4 (8%)	2 (4%)		2 (4%)
Mineralization		1 (2%)		
Thrombosis			1 (2%)	
Endometrium, hyperplasia, cystic	44 (88%)	45 (90%)	37 (74%)	38 (76%)
Endometrium, hyperplasia, reticulum cell	1 (2%)			
Vagina	(0)	(0)	(0)	(1)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Angiectasis				1 (2%)
Hyperplasia	1 (2%)			
Necrosis			1 (2%)	
Lymph node	(2)	(3)	(3)	(5)
Inguinal, hyperplasia				1 (20%)
Lumbar, hyperplasia, plasma cell		1 (33%)	1 (33%)	
Mediastinal, hyperplasia, plasma cell		1 (33%)		
Mediastinal, inflammation				1 (20%)
Renal, degeneration, cystic		1 (33%)		

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Hematopoietic System (continued)				
Lymph node, mandibular	(48)	(49)	(44)	(45)
Hematopoietic cell proliferation		1 (2%)		
Hyperplasia, lymphoid	1 (2%)	2 (4%)		
Inflammation	1 (2%)			
Lymph node, mesenteric	(48)	(50)	(47)	(47)
Degeneration, cystic		1 (2%)		
Hematopoietic cell proliferation		2 (4%)		
Hyperplasia, lymphoid	1 (2%)	2 (4%)	1 (2%)	
Hyperplasia, plasma cell		3 (6%)		
Inflammation		2 (4%)	1 (2%)	
Spleen	(49)	(50)	(50)	(48)
Accessory spleen		1 (2%)		
Hematopoietic cell proliferation	5 (10%)	4 (8%)	2 (4%)	4 (8%)
Hyperplasia, lymphoid	16 (33%)	16 (32%)	11 (22%)	10 (21%)
Infarct	1 (2%)			
Necrosis				1 (2%)
Pigmentation, hemosiderin	2 (4%)	4 (8%)		
Thymus	(50)	(50)	(46)	(46)
Hyperplasia, lymphoid	1 (2%)			
Integumentary System				
Mammary gland	(50)	(50)	(49)	(50)
Hyperplasia			1 (2%)	
Inflammation				1 (2%)
Skin	(50)	(50)	(50)	(50)
Inflammation, granulomatous				1 (2%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Fibro-osseous lesion				3 (6%)
Osteopetrosis				1 (2%)
Maxilla, osteomalacia	1 (2%)			
Vertebra, fibrosis			1 (2%)	
Skeletal muscle	(1)	(2)	(2)	(1)
Nervous System				
Brain	(49)	(50)	(47)	(49)
Cyst epithelial inclusion			1 (2%)	
Arteriole, meninges, inflammation				1 (2%)
Respiratory System				
Lung	(50)	(50)	(48)	(48)
Inflammation	4 (8%)	5 (10%)	2 (4%)	3 (6%)
Thrombosis				1 (2%)
Alveolar epithelium, hyperplasia	3 (6%)	2 (4%)	2 (4%)	1 (2%)
Alveolus, infiltration cellular, histiocyte	3 (6%)	2 (4%)		3 (6%)
Perivascular, infiltration cellular, mononuclear cell		4 (8%)	1 (2%)	2 (4%)

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Respiratory System (continued)				
Nose	(50)	(50)	(50)	(50)
Accumulation, hyaline droplet	1 (2%)	2 (4%)		1 (2%)
Inflammation	3 (6%)	4 (8%)	7 (14%)	4 (8%)
Glands, dilatation	1 (2%)			
Glands, hyperplasia			1 (2%)	1 (2%)
Goblet cell, hypertrophy		2 (4%)		1 (2%)
Respiratory epithelium, hyperplasia	1 (2%)			1 (2%)
Trachea	(50)	(50)	(46)	(46)
Inflammation				1 (2%)
Special Senses System				
Eye	(48)	(50)	(46)	(46)
Cataract	2 (4%)	1 (2%)		
Cornea, hyperplasia, squamous		1 (2%)		
Cornea, inflammation	1 (2%)	3 (6%)	1 (2%)	
Retina, dysplasia	1 (2%)	1 (2%)		
Retrolbulbar, inflammation				1 (2%)
Harderian gland	(48)	(50)	(46)	(46)
Inflammation				1 (2%)
Epithelium, hyperplasia	2 (4%)	3 (6%)	1 (2%)	2 (4%)
Urinary System				
Kidney	(50)	(50)	(50)	(49)
Hydronephrosis	3 (6%)	2 (4%)	2 (4%)	1 (2%)
Infarct	4 (8%)	2 (4%)	5 (10%)	7 (14%)
Infiltration cellular, mononuclear cell	2 (4%)		2 (4%)	1 (2%)
Inflammation	3 (6%)	4 (8%)	1 (2%)	1 (2%)
Metaplasia, osseous	1 (2%)	3 (6%)		1 (2%)
Mineralization		2 (4%)	1 (2%)	
Nephropathy	40 (80%)	38 (76%)	31 (62%)	30 (61%)
Glomerulus, amyloid deposition		1 (2%)	1 (2%)	
Papilla, necrosis		1 (2%)		
Renal tubule, cyst	1 (2%)	1 (2%)		
Renal tubule, hyperplasia		1 (2%)		
Renal tubule, necrosis	1 (2%)			
Urinary bladder	(50)	(50)	(50)	(50)
Hyperplasia, lymphoid		1 (2%)		
Infiltration cellular, mononuclear cell	1 (2%)			
Inflammation		2 (4%)	1 (2%)	
Transitional epithelium, hyperplasia				1 (2%)

APPENDIX E

GENETIC TOXICOLOGY

BACTERIAL MUTAGENICITY TEST PROTOCOL	E-2
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GENETIC TOXICOLOGY

BACTERIAL MUTAGENICITY TEST PROTOCOL

Bromodichloroacetic acid was tested in an independent bacterial gene mutation assay. Testing procedures were similar to those reported by Zeiger *et al.* (1992). A sample of the same lot of bromodichloroacetic acid that was used in the 2-year bioassay was sent to the testing laboratory for assessment of mutagenicity in *Salmonella typhimurium* strains TA97, TA98, and TA100 and in *Escherichia coli* strain WP2 *uvrA*/pKM101.

Bromodichloroacetic acid was incubated with tester strains in either buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague Dawley rat liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent (for the *S. typhimurium* strains) or tryptophan-independent (for the *E. coli* strain) mutant colonies arising on these plates were counted following 2 days incubation at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and at least five doses of bromodichloroacetic acid; the highest concentration tested was 6,000 µg/plate.

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

MOUSE PERIPHERAL BLOOD MICRONUCLEUS TEST PROTOCOL

A detailed discussion of this assay is presented by MacGregor *et al.* (1990). At the end of the 3-month toxicity study, peripheral blood samples were obtained from male and female mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes (NCEs) in each of nine or 10 animals per exposure group. In addition, the percentage of polychromatic erythrocytes (PCEs) in a population of 1,000 erythrocytes was determined for each exposure group as a measure of bone marrow toxicity.

The results were tabulated as the mean of the pooled results from all animals within a treatment group plus or minus the standard error of the mean. The frequency of micronucleated cells among NCEs was analyzed by a statistical software package that tested for increasing trend over exposure groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each exposed group and the control group. In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single exposed group is less than or equal to 0.025 divided by the number of exposed groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Results of the 3-month studies were accepted without repeat tests, because additional test data could not be obtained. Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects.

EVALUATION PROTOCOL

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple samples of a chemical were tested in the same assay, and different results were obtained among these

samples and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The summary table in the Abstract of this Technical Report presents a result that represents a scientific judgment of the overall evidence for activity of the chemical in an assay.

RESULTS

Bromodichloroacetic acid was tested in bacterial gene mutation assays. These *in vitro* assays were conducted with the same lot of bromodichloroacetic acid that was used in the 2-year bioassays, positive responses were seen in *S. typhimurium* strains TA97, TA98, and TA100 and the *E. coli* strain WP2 *uvrA*/pkM101 over a concentration range of 500 to 6,000 µg/plate in the absence of S9 (Table E1). With S9, equivocal responses were seen with the three *S. typhimurium* strains, and a positive response was observed in the *E. coli* strain. Thus, these two different samples of bromodichloroacetic acid gave different results in bacterial gene mutation assays, but the clearest indication of mutagenic activity was seen in the second assay conducted with the bioassay lot of bromodichloroacetic acid.

In vivo, no significant increases in the frequencies of micronucleated NCEs were observed in blood samples from male or female B6C3F1/N mice administered bromodichloroacetic acid (concentration range of 62.5 to 1,000 mg/L) in drinking water for 3 months (Table E2). Small increases were seen in the percentage of reticulocytes (PCEs) among total red blood cells in the highest exposure concentration groups of male and female mice, but these were not significant.

TABLE E1
Mutagenicity of Bromodichloroacetic Acid in Bacterial Tester Strains^a

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% rat S9	With 10% rat S9
TA100	0	89 ± 6	103 ± 6	110 ± 6	114 ± 4
	500	112 ± 1	127 ± 6	113 ± 3	121 ± 1
	1,000	170 ± 9	151 ± 16	127 ± 1	134 ± 10
	1,500	281 ± 13	307 ± 17	126 ± 14	138 ± 7 ^b
	3,000	500 ± 12	986 ± 21	129 ± 2	126 ± 14
	4,500	664 ± 30	1,216 ± 35	138 ± 6	140 ± 8
	6,000	623 ± 38	1,203 ± 22	156 ± 9	166 ± 6
	Trial summary		Positive	Positive	Equivocal
Positive control ^c		591 ± 24	763 ± 23	634 ± 25	856 ± 26 ^b
TA97	0	111 ± 11	120 ± 8	151 ± 4	172 ± 8
	500	147 ± 5	119 ± 7	181 ± 3	173 ± 0
	1,000	139 ± 10	121 ± 6	164 ± 5	174 ± 7
	1,500	169 ± 1	149 ± 16	166 ± 4	184 ± 2
	3,000	312 ± 17	229 ± 7	168 ± 14	169 ± 14
	4,500	452 ± 24	354 ± 32	232 ± 15	214 ± 10
	6,000	528 ± 10	354 ± 14	217 ± 12	232 ± 1
	Trial summary		Positive	Positive	Equivocal
Positive control		1,539 ± 44	2,400 ± 70	2,744 ± 138	1,854 ± 82
TA98	0	24 ± 1	29 ± 3	27 ± 1	35 ± 8
	500	32 ± 3	37 ± 5	28 ± 2	38 ± 3
	1,000	19 ± 2	33 ± 1	39 ± 5	44 ± 1
	1,500	30 ± 4	42 ± 8	28 ± 3	47 ± 7
	3,000	47 ± 7	64 ± 5	28 ± 2	51 ± 5
	4,500	59 ± 2	106 ± 12	41 ± 7	62 ± 4
	6,000	73 ± 5	149 ± 3	41 ± 3	65 ± 5
	Trial summary		Positive	Positive	Equivocal
Positive control		622 ± 69	740 ± 11	1,476 ± 25	2,066 ± 28
<i>Escherichia coli</i> WP2 <i>uvrA</i>/pKM101	0	159 ± 3	168 ± 23	166 ± 9	157 ± 16
	500	182 ± 7	213 ± 7	209 ± 5	226 ± 5
	1,000	203 ± 17	199 ± 28	236 ± 14	195 ± 33
	1,500	225 ± 19	239 ± 4	246 ± 6	274 ± 12
	3,000	274 ± 6	319 ± 12	273 ± 6	335 ± 6
	4,500	373 ± 16	454 ± 22	315 ± 7	381 ± 9
	6,000	586 ± 12	619 ± 22	380 ± 16	356 ± 46
	Trial summary		Positive	Positive	Positive
Positive control		1,803 ± 88	1,924 ± 29	1,259 ± 12	1,657 ± 20

^a Study was performed at ILS, Inc., using lot NJ 87-90/9/2005 and a modification of the protocol presented by Zeiger *et al.* (1992). Data are presented as revertants/plate (mean ± standard error) from three plates. 0 µg/plate was the solvent control.

^b Contamination

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

TABLE E2
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice Following Administration of Bromodichloroacetic Acid in Drinking Water for 3 Months^a

	Exposure Concentration (mg/L)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/1,000 NCEs ^b	P Value ^c	PCEs ^b (%)
Male					
Water ^d	0	10	3.50 ± 0.56		3.91 ± 0.25
Bromodichloroacetic acid	62.5	9	3.11 ± 0.45	0.6792	4.71 ± 0.23
	125	10	3.40 ± 0.69	0.5480	4.18 ± 0.26
	250	10	3.30 ± 0.37	0.5960	3.88 ± 0.18
	500	10	4.20 ± 0.49	0.2121	4.57 ± 0.24
	1,000	10	4.40 ± 0.52	0.1552	4.64 ± 0.21
			P=0.045 ^e		
Female					
Water	0	10	4.20 ± 0.51		4.52 ± 0.35
Bromodichloroacetic acid	62.5	10	2.40 ± 0.27	0.9868	4.98 ± 0.28
	125	10	2.40 ± 0.45	0.9868	4.80 ± 0.30
	250	10	2.50 ± 0.40	0.9813	4.52 ± 0.37
	500	10	3.20 ± 0.39	0.8779	4.59 ± 0.33
	1,000	10	3.60 ± 0.43	0.7520	5.70 ± 0.37
			P=0.215		

^a Study was performed at ILS, Inc. The detailed protocol is presented by MacGregor *et al.* (1990). NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte

^b Mean ± standard error

^c Pairwise comparison with the control group; exposed group values are significant at P≤0.005

^d Control

^e Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test; significant at P≤0.025

APPENDIX F

CLINICAL PATHOLOGY RESULTS

TABLE F1	Hematology and Clinical Chemistry Data for F344/N Rats in the 3-Month Drinking Water Study of Bromodichloroacetic Acid	F-2
TABLE F2	Hematology Data for Mice in the 3-Month Drinking Water Study of Bromodichloroacetic Acid	F-7

TABLE F1
Hematology and Clinical Chemistry Data for F344/N Rats in the 3-Month Drinking Water Study
of Bromodichloroacetic Acid^a

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male						
Hematology						
n						
Day 3	10	10	10	10	10	10
Day 21	9	10	10	10	10	10
Week 14	10	10	10	10	10	10
Hematocrit (auto) (%)						
Day 3	46.5±1.1	45.5±0.6	44.3±0.7	45.2±0.4	46.2±0.5	45.8±0.8
Day 21	46.8±0.5	45.9±0.6	45.7±0.5	46.3±0.6	48.3±0.7	46.3±0.5
Week 14	44.9±0.5	45.2±0.6	44.8±0.5	44.6±0.4	44.2±0.5	44.7±0.5
Hematocrit (spun) (%)						
Day 3	45.4±1.0	44.2±0.6	43.3±0.6	43.7±0.4	45.2±0.6	45.0±0.7
Day 21	47.3±0.5	45.6±0.6	45.7±0.2	46.3±0.6	48.5±0.7	45.9±0.5
Week 14	45.4±0.4	45.5±0.5	44.7±0.5	45.0±0.5	44.6±0.4	44.7±0.5
Hemoglobin (g/dL)						
Day 3	15.4±0.3	15.1±0.2	14.8±0.2	15.0±0.2	15.4±0.2	15.2±0.2
Day 21	14.8±0.2	14.6±0.2	14.6±0.1	14.8±0.2	15.5±0.3	14.8±0.1
Week 14	15.3±0.1	15.2±0.2	15.1±0.1	15.0±0.1	15.1±0.2	15.1±0.2
Erythrocytes (10 ⁶ /μL)						
Day 3	7.77±0.18	7.52±0.10	7.41±0.12	7.51±0.06	7.69±0.10	7.70±0.12
Day 21	7.87±0.12	7.65±0.11	7.69±0.12	7.71±0.12	8.17±0.16	7.83±0.09
Week 14	9.00±0.08	9.06±0.11	9.03±0.10	8.90±0.07	8.87±0.10	9.00±0.08
Reticulocytes (10 ⁵ /μL)						
Day 3	6.9±0.2	7.1±0.2	6.7±0.2	6.8±0.2	6.4±0.2	6.8±0.2
Day 21	4.2±0.1	4.2±0.1	4.1±0.1	4.1±0.1	3.7±0.1	4.0±0.1
Week 14	3.0±0.1	3.0±0.1	3.0±0.1	3.0±0.1	2.8±0.1	2.8±0.1
Nucleated erythrocytes/100 leukocytes						
Day 3	1.7±0.5	1.3±0.4	1.2±0.4	0.5±0.3	0.6±0.2	2.2±0.5
Day 21	0.1±0.1	0.2±0.1	0.1±0.1	0.0±0.0	0.1±0.1	0.1±0.1
Week 14	0.1±0.1	0.1±0.1	0.0±0.0	0.2±0.1	0.0±0.0	0.0±0.0
Mean cell volume (fL)						
Day 3	59.8±0.3	60.6±0.3	59.9±0.3	60.2±0.3	60.2±0.4	59.5±0.2
Day 21	59.6±0.5	60.0±0.4	59.5±0.4	60.1±0.3	59.2±0.4	59.1±0.3
Week 14	49.8±0.2	49.9±0.1	49.5±0.2	50.1±0.2	49.8±0.2	49.6±0.2
Mean cell hemoglobin (pg)						
Day 3	19.8±0.2	20.0±0.1	20.0±0.2	20.0±0.2	20.1±0.1	19.7±0.1
Day 21	18.8±0.1	19.0±0.1	19.0±0.2	19.2±0.2	19.0±0.1	18.9±0.1
Week 14	17.0±0.1	16.8±0.1	16.7±0.1	16.8±0.1	17.0±0.1	16.8±0.1
Mean cell hemoglobin concentration (g/dL)						
Day 3	33.1±0.2	33.1±0.2	33.4±0.3	33.2±0.2	33.4±0.2	33.2±0.2
Day 21	31.7±0.2	31.7±0.1	31.9±0.2	31.9±0.2	32.1±0.2	32.0±0.2
Week 14	34.0±0.2	33.7±0.2	33.7±0.2	33.6±0.2	34.1±0.2	33.8±0.1
Platelets (10 ³ /μL)						
Day 3	833±22	859±15	877±20	836±27	794±30	839±14
Day 21	666±14	717±14*	721±20	694±14	691±12	716±6*
Week 14	574±13	600±18	590±42	616±13	609±12	614±13
Leukocytes (10 ³ /μL)						
Day 3	9.96±0.33	10.09±0.38	9.22±0.25	9.90±0.38	10.69±0.22	9.78±0.26
Day 21	11.22±0.36	10.24±0.30	10.81±0.51	10.77±0.44	12.23±0.64	10.89±0.29
Week 14	10.34±0.76	10.34±0.79	10.38±0.66	10.37±0.67	10.74±0.40	9.79±0.42
Segmented neutrophils (10 ³ /μL)						
Day 3	1.05±0.06	1.04±0.05	0.94±0.03	1.00±0.04	1.06±0.04	1.01±0.03
Day 21	1.02±0.05	1.02±0.06	1.10±0.03	0.99±0.03	1.14±0.06	1.00±0.04
Week 14	1.31±0.08	1.41±0.09	1.71±0.12*	1.35±0.05	1.48±0.09	1.55±0.08

TABLE F1
Hematology and Clinical Chemistry Data for F344/N Rats in the 3-Month Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male (continued)						
Hematology (continued)						
n						
Day 3	10	10	10	10	10	10
Day 21	9	10	10	10	10	10
Week 14	10	10	10	10	10	10
Lymphocytes (10 ³ /μL)						
Day 3	8.21±0.28	8.27±0.30	7.55±0.21	8.11±0.32	8.88±0.21	8.09±0.24
Day 21	9.34±0.29	8.31±0.21	8.82±0.43	8.86±0.39	10.23±0.57	9.06±0.22
Week 14	7.85±0.61	7.79±0.60	7.51±0.46	7.96±0.59	8.02±0.34	7.13±0.39
Monocytes (10 ³ /μL)						
Day 3	0.16±0.01	0.16±0.01	0.16±0.01	0.16±0.01	0.17±0.01	0.16±0.01
Day 21	0.15±0.01	0.16±0.02	0.19±0.01	0.16±0.01	0.14±0.01	0.13±0.01
Week 14	0.13±0.01	0.14±0.02	0.14±0.02	0.13±0.01	0.14±0.01	0.12±0.01
Basophils (10 ³ /μL)						
Day 3	0.056±0.006	0.071±0.012	0.076±0.013	0.073±0.008	0.070±0.008	0.055±0.007
Day 21	0.056±0.004	0.057±0.009	0.055±0.004	0.056±0.006	0.063±0.007	0.060±0.004
Week 14	0.097±0.012	0.083±0.016	0.082±0.009	0.083±0.014	0.099±0.014	0.087±0.013
Eosinophils (10 ³ /μL)						
Day 3	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.01
Day 21	0.04±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.04±0.00	0.03±0.00
Week 14	0.08±0.01	0.08±0.01	0.09±0.01	0.08±0.01	0.09±0.01	0.09±0.01
Large unstained cells (10 ³ /μL)						
Day 3	0.460±0.022	0.516±0.059	0.460±0.045	0.521±0.045	0.481±0.030	0.432±0.037
Day 21	0.618±0.057	0.662±0.089	0.622±0.058	0.661±0.046	0.616±0.044	0.602±0.027
Week 14	0.874±0.133	0.842±0.124	0.847±0.084	0.769±0.070	0.909±0.070	0.823±0.063
Clinical Chemistry						
n						
Day 3	10	10	10	10	10	10
Day 21	9	10	10	10	10	10
Week 14	10	10	10	10	10	10
Urea nitrogen (mg/dL)						
Day 3	12.2±0.4	12.4±0.3	12.8±0.3	11.8±0.5	12.2±0.5	13.5±0.5
Day 21	17.1±1.2	13.6±0.5	13.2±0.4*	14.4±0.3	18.7±1.6	15.9±0.8
Week 14	14.0±0.6	14.0±0.7	13.8±0.4	13.6±0.5	13.7±0.4	13.6±0.4
Creatinine (mg/dL)						
Day 3	0.52±0.01	0.53±0.02	0.50±0.00	0.50±0.00	0.49±0.01	0.51±0.01
Day 21	0.56±0.02	0.58±0.01	0.59±0.01	0.58±0.01	0.60±0.01	0.57±0.02
Week 14	0.73±0.03	0.72±0.01	0.70±0.02	0.69±0.03	0.68±0.02	0.69±0.02
Total protein (g/dL)						
Day 3	5.9±0.1	5.8±0.1	5.7±0.1	5.8±0.1	5.9±0.0	5.8±0.1
Day 21	6.7±0.1	6.6±0.1	6.5±0.1	6.8±0.1	6.7±0.1	6.5±0.1
Week 14	6.9±0.1	6.8±0.1	6.8±0.1	6.7±0.1	6.7±0.1	6.7±0.1
Albumin (g/dL)						
Day 3	4.0±0.0	4.0±0.0	3.9±0.0	3.9±0.1	4.0±0.0	4.0±0.0
Day 21	4.3±0.0	4.2±0.1	4.2±0.1	4.3±0.0	4.4±0.1	4.3±0.0
Week 14	4.3±0.0	4.4±0.0	4.3±0.0	4.3±0.0	4.3±0.0	4.3±0.0
Alanine aminotransferase (IU/L)						
Day 3	61±2	65±3	60±2	62±2	63±2	66±1
Day 21	43±6	43±5	47±3	45±4	52±5	51±2
Week 14	78±4 ^b	70±3	62±3**	62±3**	65±4**	60±3**

TABLE F1
Hematology and Clinical Chemistry Data for F344/N Rats in the 3-Month Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male (continued)						
Clinical Chemistry (continued)						
n						
Day 3	10	10	10	10	10	10
Day 21	9	10	10	10	10	10
Week 14	10	10	10	10	10	10
Alkaline phosphatase (IU/L)						
Day 3	728 ± 21	687 ± 14	679 ± 11	692 ± 16	668 ± 27	698 ± 23
Day 21	417 ± 22	446 ± 7	437 ± 11	432 ± 8	378 ± 23	440 ± 15
Week 14	187 ± 6 ^b	199 ± 7	183 ± 6	187 ± 6	182 ± 7	189 ± 5
Creatine kinase (IU/L)						
Day 3	437 ± 43	401 ± 35	435 ± 37	413 ± 36	432 ± 36	469 ± 51
Day 21	398 ± 33	362 ± 28	400 ± 69	317 ± 18	365 ± 34	334 ± 29
Week 14	291 ± 64 ^b	367 ± 74	626 ± 213	288 ± 36	258 ± 38	562 ± 203
Sorbitol dehydrogenase (IU/L)						
Day 3	20 ± 1	20 ± 1	19 ± 1	18 ± 1	20 ± 1	18 ± 1
Day 21	32 ± 1	31 ± 1	32 ± 2	31 ± 1	29 ± 1	28 ± 1*
Week 14	37 ± 3 ^c	31 ± 1	32 ± 2 ^b	32 ± 3	30 ± 2	27 ± 2**
Bile acids (μmol/L)						
Day 3	23.3 ± 2.3	21.7 ± 1.4	22.0 ± 1.3	23.9 ± 1.8	23.1 ± 1.4	27.3 ± 1.6
Day 21	31.6 ± 3.2	34.3 ± 3.2	28.7 ± 4.0	31.8 ± 4.2	28.7 ± 3.5	34.9 ± 2.7
Week 14	25.6 ± 5.6 ^b	24.6 ± 2.4	25.6 ± 4.0	24.6 ± 2.8	18.8 ± 2.5	19.7 ± 2.5
Female						
Hematology						
n						
Day 3	10	10	10	10	10	10
Day 21	10	10	10	10	10	10
Week 14	9	9	10	10	10	10
Hematocrit (auto) (%)						
Day 3	44.6 ± 0.5	45.5 ± 0.5	46.5 ± 0.7	45.5 ± 0.8	46.0 ± 0.5	49.2 ± 1.5*
Day 21	48.1 ± 0.4	49.0 ± 0.6	48.6 ± 0.4	49.4 ± 0.5	48.0 ± 0.3	47.8 ± 0.4
Week 14	45.0 ± 0.6	45.3 ± 0.5	46.0 ± 0.3	45.9 ± 0.4	45.1 ± 0.3	45.0 ± 0.5
Hematocrit (spun) (%)						
Day 3	44.0 ± 0.6	44.8 ± 0.6	45.6 ± 0.6	44.3 ± 0.8	45.1 ± 0.3	47.7 ± 1.3
Day 21	47.2 ± 0.5	48.5 ± 0.7	48.0 ± 0.5	48.6 ± 0.5	47.5 ± 0.3	47.5 ± 0.5
Week 14	43.8 ± 0.6	44.6 ± 0.6	45.6 ± 0.3	45.1 ± 0.3	43.9 ± 0.4	44.3 ± 0.4
Hemoglobin (g/dL)						
Day 3	14.6 ± 0.1	15.0 ± 0.1	15.3 ± 0.2	15.0 ± 0.3	15.2 ± 0.1*	16.2 ± 0.4**
Day 21	15.8 ± 0.1	16.4 ± 0.2	16.1 ± 0.1	16.4 ± 0.2	15.9 ± 0.1	16.0 ± 0.2
Week 14	14.8 ± 0.2	14.7 ± 0.1	15.0 ± 0.1	15.0 ± 0.1	14.8 ± 0.1	14.7 ± 0.1
Erythrocytes (10 ⁶ /μL)						
Day 3	7.48 ± 0.07	7.59 ± 0.06	7.78 ± 0.10	7.64 ± 0.14	7.71 ± 0.08	8.22 ± 0.22**
Day 21	8.18 ± 0.07	8.40 ± 0.13	8.31 ± 0.09	8.44 ± 0.11	8.22 ± 0.05	8.17 ± 0.07
Week 14	8.42 ± 0.11	8.41 ± 0.11	8.54 ± 0.07	8.55 ± 0.07	8.41 ± 0.05	8.40 ± 0.08
Reticulocytes (10 ⁵ /μL)						
Day 3	5.5 ± 0.2	5.0 ± 0.2	5.5 ± 0.2	5.0 ± 0.1	4.9 ± 0.2	5.5 ± 0.2
Day 21	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.9 ± 0.1**
Week 14	2.3 ± 0.0	2.1 ± 0.1	2.4 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	2.4 ± 0.1

TABLE F1
Hematology and Clinical Chemistry Data for F344/N Rats in the 3-Month Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Female (continued)						
Hematology (continued)						
n						
Day 3	10	10	10	10	10	10
Day 21	10	10	10	10	10	10
Week 14	9	9	10	10	10	10
Nucleated erythrocytes/100 leukocytes						
Day 3	0.5±0.2	0.7±0.3	0.1±0.1	0.2±0.1	0.3±0.2	0.0±0.0
Day 21	0.3±0.2	0.1±0.1	0.1±0.1	0.1±0.1	0.0±0.0	0.1±0.1
Week 14	0.1±0.1	0.0±0.0	0.1±0.1	0.3±0.2	0.0±0.0	0.0±0.0
Mean cell volume (fL)						
Day 3	59.6±0.4	59.9±0.3	59.7±0.3	59.6±0.3	59.6±0.3	59.9±0.3
Day 21	58.8±0.3	58.4±0.5	58.5±0.3	58.6±0.3	58.5±0.2	58.5±0.3
Week 14	53.5±0.1	53.8±0.2	53.9±0.2	53.7±0.2	53.7±0.1	53.6±0.1
Mean cell hemoglobin (pg)						
Day 3	19.6±0.1	19.8±0.1	19.7±0.1	19.6±0.1	19.7±0.1	19.6±0.1
Day 21	19.4±0.1	19.5±0.1	19.4±0.1	19.5±0.2	19.3±0.1	19.5±0.1
Week 14	17.6±0.1	17.5±0.1	17.6±0.1	17.5±0.1	17.6±0.1	17.4±0.1
Mean cell hemoglobin concentration (g/dL)						
Day 3	32.9±0.2	33.0±0.2	32.9±0.2	32.9±0.1	33.0±0.1	32.8±0.2
Day 21	32.9±0.1	33.4±0.2	33.1±0.1	33.2±0.2	33.0±0.1	33.4±0.2
Week 14	32.8±0.3	32.5±0.2	32.5±0.3	32.6±0.2	32.9±0.3	32.6±0.2
Platelets (10 ³ /μL)						
Day 3	846±20	868±17	857±12	892±20	897±23	867±25
Day 21	710±12	727±21	727±19	736±22	777±7**	763±23*
Week 14	551±23	589±9	582±24	637±16**	659±15**	680±14**
Leukocytes (10 ³ /μL)						
Day 3	10.15±0.49	9.97±0.37	10.72±0.36	10.85±0.37	10.40±0.53	11.33±0.48
Day 21	11.11±0.37	10.29±0.42	10.71±0.40	10.22±0.39	9.81±0.23	10.44±0.46
Week 14	7.35±0.45	8.91±0.46	8.29±0.80	9.34±0.79	9.21±0.61	9.25±0.68
Segmented neutrophils (10 ³ /μL)						
Day 3	1.12±0.06	0.86±0.05*	0.92±0.04	0.96±0.05	0.89±0.05*	0.91±0.05*
Day 21	1.04±0.07	0.89±0.05	0.94±0.05	0.94±0.09	0.84±0.04	0.99±0.06
Week 14	1.04±0.08	1.59±0.10**	1.38±0.14	1.38±0.09	1.29±0.12	1.62±0.17*
Lymphocytes (10 ³ /μL)						
Day 3	8.32±0.41	8.41±0.31	9.02±0.32	9.14±0.37	8.81±0.48	9.61±0.40
Day 21	9.32±0.3	8.73±0.38	8.90±0.36	8.46±0.30	8.25±0.23	8.79±0.41
Week 14	5.46±0.34	6.08±0.34	5.89±0.61	6.88±0.62	6.82±0.38	6.55±0.44
Monocytes (10 ³ /μL)						
Day 3	0.16±0.01	0.16±0.01	0.17±0.01	0.16±0.01	0.17±0.02	0.20±0.02
Day 21	0.16±0.02	0.14±0.02	0.19±0.04	0.15±0.01	0.13±0.01	0.12±0.01
Week 14	0.11±0.02	0.18±0.02	0.14±0.02	0.12±0.01	0.13±0.02	0.14±0.02
Basophils (10 ³ /μL)						
Day 3	0.065±0.010	0.059±0.007	0.070±0.008	0.072±0.006	0.061±0.008	0.075±0.007
Day 21	0.053±0.004	0.044±0.004	0.054±0.004	0.055±0.007	0.044±0.003	0.047±0.003
Week 14	0.054±0.009	0.080±0.011	0.063±0.013	0.079±0.011	0.076±0.017	0.077±0.016
Eosinophils (10 ³ /μL)						
Day 3	0.04±0.00	0.04±0.00	0.04±0.00	0.05±0.00	0.05±0.01	0.05±0.00
Day 21	0.04±0.00	0.04±0.00	0.04±0.00	0.04±0.00	0.04±0.00	0.05±0.00
Week 14	0.05±0.01	0.07±0.01	0.07±0.01	0.06±0.01	0.06±0.01	0.06±0.01
Large unstained cells (10 ³ /μL)						
Day 3	0.437±0.037	0.441±0.035	0.494±0.048	0.470±0.032	0.426±0.028	0.479±0.039
Day 21	0.500±0.040	0.438±0.036	0.587±0.054	0.569±0.041	0.503±0.031	0.445±0.024
Week 14	0.629±0.056	0.912±0.087	0.747±0.095	0.815±0.109	0.830±0.122	0.806±0.113

TABLE F1
Hematology and Clinical Chemistry Data for F344/N Rats in the 3-Month Drinking Water Study
of Bromodichloroacetic Acid

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Female (continued)						
Clinical Chemistry						
n	10	10	10	10	10	10
Day 3	10	10	10	10	10	10
Day 21	9	10	10	10	10	10
Week 14						
Urea nitrogen (mg/dL)						
Day 3	11.0±0.3	12.5±0.5*	12.3±0.5*	12.6±0.4*	12.8±0.5**	13.2±0.5**
Day 21	14.6±0.3	14.6±0.3	15.2±0.3	16.0±0.4*	15.8±0.3*	17.5±0.6**
Week 14	12.6±0.5	13.4±0.8	13.6±0.6	11.9±0.5	12.9±0.5	14.4±0.5
Creatinine (mg/dL)						
Day 3	0.45±0.02	0.48±0.01	0.50±0.02	0.46±0.02	0.47±0.02	0.48±0.01
Day 21	0.51±0.01	0.50±0.02	0.51±0.01	0.49±0.02	0.49±0.01	0.49±0.01
Week 14	0.63±0.03	0.60±0.03	0.60±0.02	0.60±0.01	0.60±0.01	0.61±0.01
Total protein (g/dL)						
Day 3	6.1±0.1	6.2±0.1	6.2±0.1	6.1±0.1	6.1±0.1	6.3±0.2
Day 21	6.3±0.1	6.4±0.1	6.4±0.1	6.7±0.2	6.4±0.1	6.5±0.1
Week 14	7.3±0.2	7.0±0.1	7.0±0.1	7.0±0.1	6.8±0.1**	6.6±0.1**
Albumin (g/dL)						
Day 3	4.1±0.1	4.2±0.1	4.2±0.1	4.2±0.1	4.2±0.1	4.3±0.1
Day 21	4.4±0.0	4.4±0.1	4.4±0.0	4.5±0.1	4.4±0.0	4.3±0.0
Week 14	5.0±0.1	4.7±0.1*	4.7±0.1*	4.7±0.1*	4.6±0.1**	4.5±0.1**
Alanine aminotransferase (IU/L)						
Day 3	53±2	55±1	56±2	53±2	53±2	49±2
Day 21	41±4	38±3 ^b	38±4	33±4 ^b	40±4	37±5 ^c
Week 14	57±4	77±9	61±5	45±1*	48±4*	47±4*
Alkaline phosphatase (IU/L)						
Day 3	517±13	503±11	513±13	495±11	490±10	478±7**
Day 21	328±9	336±13	335±12	305±9	300±7	282±12**
Week 14	143±7	149±8	147±6	127±5	130±7	121±9
Creatine kinase (IU/L)						
Day 3	270±11	228±18	232±13	228±10	235±21	276±19
Day 21	262±25	304±34	326±28	318±28	342±59	317±33
Week 14	414±88	322±58	459±90	285±33	352±95	272±57
Sorbitol dehydrogenase (IU/L)						
Day 3	18±1	19±0	18±0	18±0	18±1	19±1
Day 21	32±2	32±2	32±3	33±3	32±2	35±3
Week 14	23±1	28±3	24±2	19±1	20±2	19±1
Bile acids (µmol/L)						
Day 3	13.9±0.8	12.4±0.7	15.3±1.2	13.7±1.0	14.0±1.3	15.1±1.2
Day 21	25.0±1.6	29.3±3.9	55.8±25.7	32.7±7.6 ^b	24.7±2.6	103.6±50.8
Week 14	25.5±3.4	32.8±5.1	22.8±2.2	23.7±1.4	30.3±3.2	25.9±2.8

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test

** P≤0.01

^a Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.

^b n=9

^c n=8

TABLE F2
Hematology Data for Mice in the 3-Month Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
n	10	10	10	10	10	10
Male						
Hematocrit (auto) (%)	49.5±0.5	50.2±0.6	49.3±0.4	50.3±0.4	48.8±0.6	49.8±0.7
Hematocrit (spun) (%)	50.2±0.6	50.8±0.6	49.9±0.4	50.6±0.6	49.4±0.5	50.3±0.7
Hemoglobin (g/dL)	16.6±0.2	17.2±0.3	16.6±0.1	17.0±0.2	16.5±0.2	16.8±0.2
Erythrocytes (10 ⁶ /μL)	11.10±0.09	11.31±0.14	11.08±0.11	11.24±0.08	10.79±0.10	11.00±0.14
Reticulocytes (10 ⁵ /μL)	4.0±0.1	4.1±0.1	3.9±0.1	4.5±0.6	4.1±0.1	4.3±0.1
Nucleated erythrocytes /100 leukocytes	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1
Mean cell volume (fL)	44.6±0.1	44.4±0.2	44.5±0.2	44.8±0.2	45.2±0.2	45.2±0.2
Mean cell hemoglobin (pg)	15.0±0.1	15.2±0.1	15.0±0.1	15.1±0.1	15.3±0.1**	15.3±0.1**
Mean cell hemoglobin concentration (g/dL)	33.5±0.2	34.3±0.2*	33.7±0.2	33.7±0.2	33.9±0.1	33.8±0.1
Platelets (10 ³ /μL)	827±32	812±39	816±34	789±49	886±40	799±26
Leukocytes (10 ³ /μL)	6.15±0.39	5.37±0.31	5.87±0.32	6.72±0.22	6.03±0.41	5.63±0.33
Segmented neutrophils (10 ³ /μL)	0.66±0.06	0.62±0.06	0.61±0.06	0.71±0.05	0.57±0.04	0.58±0.05
Lymphocytes (10 ³ /μL)	5.12±0.31	4.42±0.25	4.92±0.25	5.60±0.18	5.09±0.37	4.68±0.27
Monocytes (10 ³ /μL)	0.08±0.01	0.08±0.01	0.08±0.01	0.09±0.01	0.09±0.01	0.06±0.01
Basophils (10 ³ /μL)	0.030±0.006	0.023±0.005	0.028±0.006	0.029±0.007	0.022±0.003	0.026±0.005
Eosinophils (10 ³ /μL)	0.12±0.01	0.10±0.02	0.11±0.01	0.11±0.01	0.11±0.01	0.13±0.02
Large unstained cells (10 ³ /μL)	0.138±0.016	0.118±0.014	0.133±0.007	0.177±0.015	0.150±0.011	0.154±0.014
Female						
Hematocrit (auto) (%)	46.2±1.4	45.8±0.9	48.5±0.6	47.0±0.5	46.8±0.4	46.9±0.3
Hematocrit (spun) (%)	46.9±1.5	46.5±0.9	48.7±0.7	47.6±0.5	47.1±0.5	47.2±0.3
Hemoglobin (g/dL)	15.0±0.5	14.7±0.3	15.5±0.2	15.2±0.1	15.2±0.1	15.2±0.1
Erythrocytes (10 ⁶ /μL)	10.02±0.29	9.88±0.20	10.52±0.09	10.18±0.09	10.14±0.08	10.05±0.08
Reticulocytes (10 ⁵ /μL)	4.0±0.2	4.1±0.1	4.4±0.2	4.0±0.3	3.7±0.2	4.2±0.1
Nucleated erythrocytes /100 leukocytes	0.1±0.1	0.1±0.1	0.1±0.1	0.2±0.1	0.0±0.0	0.1±0.1
Mean cell volume (fL)	46.1±0.2	46.3±0.2	46.0±0.2	46.2±0.2	46.2±0.2	46.7±0.2
Mean cell hemoglobin (pg)	15.0±0.1	14.9±0.0	14.8±0.1	14.9±0.1	15.0±0.1	15.1±0.1
Mean cell hemoglobin concentration (g/dL)	32.5±0.2	32.1±0.2	32.0±0.3	32.3±0.2	32.4±0.3	32.3±0.2
Platelets (10 ³ /μL)	776±55	854±43	957±35*	927±45	840±39	912±48
Leukocytes (10 ³ /μL)	5.19±0.55	5.40±0.28	4.93±0.26	4.72±0.33	5.46±0.37	5.24±0.28
Segmented neutrophils (10 ³ /μL)	0.79±0.19	0.65±0.10	0.56±0.06	0.57±0.06	0.59±0.05	0.63±0.07
Lymphocytes (10 ³ /μL)	4.03±0.39	4.36±0.21	4.06±0.20	3.80±0.25	4.51±0.32	4.26±0.22
Monocytes (10 ³ /μL)	0.09±0.01	0.09±0.01	0.07±0.01	0.07±0.01	0.07±0.01	0.06±0.00**
Basophils (10 ³ /μL)	0.020±0.005	0.023±0.004	0.018±0.005	0.037±0.013	0.025±0.007	0.021±0.003
Eosinophils (10 ³ /μL)	0.08±0.02	0.12±0.01	0.07±0.02	0.08±0.01	0.11±0.01	0.10±0.01
Large unstained cells (10 ³ /μL)	0.173±0.016	0.162±0.008	0.147±0.020	0.179±0.018	0.159±0.014	0.182±0.011

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test

** P≤0.01

^a Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.

APPENDIX G

HEPATIC ACETYL-CoA HYDROLASE ACTIVITY

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HEPATIC ACETYL-CoA HYDROLASE ACTIVITY

INTRODUCTION

Activation of PPAR α is one of the hypotheses of the mechanism of hepatic carcinogenicity of haloacetic acids. Hepatic acetyl-CoA hydrolase activity is inducible by PPAR α activators (Nakanishi *et al.*, 1993). To evaluate the role of PPAR in the potential hepatocarcinogenicity of bromodichloroacetic acid, hepatic acetyl-CoA hydrolase activity was evaluated at the end of the 2-week study.

RESULTS

No increases in acetyl-CoA hydrolase activities were observed in either male or female rats, although there were exposure concentration-dependent decreases in acetyl-CoA hydrolase activities in both males and females (Table G1). Significantly decreased acetyl-CoA hydrolase activities occurred in males exposed to 125, 250, or 500 mg/L, and significantly increased activities occurred in females administered 125 or 500 mg/L (Table G2). These data suggest that bromodichloroacetic acid generally does not activate PPAR α , and these assays were not performed in the 3-month and 2-year studies.

TABLE G1
Hepatic Acetyl-CoA Hydrolase Activity in F344/N Rats in the 2-Week Drinking Water Study
of Bromodichloroacetic Acid^a

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
n	5	5	5	5	5	5
Male						
Acetyl-CoA hydrolase (nmol/mg protein per minute)	52.1±4.3	40.0±2.1	37.2±2.3	33.5±2.1**	33.3±2.1**	47.0±4.5
Female						
Acetyl-CoA hydrolase (nmol/mg protein per minute)	29.2±2.5	27.5±1.0	32.7±1.3	27.7±2.4	21.0±1.8*	14.0±1.1**

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

** $P \leq 0.01$

^a Data are presented as mean ± standard error.

TABLE G2
Hepatic Acetyl-CoA Hydrolase Activity in Mice in the 2-Week Drinking Water Study
of Bromodichloroacetic Acid^a

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
n	5	5	5	5	5	5
Male						
Acetyl-CoA hydrolase (nmol/mg protein per minute)	23.2±2.1	18.2±1.5	7.5±0.7**	8.7±1.1*	7.8±1.2**	16.5±1.5
Female						
Acetyl-CoA hydrolase (nmol/mg protein per minute)	5.5±0.4	5.4±0.9	15.7±1.6**	9.7±0.8	11.5±0.7*	6.5±0.8

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

** $P \leq 0.01$

^a Data are presented as mean ± standard error.

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

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TABLE H1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 2-Week Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
n	5	5	5	5	5	5
Male						
Necropsy body wt	163 ± 5	170 ± 4	165 ± 4	170 ± 7	158 ± 5	164 ± 3
Heart						
Absolute	0.59 ± 0.02	0.59 ± 0.01	0.58 ± 0.02	0.62 ± 0.03	0.57 ± 0.02	0.60 ± 0.02
Relative	3.612 ± 0.074	3.462 ± 0.082	3.534 ± 0.048	3.642 ± 0.076	3.589 ± 0.043	3.679 ± 0.09
R. Kidney						
Absolute	0.72 ± 0.03	0.74 ± 0.02	0.71 ± 0.04	0.74 ± 0.02	0.70 ± 0.01	0.74 ± 0.02
Relative	4.440 ± 0.072	4.339 ± 0.092	4.287 ± 0.136	4.359 ± 0.072	4.460 ± 0.053	4.507 ± 0.033
Liver						
Absolute	7.74 ± 0.37	8.25 ± 0.19	7.96 ± 0.29	8.18 ± 0.35	7.60 ± 0.39	8.31 ± 0.31
Relative	47.451 ± 1.121	48.500 ± 0.503	48.148 ± 0.564	48.046 ± 0.379	48.139 ± 1.051	50.608 ± 1.196
Lung						
Absolute	0.97 ± 0.05	0.88 ± 0.02	0.98 ± 0.1 ^b	1.01 ± 0.04	0.90 ± 0.04	0.94 ± 0.05
Relative	5.938 ± 0.205	5.18 ± 0.129	5.857 ± 0.434 ^b	5.945 ± 0.144	5.686 ± 0.157	5.723 ± 0.266
R. Testis						
Absolute	0.962 ± 0.021	1.014 ± 0.036	1.031 ± 0.020	1.015 ± 0.022	0.810 ± 0.074*	0.974 ± 0.027
Relative	5.918 ± 0.071	5.965 ± 0.192	6.257 ± 0.172	5.988 ± 0.152	5.130 ± 0.428	5.935 ± 0.084
Thymus						
Absolute	0.410 ± 0.016	0.409 ± 0.008	0.402 ± 0.042	0.400 ± 0.014	0.385 ± 0.026	0.361 ± 0.022
Relative	2.527 ± 0.126	2.410 ± 0.082	2.419 ± 0.213	2.373 ± 0.141	2.446 ± 0.159	2.195 ± 0.103
Female						
Necropsy body wt	124 ± 2	125 ± 3	124 ± 2	123 ± 2	118 ± 3	120 ± 3
Heart						
Absolute	0.45 ± 0.01	0.47 ± 0.01	0.47 ± 0.01	0.43 ± 0.01	0.44 ± 0.01	0.43 ± 0.01
Relative	3.632 ± 0.048	3.716 ± 0.078	3.766 ± 0.052	3.468 ± 0.035	3.709 ± 0.094	3.57 ± 0.092
R. Kidney						
Absolute	0.55 ± 0.01	0.57 ± 0.03	0.57 ± 0.01	0.54 ± 0.01	0.55 ± 0.02	0.55 ± 0.03
Relative	4.459 ± 0.074	4.571 ± 0.144	4.596 ± 0.086	4.383 ± 0.103	4.662 ± 0.045	4.581 ± 0.147
Liver						
Absolute	4.95 ± 0.09	5.21 ± 0.19	5.17 ± 0.12	5.01 ± 0.14	4.98 ± 0.20	5.23 ± 0.21
Relative	39.985 ± 0.607	41.515 ± 0.963	41.792 ± 0.411	40.726 ± 0.711	42.077 ± 0.599	43.559 ± 0.695**
Lung						
Absolute	0.83 ± 0.06	0.79 ± 0.02	0.83 ± 0.04	0.75 ± 0.02	0.75 ± 0.04	0.74 ± 0.04
Relative	6.691 ± 0.502	6.301 ± 0.107	6.740 ± 0.362	6.122 ± 0.118	6.328 ± 0.283	6.144 ± 0.299
Thymus						
Absolute	0.356 ± 0.011	0.339 ± 0.024	0.344 ± 0.003	0.349 ± 0.009	0.319 ± 0.015	0.305 ± 0.004*
Relative	2.881 ± 0.098	2.691 ± 0.144	2.785 ± 0.054	2.844 ± 0.077	2.699 ± 0.102	2.548 ± 0.064

* Significantly different (P≤0.05) from the control group by Williams' or Dunnett's test

** P≤0.01

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

^b n=4

TABLE H2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 3-Month Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Male						
n	10	10	10	10	10	10
Necropsy body wt	341 ± 5	349 ± 8	349 ± 6	356 ± 5	350 ± 6	336 ± 6
Heart						
Absolute	0.89 ± 0.01	0.9 ± 0.02	0.89 ± 0.03	0.92 ± 0.02	0.89 ± 0.02	0.89 ± 0.01
Relative	2.60 ± 0.03	2.59 ± 0.02	2.56 ± 0.04	2.59 ± 0.03	2.55 ± 0.04	2.65 ± 0.03
R. Kidney						
Absolute	0.98 ± 0.03	1.01 ± 0.02	1.00 ± 0.03	1.00 ± 0.02	0.95 ± 0.02	0.95 ± 0.02
Relative	2.86 ± 0.04	2.90 ± 0.03	2.86 ± 0.04	2.80 ± 0.02	2.71 ± 0.04*	2.83 ± 0.03
Liver						
Absolute	10.60 ± 0.34	11.01 ± 0.30	10.79 ± 0.19	11.25 ± 0.28	10.91 ± 0.24	11.33 ± 0.33
Relative	31.05 ± 0.57	31.53 ± 0.45	30.99 ± 0.36	31.59 ± 0.42	31.20 ± 0.66	33.73 ± 0.69**
Lung						
Absolute	1.43 ± 0.04	1.60 ± 0.05	1.55 ± 0.04	1.57 ± 0.06	1.56 ± 0.09	1.48 ± 0.08
Relative	4.19 ± 0.08	4.59 ± 0.09	4.47 ± 0.19	4.40 ± 0.13	4.47 ± 0.26	4.41 ± 0.21
R. Testis						
Absolute	1.446 ± 0.022	1.476 ± 0.019	1.475 ± 0.026	1.461 ± 0.027	1.403 ± 0.022	1.391 ± 0.012
Relative	4.249 ± 0.073	4.236 ± 0.049	4.239 ± 0.071	4.108 ± 0.051	4.013 ± 0.053*	4.151 ± 0.062
Thymus						
Absolute	0.244 ± 0.009	0.241 ± 0.007	0.240 ± 0.006	0.261 ± 0.012	0.241 ± 0.010	0.235 ± 0.007
Relative	0.718 ± 0.026	0.694 ± 0.023	0.691 ± 0.019	0.733 ± 0.031	0.690 ± 0.026	0.699 ± 0.023
Female						
n	9	10	10	10	10	10
Necropsy body wt	194 ± 3	206 ± 3	203 ± 2	193 ± 3	197 ± 2	184 ± 2**
Heart						
Absolute	0.58 ± 0.01	0.60 ± 0.02	0.62 ± 0.03	0.57 ± 0.01	0.58 ± 0.01	0.56 ± 0.01
Relative	3.01 ± 0.04	2.91 ± 0.08	3.06 ± 0.15	2.96 ± 0.04	2.95 ± 0.03	3.05 ± 0.05
R. Kidney						
Absolute	0.61 ± 0.01	0.67 ± 0.01*	0.65 ± 0.01	0.65 ± 0.01	0.65 ± 0.02	0.66 ± 0.02*
Relative	3.15 ± 0.05	3.24 ± 0.06	3.23 ± 0.04	3.36 ± 0.03*	3.31 ± 0.07*	3.62 ± 0.09**
Liver						
Absolute	5.85 ± 0.20	6.29 ± 0.07	6.12 ± 0.12	5.70 ± 0.10	6.13 ± 0.17	5.77 ± 0.15
Relative	30.16 ± 0.81	30.61 ± 0.51	30.30 ± 0.78	29.57 ± 0.45	31.16 ± 0.72	31.43 ± 0.64
Lung						
Absolute	1.03 ± 0.03	1.12 ± 0.06	1.11 ± 0.05	1.06 ± 0.03	1.13 ± 0.02	1.08 ± 0.03
Relative	5.32 ± 0.08	5.43 ± 0.30	5.52 ± 0.29	5.49 ± 0.17	5.73 ± 0.13	5.87 ± 0.17
Thymus						
Absolute	0.205 ± 0.008	0.212 ± 0.008	0.220 ± 0.007	0.200 ± 0.006	0.214 ± 0.005	0.210 ± 0.006
Relative	1.060 ± 0.040	1.031 ± 0.039	1.086 ± 0.031	1.037 ± 0.028	1.091 ± 0.035	1.144 ± 0.031

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE H3
Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/NTac Rats
at the 6-Month Interim Evaluation in the 2-Year Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
n	8	8	8	8
Male				
Necropsy body wt	454 ± 6	425 ± 7**	411 ± 7**	398 ± 3**
L. Kidney				
Absolute	1.381 ± 0.024	1.273 ± 0.030**	1.221 ± 0.022**	1.184 ± 0.011**
Relative	3.04 ± 0.04	2.99 ± 0.05	2.97 ± 0.04	2.98 ± 0.04
R. Kidney				
Absolute	1.35 ± 0.03	1.24 ± 0.02**	1.18 ± 0.03**	1.13 ± 0.02**
Relative	2.96 ± 0.04	2.91 ± 0.04	2.87 ± 0.06	2.85 ± 0.03
Liver				
Absolute	18.57 ± 0.42	16.87 ± 0.49*	17.03 ± 0.42*	16.70 ± 0.32**
Relative	40.90 ± 0.49	39.67 ± 0.74	41.43 ± 0.43	41.93 ± 0.68
Female				
Necropsy body wt	230 ± 5	213 ± 5**	217 ± 3**	192 ± 2**
L. Kidney				
Absolute	0.749 ± 0.014	0.737 ± 0.011	0.778 ± 0.018	0.746 ± 0.018
Relative	3.25 ± 0.05	3.47 ± 0.05*	3.59 ± 0.08**	3.87 ± 0.08**
R. Kidney				
Absolute	0.73 ± 0.02	0.74 ± 0.01	0.76 ± 0.02	0.72 ± 0.01
Relative	3.19 ± 0.07	3.47 ± 0.07**	3.49 ± 0.08**	3.76 ± 0.05**
Liver				
Absolute	8.12 ± 0.21	7.88 ± 0.19	8.83 ± 0.19*	7.65 ± 0.20
Relative	35.28 ± 0.80	37.10 ± 0.65	40.71 ± 0.76**	39.72 ± 0.77**

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE H4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 2-Week Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
n	5	5	5	5	5	5
Male						
Necropsy body wt	26.2 ± 0.4	26.4 ± 0.5	25.9 ± 0.3	25.9 ± 0.9	26.9 ± 0.7	26.2 ± 0.5
Heart						
Absolute	0.13 ± 0.01	0.12 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	0.12 ± 0.00	0.12 ± 0.01
Relative	4.880 ± 0.183	4.470 ± 0.122	4.935 ± 0.132	4.898 ± 0.269	4.620 ± 0.124	4.422 ± 0.140
R. Kidney						
Absolute	0.26 ± 0.00	0.26 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	0.25 ± 0.01	0.26 ± 0.00
Relative	9.920 ± 0.169	9.979 ± 0.134	10.101 ± 0.260	10.442 ± 0.179	9.374 ± 0.239	9.858 ± 0.187
Liver						
Absolute	1.39 ± 0.05	1.40 ± 0.04	1.35 ± 0.05	1.42 ± 0.06	1.53 ± 0.06	1.57 ± 0.06
Relative	52.920 ± 0.942	52.796 ± 1.057	52.013 ± 1.697	54.681 ± 1.497	56.745 ± 0.857	59.683 ± 1.246**
Lung						
Absolute	0.16 ± 0.00	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.00
Relative	6.101 ± 0.119	5.830 ± 0.207	6.013 ± 0.179	6.265 ± 0.253	5.810 ± 0.140	5.875 ± 0.059
R. Testis						
Absolute	0.104 ± 0.001	0.105 ± 0.004	0.104 ± 0.001	0.109 ± 0.004	0.099 ± 0.004	0.095 ± 0.003
Relative	3.983 ± 0.064	3.977 ± 0.104	4.011 ± 0.065	4.217 ± 0.115	3.698 ± 0.081	3.638 ± 0.139
Thymus						
Absolute	0.042 ± 0.003	0.048 ± 0.002	0.041 ± 0.002	0.040 ± 0.003	0.042 ± 0.005	0.039 ± 0.002
Relative	1.624 ± 0.149	1.808 ± 0.071	1.584 ± 0.086	1.558 ± 0.076	1.555 ± 0.155	1.502 ± 0.087
Female						
Necropsy body wt	21.5 ± 0.4	21.9 ± 0.4	20.9 ± 0.5	21.3 ± 0.4	21.3 ± 0.2	21.3 ± 0.2
Heart						
Absolute	0.11 ± 0.00	0.11 ± 0.00	0.10 ± 0.00	0.11 ± 0.00	0.11 ± 0.01	0.10 ± 0.00
Relative	5.109 ± 0.139	4.852 ± 0.121	4.883 ± 0.112	5.176 ± 0.197	5.067 ± 0.202	4.874 ± 0.187
R. Kidney						
Absolute	0.17 ± 0.00	0.16 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.00	0.17 ± 0.00
Relative	7.898 ± 0.155	7.500 ± 0.174	7.748 ± 0.222	7.807 ± 0.360	7.605 ± 0.154	7.784 ± 0.225
Liver						
Absolute	1.02 ± 0.02	1.00 ± 0.04	0.95 ± 0.03	0.99 ± 0.03	1.06 ± 0.03	1.09 ± 0.02
Relative	47.533 ± 0.370	45.523 ± 1.275	45.580 ± 0.585	46.354 ± 0.517	49.754 ± 1.180	50.964 ± 0.694*
Lung						
Absolute	0.16 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
Relative	7.344 ± 0.340	6.956 ± 0.258	6.982 ± 0.261	7.171 ± 0.450	6.944 ± 0.239	6.938 ± 0.286
Thymus						
Absolute	0.055 ± 0.005	0.064 ± 0.003	0.059 ± 0.004	0.057 ± 0.004	0.063 ± 0.002	0.062 ± 0.003
Relative	2.558 ± 0.257	2.944 ± 0.103	2.810 ± 0.161	2.685 ± 0.189	2.959 ± 0.107	2.898 ± 0.127

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE H5
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 3-Month Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	62.5 mg/L	125 mg/L	250 mg/L	500 mg/L	1,000 mg/L
n	10	10	10	10	10	10
Male						
Necropsy body wt	39.3 ± 1.0	40.0 ± 0.6	39.6 ± 0.8	40.1 ± 1.0	39.8 ± 0.8	36.8 ± 0.7
Heart						
Absolute	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.00	0.16 ± 0.00	0.15 ± 0.01
Relative	3.94 ± 0.07	4.01 ± 0.14	4.03 ± 0.19	4.00 ± 0.18	4.00 ± 0.09	4.16 ± 0.15
R. Kidney						
Absolute	0.29 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	0.29 ± 0.01	0.27 ± 0.00	0.26 ± 0.00**
Relative	7.28 ± 0.07	7.01 ± 0.11	7.08 ± 0.16	7.12 ± 0.19	6.88 ± 0.15	7.10 ± 0.14
Liver						
Absolute	1.53 ± 0.07	1.61 ± 0.07	1.61 ± 0.06	1.68 ± 0.03	1.79 ± 0.05**	1.74 ± 0.03**
Relative	38.60 ± 1.04	40.28 ± 1.41	40.75 ± 1.01	42.07 ± 1.15*	45.05 ± 0.98**	47.41 ± 1.16**
Lung						
Absolute	0.28 ± 0.01	0.29 ± 0.01	0.27 ± 0.01	0.29 ± 0.02	0.27 ± 0.01	0.27 ± 0.01
Relative	7.07 ± 0.34	7.30 ± 0.25	6.92 ± 0.19	7.18 ± 0.49	6.74 ± 0.33	7.46 ± 0.30
R. Testis						
Absolute	0.123 ± 0.002	0.123 ± 0.002	0.119 ± 0.002	0.128 ± 0.003	0.124 ± 0.002	0.120 ± 0.002
Relative	3.137 ± 0.068	3.081 ± 0.081	3.022 ± 0.079	3.209 ± 0.121	3.119 ± 0.068	3.256 ± 0.082
Thymus						
Absolute	0.038 ± 0.002	0.040 ± 0.001	0.037 ± 0.002	0.038 ± 0.002	0.041 ± 0.002	0.040 ± 0.002
Relative	0.971 ± 0.039	0.999 ± 0.023	0.934 ± 0.065	0.946 ± 0.044	1.026 ± 0.028	1.096 ± 0.064
Female						
Necropsy body wt	33.8 ± 0.6	32.8 ± 1.3	35.4 ± 0.7	32.6 ± 0.7	33.2 ± 0.9	30.7 ± 0.7
Heart						
Absolute	0.13 ± 0.00	0.13 ± 0.00	0.12 ± 0.00	0.13 ± 0.00	0.12 ± 0.00	0.12 ± 0.00
Relative	3.83 ± 0.11	4.07 ± 0.16	3.50 ± 0.12	3.88 ± 0.10	3.76 ± 0.12	4.01 ± 0.10
R. Kidney						
Absolute	0.18 ± 0.00	0.19 ± 0.01	0.18 ± 0.00	0.18 ± 0.01	0.18 ± 0.00	0.18 ± 0.00
Relative	5.37 ± 0.09	5.77 ± 0.16	5.16 ± 0.13	5.57 ± 0.13	5.39 ± 0.17	6.00 ± 0.07**
Liver						
Absolute	1.32 ± 0.04	1.29 ± 0.05	1.38 ± 0.02	1.28 ± 0.05	1.31 ± 0.03	1.29 ± 0.05
Relative	39.08 ± 0.79	39.43 ± 1.45	39.14 ± 0.95	39.29 ± 0.72	39.52 ± 1.03	41.78 ± 0.78
Lung						
Absolute	0.21 ± 0.02	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.18 ± 0.01
Relative	6.22 ± 0.42	5.91 ± 0.43	5.41 ± 0.31	5.85 ± 0.36	5.55 ± 0.42	5.88 ± 0.23
Thymus						
Absolute	0.044 ± 0.002	0.048 ± 0.003	0.048 ± 0.003	0.044 ± 0.002	0.044 ± 0.002	0.047 ± 0.002
Relative	1.319 ± 0.058	1.455 ± 0.066	1.345 ± 0.072	1.358 ± 0.058	1.337 ± 0.052	1.540 ± 0.064

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P < 0.01$

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE H6
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice at the 6-Month Interim Evaluation
in the 2-Year Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
n	8	8	8	8
Male				
Necropsy body wt	50.9 ± 0.9	50.4 ± 1.0	49.5 ± 1.1	49.5 ± 0.5
L. Kidney				
Absolute	0.405 ± 0.013	0.418 ± 0.011	0.373 ± 0.011	0.354 ± 0.011**
Relative	7.96 ± 0.21	8.29 ± 0.13	7.52 ± 0.16	7.16 ± 0.23**
R. Kidney				
Absolute	0.43 ± 0.01	0.45 ± 0.02	0.40 ± 0.01	0.37 ± 0.01**
Relative	8.42 ± 0.11	8.97 ± 0.15	8.09 ± 0.15	7.47 ± 0.14**
Liver				
Absolute	3.19 ± 0.22	3.06 ± 0.20	2.86 ± 0.15	2.95 ± 0.09
Relative	62.29 ± 3.10	60.46 ± 2.59	57.49 ± 2.06	59.50 ± 1.43
Female				
Necropsy body wt	50.4 ± 1.1	47.0 ± 1.7	44.9 ± 1.3*	43.6 ± 2.0**
L. Kidney				
Absolute	0.210 ± 0.005	0.209 ± 0.008	0.205 ± 0.005	0.195 ± 0.005
Relative	4.19 ± 0.10	4.47 ± 0.14	4.60 ± 0.19	4.52 ± 0.15
R. Kidney				
Absolute	0.22 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.22 ± 0.01
Relative	4.37 ± 0.10	4.81 ± 0.15	4.81 ± 0.17	4.99 ± 0.17**
Liver				
Absolute	1.79 ± 0.06	1.70 ± 0.12	1.65 ± 0.05	1.83 ± 0.08
Relative	35.59 ± 0.80	35.95 ± 1.62	36.77 ± 0.80	42.05 ± 1.25**

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX I

REPRODUCTIVE TISSUE EVALUATIONS AND ESTROUS CYCLE CHARACTERIZATION

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TABLE II
Summary of Reproductive Tissue Evaluations for Male F344/N Rats in the 3-Month Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
n	10	10	10	10
Weights (g)				
Necropsy body wt	341 ± 5	356 ± 5	350 ± 6	336 ± 6
L. Cauda epididymis wt	0.1984 ± 0.0051	0.2054 ± 0.0059	0.1985 ± 0.0063	0.1927 ± 0.0059
L. Epididymis	0.4881 ± 0.0131	0.4901 ± 0.0105	0.4812 ± 0.0077	0.4659 ± 0.0092
L. Testis	1.5659 ± 0.0309	1.5448 ± 0.0236	1.4900 ± 0.0204	1.4853 ± 0.0145*
Spermatid measurements				
Spermatid heads (10 ⁶ /testis)	188.00 ± 6.63	189.69 ± 5.84	186.75 ± 5.73	179.50 ± 5.81
Spermatid heads (10 ⁶ /g testis)	132.7 ± 4.3	134.9 ± 4.8	136.4 ± 3.7	132.4 ± 4.5
Epididymal spermatozoal measurements				
Sperm motility (%)	74.3 ± 0.9	71.0 ± 1.4	71.0 ± 1.1	70.8 ± 1.5
Sperm (10 ⁶ /cauda epididymis)	92.65 ± 5.89	102.89 ± 2.98	98.66 ± 5.43	95.64 ± 4.51
Sperm (10 ⁶ /g cauda epididymis)	467 ± 26	504 ± 20	499 ± 27	502 ± 31

* Significantly different (P≤0.05) from the control group by Dunnett's test

^a Data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (body, cauda epididymis, and epididymis weights) or Dunn's test (spermatid and epididymal spermatozoal measurements).

TABLE I2
Estrous Cycle Characterization for Female F344/N Rats in the 3-Month Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Number weighed at necropsy	9	10	10	10
Necropsy body wt (g)	194 ± 3	193 ± 3	197 ± 2	184 ± 2*
Proportion of regular cycling females ^b	6/9	6/10	7/10	6/10
Estrous cycle length (days)	4.78 ± 0.28	5.33 ± 0.48 ^c	4.85 ± 0.18	4.65 ± 0.25
Estrous stages (% of cycle)				
Diestrus	48.1	35.8	46.7	36.7
Proestrus	3.7	5.8	6.7	5.0
Estrus	32.4	44.2	30.0	40.8
Metestrus	15.7	14.2	16.7	17.5

* Significantly different (P≤0.05) from the control group by Dunnett's test

^a Necropsy body weights and estrous cycle length data are presented as mean ± standard error. Differences from the control group are not significant by Dunn's test (estrous cycle length). By multivariate analysis of variance, exposed females do not differ significantly from the control females in the relative length of time spent in the estrous stages. Tests for equality of transition probability matrices among all groups and between the control group and each exposed group indicated that 250 mg/L females had a higher probability of extended estrus than did the controls.

^b Number of females with a regular cycle/number of females cycling

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

TABLE I3
Summary of Reproductive Tissue Evaluations for Male Mice in the 3-Month Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
n	10	10	10	10
Weights (g)				
Necropsy body wt	39.3 ± 1.0	40.1 ± 1.0	39.8 ± 0.8	36.8 ± 0.7
L. Cauda epididymis wt	0.0281 ± 0.0027	0.0272 ± 0.0017	0.0236 ± 0.0013	0.0265 ± 0.0017
L. Epididymis	0.0609 ± 0.0032	0.0590 ± 0.0019	0.0585 ± 0.0024	0.0559 ± 0.0015
L. Testis	0.1228 ± 0.0018	0.1251 ± 0.0042	0.1251 ± 0.0017	0.1190 ± 0.0024
Spermatid measurements				
Spermatid heads (10 ⁶ /testis)	21.17 ± 1.14	21.88 ± 0.92	22.01 ± 1.32	20.88 ± 0.97
Spermatid heads (10 ⁶ /g testis)	189.8 ± 10.6	200.0 ± 10.1	200.4 ± 10.3	192.6 ± 7.8
Epididymal spermatozoal measurements				
Sperm motility (%)	69.3 ± 1.4	71.0 ± 1.1	62.0 ± 5.2	68.9 ± 1.8
Sperm (10 ⁶ /cauda epididymis)	18.40 ± 0.99	21.04 ± 0.85	17.57 ± 2.30	17.65 ± 0.79
Sperm (10 ⁶ /g cauda epididymis)	698 ± 60	803 ± 63	777 ± 119	682 ± 41

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

TABLE I4
Estrous Cycle Characterization for Female Mice in the 3-Month Drinking Water Study of Bromodichloroacetic Acid^a

	0 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Number weighed at necropsy	10	10	10	10
Necropsy body wt (g)	33.8 ± 0.6	32.6 ± 0.7	33.2 ± 0.9	30.7 ± 0.7*
Proportion of regular cycling females ^b	2/10	1/10	2/10	4/10
Estrous cycle length (days)	5.22 ± 0.68 ^c	5.94 ± 0.59 ^d	4.16 ± 0.19	4.83 ± 0.52 ^d
Estrous stages (% of cycle)				
Diestrus	25.8	27.5	25.0	27.5
Proestrus	0.0	1.7	2.5	0.8
Estrus	60.8	56.7	55.0	54.2
Metestrus	13.3	14.2	17.5	17.5

* Significantly different (P≤0.05) from the control group by Dunnett's test

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

^b Number of females with a regular cycle/number of females cycling

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

^d Estrous cycle was longer than 12 days or unclear in 2 of 10 animals.

APPENDIX J

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

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CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF BROMODICHLOROACETIC ACID

Bromodichloroacetic acid was obtained from Radian International, LLC (Austin, TX), in one lot (31542-80) and from Chemfinet Services, Inc. (Tarrytown, NY), in one lot (NJ 87-90/9/2005). Lot 31542-80 was used in the 2-week and 3-month studies, and lot NJ 87-90/9/2005 was used in the 2-year studies and in the genetic toxicology. Identity and purity analyses were conducted by the analytical chemistry laboratory at Battelle Chemistry Support Services (Columbus, OH) and the study laboratories at Southern Research Institute (SRI) (Birmingham, AL) for the 2-week and 3-month studies and at Battelle Columbus Operations (Columbus, OH) for the 2-year studies. Karl Fischer titration was performed by Galbraith Laboratories (Knoxville, TN), and elemental analyses were performed by Prevalere Life Sciences, Inc. (Whitesboro, NY) and Elemental Analysis Corp. (Lexington, KY). Reports on analyses performed in support of the bromodichloroacetic acid studies are on file at the National Institute of Environmental Health Sciences.

Both lots of the chemical, an off-white crystalline solid, were identified as bromodichloroacetic acid by the analytical chemistry laboratory using infrared (IR) and proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy and by the study laboratories using IR and NMR (SRI only) spectroscopy. All spectra were consistent with the literature spectra (Aldrich, 1985) and for the structure of bromodichloroacetic acid. Representative IR and proton NMR spectra are presented in Figures J1 and J2, respectively.

For lot 31542-80, purity was determined using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection by system A (Table J1) and ion chromatography (IC) by a system including a Dionex Ionpac AS11 (4 × 250 mm) reverse phase column (Thermo Scientific Inc., Bannockburn, IL) with suppressed conductivity detection, a mobile phase of A) 3 mM NaOH, and B) 50 mM NaOH, beginning with 100% A for 9 minutes (1.5 mL/minute flow rate), then to 0% A in 15 minutes (2 mL/minute flow rate), held for 31.9 minutes, then to 100% A in 0.1 minute, held for 5 minutes (flow rate 1.5 mL/minute). Titration of the acid functional group was performed using a Brinkman (Westbury, NY) Metrohm 702 M Titrino automatic titrator with a Metrohm combined pH glass electrode, titrated with NaOH (0.1 N).

For lot NJ 87-90/9/2005, elemental analyses were performed for carbon and hydrogen content using a LECO CHNS-932 analyzer (St. Joseph, MI). Elements from sodium through uranium (72 elements) were determined by the analytical chemistry laboratory using PIXE spectroscopy on a system including a General Ionex (Bellaire, MI) 4 MV tandem accelerator. The purity was determined using HPLC/UV by system A, gas chromatography (GC) with flame ionization detection (FID) by the method described below, and titration of the acid functional group by the method previously described.

GC/FID system (Agilent Technologies, Inc., Palo Alto, CA) included a Stabilwax DA, 30 m × 0.32 mm, 1.0 μm film (Restek, Bellefonte, PA) column, an oven temperature program beginning with 50° C held for 2 minutes, then 10° C/minute to 250° C, held for 2 minutes, and helium carrier gas at a flow rate of 2 mL/minute.

For lot 31542-80, Karl Fischer titration indicated 440 ppm water. HPLC/UV indicated one major peak and no impurities greater than 0.1% of the total peak area. IC indicated one major peak and four impurities, each greater than 0.1% of the total peak area, with a combined area of 1.2%. Titration of the acid functional group indicated a purity of 100%, assuming all titratable acid was present as bromodichloroacetic acid. The overall purity of lot 31542-80 was estimated to be approximately 99%.

For lot NJ 87-90/9/2005, Karl Fischer titration indicated approximately 0.4% water; elemental analyses for carbon and hydrogen were in agreement with the theoretical values; PIXE analysis indicated the presence of carbon (13%), hydrogen (0.5%), oxygen (17%), chlorine (31%), and bromine (38%), consistent with the theoretical values; no significant impurities were detected. HPLC/UV indicated one major peak with two impurities greater than 0.1% of the major peak area with a combined area of 0.6% (retention times 5.75 and 12.02 minutes). GC/FID indicated one major peak and two impurities with areas greater than 0.1% of the total peak area, with a combined area of 1.3%

(retention times 11.17 and 12.29 minutes). Functional group titration indicated a purity of approximately 95%, assuming all titratable acid was present as bromodichloroacetic acid.

In an attempt to identify the impurities, commercially obtained standards of bromodichloroacetic acid, dibromochloroacetic acid, monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, tribromoacetic acid, monobromoacetic acid, and dibromoacetic acid were analyzed using HPLC/UV by system A and GC/FID by the system previously described. Using HPLC spectra, the two impurities were identified as monochloroacetic acid (impurity one) and dibromochloroacetic acid (impurity two) by matching the retention times of the impurities to those of the standards. Spectra obtained using the GC/FID method detected only bromodichloroacetic acid (the test chemical) and dibromochloroacetic acid (impurity two); an additional impurity (impurity three) was detected at 12.29 minutes. Standard addition was used to quantitate the amount of dibromochloroacetic acid (impurity two) in the test chemical using the same GC/FID method; the amount present was determined to be 2.7%. Using the GC method previously described except with mass spectrometry detection, spectra were obtained of the test chemical and of commercially obtained standards for bromodichloroacetic acid and dibromochloroacetic acid and these spectra were compared to the National Institute of Standards and Technology library for mass spectrometry; results supported the tentative identity of impurity two as dibromochloroacetic acid; impurity three could not be identified because the peak was too small to give a useful mass spectrum. The overall purity of lot NJ 87-90/9/2005 was determined to be greater than 97%.

To ensure stability, the test chemical was stored at approximately -20°C in amber glass bottles sealed with Teflon[®]-lined lids protected from light and moisture for the 2-week and 3-month studies. Prior to the 2-year study, the physical appearance of a frozen reference standard of the test chemical kept at room temperature and exposed to air for approximately 3 days changed from “off white crystals” to a “very pale yellow liquid.” A forced degradation study of a frozen reference standard was performed by the study laboratory (Battelle Columbus Operations) using HPLC/UV by system B; no degradation occurred; the physical change was attributed to the hygroscopic nature of the test chemical. For the 2-year studies, the bulk chemical was stored at -20°C in sealed amber glass bottles with Teflon[®]-lined lids, secured by tape, and placed in a sealed glove bag filled with nitrogen, or repackaged in sealed amber glass bottles under a nitrogen headspace. Periodic reanalyses of the test chemical were performed twice during the 3-month studies and at least every 6 months during the 2-year studies using HPLC/UV by systems B or C; no degradation of the test chemical was observed.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared once during the 2-week studies, four times during the 3-month studies, and approximately every 6 weeks during the 2-year studies. The dose formulations were prepared by mixing bromodichloroacetic acid with tap water (Table J2) and stored in sealed Nalgene[®] containers with a minimal headspace at 5°C for up to 40 (3-month studies) or 42 (2-week and 2-year studies) days.

Stability studies of the 62.5 mg/L dose formulation were performed by the analytical chemistry laboratory using HPLC/UV by system C (Table J1). Stability was confirmed for dose formulations stored in sealed amber glass or Nalgene[®] high density polyethylene containers at 5°C for at least 42 days and for 7 days under simulated animal room conditions.

Periodic analyses of the dose formulations of bromodichloroacetic acid were conducted by the study laboratories using HPLC/UV by system D (2-week and 3-month studies) or system B (2-year studies). During the 2-week studies, the dose formulations were analyzed once; all five dose formulations for rats and mice were within 10% of the target concentrations (Table J3). Animal room samples of these dose formulations were also analyzed; all five samples for rats and all five for mice were within 10% of the target concentrations. During the 3-month studies, the dose formulations were analyzed at the beginning, midpoint, and end of the studies; all 15 dose formulations for rats and mice were within 10% of the target concentrations (Table J4). Animal room samples of these dose formulations were analyzed, and all 15 for rats and all 14 for mice were within 10% of the target concentrations. During the 2-year studies, the dose formulations were analyzed approximately every 6 weeks, and all 63 dose formulations for rats and all 57 for mice were within 10% of the target concentrations (Table J5). Animal room samples were also analyzed; all 12 for rats and all 12 for mice were within 10% of the target concentrations.

Tap water from the City of Columbus municipal supply was used as the vehicle. Samples of the tap water were taken within 30 days prior to the study start at 3-month intervals throughout the study and within 30 days of the end of the in-life phase and analyzed by an NIEHS analytical contractor for total chlorates, trihalomethanes, and haloacetic acids. Bromodichloroacetic acid was measured in the tap water and was found below the detection limit (approximately 0.6 µg/mL) in all samples analyzed. This detection limit was 100 times lower than the lowest concentration in the exposed animals in the 2-week and 3-month studies and 400 times lower than the lowest concentrations in the exposed animals in the 2-year studies. Note that in a nationwide study, bromodichloroacetic acid levels ranged from below detection limits to 15 ng/mL (Weinberg *et al.*, 2002) and that the highest concentration reported is approximately 4,200 times lower than the lowest concentration used in the 2-week and 3-month studies and 16,666 times lower than the lowest concentration used in the 2-year studies.

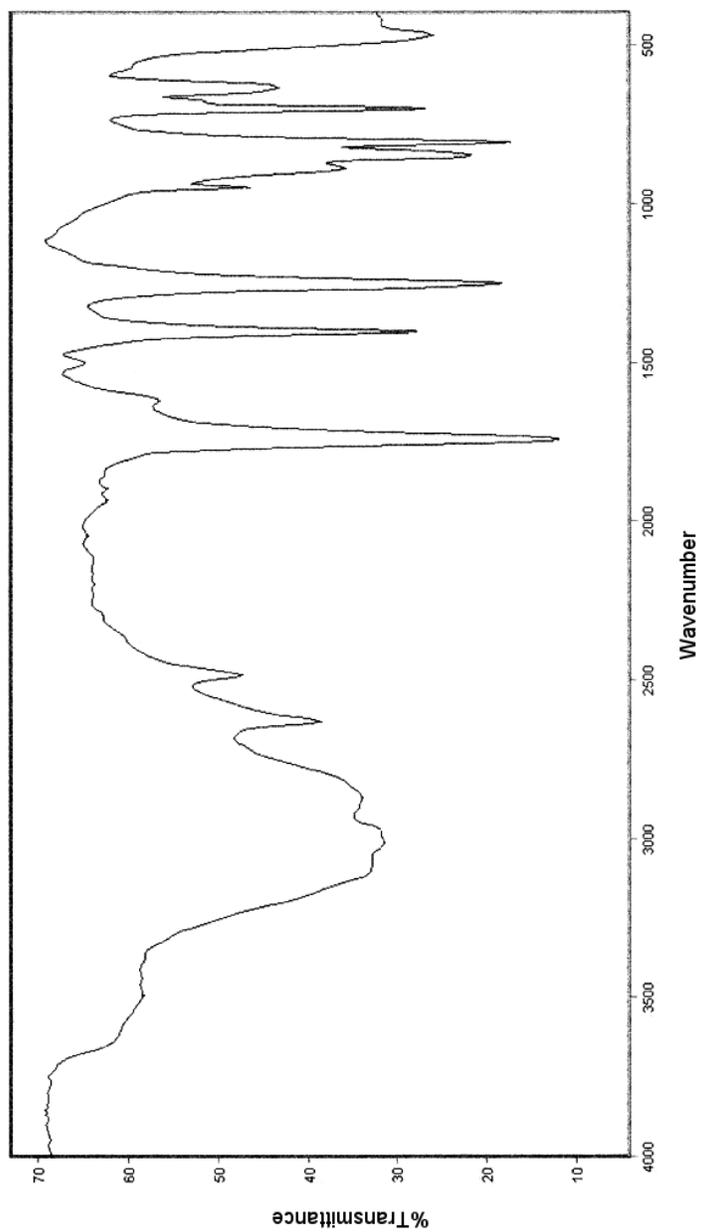


FIGURE J1
Infrared Absorption Spectrum of Bromodichloroacetic Acid

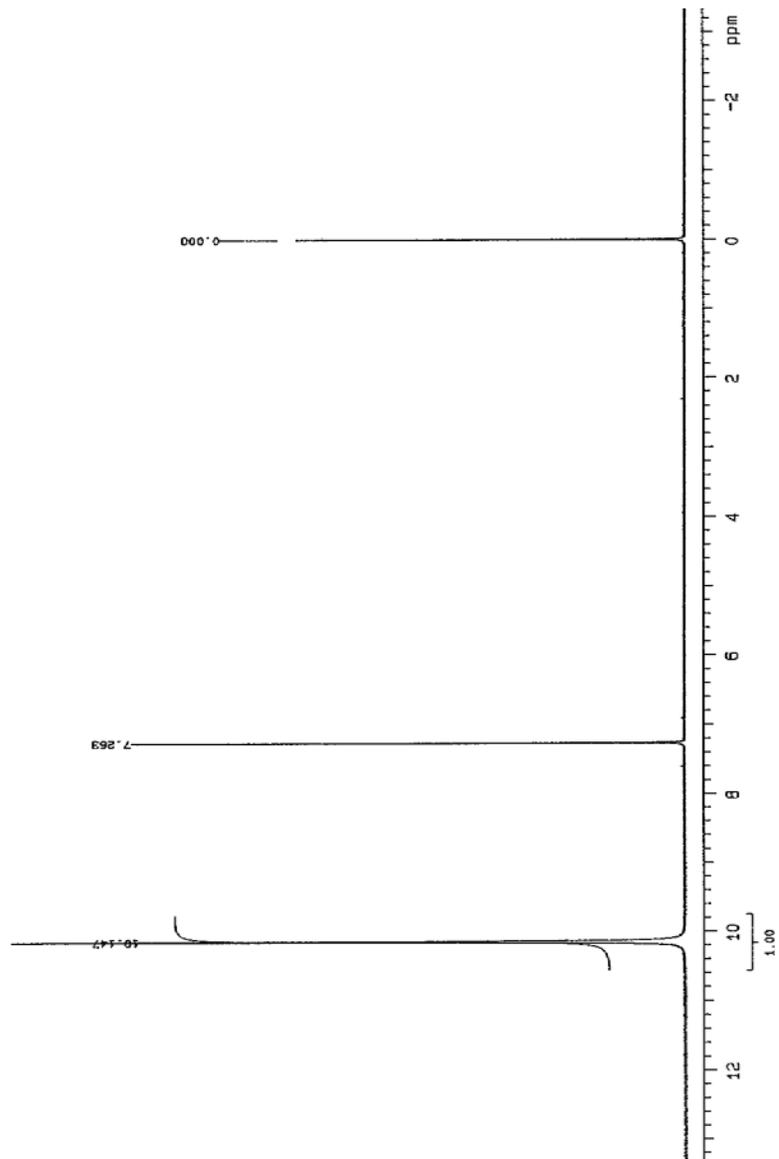


FIGURE J2
Proton Nuclear Magnetic Resonance Spectrum of Bromodichloroacetic Acid

TABLE J1
High-Performance Liquid Chromatography Systems Used in the Drinking Water Studies
of Bromodichloroacetic Acid^a

Detection System	Column	Solvent System
System A Ultraviolet (220 nm) light	Prodigy-5 ODS-3, 150 mm × 4.6 mm, 5.0 μm (Phenomenex, Torrance, CA)	A) 15 mM phosphoric acid and B) 1:1 30 mM phosphoric acid:acetonitrile, linear gradient from 100% A to 0% A in 20 minutes, held for 15 minutes, linear gradient to 100% A in 5 minutes, held for 10 (2-week and 3-month studies) or 25 (2-year studies) minutes; flow rate 1.0 mL/minute
System B Ultraviolet (220 nm) light	Aqua C ₁₈ , 150 mm × 4.6 mm, 3.0 μm (Phenomenex)	A) 80:20 0.1 M phosphoric acid:acetonitrile B) 10:90 0.1 M phosphoric acid:acetonitrile, beginning with 100% A, held for 2 minutes, linear gradient to 80% A in 2 minutes, held for 11 minutes, linear gradient to 100% A in 5 minutes; flow rate 1.0 mL/minute
System C Ultraviolet (220 nm) light	Aqua C ₁₈ , 150 mm × 4.6 mm, 5.0 μm (Phenomenex)	A) 80:20 0.1 M phosphoric acid:acetonitrile B) 10:90 0.1 M phosphoric acid:acetonitrile, beginning with 100% A, held for 2 minutes, linear gradient to 0% A in 4 minutes, held for 9 minutes, linear gradient to 100% A in 5 minutes, held 5 minutes; flow rate 1.0 mL/minute
System D Ultraviolet (220 nm) light	Aqua C ₁₈ , 150 mm × 4.6 mm, 3.0 μm (Phenomenex)	80:20 0.1 M phosphoric acid:acetonitrile, isocratic; flow rate 1.0 mL/minute

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

TABLE J2
Preparation and Storage of Dose Formulations in the Drinking Water Studies of Bromodichloroacetic Acid

2-Week Studies	3-Month Studies	2-Year Studies
<p>Preparation The appropriate amount of bromodichloroacetic acid was weighed in a graduated flask or beaker, tap water was added and stirred with a stir-bar until in solution, then transferred with five tap-water rinses to a mixing container filled to 2/3 volume with tap water, mixed for approximately 2 minutes with a stir bar, filled to final volume and mixed for at least 5 minutes; the pH was determined and, if necessary, adjusted to pH 5 using NaOH or HCl, then mixed an additional 10 minutes. Formulations were prepared once during the 2-week studies.</p>	<p>Same as 2-week studies. Formulations were prepared four times during the 3-month studies.</p>	<p>Bromodichloroacetic acid was removed from the freezer the day before and allowed to equilibrate to room temperature. The appropriate amount of bromodichloroacetic acid was weighed and transferred with three tap-water rinses to a calibrated Nalgene® container half-filled with tap water, filled to 90% volume and stirred with an overhead drum stirrer for approximately 10 minutes; the pH was determined and, if necessary, adjusted with NaOH to a pH between 6 and 7.5, diluted to final volume, and stirred for approximately 5 minutes. Formulations were prepared approximately every 6 weeks during the 2-year studies.</p>
<p>Chemical Lot Number 31542-80</p>	<p>31542-80</p>	<p>NJ 87-90/9/2005</p>
<p>Maximum Storage Time 42 days</p>	<p>40 days</p>	<p>42 days</p>
<p>Storage Conditions Stored in Nalgene® carboys, protected from light at approximately 5° C.</p>	<p>Stored in Nalgene® carboys, protected from light at approximately 5° C.</p>	<p>Stored in Nalgene® carboys, protected from light at approximately 5° C.</p>
<p>Study Laboratory Southern Research Institute (Birmingham, AL)</p>	<p>Southern Research Institute (Birmingham, AL)</p>	<p>Battelle Columbus Operations (Columbus, OH)</p>

TABLE J3
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 2-Week Drinking Water Studies of Bromodichloroacetic Acid

Date Prepared	Date Analyzed	Target Concentration (mg/L)	Determined Concentration ^a (mg/L)	Difference from Target (%)
Rats and Mice				
August 15, 2000	August 16, 2000	62.5	62.7 ± 1.0	0
		125	123 ± 0.9	-2
		250	242 ± 2.9	-3
		500	498 ± 14	0
		1,000	997 ± 21	0
Animal Room Samples				
Rats				
August 15, 2000	September 4, 2000	62.5	61.4 ± 0.8	-2
		125	123 ± 1.5	-2
		250	239 ± 3.0	-4
		500	488 ± 2.4	-2
		1,000	989 ± 7.5	-1
Mice				
August 15, 2000	September 5, 2000	62.5	61.1 ± 0.2	0
		125	124 ± 0.4	-1
		250	242 ± 2.3	-3
		500	498 ± 2.1	0
		1,000	999 ± 6.6	0

^a Results of duplicate analyses

TABLE J4
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 3-Month Drinking Water Studies of Bromodichloroacetic Acid

Date Prepared	Date Analyzed	Target Concentration (mg/L)	Determined Concentration ^a (mg/mL)	Difference from Target (%)
Rats				
November 2, 2000	November 3, 2000	62.5	60.1 ± 0.7	-4
		125	120 ± 2.2	-4
		250	246 ± 2.4	-2
		500	493 ± 7.9	-1
		1,000	981 ± 10.5	-2
	December 5, 2000 ^b	62.5	61.0 ± 0.6	-2
		125	120 ± 1.0	-4
		250	246 ± 0.2	-2
		500	495 ± 3.8	-1
		1,000	978 ± 2.3	-2
November 30, 2000	December 1, 2000	62.5	61.3 ± 0.3	-2
		125	129 ± 1.1	+3
		250	246 ± 1.1	-2
		500	497 ± 1.6	-1
		1,000	998 ± 5.8	0
	January 9, 2001 ^b	62.5	60.7 ± 0.6	-3
		125	128 ± 0.5	+2
		250	244 ± 2.5	-2
		500	495 ± 3.2	-1
		1,000	994 ± 5.4	-1
January 25, 2001	January 26, 2001	62.5	59.2 ± 0.5	-5
		125	122 ± 0.3	-2
		250	239 ± 2.7	-4
		500	480 ± 0.7	-4
		1,000	973 ± 14.1	-3
	February 15-16, 2001 ^b	62.5	63.2 ± 0.1	+1
		125	124 ± 1.3	-1
		250	245 ± 1.7	-2
		500	492 ± 2.8	-2
		1,000	994 ± 3.7	-1
Mice				
November 2, 2000	November 3, 2000	62.5	60.1 ± 0.7	-4
		125	120 ± 2.2	-4
		250	246 ± 2.4	-2
		500	493 ± 7.9	-1
		1,000	981 ± 10.5	-2
	December 5, 2000 ^b	62.5	61.0 ± 0.4	-2
		125	122 ± 0.8	-2
		250	249 ± 4.0	0
		500	501 ± 0.4	0

TABLE J4
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 3-Month Drinking Water Studies of Bromodichloroacetic Acid

Date Prepared	Date Analyzed	Target Concentration (mg/L)	Determined Concentration (mg/L)	Difference from Target (%)
Mice (continued)				
November 30, 2000	December 1, 2000	62.5	61.3 ± 0.3	-2
		125	129 ± 1.1	+3
		250	246 ± 1.1	-2
		500	497 ± 1.6	-1
		1,000	998 ± 5.8	0
	January 9, 2001 ^b	62.5	60.6 ± 0.8	-3
		125	127 ± 0.5	+2
		250	244 ± 1.7	-2
		500	493 ± 3.0	-1
		1,000	987 ± 6.5	-1
January 25, 2001	January 26, 2001	62.5	59.2 ± 0.5	-5
		125	122 ± 0.3	-2
		250	239 ± 2.7	-4
		500	480 ± 0.7	-4
		1,000	973 ± 14.1	-3
	February 15-16, 2001 ^b	62.5	62.0 ± 0.2	-1
		125	123 ± 3.1	-2
		250	248 ± 2.4	-1
		500	494 ± 15.8	-1
		1,000	985 ± 17.3	-2

^a Results of duplicate analyses

^b Animal room samples

TABLE J5
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 2-Year Drinking Water Studies of Bromodichloroacetic Acid

Date Prepared	Date Analyzed	Target Concentration (mg/L)	Determined Concentration ^a (mg/L)	Difference from Target (%)
Rats				
September 19, 2006	September 19, 2006	250	248 ± 0	-1
		500	482 ± 1	-4
		1,000	1,000 ± 0	0
	November 2, 2006 ^b	250	243 ± 1	-3
		500	479 ± 1	-4
		1,000	989 ± 0	-1
November 21, 2006	November 21, 2006	250	252 ± 0	+1
		250	245 ± 0	-2
		500	498 ± 2	0
		500	503 ± 0	+1
		1,000	1,020 ± 0	+2
February 13, 2007	February 15, 2007	1,000	1,000 ± 0	0
		250	252 ± 0	+1
		250	253 ± 1	+1
		500	505 ± 1	+1
		500	508 ± 0	+2
May 8, 2007	May 9, 2007	1,000	1,020 ± 0	+2
		1,000	1,030 ± 0	+3
		250	247 ± 0	-1
		250	248 ± 0	-1
		500	488 ± 1	-2
	June 20, 2007 ^b	500	496 ± 1	-1
		1,000	1,010 ± 0	+1
		1,000	1,000 ± 0	0
		250	259 ± 2	+4
		500	509 ± 1	+2
July 31, 2007	August 2, 2007	1,000	1,020 ± 0	+2
		250	254 ± 1	+2
		250	252 ± 1	+1
		500	505 ± 3	+1
		500	501 ± 0	0
September 25, 2007	September 28, 2007	1,000	1,080 ± 40	+8
		1,000	1,040 ± 0	+4
		250	252 ± 1	+1
		250	252 ± 1	+1
		500	512 ± 2	+2
		500	498 ± 1	0
		1,000	1,040 ± 0	+4
		1,000	1,030 ± 0	+3
		500	498 ± 1	0
		1,000	1,040 ± 0	+4

TABLE J5
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 2-Year Drinking Water Studies of Bromodichloroacetic Acid

Date Prepared	Date Analyzed	Target Concentration (mg/L)	Determined Concentration (mg/L)	Difference from Target (%)	
Rats (continued)					
December 18, 2007	December 19, 2007	250	254 ± 1	+2	
		250	250 ± 1	0	
		500	504 ± 2	+1	
		500	497 ± 2	-1	
		1,000	1,040 ± 10	+4	
		1,000	1,030 ± 0	+3	
December 18, 2007	January 29, 2008 ^b	250	247 ± 3	-1	
		500	493 ± 0	-1	
		1,000	1,030 ± 0	+3	
March 11, 2008	March 12, 2008	250	253 ± 1	+1	
		250	250 ± 1	0	
		500	503 ± 2	+1	
		500	507 ± 1	+1	
		1,000	1,030 ± 10	+3	
		1,000	1,020 ± 0	+2	
June 3, 2008	June 3, 2008	250	260 ± 1	+4	
		250	257 ± 1	+3	
		500	518 ± 0	+4	
		500	523 ± 1	+5	
		1,000	1,070 ± 0	+7	
		1,000	1,060 ± 0	+6	
	July 17, 2008 ^b	July 17, 2008 ^b	250	250 ± 1	0
			500	504 ± 1	+1
			1,000	1,030 ± 20	+3
			250	254 ± 1	+2
			250	247 ± 1	-1
			500	508 ± 0	+2
July 29, 2008	July 31, 2008	500	496 ± 0	-1	
		1,000	1,050 ± 0	+5	
		1,000	1,030 ± 0	+3	
		250	251 ± 0	0	
		250	249 ± 1	0	
		500	508 ± 2	+2	
September 23, 2008	September 24, 2008	500	498 ± 1	0	
		1,000	1,040 ± 10	+4	
		1,000	1,020 ± 0	+2	
		250	248 ± 0	-1	
		500	482 ± 1	-4	
		1,000	1,000 ± 0	0	
September 19, 2006	November 2, 2006 ^b	250	245 ± 1	-2	
		500	479 ± 1	-4	
		1,000	988 ± 20	-1	
	Mice				

TABLE J5
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 2-Year Drinking Water Studies of Bromodichloroacetic Acid

Date Prepared	Date Analyzed	Target Concentration (mg/L)	Determined Concentration (mg/L)	Difference from Target (%)	
Mice (continued)					
November 21, 2006	November 21, 2006	250	252 ± 0	+1	
		250	245 ± 0	-2	
		500	498 ± 2	0	
		500	503 ± 0	+1	
		1,000	1,020 ± 0	+2	
		1,000	1,000 ± 0	0	
February 13, 2007	February 15, 2007	250	252 ± 0	+1	
		250	253 ± 1	+1	
		500	505 ± 1	+1	
		500	508 ± 0	+2	
		1,000	1,020 ± 0	+2	
		1,000	1,030 ± 0	+3	
May 8, 2007	May 9, 2007	250	247 ± 0	-1	
		250	248 ± 0	-1	
		500	488 ± 1	-2	
		500	496 ± 1	-1	
		1,000	1,010 ± 0	+1	
		1,000	1,000 ± 0	0	
	June 20, 2007 ^b	June 20, 2007 ^b	250	255 ± 1	+2
			500	508 ± 0	+2
			1,000	1,030 ± 30	+3
			1,000	1,030 ± 30	+3
July 31, 2007	August 2, 2007	250	254 ± 1	+2	
		250	252 ± 1	+1	
		500	505 ± 3	+1	
		500	501 ± 0	0	
		1,000	1,080 ± 40	+8	
		1,000	1,040 ± 0	+4	
September 25, 2007	September 28, 2007	250	252 ± 1	+1	
		250	252 ± 1	+1	
		500	512 ± 2	+2	
		500	498 ± 1	0	
		1,000	1,040 ± 0	+4	
		1,000	1,030 ± 0	+3	
December 18, 2007	December 19, 2007	250	254 ± 1	+2	
		250	250 ± 1	0	
		500	504 ± 2	+1	
		500	497 ± 2	-1	
		1,000	1,040 ± 10	+4	
		1,000	1,030 ± 0	+3	
	January 29, 2008 ^b	January 29, 2008 ^b	250	248 ± 1	-1
			500	493 ± 2	-1
			1,000	1,020 ± 10	+2
			1,000	1,020 ± 10	+2

TABLE J5
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 2-Year Drinking Water Studies of Bromodichloroacetic Acid

Date Prepared	Date Analyzed	Target Concentration (mg/L)	Determined Concentration (mg/L)	Difference from Target (%)
Mice (continued)				
March 11, 2008	March 12, 2008	250	253 ± 1	+1
		250	250 ± 1	0
		500	503 ± 2	+1
		500	507 ± 1	+1
		1,000	1,030 ± 10	+3
		1,000	1,020 ± 0	+2
June 3, 2008	June 3, 2008	250	260 ± 1	+4
		250	257 ± 1	+3
		500	518 ± 0	+4
		500	523 ± 1	+5
		1,000	1,070 ± 0	+7
		1,000	1,060 ± 0	+6
June 3, 2008	July 17, 2008 ^b	250	249 ± 1	0
		500	505 ± 1	+1
		1,000	1,030 ± 0	+3
July 29, 2008	July 31, 2008	250	254 ± 1	+2
		250	247 ± 1	-1
		500	508 ± 0	+2
		500	496 ± 0	-1
		1,000	1,050 ± 0	+5
		1,000	1,030 ± 0	+3

^a Results of duplicate analyses

^b Animal room samples

APPENDIX K

WATER AND COMPOUND CONSUMPTION IN THE 2-YEAR DRINKING WATER STUDIES OF BROMODICHLOROACETIC ACID

TABLE K1	Water and Compound Consumption by Male F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid	K-2
TABLE K2	Water and Compound Consumption by Female F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid	K-3
TABLE K3	Water and Compound Consumption by Male Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid	K-4
TABLE K4	Water and Compound Consumption by Female Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid	K-5

TABLE K1
Water and Compound Consumption by Male F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

Week	0 mg/L		250 mg/L			500 mg/L			1,000 mg/L		
	Water ^a (g/day)	Body Weight (g)	Water (g/day)	Body Weight (g)	Dose ^b (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)
5	18.5	211	18.3	206	22.2	17.7	205	43.3	16.8	201	83.7
9	18.0	282	17.7	276	16.0	17.1	270	31.7	16.5	267	61.9
13	17.6	330	17.2	321	13.4	16.1	312	25.8	15.9	309	51.4
17	17.7	365	18.2	356	12.8	16.7	345	24.2	16.3	342	47.7
21	18.0	395	18.5	382	12.1	17.2	370	23.2	16.7	367	45.5
25	18.8	420	18.0	405	11.1	17.3	393	22.0	16.4	387	42.4
29	19.1	431	19.1	420	11.4	18.5	407	22.7	18.0	400	45.0
33	17.4	449	17.5	438	10.0	16.7	425	19.6	16.0	417	38.4
37	18.6	464	17.8	449	9.9	17.0	438	19.4	17.2	430	40.0
41	19.3	478	18.8	464	10.1	18.2	452	20.1	17.8	440	40.5
45	18.9	490	18.4	474	9.7	17.7	462	19.2	17.8	451	39.5
49	18.7	500	19.1	486	9.8	18.1	474	19.1	17.5	457	38.3
53	19.6	510	19.0	491	9.7	18.0	479	18.8	17.9	463	38.7
57	19.8	517	19.0	498	9.5	18.4	484	19.0	17.8	470	37.9
61	19.2	520	18.9	502	9.4	18.5	486	19.0	17.6	472	37.3
65	20.4	524	19.5	505	9.7	18.7	490	19.1	18.6	472	39.4
69	20.6	527	20.0	506	9.9	19.3	492	19.6	19.3	473	40.8
73	20.7	526	20.1	511	9.8	19.9	497	20.0	18.8	473	39.8
77	21.8	528	19.7	512	9.6	19.6	500	19.6	18.9	470	40.2
81	21.9	517	20.2	507	10.0	19.5	496	19.7	18.5	470	39.4
85	21.2	508	20.3	504	10.1	20.4	496	20.6	19.1	470	40.7
89	22.7	505	21.8	503	10.8	20.7	491	21.1	20.0	468	42.8
93	21.8	512	22.3	498	11.2	21.0	482	21.8	20.1	457	43.9
97	23.1	494	23.3	479	12.1	21.9	474	23.1	20.0	445	44.9
101	23.8	486	22.7	465	12.2	21.7	468	23.2	20.5	434	47.2
Mean for Weeks											
1-13	18.0	274	17.7	268	17.2	17.0	262	33.6	16.4	259	65.7
14-52	18.5	444	18.4	430	10.8	17.5	418	21.1	17.1	410	41.9
53-101	21.3	513	20.5	499	10.3	19.8	487	20.4	19.0	464	41.0

^a Grams of water consumed per animal per day

^b Milligrams of bromodichloroacetic acid consumed per kilogram body weight per day

TABLE K2
Water and Compound Consumption by Female F344/NTac Rats in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

Week	0 mg/L		250 mg/L			500 mg/L			1,000 mg/L		
	Water ^a (g/day)	Body Weight (g)	Water (g/day)	Body Weight (g)	Dose ^b (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)
5	14.0	147	13.6	145	23.4	13.6	143	47.5	10.8	138	78.4
9	13.2	175	12.3	171	18.0	12.8	167	38.3	10.1	159	63.5
13	12.0	189	11.6	184	15.7	12.2	180	33.9	9.8	171	57.5
17	12.9	202	12.1	195	15.5	12.2	191	32.0	10.1	178	56.9
21	12.7	213	12.0	204	14.7	12.4	198	31.3	10.2	186	54.9
25	12.8	223	11.2	212	13.2	11.3	206	27.5	9.3	191	48.7
29	12.5	231	12.2	220	13.9	12.1	212	28.6	10.5	197	53.4
33	11.9	241	11.6	229	12.7	12.2	221	27.6	10.5	204	51.5
37	12.6	250	12.6	238	13.2	12.7	227	28.0	11.4	210	54.4
41	12.6	258	12.7	246	12.9	12.9	235	27.5	11.8	214	55.1
45	13.0	266	13.2	255	13.0	13.7	241	28.4	12.3	218	56.5
49	13.0	277	12.9	263	12.3	13.9	252	27.6	12.5	224	55.7
53	13.3	285	13.4	272	12.3	13.9	258	27.0	12.9	229	56.4
57	13.9	295	13.2	282	11.7	14.3	269	26.6	13.2	238	55.4
61	14.0	304	14.1	293	12.0	14.6	276	26.4	13.8	244	56.5
65	14.2	313	14.3	300	11.9	14.7	280	26.3	14.6	247	59.1
69	15.2	321	14.5	306	11.9	15.7	290	27.1	15.1	251	60.2
73	14.9	327	15.4	314	12.3	16.0	294	27.2	15.1	256	59.1
77	15.1	334	14.9	320	11.6	15.3	300	25.5	14.7	260	56.6
81	14.1	340	15.6	327	11.9	16.0	304	26.3	14.8	265	56.0
85	14.8	344	15.2	328	11.6	15.8	307	25.7	15.2	272	55.8
89	16.2	349	16.5	335	12.3	16.1	313	25.7	15.4	266	58.0
93	16.9	352	17.9	336	13.3	16.5	311	26.5	19.1	268	71.2
97	17.3	350	18.8	340	13.8	17.2	306	28.1	23.5	266	88.4
101	19.3	348	18.6	342	13.6	16.9	312	27.1	18.4	265	69.4
Mean for Weeks											
1-13	13.1	170	12.5	167	19.0	12.9	163	39.9	10.2	156	66.5
14-52	12.7	240	12.3	229	13.5	12.6	220	28.7	11.0	202	54.1
53-101	15.3	328	15.6	315	12.3	15.6	294	26.6	15.8	256	61.7

^a Grams of water consumed per animal per day

^b Milligrams of bromodichloroacetic acid consumed per kilogram body weight per day

TABLE K3
Water and Compound Consumption by Male Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

Week	0 mg/L		250 mg/L			500 mg/L			1,000 mg/L		
	Water ^a (g/day)	Body Weight (g)	Water (g/day)	Body Weight (g)	Dose ^b (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)
5	3.4	30.1	3.3	30.1	27.4	3.3	29.9	55.2	3.2	28.8	110.9
9	3.5	37.2	3.4	37.1	22.9	3.3	36.5	45.2	3.4	35.2	96.7
13	3.4	42.3	3.5	42.5	20.6	3.3	41.6	39.6	3.2	39.6	80.8
17	3.3	46.6	3.2	46.3	17.3	3.2	46.2	34.6	3.1	44.5	69.6
21	3.7	48.4	3.6	48.3	18.6	3.5	48.3	36.2	3.3	47.2	70.0
25	3.8	49.7	3.8	49.5	19.2	3.6	49.9	36.1	3.3	49.3	67.0
29	4.0	50.9	3.7	50.7	18.2	3.6	50.7	35.5	3.2	50.2	63.7
33	4.4	52.3	4.3	51.7	20.8	4.0	51.9	38.6	3.7	51.1	72.4
37	4.0	53.1	3.9	52.3	18.6	3.8	52.5	36.2	3.5	51.8	67.6
41	4.3	54.1	4.2	53.3	19.7	4.1	53.4	38.4	3.9	52.5	74.3
45	4.3	54.5	4.2	53.6	19.6	4.0	53.8	37.2	3.8	52.3	72.6
49	4.9	55.6	4.7	54.7	21.5	4.5	54.8	41.0	4.5	53.1	84.8
53	4.2	55.6	4.3	54.7	19.6	4.2	54.6	38.4	4.1	52.3	78.5
57	5.0	55.8	5.1	54.9	23.2	4.9	55.2	44.4	4.9	51.2	95.7
61	4.8	55.9	4.9	55.3	22.2	5.0	54.7	45.7	4.9	50.4	97.2
65	5.0	55.4	5.0	54.6	22.9	5.0	53.4	46.8	5.4	47.8	112.9
69	5.4	55.3	5.2	53.3	24.4	5.4	51.1	52.8	5.2	44.2	117.8
73	5.2	55.2	5.4	53.3	25.3	5.4	50.8	53.2	5.8	42.8	135.5
77	5.5	54.9	5.9	53.4	27.6	7.4	47.3	78.2	6.3	39.6	159.1
81	5.2	54.6	6.3	52.3	30.1	7.5	44.8	83.7	6.5	38.3	169.8
85	5.3	54.7	6.0	51.2	29.3	7.0	44.0	79.6	6.0	36.8	163.3
89	5.2	54.3	6.2	49.5	31.3	6.6	41.4	79.8	5.8	34.2	169.8
93	4.9	52.9	5.8	47.8	30.3	6.9	38.8	88.9	5.9	32.2	183.5
97	5.2	50.1	6.8	45.1	37.7	6.6	37.3	88.5	6.3	31.7	198.6
101	5.5	48.1	6.2	43.1	35.9	6.1	35.7	85.3	5.9	30.3	194.4
Mean for Weeks											
1-13	3.4	36.5	3.4	36.6	23.6	3.3	36.0	46.7	3.3	34.5	96.1
14-52	4.1	51.7	4.0	51.2	19.3	3.8	51.3	37.1	3.6	50.2	71.3
53-101	5.1	54.1	5.6	51.4	27.7	6.0	46.9	66.6	5.6	40.9	144.3

^a Grams of water consumed per animal per day

^b Milligrams of bromodichloroacetic acid consumed per kilogram body weight per day

TABLE K4
Water and Compound Consumption by Female Mice in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

Week	0 mg/L		250 mg/L			500 mg/L			1,000 mg/L		
	Water ^a (g/day)	Body Weight (g)	Water (g/day)	Body Weight (g)	Dose ^b (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)
5	2.6	22.7	2.6	22.8	28.6	2.6	22.5	57.7	2.4	22.4	106.9
9	2.7	27.8	2.7	28.1	24.0	2.7	27.3	49.4	2.6	27.5	94.7
13	2.7	33.0	2.6	33.4	19.4	2.5	32.2	38.8	2.5	32.0	78.2
17	2.5	38.4	2.5	38.9	16.1	2.5	36.8	34.0	2.4	36.7	65.4
21	2.4	42.7	2.5	43.3	14.4	2.6	42.3	30.7	2.4	40.4	59.4
25	2.6	46.6	2.6	47.1	13.8	2.6	45.7	28.5	2.5	43.9	57.0
29	2.4	50.1	2.3	50.0	11.5	2.5	49.5	25.2	2.3	46.9	49.0
33	2.7	53.2	2.8	52.6	13.3	2.8	52.2	26.8	2.6	49.7	52.4
37	2.3	54.5	2.3	53.2	10.8	2.3	53.2	21.6	2.1	50.9	41.3
41	2.4	56.3	2.7	55.7	12.1	2.5	55.5	22.5	2.4	53.8	44.6
45	2.8	57.2	2.9	56.5	12.8	2.7	56.3	24.0	2.6	54.8	47.4
49	2.7	58.5	2.9	57.8	12.5	2.7	57.5	23.5	2.6	56.1	46.4
53	2.6	59.5	2.8	59.0	11.9	2.6	58.4	22.3	2.4	56.5	42.5
57	2.8	60.7	3.0	59.7	12.6	2.7	59.3	22.8	2.6	57.8	45.0
61	2.9	61.3	3.0	60.0	12.5	2.8	60.3	23.2	2.5	58.5	42.8
65	2.7	61.4	3.0	59.9	12.5	2.8	60.8	23.0	2.6	58.0	44.8
69	3.2	61.6	3.4	59.9	14.2	3.1	61.0	25.4	3.0	56.7	52.9
73	3.3	61.9	3.8	60.0	15.8	3.1	61.1	25.4	3.3	55.8	59.1
77	3.8	62.1	3.8	60.6	15.7	3.5	60.8	28.8	3.7	52.6	70.4
81	4.0	62.3	4.3	60.3	17.8	4.3	60.4	35.6	4.1	50.8	80.7
85	4.1	63.0	5.2	58.5	22.2	4.3	58.2	36.9	3.9	48.5	80.4
89	3.8	63.5	6.4	55.9	28.6	4.9	56.4	43.4	3.9	45.5	85.6
93	4.6	63.5	6.5	53.3	30.5	5.5	52.8	52.1	4.3	42.1	102.2
97	4.6	62.5	5.9	51.8	28.5	6.1	47.9	63.7	4.7	38.6	121.8
101	5.4	59.5	5.4	49.6	27.2	6.9	42.4	81.5	4.8	36.3	132.1
Mean for Weeks											
1-13	2.7	27.8	2.6	28.1	24.0	2.6	27.3	48.6	2.5	27.3	93.3
14-52	2.5	50.8	2.6	50.6	13.0	2.6	49.9	26.3	2.4	48.1	51.4
53-101	3.7	61.8	4.3	57.6	19.2	4.0	56.9	37.2	3.5	50.6	73.9

^a Grams of water consumed per animal per day

^b Milligrams of bromodichloroacetic acid consumed per kilogram body weight per day

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

TABLE L1	Ingredients of NTP-2000 Rat and Mouse Ration	L-2
TABLE L2	Vitamins and Minerals in NTP-2000 Rat and Mouse Ration	L-2
TABLE L3	Nutrient Composition of NTP-2000 Rat and Mouse Ration	L-3
TABLE L4	Contaminant Levels in NTP-2000 Rat and Mouse Ration	L-4

TABLE L1
Ingredients of NTP-2000 Rat and Mouse Ration

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

^a Wheat middlings as carrier

^b Calcium carbonate as carrier

TABLE L2
Vitamins and Minerals in NTP-2000 Rat and Mouse Ration^a

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

^a Per kg of finished product

TABLE L3
Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Nutrient			
Protein (% by weight)	14.7 ± 0.57	13.8 - 15.9	24
Crude fat (% by weight)	8.2 ± 0.27	7.7 - 8.8	24
Crude fiber (% by weight)	9.1 ± 0.57	8.1 - 10.3	24
Ash (% by weight)	5.0 ± 0.29	4.4 - 5.4	24
Amino Acids (% of total diet)			
Arginine	0.783 ± 0.070	0.67 - 0.97	22
Cystine	0.220 ± 0.024	0.15 - 0.25	22
Clycine	0.701 ± 0.041	0.62 - 0.80	22
Histidine	0.352 ± 0.077	0.27 - 0.68	22
Isoleucine	0.546 ± 0.044	0.43 - 0.66	22
Leucine	1.095 ± 0.067	0.96 - 1.24	22
Lysine	0.711 ± 0.114	0.31 - 0.86	22
Methionine	0.409 ± 0.046	0.26 - 0.49	22
Phenylalanine	0.628 ± 0.040	0.54 - 0.72	22
Threonine	0.505 ± 0.043	0.43 - 0.61	22
Tryptophan	0.150 ± 0.028	0.11 - 0.20	22
Tyrosine	0.401 ± 0.061	0.28 - 0.54	22
Valine	0.665 ± 0.043	0.55 - 0.73	22
Essential Fatty Acids (% of total diet)			
Linoleic	3.95 ± 0.259	3.49 - 4.55	22
Linolenic	0.30 ± 0.032	0.21 - 0.35	22
Vitamins			
Vitamin A (IU/kg)	3,590 ± 47	2,790 - 4,780	24
Vitamin D (IU/kg)	1,000 ^a		
α-Tocopherol (ppm)	80.6 ± 22.03	27.0 - 124.0	22
Thiamine (ppm) ^b	7.0 ± 1.18	5.1 - 9.3	24
Riboflavin (ppm)	7.6 ± 2.89	4.20 - 17.50	22
Niacin (ppm)	78.9 ± 9.08	66.4 - 98.2	22
Pantothenic acid (ppm)	26.9 ± 12.63	17.4 - 81.0	22
Pyridoxine (ppm) ^b	9.54 ± 1.99	6.44 - 13.7	22
Folic acid (ppm)	1.62 ± 0.48	1.15 - 3.27	22
Biotin (ppm)	0.32 ± 0.10	0.20 - 0.704	22
Vitamin B ₁₂ (ppb)	53.6 ± 39.6	18.3 - 174.0	22
Choline (ppm) ^b	2,846 ± 485	1,820 - 3,790	22
Minerals			
Calcium (%)	0.925 ± 0.051	0.808 - 1.02	24
Phosphorus (%)	0.551 ± 0.065	0.471 - 0.822	24
Potassium (%)	0.666 ± 0.030	0.626 - 0.733	22
Chloride (%)	0.386 ± 0.039	0.300 - 0.474	22
Sodium (%)	0.189 ± 0.016	0.160 - 0.222	22
Magnesium (%)	0.216 ± 0.062	0.185 - 0.490	22
Sulfur (%)	0.170 ± 0.029	0.116 - 0.209	14
Iron (ppm)	186 ± 39.2	135 - 311	22
Manganese (ppm)	51.4 ± 10.28	21.0 - 73.1	22
Zinc (ppm)	53.4 ± 8.46	43.3 - 78.5	22
Copper (ppm)	7.01 ± 2.562	3.21 - 16.3	22
Iodine (ppm)	0.503 ± 0.206	0.158 - 0.972	22
Chromium (ppm)	0.694 ± 0.276	0.330 - 1.380	22
Cobalt (ppm)	0.256 ± 0.164	0.098 - 0.864	22

^a From formulation

^b As hydrochloride (thiamine and pyridoxine) or chloride (choline)

TABLE L4
Contaminant Levels in NTP-2000 Rat and Mouse Ration^a

	Mean ± Standard Deviation ^b	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.25 ± 0.050	0.16 – 0.40	24
Cadmium (ppm)	0.06 ± 0.011	0.04 – 0.10	24
Lead (ppm)	0.10 ± 0.020	0.07 – 0.16	24
Mercury (ppm)	<0.02		24
Selenium (ppm)	0.31 ± 0.253	0.16 – 1.02	24
Aflatoxins (ppb)	<5.00		24
Nitrate nitrogen (ppm) ^c	17.9 ± 8.99	10.0 – 42.3	24
Nitrite nitrogen (ppm) ^c	0.65 ± 0.26	0.30 – 1.84	24
BHA (ppm) ^d	1.17 ± 0.82	1.0 – 5.0	24
BHT (ppm) ^d	1.17 ± 0.82	1.0 – 5.0	24
Aerobic plate count (CFU/g)	10 ± 0	10	24
Coliform (MPN/g)	3.0 ± 0	3.0	24
<i>Escherichia coli</i> (MPN/g)	<10		24
<i>Salmonella</i> (MPN/g)	Negative		24
Total nitrosamines (ppb) ^e	7.7 ± 6.34	2.0 – 28.0	24
<i>N</i> -Nitrosodimethylamine (ppb) ^e	2.3 ± 2.17	1.0 – 10.3	24
<i>N</i> -Nitrosopyrrolidine (ppb) ^e	5.4 ± 5.18	1.0 – 17.7	24
Pesticides (ppm)			
α-BHC	<0.01		24
β-BHC	<0.02		24
γ-BHC	<0.01		24
δ-BHC	<0.01		24
Heptachlor	<0.01		24
Aldrin	<0.01		24
Heptachlor epoxide	<0.01		24
DDE	<0.01		24
DDD	<0.01		24
DDT	<0.01		24
HCB	<0.01		24
Mirex	<0.01		24
Methoxychlor	<0.05		24
Dieldrin	<0.01		24
Endrin	<0.01		24
Telodrin	<0.01		24
Chlordane	<0.05		24
Toxaphene	<0.10		24
Estimated PCBs	<0.20		24
Ronnel	<0.01		24
Ethion	<0.02		24
Trithion	<0.05		24
Diazinon	<0.10		24
Methyl chlorpyrifos	0.05 ± 0.039	0.02 – 0.139	24
Methyl parathion	<0.02		24
Ethyl parathion	<0.02		24
Malathion	0.068 ± 0.079	0.020 – 0.317	24
Endosulfan I	<0.01		24
Endosulfan II	<0.01		24
Endosulfane sulfate	<0.03		24

^a All samples were irradiated. CFU=colony-forming units; MPN=most probable number; BHC=hexachlorocyclohexane or benzene hexachloride

^b For values less than the limit of detection, the detection limit is given as the mean.

^c Sources of contamination: alfalfa, grains, and fish meal

^d Sources of contamination: soy oil and fish meal

^e All values were corrected for percent recovery.

APPENDIX M

SENTINEL ANIMAL PROGRAM

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SENTINEL ANIMAL PROGRAM

METHODS

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

Blood samples were collected and allowed to clot, and the serum was separated. Additionally, fecal samples were collected and tested for *Helicobacter* species. All samples were processed appropriately and sent to BioReliance Corporation, Rockville, MD, or Research Animal Diagnostic Laboratory, University of Missouri, Columbia, MO, for determination of the presence of pathogens. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

Method and Test

Time of Collection

RATS

3-Month Study

ELISA

PVM (pneumonia virus of mice)

Study termination

RCV/SDA

(rat coronavirus/sialodacryoadenitis virus)

Study termination

Sendai

Study termination

Immunofluorescence Assay

Parvovirus

Study termination

2-Year Study

ELISA

Mycoplasma arthritidis

12 months

Mycoplasma pulmonis

12 months

PVM

Study start, 1, 6, and 12 months

RCV/SDA

Study start, 1, 6, and 12 months

Sendai

Study start, 1, 6, and 12 months

Immunofluorescence Assay

Parvovirus

1, 6, and 12 months

PVM

1 month

RCV/SDA

1 month

Sendai

1 month

Method and Test**Time of Collection****RATS** (continued)**2-Year Study** (continued)

Multiplex Fluorescent Immunoassay

H1 (Toolan's H-1 virus)	18 months, study termination
KRV (Kilham's rat virus)	18 months, study termination
<i>M. pulmonis</i>	18 months, study termination
Parvovirus NS-1	18 months, study termination
PVM	18 months, study termination
RCV/SDA	18 months, study termination
RMV (rat minute virus)	18 months, study termination
RPV (rat parvovirus)	18 months, study termination
RTV (rat theilovirus)	18 months, study termination
Sendai	18 months, study termination
TMEV (Theiler's murine encephalomyelitis virus)	18 months, study termination

Polymerase Chain Reaction

<i>Helicobacter</i> species	12 months
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MICE**3-Month Study**

ELISA

Ectromelia virus	Study termination
EDIM (epizootic diarrhea of infant mice)	Study termination
GDVII (mouse encephalomyelitis virus)	Study termination
LCM (lymphocytic choriomeningitis virus)	Study termination
Mouse adenoma virus-FL	Study termination
MHV (mouse hepatitis virus)	Study termination
PVM	Study termination
Reovirus 3	Study termination
Sendai	Study termination

Immunofluorescence Assay

Parvovirus	Study termination
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2-Year Study

ELISA

Ectromelia virus	Study start, 1, 6, and 12 months
EDIM	Study start, 1, 6, and 12 months
GDVII	Study start, 1, 6, and 12 months
LCM	Study start, 1, 6, and 12 months
Mouse adenoma virus-1	Study start, 1, 6, and 12 months
MHV	Study start, 1, 6, and 12 months
MPV VP2 (mouse parvovirus viral protein 2)	Study start, 1, 6, and 12 months
MVM VP2 (minute virus of mice viral protein 2)	Study start, 1, 6, and 12 months
<i>M. arthritidis</i>	12 months
<i>M. pulmonis</i>	12 months
PVM	Study start, 1, 6, and 12 months
Reovirus 3	Study start, 1, 6, and 12 months
Sendai	Study start, 1, 6, and 12 months

Method and Test**Time of Collection****MICE** (continued)**2-Year Study** (continued)

Immunofluorescence Assay

EDIM

6 months

Mouse adenoma virus-1

6 months

Multiplex Fluorescent Immunoassay

Ectromelia virus

18 months, study termination

EDIM

18 months, study termination

LCM

18 months, study termination

MHV

18 months, study termination

MNV (mouse norovirus)

18 months, study termination

MPV

18 months, study termination

MVM

18 months, study termination

M. pulmonis

18 months, study termination

Parvovirus NS-1

18 months, study termination

PVM

18 months, study termination

Reovirus 3

18 months, study termination

TMEV GDVII

18 months, study termination

Sendai

18 months, study termination

Polymerase Chain Reaction

Helicobacter species

12 and 18 months

RESULTS

All test results were negative.

APPENDIX N
INVOLVEMENT OF *Tgfβ* SIGNALING
IN MAMMARY GLAND ADENOCARCINOMAS
IN F344/NTac RATS EXPOSED
TO BROMODICHLOROACETIC ACID
IN DRINKING WATER FOR 2 YEARS

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INVOLVEMENT OF *Tgfb* SIGNALING IN MAMMARY GLAND ADENOCARCINOMAS IN F344/NTac RATS EXPOSED TO BROMODICHLOROACETIC ACID IN DRINKING WATER FOR 2 YEARS

INTRODUCTION

While mammary gland fibroadenomas are one of the most common background neoplasms in female F344/N rats, occurring at an incidence of about 51% in the National Toxicology Program (NTP) historical control database (all routes, all vehicles), adenocarcinomas of the mammary gland are fairly uncommon, occurring at an incidence of less than 4% in NTP studies. The concurrent NTP 2-year bioassay indicated significantly increased incidences of mammary gland proliferative lesions, including hyperplasia, fibroadenoma, and adenocarcinoma in female F344/NTac rats exposed to bromodichloroacetic acid in drinking water. The NTP has identified 51 chemicals that have been associated with the induction of mammary gland neoplasms in rodents, with 24 of these exhibiting clear evidence of carcinogenicity in either the F344/N rat or B6C3F1 mouse (NTP, 2012), including another drinking water disinfection by-product, bromochloroacetic acid, which showed clear evidence of mammary gland carcinogenesis in male and female B6C3F1 mice and F344/N rats (NTP, 2009). The molecular mechanisms underlying the pathogenesis of these lesions are poorly understood, and the effects of bromodichloroacetic acid exposure on the mammary gland in the context of tumorigenesis have not been explored. In addition, separation of background spontaneous tumors from treatment-related tumors can be difficult, particularly when a tumor response to chemical exposure is not robust. Thus, the identification of target pathways that inform on mechanisms of tumorigenesis as well as differentiate between spontaneous and chemically induced tumors is of critical importance. In the current study, real-time quantitative polymerase chain reaction (qPCR) arrays targeting pathways relevant for mammary gland tumorigenesis were employed to investigate differential gene expression between mammary gland adenocarcinomas arising in bromodichloroacetic acid-exposed animals compared to those occurring in vehicle and naïve controls.

MATERIALS AND METHODS

Animals and Tumor Sampling

Four frozen mammary gland adenocarcinomas from female F344/NTac rats exposed to bromodichloroacetic acid were available for analysis from the concurrent 2-year drinking water study of bromodichloroacetic acid (Table N1). Collection of tumors for molecular biology analysis occurred during the in-life phase of the 2-year study and was triggered when a potential chemical-related response was noted in moribund animals or at terminal kill. Tumors of sufficient size were sectioned in half; one half was flash frozen in liquid nitrogen, and the other half was placed in 10% neutral buffered formalin for histopathology examination. Low numbers of frozen tumors relative to histologically diagnosed adenocarcinomas from bromodichloroacetic acid exposed animals and the lack of frozen tumors from the concurrent vehicle treated animals may be due to small size (less than 0.5 cm) of tumors or the lack of an apparent chemical-related effect until scheduled terminal kill of the exposed groups. Because the primary components of mammary gland adenocarcinomas are epithelial, to obtain appropriate age-matched naïve control samples, mammary gland epithelium was laser-capture microdissected from frozen mammary gland samples (embedded in optimal cutting temperature medium) from five age-matched female F344/N rats obtained from the National Institute on Aging. Because frozen spontaneous adenocarcinomas were not available from vehicle control animals from the concurrent 2-year study of bromodichloroacetic acid, five spontaneous tumors were obtained from age-matched vehicle control female rats from four other 2-year NTP studies available in the NTP frozen tissue repository. Two mammary gland tumors from female F344/N rats were obtained from the vehicle controls from the 2,2-bis(bromomethyl)-1,3-propanediol feed study (NTP, 1996a). One mammary gland tumor from one female F344/N rat was obtained from the vehicle controls from the inhalation study of nickel sulfate hexahydrate (NTP, 1996b), and one from the vehicle controls from the D&C Yellow No. #11 feed study (NTP, 1997). One mammary gland tumor from one female Sprague Dawley rat was obtained from the vehicle control group from the

3,3',4,4'-tetrachloroazobenzene gavage study (NTP, 2010). For the purposes of this report, these are described as vehicle control tumors (Table N1).

Real Time qPCR Arrays and Data Analysis

RNA was extracted from five laser-captured samples of mammary gland epithelium from vehicle control, age-matched female mammary glands, five spontaneous adenocarcinomas from vehicle control females, and four adenocarcinomas from bromodichloroacetic acid-exposed females using the TRIzol[®] kit (Invitrogen Corporation, Carlsbad, CA). Fold changes in gene expression were determined by cDNA quantification of spontaneous and bromodichloroacetic acid-exposed adenocarcinoma relative to vehicle control mammary gland epithelium from age-matched naïve control rats. The 18s RNA gene was used for the endogenous control for normalization of RNA levels.

A rat-specific PCR array (PARN-131Z, SABiosciences, Frederick, MD) was used to identify differential expression of genes relevant for mammary gland tumorigenesis (84 genes represented) between mammary gland adenocarcinomas in vehicle control and bromodichloroacetic acid-exposed animals. Quantitative differential gene expression changes were identified using arrays containing corresponding PCR primers and SABiosciences' SYBR[®] Green qPCR master mix, and the reactions were run on an ABI PRISM[®] 7900HT Sequence Detection System (Applied BioSystems, Inc., Foster City, CA) using the manufacturer's protocols. Gene expression was normalized to beta-actin (*Actb*), and fold changes were calculated using the $\Delta\Delta Ct$ method (Pfaffl, 2001). To identify significant gene expression changes between spontaneous and bromodichloroacetic acid-exposed adenocarcinomas, pairwise comparisons were performed using a residual bootstrap-based methodology (Efron and Tibshirani, 1994) to compute P values using 10,000 bootstrap samples and controlling the false discovery rate at 5%. Pairwise comparisons were performed using ORIOGEN, version 4.01 (Peddada *et al.*, 2003, 2005; Guo *et al.*, 2010). These statistical methods have been reviewed and approved by the NTP Board of Scientific Counselors during the 2012 Biostatistics Branch Board Review.

RESULTS

The *Tgfb* pathway and its downstream mediators are overrepresented in mammary gland adenocarcinomas from bromodichloroacetic acid-exposed female F344/NTac rats. Using a pairwise comparison analysis to identify differential gene expression between mammary gland adenocarcinomas from vehicle control and bromodichloroacetic acid-exposed female F344/NTac rats, of the 84 genes present on the PCR array relevant to mammary gland carcinogenesis, eight were identified as significantly ($P < 0.05$) upregulated in mammary gland adenocarcinomas from rats exposed to bromodichloroacetic acid compared to spontaneous adenocarcinomas in vehicle control rats (Table N1). These genes included *Mmp9*, *Mmp2*, inhibitor of differentiation (*Id1*), *Hic1*, *Adam23*, vascular endothelial growth factor a (*Vegfa*), thrombospondin 1 (*Thbs1*), and *Cdh13*. Five of these eight genes are associated with *Tgfb* pathway signaling, including its effects on matrix remodeling, mammary gland cancer progression, tumor invasion, and metastasis (*Mmp9*, *Mmp2*, *Id1*, *Vegfa*, and *Thbs1*) (Table N2).

DISCUSSION

The *Tgfb* gene and downstream signaling pathway is known to play a significant role in a variety of biologic processes, including cell differentiation, inflammation, autoimmunity, and carcinogenesis in a variety of tissues (Moustakas *et al.*, 2002). The differential expression of this gene has been associated with tumor suppression early in the course of mammary gland carcinogenesis, while late in the course of disease it is known to promote invasion and metastasis (Akhurst and Derynck, 2001; Tang *et al.*, 2003). Not only is it overexpressed in mammary gland carcinogenesis, it is also associated with increased angiogenesis, epithelial-mesenchymal transition, and increased tumor cell invasion and proliferation, tumor progression, and evasion of apoptosis (Ivanović *et al.*, 2003; Moore-Smith and Pasche, 2011).

Tgfb regulates the expression of a large number of downstream mediators, including growth and differentiation factors, apoptosis mediators, cytokines, and matrix metalloproteinases. Two matrix metalloproteinases found significantly upregulated in adenocarcinomas from rats exposed to bromodichloroacetic acid (compared to spontaneous adenocarcinomas) included *Mmp2* and *Mmp9*. The expression of these metalloproteinases is regulated by upstream expression of *Tgfb* (Wiercinska *et al.*, 2011), and they function to degrade extracellular matrix

components such as type IV collagen, the primary collagen in the basement membrane (Stetler-Stevenson, 1994). Conversely, these mediators also activate various growth factors, cleave adhesion molecules, and act to activate *Tgfb* (Egeblad and Werb, 2002). Overexpression of *Mmp2* and *Mmp9* is also associated with mammary gland cancer cell growth, migration, and invasion (Cupić *et al.*, 2011).

Id1, another gene regulated by *Tgfb* (Liang *et al.*, 2009), was also shown to be significantly upregulated in mammary gland adenocarcinomas from rats exposed to bromodichloroacetic acid. Overexpression of this gene in mammary gland cancer is associated with an invasive phenotype including the process of epithelial-to-mesenchymal transition (Tobin *et al.*, 2011). *Thbs1* is a member of a group of mediators that act to modulate cell differentiation and matrix structure during tissue development and remodeling; it has been shown to interact with a variety of other mediators in the tumor microenvironment to increase *Tgfb* expression (Yee *et al.*, 2009), and it is also secreted in response to *Tgfb* stimulation. *Thbs1* is overexpressed in various types of cancers, including mammary gland cancer (Roberts, 1996).

Members of the *Vegf* family have a critical role in the regulation of angiogenesis, which is very important in the pathogenesis of neoplasia. Angiogenesis is also regulated by a number of secreted growth factors, including members of the *Tgfb* family (Soufla *et al.*, 2006), and the induction of *Vegf* during tumor angiogenesis is mediated in part through *Tgfb* stimulation (Breier *et al.*, 2002). *Vegfa* is released by tumor cells to promote tumor angiogenesis, and it is also released from the extracellular matrix during tissue remodeling by *Mmp9* to induce the angiogenesis that promotes tumor progression (Sakurai and Kudo, 2011).

The overrepresentation of *Tgfb* mediators that were significantly upregulated in mammary gland adenocarcinomas from bromodichloroacetic acid-exposed female F344/NTac rats compared to spontaneous mammary gland adenocarcinomas may suggest a correlation between this pathway and the increased incidences of proliferative mammary gland lesions observed in the females. These data suggest that mammary gland carcinogenesis in animals exposed to bromodichloroacetic acid may be influenced in part by *Tgfb*-dependent mechanisms.

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TABLE N1
Frozen Mammary Gland and Adenocarcinoma Samples
from Control and Bromodichloroacetic Acid-Exposed Female Rats

Animal Number	Histology Number	Frozen ID	Chemical	Dose	Diagnosis	Collection
1	NA	Control-1LCM	NA	Control	Normal mammary gland	Frozen - LCM
2	NA	Control-2LCM	NA	Control	Normal mammary gland	Frozen - LCM
3	NA	Control-3LCM	NA	Control	Normal mammary gland	Frozen - LCM
4	NA	Control-4LCM	NA	Control	Normal mammary gland	Frozen - LCM
5	NA	Control-5LCM	NA	Control	Normal mammary gland	Frozen - LCM
DF401	91-57762	SO905	2,2-Bis(bromomethyl)- 1,3-propanediol	Control	Adenocarcinoma	Frozen
250	500250	BC12350	3,3',4,4'-Tetrachloroazo- benzene	Control	Adenocarcinoma	Frozen
LF325	92-61871	SO1427	D&C Yellow #11	Control	Adenocarcinoma	Frozen
1033	rz107-i033	DV272	Nickel sulfate hexahydrate	Control	Adenocarcinoma	Frozen
DF427	91-57771	SO889	2,2-Bis(bromomethyl)- 1,3-propanediol	Control	Adenocarcinoma	Frozen
649	0801003-14	BC18860	Bromodichloroacetic acid	500 mg/L	Adenocarcinoma	Frozen
718	0801038-13	BC18877	Bromodichloroacetic acid	1,000 mg/L	Adenocarcinoma	Frozen
708	0801028-13	BC18846	Bromodichloroacetic acid	1,000 mg/L	Adenocarcinoma	Frozen
559	0800947-13	BC18879	Bromodichloroacetic acid	250 mg/L	Adenocarcinoma	Frozen

NA=Not applicable; LCM=Laser-capture microdissected

TABLE N2
Upregulated Genes Associated with *Tgfb* Signaling, Breast Cancer Growth, Invasion, and Metastasis
in Mammary Gland Adenocarcinomas from Exposed F344/NTac Female Rats
in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

Gene	Fold Change	P Value	Function	Role in Cancer	References
<i>Mmp9</i>	11.232	0.0164	Extracellular matrix remodeling	Cancer growth, migration, invasion	Cupić <i>et al.</i> , 2011
<i>Mmp2</i>	4.69	0.0110	Extracellular matrix remodeling	Cancer growth, migration, invasion	Cupić <i>et al.</i> , 2011
<i>Id1</i>	3.055	0.0154	Inhibition of differentiation	Tumor invasion, metastasis, EMT	Tobin <i>et al.</i> , 2011
<i>Vegfa</i>	2.4	0.0381	Proangiogenic factor	Cancer growth and metastasis	Breier <i>et al.</i> , 2002
<i>Thbs1</i>	2.04	0.0307	Extracellular matrix remodeling	Tumor growth and metastasis	Roberts, 1996

EMT=Epithelial-mesenchymal transition

APPENDIX O

TRANSCRIPTOMIC AND MUTATIONAL ANALYSIS OF BROMODICHLOROACETIC ACID TREATED NONTUMOR LIVER AND HEPATOCELLULAR TUMORS IN B6C3F1/N MICE

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TRANSCRIPTOMIC AND MUTATIONAL ANALYSIS OF BROMODICHLOROACETIC ACID TREATED NONTUMOR LIVER AND HEPATOCELLULAR TUMORS IN B6C3F1/N MICE

INTRODUCTION

The National Toxicology Program (NTP) bioassay of bromodichloroacetic acid in B6C3F1/N mice resulted in statistically significant increases in the incidences of hepatoblastoma in male mice and hepatocellular carcinoma in both male and female mice. Currently, very little information is available regarding bromodichloroacetic acid-induced mechanisms of toxicity and tumorigenesis. The current study compares global gene profiles between laser capture microdissected hepatoblastoma, associated hepatocellular carcinoma, adjacent nontumor liver, and age-matched vehicle normal liver in order to identify global gene expression changes related to bromodichloroacetic acid exposure and mechanisms of hepatocarcinogenesis in bromodichloroacetic acid-exposed B6C3F1/N mice.

The specific objectives of this study were to 1) identify significant differences in global gene expression between bromodichloroacetic acid-treated adjacent nontumor and vehicle control liver, 2) identify genomic alterations in bromodichloroacetic acid-treated hepatocellular carcinoma compared to its adjacent nontumor liver, and 3) identify genomic alterations in bromodichloroacetic acid-induced hepatoblastoma compared to hepatocellular carcinoma. The first objective addresses the chemical effect on the liver resulting from bromodichloroacetic acid exposure, and the second objective provides important information on the molecular characterization of hepatocellular carcinoma resulting from bromodichloroacetic acid exposure in the B6C3F1/N mouse. The third objective addresses the genomic alterations between bromodichloroacetic acid-treated hepatoblastoma compared to its associated hepatocellular carcinoma and molecular pathogenesis of mice hepatoblastoma response in NTP rodent bioassays.

MATERIALS AND METHODS

Animals and Tissue Sampling

Frozen tissues from spontaneous and treatment-related hepatocellular tumors were collected from the 2-year NTP bioassay of bromodichloroacetic acid and used for molecular biology analysis. The frozen sample selection was based on tumor size, i.e. when a tumor was at least 0.5 cm in diameter, one-half of that tumor was collected for fixation in 10% neutral buffered formalin, and the other corresponding half was flash frozen in liquid nitrogen. RNA isolated from frozen tissue was utilized for quantitative real-time PCR (qPCR) and microarray analysis. DNA isolated from either the frozen tissues or from formalin-fixed paraffin-embedded (FFPE) tissues was used for mutation analysis. In the current study, hepatoblastoma, hepatocellular carcinoma, and adjacent nontumor liver were sampled from bromodichloroacetic acid-treated B6C3F1/N male mice and normal liver from two male and four female age-matched vehicle control mice were collected at terminal sacrifice of the 2-year NTP bioassay. Histopathology was conducted on all the samples used in the molecular analysis. The samples from bromodichloroacetic acid-exposed and vehicle control B6C3F1/N mice are listed in Table O1.

Laser Capture Microdissection, RNA Isolation, and Amplification

All the tissue samples considered for laser capture microdissection (LCM) were microscopically examined for the lack of autolysis, necrosis, and hemorrhage and the presence of adequate adjacent nontumor liver. Following histopathology, identification of samples that had a good representation of hepatoblastoma, hepatocellular carcinoma, and adjacent nontumor liver, frozen samples were obtained from the NTP frozen tissue repository, removed from cryovials, and embedded in OCT medium on dry ice. Following embedding, cryosections were prepared, stained with hematoxylin and eosin (H&E), and evaluated for adequate amounts of each tissue component (e.g. hepatoblastoma, hepatocellular carcinoma, and adjacent nontumor liver). Next, hepatoblastomas (N=6), hepatocellular carcinomas (N=6), adjacent nontumor liver (N=6), and age-matched vehicle normal liver (N=6) were LCMed from two to five serial 10 mm cryosections for microarray analysis (Table O1). LCM was performed on the above tissues using MMI CellCut Plus (MMI, Haslett, MI). RNA extraction and whole genome amplification was

performed using Ovation Pico WTA System V2 (NuGEN, San Carlos, CA) following manufacturer's recommendation and RNA integrity was measured with Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

Microarray Hybridizations

Gene expression analysis was conducted on bromodichloroacetic acid-treated hepatoblastoma (N=6), bromodichloroacetic acid-treated hepatocellular carcinoma (N=6), adjacent nontumor liver (N=6), and age-matched vehicle normal liver (N=6) using Affymetrix Mouse Genome 430 2.0 GeneChip® arrays (Affymetrix, Santa Clara, CA). Total RNA (10 ng) was amplified as directed in the WT-Ovation Pico RNA Amplification System protocol, and labeling with biotin following the Encore Biotin Module. Amplified biotin-aRNAs (5µg) were fragmented and hybridized to each array for 18 hours at 45° C in a rotating hybridization. Array slides were stained with streptavidin/phycoerythrin utilizing a double-antibody staining procedure and then washed for antibody amplification following manufacturer's protocol. Arrays were scanned in an Affymetrix Scanner 3000 and data was obtained using the GeneChip® Command Console Software (AGCC; Version 1.1) using the MAS5 algorithm to generate .CHP files.

Data Processing and Identification of Differentially Expressed Genes

Data processing and identification of differentially expressed genes was done as previously described in detail (Hoenerhoff *et al.*, 2011; Pandiri *et al.*, 2012). Briefly, array fluorescent pixel intensity measurements were acquired and gene expression data were normalized across all samples using the robust multiarray analysis (RMA) methodology (Irizarry *et al.*, 2003). Using RMA-normalized data, for each probe set, pairwise comparisons were made using a bootstrap t-test while controlling the mixed directional false discovery rate (FDR) (Guo *et al.*, 2010). This methodology controls for the overall FDRs for multiple comparisons as well as directional errors when declaring a gene to be upregulated or downregulated. The level of significance was set at $P < 0.05$ and the mdFDR was set at 5%.

Bioinformatics Analyses

Partek Genomics Suite, version 6.6 (Partek Inc., St. Louis, MO) was used to perform PCA on the normalized data of hepatoblastoma, hepatocellular carcinoma, adjacent nontumor liver, and vehicle normal liver samples. PCA uses a linear transformation to reduce the dimension of the data from n probe sets to k principal components (PC). The first three PC that capture the majority of the variation in the data were used to visualize the spatial relationship of the hepatoblastoma, hepatocellular carcinoma, adjacent nontumor liver, and vehicle normal liver samples.

Ingenuity Pathway Analysis (IPA) 9.0, application build-220217, version-16542223 (Ingenuity systems Inc., Redwood City, CA), was used to evaluate the most statistically significant overrepresented canonical pathways. These canonical pathways are based on the Ingenuity knowledge base. The significant biological canonical pathways were derived from IPA and the statistical significance was assumed at a $P < 0.001$ (Fisher's exact test).

Quantitative Real-Time PCR (qPCR)

RNA extraction and amplification were performed using Ovation Pico WTA System V2 (NuGEN, San Carlos, CA) following manufacturer's recommendation. Total RNA (10 ng) was amplified using Ovation Pico WTA System and resulted in 6 µg of amplified cDNA. Relative quantitative gene expression levels were detected using real-time PCR with the ABI PRISM 7900HT Sequence Detection System (Life Technologies, Grand Island, NY) using SYBR green methodology. Primers were designed using Primer3Plus software (Rozen and Skaletsky, 2000) to span exon-exon junctions with an annealing temperature of 60° C and amplification size of less than 150 bp.

Briefly, 25 ng of cDNA were added to a 25-µL PCR reaction to get a final concentration of 1.00 ng/µL of cDNA. Forward and reverse primer final concentrations were 100 nM in the SYBR green assay. The reactions were performed using the Power SYBR® Green PCR Master Mix (Life Technologies, Grand Island, NY). 18S was chosen as the endogenous control gene in our qPCR experiments. Relative quantification of gene expression changes was recorded after normalizing for 18S expression, computed by using the $2^{-\Delta\Delta CT}$ method (user manual #2, ABI Prism 7700 SDS).

Comparative Mutation Analysis of *H-ras* and *Ctnnb1* in Hepatoblastoma and Hepatocellular Carcinoma

To study the comparative mutation profile in hepatoblastoma and adjacent hepatocellular carcinoma, *H-ras* codon 61 and *Ctnnb1* exon 2 and 3 mutation spectra were analyzed in 30 FFPE hepatoblastomas and adjacent hepatocellular carcinomas from the 2-year bromodichloroacetic acid mouse bioassay. Hepatoblastomas and adjacent hepatocellular carcinomas were LCMed as described above. DNA was isolated from LCM tissues using Ovation Pico WTA System V2 (NuGEN, San Carlos, CA) following the manufacturer's recommendation. PCR amplification reaction and sequencing was done following previously published methods (Hoenerhoff *et al.*, 2013).

RESULTS

The first objective of this study was to identify significant differences in global gene expression between bromodichloroacetic acid-treated adjacent nontumor and vehicle control liver. Principal component analysis (PCA) clearly differentiated bromodichloroacetic acid-treated hepatoblastoma, hepatocellular carcinoma, adjacent nontumor liver, and vehicle normal liver based on their respective global gene expression profiles (Figures O1, O2, and O3). Analysis of bromodichloroacetic acid-exposed adjacent nontumor liver compared to vehicle normal liver using Affymetrix Mouse Genome 430 2.0 GeneChip[®] arrays identified 841 probes (mapped to 790 genes) as differentially expressed. PCA captured 40.6% of data variation in all the genes and indicated distinct separation of the bromodichloroacetic acid-treated adjacent nontumor liver from vehicle normal liver (Figure O1). When bromodichloroacetic acid-treated hepatocellular carcinoma was compared to adjacent nontumor liver, 1,237 probes (mapped to 1,087 genes) were identified as differentially expressed. Likewise, PCA captured 38.6% of data variation in all the genes between hepatocellular carcinoma, adjacent nontumor liver, and vehicle normal liver groups and there was distinct separation of hepatocellular carcinoma samples from adjacent nontumor liver and vehicle normal liver samples (Figure O2). Analysis of hepatoblastoma compared to adjacent nontumor liver indicated 13,889 probes (mapped to 10,346 genes) as differentially expressed. PCA captured 47.2% of data variation in all the expressed genes and indicated a tight and distinct grouping of the hepatoblastomas from the associated hepatocellular carcinoma, adjacent nontumor liver, and vehicle normal liver suggesting marked differences in global gene expression between hepatoblastoma and other sample types (Figure O3).

IPA comparison analysis of bromodichloroacetic acid-treated adjacent nontumor liver with vehicle normal liver indicated altered oncogenic, metabolic, and hepatic function related pathways. IPA analysis showed that top biological functions that were altered in adjacent nontumor liver compared to vehicle normal liver included molecular pathways involved in cancer, cellular function and maintenance, and cell morphology (Figure O4). Top toxicologic functions perturbed in adjacent nontumor liver included molecular pathways associated with liver necrosis/cell death, regeneration and proliferation (Figure O5). Finally, top canonical pathways altered in adjacent nontumor liver included metabolic pathways such as cholesterol and retinol biosynthesis, and cancer pathways such as ephrin receptor, insulin receptor, and protein kinase A signaling (data not shown). Top genes differentially expressed in bromodichloroacetic acid-treated adjacent nontumor liver were involved in cell growth, cell proliferation, neoplasia, and transcriptional regulation (Table O2).

In bromodichloroacetic acid-treated hepatocellular carcinoma, IPA analysis indicated dysregulation of a variety of metabolic and cancer related pathways. Overrepresented pathways altered in bromodichloroacetic acid-treated hepatocellular carcinoma included biological functions such as organismal function, cell movement, cancer signaling, cellular development, and cell growth and proliferation. Top overrepresented canonical pathways in hepatocellular carcinoma included fatty acid metabolism, xenobiotic signaling, cell cycle: G2/M DNA damage checkpoint regulation, aryl hydrocarbon receptor signaling, and apoptosis signaling (data not shown). In bromodichloroacetic acid-treated hepatocellular carcinoma, many of the top dysregulated cancer pathways included upregulation of oncogenes (*Gpc3*, *Akr1c3*, *Plat*, *Itih5*, *Areg*, *Tff3*, *Afp*) and downregulation of tumor suppressor genes (*Dct*, *Gas1*, *Prlr*, *Socs2*, *Wnt5b*) (Table O3). Directionality of fold changes of a number of relevant genes observed on microarray were validated by qPCR (Table O4).

Hepatoblastomas are characterized by dysregulation of Wnt/Ctnnb1 targets, embryonic development, and genomic imprinting. IPA analysis was performed to identify pathways dysregulated in hepatoblastoma compared to adjacent nontumor liver. Genes involved in Wnt/*Ctnnb1* pathway signaling were dysregulated in hepatoblastoma, including upregulation of various Wnt signaling genes (*Wnt9a*, *Wnt10a*, *Wnt7a*), genes involved in Wnt feedback-regulation

(*Axin2*, *Nkd1*), positive effectors (*Lef1*, *Dvl3*), and *Wnt* antagonists (*Dkk2*, *Dkk3*, *Wif1*; Table O5). Secondly, there was significant upregulation of a number of genes involved in genomic imprinting (*Igf2*, *Peg1*, *Peg10*, *Bex1*, *Meg3*, *H19*, *Ndn*), which are typically expressed in fetal liver. Finally, genes involved in embryonic stem cell pluripotency (*T*, *Bmp4*, *Fzd6*, *Fzd10*, *Nog*) and stem cell-related target genes (*Sox9*, *Tbx3*, *Suz12*) were upregulated, while *Zfp42*, *Gata4*, *Fzd4*, and *Fzd8* were downregulated, indicating distinctive expression of hepatic stem/progenitor markers (Table O5).

NextBio meta-analysis software (Kupershmidt *et al.*, 2010) was used to identify the most concordant mouse microarray datasets in the curated literature compared to our hepatoblastoma microarray dataset. NextBio metaanalysis of publicly available gene expression databases indicated that hepatoblastomas in B6C3F1/N mice are genomically very similar to early embryonic mouse liver (E10.5 to E14.5) (Otu *et al.*, 2007; Chaignat *et al.*, 2011).

Hepatoblastomas show overrepresentation of pathways associated with carcinogenesis and stem/progenitor cell signaling compared to hepatocellular carcinoma. A comparison analysis between hepatoblastoma and hepatocellular carcinoma relative to adjacent nontumor liver was performed using IPA in order to identify significant differential gene expression changes between hepatoblastoma and hepatocellular carcinoma. Results of this comparison analysis indicated overrepresentation of 1) biologic functions including metabolic signaling pathways [LXR/FXR activation, fatty acid β -oxidation, xenobiotic metabolism signaling, FXR/RXR activation, triacylglycerol, and cholesterol biosynthesis (Figures O6 and O7)]; 2) canonical pathways related to carcinogenesis [cell cycle control of chromosome replication, NRF2-mediated oxidative stress response, basal cell carcinoma signaling (Figure O8)], and a number of developmental and stem cell-related regulation pathways [mouse embryonic stem cell pluripotency, DNA methylation and transcriptional repression signaling, sonic hedgehog signaling (Figure O9)] in hepatoblastoma compared to hepatocellular carcinoma. Select genes that were differentially expressed between hepatoblastoma and hepatocellular carcinoma were validated using qPCR (Table O4).

Hepatoblastoma and hepatocellular carcinoma from B6C3F1/N mice do not share common mutations in Ctnnb1 and H-ras. In the present study, we screened for mutations in the “hot-spot” regions in mouse *Ctnnb1* (exon 2, corresponding to exon 3 in human *CTNNB1*) and *H-ras* codon 61. Bromodichloroacetic acid-treated hepatoblastoma and adjacent hepatocellular carcinoma were LCMed from FFPE B6C3F1/N mouse livers (N=30) to analyze the comparative mutation spectra of *H-ras* and *Ctnnb1* genes in bromodichloroacetic acid-treated hepatoblastoma and adjacent hepatocellular carcinoma. Bromodichloroacetic acid-treated hepatoblastoma and hepatocellular carcinoma showed a relatively lower incidence of *H-ras* mutation and a higher incidence of *Ctnnb1* mutation compared to historical spontaneous hepatocellular carcinoma (Table O6). Between bromodichloroacetic acid-treated hepatoblastoma and hepatocellular carcinoma, there was not much difference in the incidence of *H-ras* (7% and 13%, respectively) and *Ctnnb1* mutations (23% and 10%, respectively) (Table O6). Interestingly, for hepatoblastoma and its adjacent hepatocellular carcinoma, with the exception of one sample (LM122) the mutation spectra were different and there was no mutation overlap for *H-ras* and *Ctnnb1* genes (Table O7).

DISCUSSION

Global gene expression profiling was used to identify genomic alterations in bromodichloroacetic acid-treated adjacent nontumor liver, hepatocellular carcinoma, and hepatoblastoma compared to vehicle normal livers. PCA analysis done on normalized gene expression data clearly distinguished adjacent nontumor liver from vehicle normal liver indicating distinct gene expression changes between groups related to bromodichloroacetic acid exposure. These data indicate that there are distinct genomic alterations at 2 years in bromodichloroacetic acid-treated liver that can be related to exposure. For example, when bromodichloroacetic acid-treated adjacent nontumor liver was compared to vehicle normal liver, a number of biological functions involved in cancer, cellular function and maintenance, cell death, and survival were perturbed in bromodichloroacetic acid-treated livers. Similarly, toxicologic functions perturbed in adjacent nontumor liver were associated with liver necrosis/cell death, liver proliferation, and liver damage. In the present study, bromodichloroacetic acid-treated adjacent nontumor liver were associated with changes in expression of genes involved in cell growth and proliferation (*Lcn2*, *Lepr*, *Gpx3*, *Gas1*), neoplasia (*Socs3*, *Gsk3 β* , *Id2*, *Fat4*, *Dpt*). Specifically, genes involved in the process of tumorigenesis, like *Mdm2* oncogene, were overexpressed in adjacent nontumor liver, which is also overexpressed in human hepatocellular carcinoma (Jablkowski *et al.*, 2005), and the overexpression of *Mdm2* can result in excessive inactivation of tumor protein p53, diminishing its tumor suppressor function (Jablkowski *et al.*, 2005). Similarly, Ca²⁺-regulated

actin-binding gene gelsolin (*Gsn*) was also found to be downregulated in adjacent nontumor liver compared to vehicle normal livers. Dysregulation of *Gsn* has been reported in a number of cancer types, including human colorectal, gastric, bladder, lung, prostate, and kidney, where gelsolin was downregulated, suggesting that it might act as a tumor suppressor (Megger *et al.*, 2013). Although these changes are representative of gene expression changes between bromodichloroacetic acid-treated adjacent nontumor liver and vehicle normal liver, the influence of the adjacent tumor microenvironment and its influence on gene expression in adjacent nontumor liver cannot be ignored. Therefore, it is possible that some of the expression changes observed may be influenced by the presence of hepatocellular carcinoma adjacent to the adjacent nontumor liver and are likely not solely due to bromodichloroacetic acid toxicologic effects on the liver. This phenomenon of ‘field cancerization’ is defined as the presence of molecular changes associated with tumorigenesis in histologically normal tissues that are in the immediate vicinity of the tumor and could help to identify the potential molecular mechanisms responsible for hepatocarcinogenesis (Utsunomiya and Shimada, 2011).

The second objective of our study was to identify genomic alterations in bromodichloroacetic acid-treated hepatocellular carcinoma compared to its adjacent nontumor liver. PCA analysis showed clear separation of bromodichloroacetic acid-treated hepatocellular carcinoma from adjacent nontumor liver and vehicle normal livers, as expected when making nontumor to tumor comparisons in gene expression. Moreover, a number of genes were altered that were either classic oncogenes involved in hepatocarcinogenesis, or alterations in genes were suggestive of loss of normal hepatic function. For example, those genes involved in fatty acid metabolism and xenobiotic metabolism that were downregulated in bromodichloroacetic acid-treated hepatocellular carcinoma suggested hepatic dysfunction or reduced metabolic capability that can be seen in hepatic tumors. In terms of dysregulated cancer pathways, well known pathways involved in mouse hepatic carcinogenesis were altered including cell cycle G2/M checkpoint regulation, aryl hydrocarbon receptor signaling, and apoptosis regulation. More importantly, upstream tumor suppressor genes such as *Tp53* and *Cdkn2a* were downregulated in hepatocellular carcinoma, while oncogenes such as Myc transcriptional regulators such as *Hif1a*, and growth factors like *Vegfa*, *Fgf2*, *Tgfb1* were upregulated in bromodichloroacetic acid-treated hepatocellular carcinoma. Loss of expression or mutations in *Tp53* gene play an important role in hepatocellular carcinoma initiation or progression, depending on the context. Loss of expression of *Tp53* with other cooperating events (oxidative stress, telomere erosion, DNA-damage signaling) have a more prominent role in hepatocellular carcinoma progression by facilitating continued proliferative potential, which could also contribute to genomic instability in hepatocellular carcinoma (Farazi and DePinho, 2006). MYC oncogene is a central mediator of human hepatocarcinogenesis and in mouse model of tumorigenesis wherein MYC activation is required for maintenance and expansion of transformed cells (Kaposi-Novak *et al.*, 2009). Angiogenesis is an important event during the neoplastic process and is induced by the secretion of numerous angiogenic growth factors like vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (FGF2), and transforming growth factor (TGF)- β 1. In human hepatocellular carcinoma, increased expression of VEGF, FGF2 and TGF β 1 has been associated with poor differentiation, portal vein invasion, and poor prognosis (Uematsu *et al.*, 2005; Harimoto *et al.*, 2010). Similarly, many of the classic oncogenes like *Afp*, *Gpc3*, *Tff3*, *Areg/Aregb*, *Scd2* and tumor suppressors like *Dct*, *Socs2*, *Gas1*, which are commonly dysregulated in human hepatocellular carcinoma, were also found to be similarly differentially expressed in BDCA-treated hepatocellular carcinoma.

The third objective of the current study was to evaluate global genomic profiles and mutation spectra of commonly altered oncogenes in hepatoblastoma compared to adjacent hepatocellular carcinoma in B6C3F1/N mice. Consistent with the histopathological findings, results of our global gene expression analysis show that mouse hepatoblastomas are markedly different and quite distinct from adjacent hepatocellular carcinomas and normal liver. In fact, it is quite remarkable to note that the spatial distribution of samples on the PCA plot suggests that there are more overlapping genes shared between hepatocellular carcinoma and normal liver than there are between hepatocellular carcinoma and hepatoblastoma (Figure O3). Several biologic pathways observed as dysregulated in hepatoblastoma provide insight in terms of their origin, relationship to hepatocellular carcinoma. In mouse hepatoblastoma, there was overrepresentation of oncogenic signaling pathways including *Wnt/Ctnnb1* target genes, hepatic metabolism targets, stem/pluripotent progenitor cell genes, and stem cell-related target genes (Table O5). It is known that these pathways play a role in murine and human hepatocellular carcinoma (Clevers, 2006; Cairo *et al.*, 2008; Monga, 2011), but surprisingly, these genes were not differentially expressed in adjacent hepatocellular carcinoma (Table O5).

The *Wnt/Ctnnb1* pathway is a master regulator of cell fate and proliferation during embryonic development, and it is essential for stem cell maintenance in a wide variety of tissues (Clevers, 2006; Haegerbarth and Clevers, 2009). In

this study, we observed upregulation of *Wnt*-pathway related genes (*Wnt9a*, *Wnt10a*, *Wnt7a*), cancer target genes (*cMyc*, *Ccnd1*), genes involved in *Wnt* feedback-regulation (*Axin2*, *Nkd1*), positive effectors (*Lef1*, *Dvl3*), and *Wnt* antagonists (*Dkk2*, *Dkk3*, *Wif1*), and many of these genes expressed at high levels in hepatoblastoma tumors were targets of *Lef1*, a known *Wnt/Ctnnb1* pathway transcription factor. Dysregulation of the *Wnt/Ctnnb1/Lef1* pathway has also been observed in human cancer, and plays an important role in cancer stem cell biology (Polyak and Weinberg, 2009), including maintenance of self-renewal and differentiation of cancer stem cells (Bisson and Prowse, 2009). Upregulation of downstream oncogene targets of *Wnt* signaling as seen in this study (*cMyc*, *Ccnd1*) have also been associated with human hepatoblastoma (Takayasu *et al.*, 2001; Cairo *et al.*, 2008).

Metabolic capacity and function of the embryonic liver is significantly decreased compared to the mature liver. A number of genes associated with normal hepatic metabolic function were significantly downregulated in hepatoblastoma compared to adjacent hepatocellular carcinoma, supporting this embryonal phenotype in hepatoblastoma. For example, there was downregulation of number of Nrf2 target genes coding for anti-oxidant enzymes, (*Sod*, *Cat*, *Ho1*, *Gsr*, *Txn*, *Prdx1*, *Ftl*), phase I and II xenobiotic enzymes (*Fmo1*, *Ugt*, *Gst*, *Ephx1*, *Gclc*, *Nqo*), xenobiotic transporter genes (*Mrp2*, *Srb1*), and chaperone and stress response (*Hsp22*, *Hsp40*, *Hsp90*, *Clpp*, *Fkbp5*, *Herpud1*) compared to hepatocellular carcinoma. Several other genes involved in LXR/RXR activation, LXR/FXR activation, fatty acid β -oxidation, FXR/RXR activation, triacylglycerol and cholesterol biosynthesis, and xenobiotic metabolism (*Cyp2e1*, *Cyp2c8*, *Cyp2b6*, *Cyp1a2*) were downregulated in mice hepatoblastoma compared to hepatocellular carcinoma, suggesting reduced metabolic function. Previous studies in human hepatoblastoma have documented reduced expression of CYP genes including CYP2C8, CYP3A4, CYP2C9 (Nagata *et al.*, 2003). Downregulation or loss of function of these metabolic enzymes may suggest a metabolically inactive, embryonal origin of hepatoblastoma compared to hepatocellular carcinoma. Further, meta-analysis examination of our mouse hepatoblastoma dataset supported the hypothesized stem/multipotent nature of these tumors. Comparison of the current mouse hepatoblastoma dataset with curated datasets using NextBio meta-analysis software indicated significant concordance with early embryonic mouse liver (ED10.5 to 14.5).

Following the somatic mutation theory, carcinogenesis occurs in a step-wise manner through the accumulation of various mutations or deletions in oncogenes and tumor-suppressor genes, respectively (Berenblum and Shubik, 1949). As such, a mutation that occurs during the transformation of a precursor lesion or early tumor should remain fixed within the genome throughout the progression to later stages of malignancy or metastasis. Therefore, mutation spectra between 30 hepatoblastomas and adjacent hepatocellular carcinomas were compared for *Hras* and *Ctnnb1* genes to see if they share common mutation spectra. Interestingly, in 11 hepatoblastoma and adjacent hepatocellular carcinoma samples that carried *Hras* or *Ctnnb1* mutations, the mutation spectra of these genes was different, and these tumors did not share common mutations with the exception of one sample. Current data are consistent with previous studies (Anna *et al.*, 2000) which show that the mutational spectrum is different in hepatoblastoma than that of adjacent hepatocellular tumors, suggesting that these tumors are distinct entities. This is consistent with our hypothesis that hepatoblastoma arises from a transformed stem or multipotent progenitor cell, rather than a transformed hepatocyte. However, more studies with greater sample sizes and other alternate testing modalities like RNA-Seq and exome sequencing are required before we can make more definitive conclusions. The current study has some limitations such as small sample size, samples from a single chemical study, and lack of frozen spontaneous hepatoblastoma samples. Moreover, factors such as tumor heterogeneity may have played a role in these results, as each tumor was sampled as a whole. Sampling multiple sites within each tumor may provide more information about mutation spectra and similarities or differences between hepatoblastoma and adjacent hepatocellular carcinoma as well as the possible cell of origin of these tumors.

In conclusion, key differences in gene expression were observed between bromodichloroacetic acid-treated adjacent nontumor compared to vehicle normal liver involving genes mostly involved in metabolic pathways, cell death, cell growth and proliferation, and neoplasia, suggesting that bromodichloroacetic acid causes specific toxic and carcinogenic effects in the liver of exposed B6C3F1/N mice. Gene changes in bromodichloroacetic acid-treated adjacent nontumor liver are consistent with neoplastic signaling and may suggest microenvironmental changes preceding neoplastic transformation due to chemical treatment, or a type of "field cancerization"; however, given the fact that these bromodichloroacetic acid-treated nontumor tissues were isolated from sections adjacent to hepatocellular carcinoma, these gene expression changes may represent microenvironmental effects of the adjacent hepatocellular carcinoma on the histologically normal adjacent tissue. Although, it is interesting to consider that bromodichloroacetic acid exposure may be eliciting a "field cancerization" situation in which chemical exposure is resulting in preneoplastic areas within regions adjacent to existing tumors (Rubin, 2011; Ushijima and Hattori,

2012), there was no evidence of dysplastic hepatic tissue in these areas, and the tumor microenvironment may be largely influencing cancer signaling in tumor-adjacent tissue.

Further, bromodichloroacetic acid-treated mouse hepatoblastomas are markedly different from adjacent hepatocellular carcinoma in terms of their morphology, global gene expression, and *Hras/Ctnnb1* mutation profiles. Mouse hepatoblastoma, similar to human hepatoblastoma, shares significant similarities in global gene expression, including dysregulation of genes involved in Wnt/ β -catenin signaling, embryonic/stem cell pluripotency pathways, metabolic dysregulation, and expression of genomic imprinting genes. Furthermore, meta-analysis shows that mouse hepatoblastoma is very similar to early embryonic liver in terms of their gene expression profile. These findings suggest that hepatoblastoma and hepatocellular carcinoma are very different entities, likely arising from the same hepatic lineage, but hepatoblastoma arising as a result of transformation of a hepatic stem or multipotential progenitor cell. However, further studies are required to further understand the molecular tumorigenesis of hepatocellular carcinoma and hepatoblastoma.

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TABLE O1
Hepatoblastomas, Hepatocellular Carcinomas, and Adjacent Nontumor Liver
from Control and Treated Mice Used for Microarray Analysis in the 2-Year Drinking Water Study
of Bromodichloroacetic Acid

Dose (mg/L)	Animal Number	Sex	Vehicle Normal Liver	BDCA-Treated Adjacent Nontumor	BDCA-Treated Hepatocellular Carcinoma	BDCA-Treated Hepatoblastoma
0	446	Female	Replicate 1	–	–	–
0	443	Female	Replicate 2	–	–	–
0	17	Male	Replicate 3	–	–	–
0	405	Female	Replicate 4	–	–	–
0	424	Female	Replicate 5	–	–	–
0	32	Male	Replicate 6	–	–	–
1,000	343	Male	–	Replicate 1	Replicate 1	Replicate 1
500	244	Male	–	Replicate 2	Replicate 2	Replicate 2
1,000	366	Male	–	Replicate 3	Replicate 3	Replicate 3
1,000	352	Male	–	Replicate 4	Replicate 4	Replicate 4
1,000	304	Male	–	Replicate 5	Replicate 5	Replicate 5
500	244	Male	–	–	Replicate 6	–
500	636	Male	–	Replicate 6	–	Replicate 6

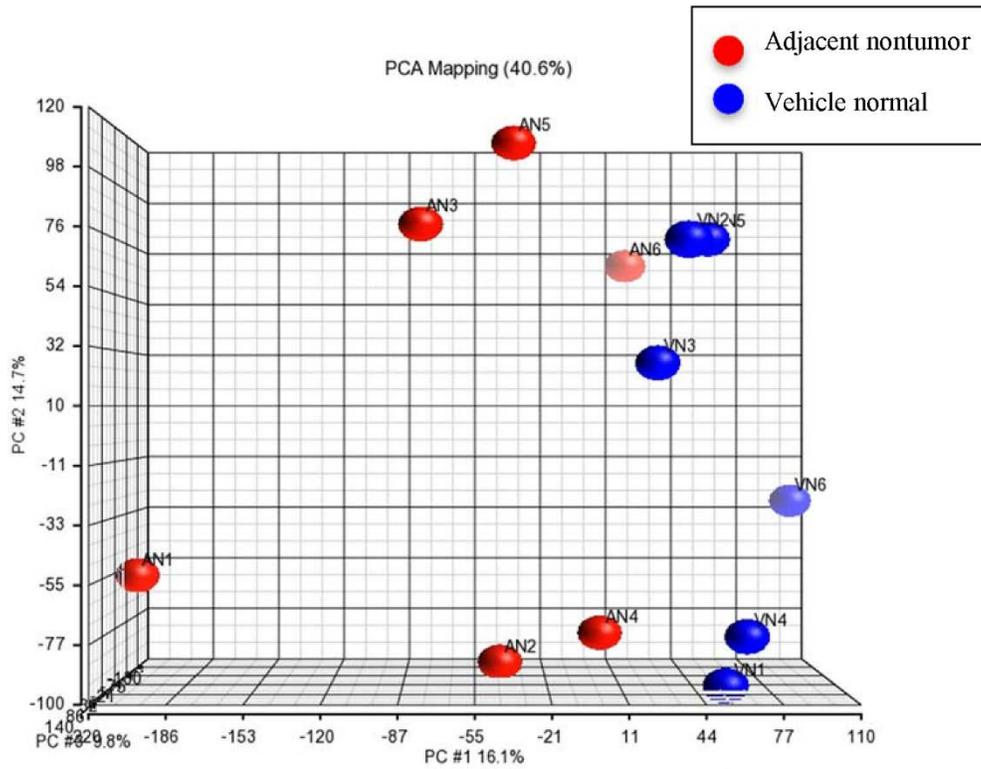


FIGURE O1
Principal Component Analysis Comparing Global Gene Expression Profiles of Adjacent Nontumor Liver from Treated B6C3F1/N Mice and Vehicle Normal Liver from Control Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

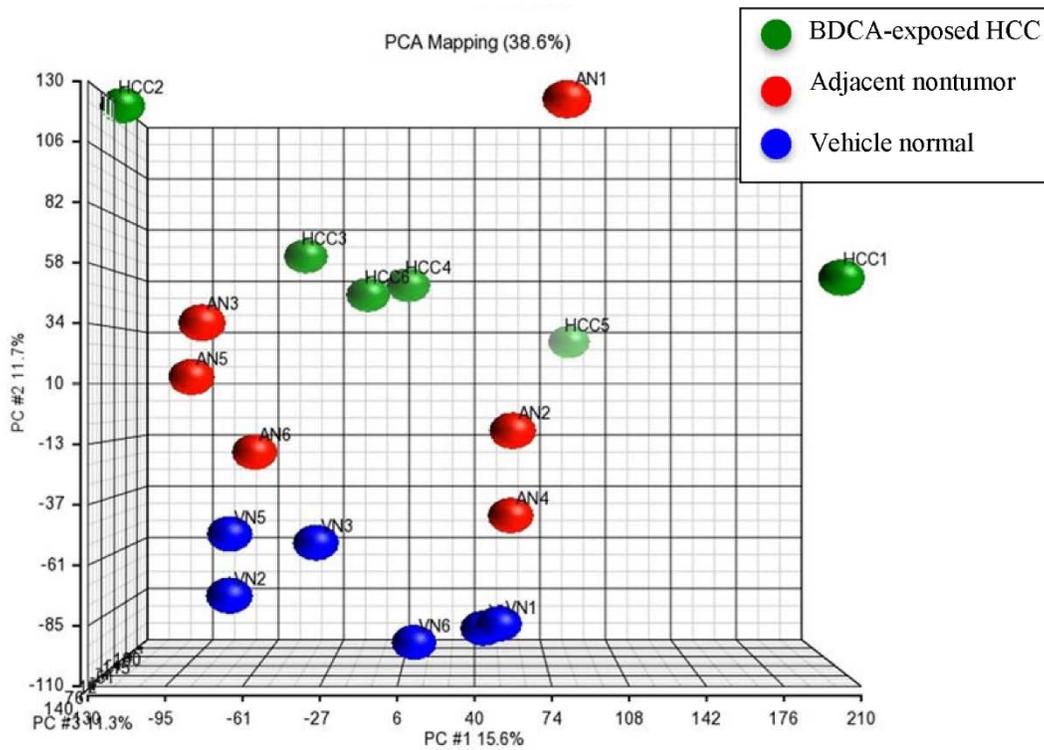


FIGURE O2
Principal Component Analysis Comparing Global Gene Expression Profiles of Hepatocellular Carcinomas (HCC) to Adjacent Nontumor Liver from Treated Mice and to Vehicle Normal Liver from Control B6C3F1/N Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

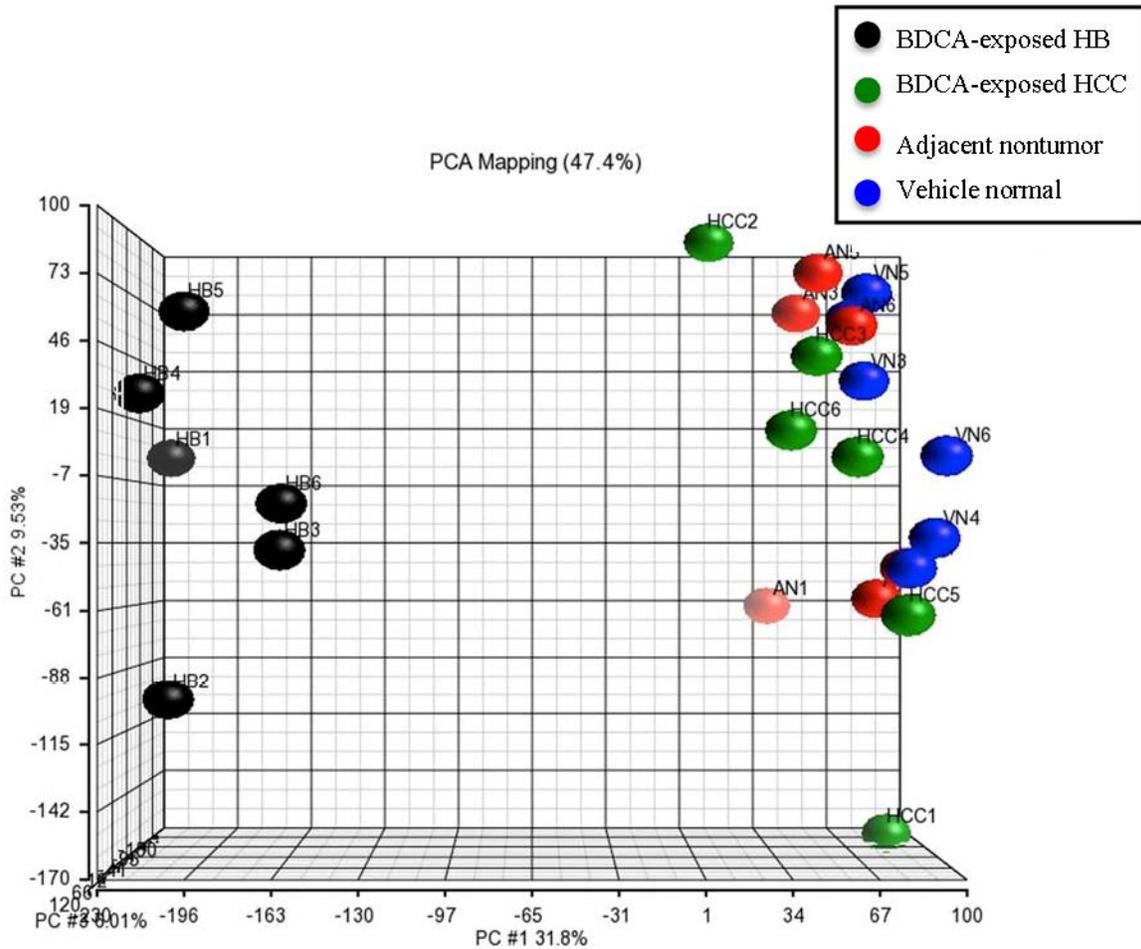
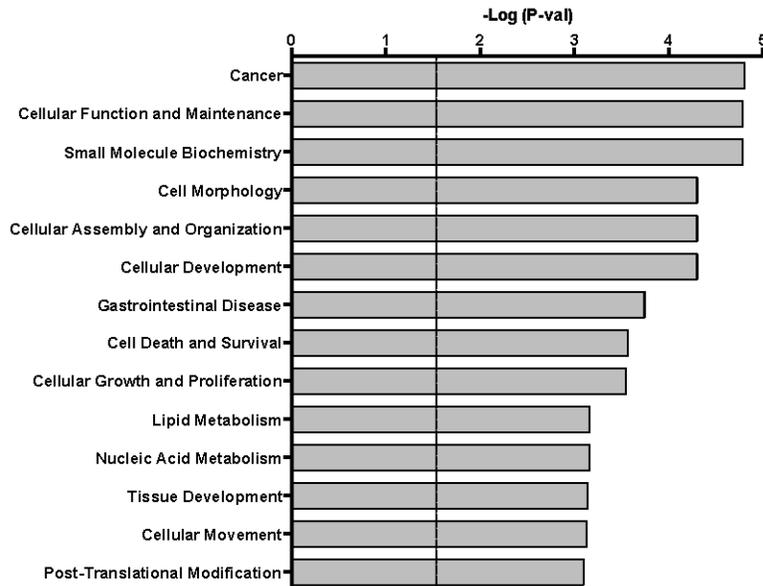


FIGURE O3
Principal Component Analysis Showing Distinct Separation, Indicating Unique Gene Expression Profile, of Hepatoblastomas (HB) from Hepatocellular Carcinomas (HCC), Adjacent Nontumor Liver (AN) from Treated Mice, and Vehicle Normal Liver (VN) from Control B6C3F1/N Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

**FIGURE O4**

Ingenuity Pathway Analysis™ of Differentially Expressed Genes Showing Top Biological Functions Perturbed in Adjacent Nontumor Liver from Treated B6C3F1/N Mice Compared to Vehicle Normal Liver from Control Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

These biological functions involved cancer, cellular function and maintenance, and cell death and survival.

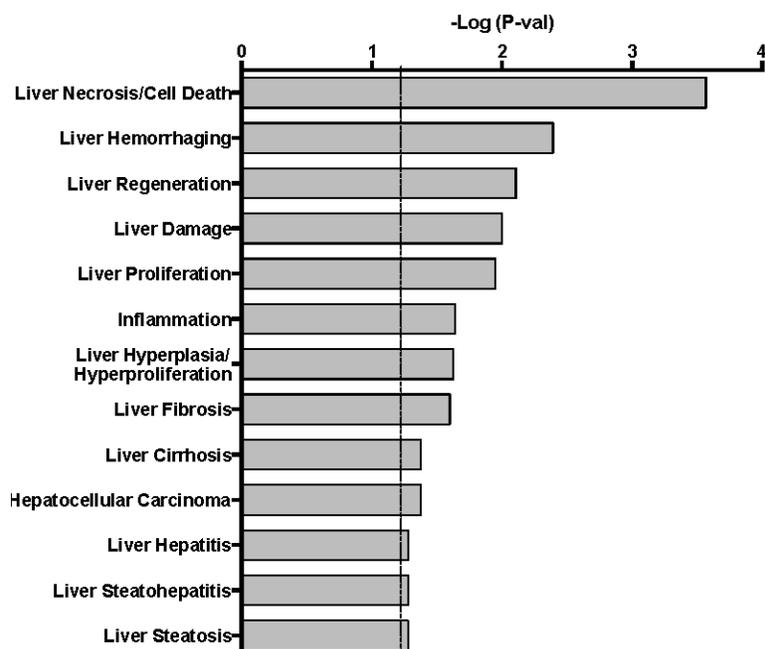


FIGURE O5

Ingenuity Pathway Analysis™ of Differently Expressed Genes Showing Top Toxicological Functions Perturbed in Adjacent Nontumor Liver from Treated B6C3F1/N Mice Compared to Vehicle Normal Liver from Control Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

These toxicological functions were associated with liver necrosis/cell death, liver proliferation, and liver damage.

TABLE O2
Dysregulated Genes in Treated Adjacent Nontumor Livers Compared to Vehicle Normal Liver
from B6C3F1/N Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

Gene Symbol	Gene Name	Fold Change
Cell growth and proliferation		
<i>Lcn2</i>	Lipocalin 2	8.74
<i>Lepr</i>	Leptin receptor	6.32
<i>Gpx3</i>	Glutathione peroxidase 3	5.06
<i>Mt1H</i>	Metallothionein 1H	3.80
<i>Igfbp1</i>	Insulin-like growth factor binding protein 1	2.50
<i>Gas1</i>	Growth arrest-specific 1	-3.50
Neoplasia		
<i>Socs3</i>	Suppressor of cytokine signaling 3	3.60
<i>Ddit4</i>	DNA-damage-inducible transcript 4	3.30
<i>Itgb3</i>	Integrin, beta 3	2.90
<i>Gsk3β</i>	Glycogen synthase kinase 3 beta	1.77
<i>Id2</i>	Inhibitor of DNA binding 2	-2.40
<i>Stat1</i>	Signal transducer and activator of transcription 1	-2.5
<i>Fzd8</i>	Frizzled family receptor 8	-2.60
<i>Fat4</i>	FAT tumor suppressor homolog 4	-3.42
<i>Clic5</i>	Chloride intracellular channel 5	-4.30
<i>Dpt</i>	Dermatopontin	-5.40
<i>Ca3</i>	Carbonic anhydrase III	-8.50
Transcriptional regulators		
<i>Nolc1</i>	Nucleolar and coiled-body phosphoprotein 1	2.50
<i>Lrp1</i>	Low density lipoprotein receptor-related protein 1	2.50
<i>Pawr</i>	PRKC, apoptosis, WT1, regulator	2.0
<i>Meis1</i>	Meis homeobox 1	-2.0
<i>Hlf</i>	Hepatic leukemia factor	-2.0

TABLE O3
Top Dysregulated Genes Involved in Cancer Pathways in Treated Hepatocellular Carcinoma
in B6C3F1/N Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

Gene Symbol	Gene Name	Fold Change
<i>Gpc3</i>	Glypican 3	39.4
<i>Akr1c3</i>	Aldo-keto reductase family 1, member C3	26.6
<i>Phgdh</i>	Phosphoglycerate dehydrogenase	23.3
<i>Plat</i>	Plasminogen activator, tissue	15.5
<i>Hsd3b1</i>	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	10.5
<i>Itih5</i>	Inter-alpha-trypsin inhibitor heavy chain family, member 5	10.4
<i>Areg/Aregb</i>	Amphiregulin	9.3
<i>Tff3</i>	Trefoil factor 3 (intestinal)	8.9
<i>Scd2</i>	Stearoyl-Coenzyme A desaturase 2	8.4
<i>Igfbp3</i>	Insulin-like growth factor binding protein 3	7.9
<i>Afp</i>	Alpha-fetoprotein	7.8
<i>Ddr1</i>	Discoidin domain receptor tyrosine kinase 1	6.9
<i>Mep1a</i>	Mepirin A, alpha (PABA peptide hydrolase)	6.1
<i>Dct</i>	Dopachrome tautomerase	-15.2
<i>Cyp3a5</i>	Cytochrome P450, family 3A, polypeptide 5	-10.2
<i>Cyp2c18</i>	Cytochrome P450, family 2C polypeptide 18	-6.2
<i>Cyp2f1</i>	Cytochrome P450, family 2F, polypeptide 1	-7.6
<i>Prlr</i>	Prolactin receptor	-7.8
<i>Gas1</i>	Growth arrest-specific 1	-4.3
<i>Socs2</i>	Suppressor of cytokine signaling 2	-2.3
<i>Mt1h</i>	Metallothionein 1H	-2.3
<i>Wnt5b</i>	wingless-type MMTV family, member 5B	-2.2

TABLE O4
Showing Validation of Microarray Gene Expression Changes Measured Using Real-time PCR
in Treated Adjacent Nontumor Liver, Hepatocellular Carcinoma, and Hepatoblastoma
from B6C3F1/N Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

Gene Symbol	Gene Name	Microarray	qPCR
BDCA-Adjacent Nontumor Liver			
<i>Gpx3</i>	Glutathione peroxidase 3	5.0	27.5
<i>Cyp2b10</i>	Cytochrome P450, family 2, subfamily b, polypeptide 10	-12.6	-9.8
<i>Fat4</i>	FAT tumor suppressor homolog 4	-3.5	-13.3
<i>Dpt</i>	Dermatopontin	-5.4	-3.3
<i>Mt1</i>	Metallothionein 1	6.3	19.8
<i>Serp1b1a</i>	Serine (or cysteine) peptidase inhibitor, clade B, member 1a	-16.2	-24.4
BDCA-Treated Hepatocellular Carcinoma			
<i>Cav1</i>	Caveolin 1	4.0	27.8
<i>Runx2</i>	Runt related transcription factor 2	3.4	8.5
<i>Prlr</i>	Prolactin receptor	-6.1	-5.4
<i>Gas1</i>	Growth arrest specific 1	-4.3	-7.2
<i>Pten</i>	Phosphatase and tensin homolog	-1.5	-1.4
BDCA-Treated Hepatoblastoma			
<i>T</i>	Brachyury	262.0	6,300
<i>Bex1</i>	Brain expressed gene 1	166	7,300
<i>Igf2</i>	Insulin-like growth factor-2	59.7	712
<i>Lef1</i>	Lymphoid enhancer binding factor 1	47.4	455
<i>Wnt6</i>	Wingless-related MMTV integration site 6	60.6	1,033
<i>Wif1</i>	Wnt inhibitory factor 1	427.5	7,575.0
<i>Cyp2e1</i>	Cytochrome P450, family 2, subfamily e, poly1	-212.6	-654.1

TABLE O5
Ingenuity Pathway Analysis of β -catenin Target Genes and Genomic Imprinted Genes Dysregulated in B6C3F1/N Mouse Hepatoblastomas in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

Gene Symbol	Gene Name	Fold Change Hepatoblastoma	Fold Change Hepatocellular Carcinoma
β-Catenin/Wnt Target Genes Significantly Dereglated in Hepatoblastoma			
<i>Axin2</i>	Axin2 (conductin)	20.3	NS*
<i>Dkk1</i>	Dickkopf homolog 1	33.0	NS
<i>Lef1</i>	Lymphoid enhancer-binding factor 1	43.1	NS
<i>Bmp4</i>	Bone morphogenetic protein 4	58.8	NS
<i>Dkk2</i>	Dickkopf 2 homolog	8.6	NS
<i>Dvl3</i>	Dishevelled, dsh homolog 3 (Drosophila)	2.0	NS
<i>Wnt 5, 6, 7, 9, 10</i>	Wingless-type MMTV integration site family	10.0 to 90.0	NS
<i>Wif1</i>	WNT inhibitory factor 1	475.0	NS
Stem-Cell Related Targets			
<i>Tbx1</i>	T-box1	37.3	NS
<i>Sox9</i>	SRY (sex determining region Y)-box 9	3.2	NS
<i>Suz12</i>	Suppressor of zeste 12 homolog (Drosophila)	3.0	NS
<i>T</i>	T, brachyury homolog (mouse)	268.0	NS
Hepatic Targets			
<i>Hdac2</i>	Histone deacetylase 2	3.2	NS
<i>Cyp2e1</i>	Cytochrome P450, family 2, subfamily E, polypeptide 1	-233.2	NS
<i>Cyp1a1</i>	Cytochrome P450, family 1, subfamily A, polypeptide 1	-42.0	NS
Upregulation of Genomic Imprinted Genes			
<i>Igf2</i>	Insulin-like growth factor 2	67.0	3.3
<i>Peg1</i>	Paternally expressed 1	5.7	NS
<i>Peg10</i>	Paternally expressed 10	3.5	NS
<i>Bex1</i>	Brain expressed, X-linked 1	166.0	44.0
<i>Meg3</i>	Maternally expressed 3	26.0	NS
<i>Ndn</i>	Necdin homolog (mouse)	6.0	-1.23
<i>H19</i>	H19, imprinted maternally expressed transcript (non-protein coding)	40.6	NS

* NS = No significant change

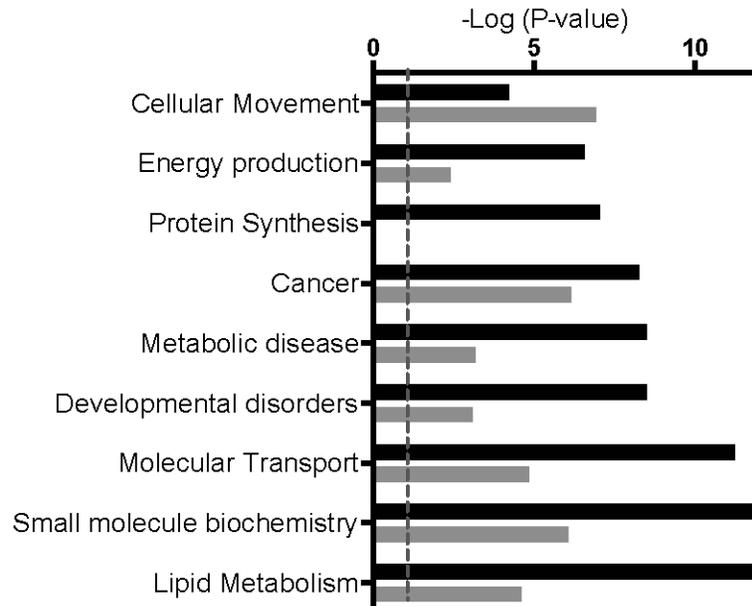


FIGURE O6

Ingenuity Pathway Analysis of Top Biological Pathways in Hepatoblastoma (black bars) Compared to Hepatocellular Carcinoma from Treated B6C3F1/N Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

The dotted line indicates the significance threshold of $-\log (P \text{ value})$.

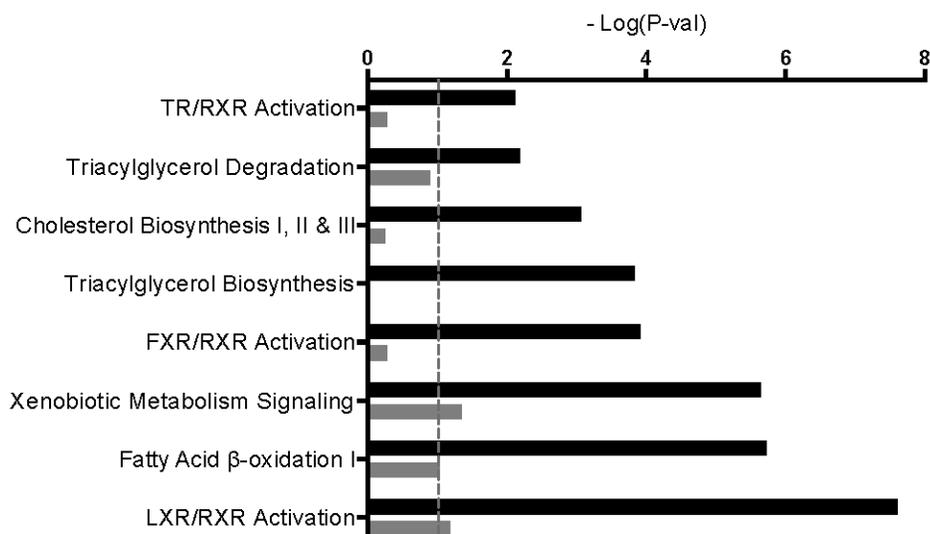


FIGURE O7
Ingenuity Pathway Analysis of Top Metabolic Pathways in Hepatoblastoma (black bars) Compared to Hepatocellular Carcinoma from Treated B6C3F1/N Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

The dotted line indicates the significance threshold of $-\log(P\text{ value})$.

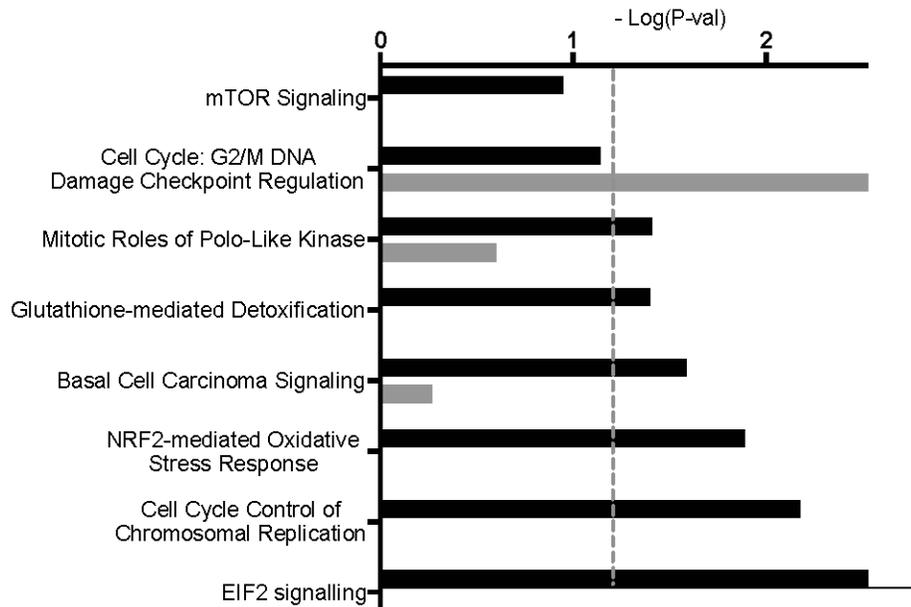


FIGURE O8

Ingenuity Pathway Analysis of Top Cancer Pathways in Hepatoblastoma (black bars) Compared to Hepatocellular Carcinoma from Treated B6C3F1/N Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

The dotted line indicates the significance threshold of $-\log(P\text{ value})$.

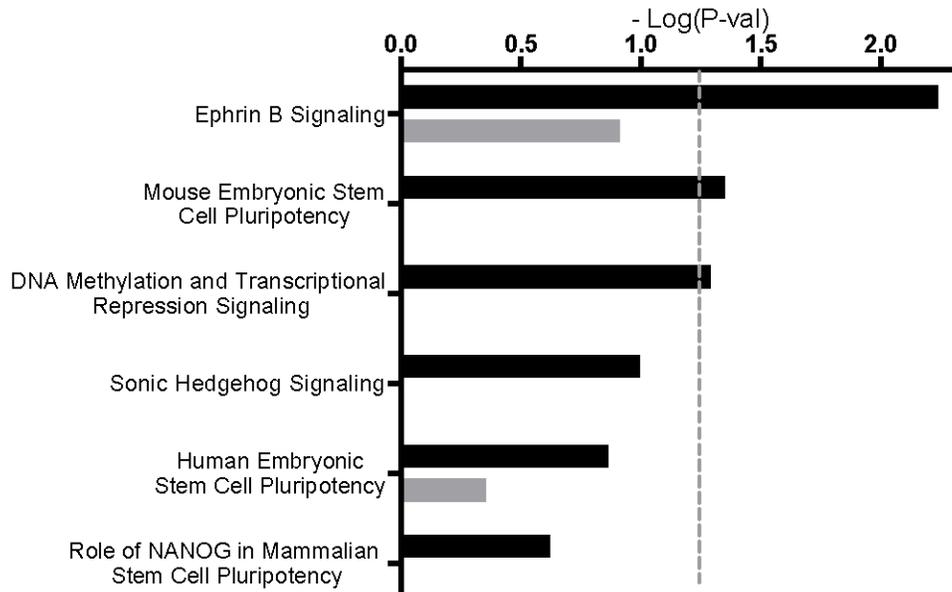


FIGURE O9

Ingenuity Pathway Analysis of Top Developmental and Stem Cell Related Pathways in Hepatoblastoma (black bars) Compared to Hepatocellular Carcinoma from Treated B6C3F1/N Mice in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

The dotted line indicates the significance threshold of $-\log(P \text{ value})$.

TABLE O6
Incidence of *H-ras* and *Ctnnb1* (β -catenin) Mutations
in Treated Hepatoblastoma and Hepatocellular Carcinoma from B6C3F1/N Mice
in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

	<i>H-ras</i>		<i>Ctnnb1</i> (β -catenin)	
	Hepatoblastoma	Hepatocellular Carcinoma	Hepatoblastoma	Hepatocellular Carcinoma
Historical spontaneous	n/a	260/473 (55%) ^a	n/a	1/59 (2%) ^b
Chemical Exposed	2/30 (7%)	4/30 (13%)	7/30 (23%)	3/30 (10%)

^a Incidence of *H-ras* mutations in spontaneous hepatocellular carcinoma of B6C3F1/N mice (Maronpot *et al.*, 1995; Sills *et al.*, 1999)

^b Incidence of β -catenin mutations in spontaneous hepatocellular carcinoma of B6C3F1/N mice (Hayashi *et al.*, 2003)

TABLE O7
Mutation Spectrum of *H-ras* and *Ctnnb1* (β -catenin) in Treated B6C3F1/N Mouse Hepatoblastoma
and Associated Hepatocellular Carcinoma in the 2-Year Drinking Water Study of Bromodichloroacetic Acid

Animal ID	<i>H-ras</i>		<i>Ctnnb1</i> (β -catenin)	
	Hepatoblastoma	Hepatocellular Carcinoma	Hepatoblastoma	Hepatocellular Carcinoma
LM101	— ^a	61 CAA → CGA	—	—
LM122	—	61 CAA → CGA	—	34 GGA → GTA
HM343	—	61 CAA → CGA	37 TCT → TTT	37 TCT → TTT
LM106	—	61 CAA → CTA	32 GAT → GTT	—
MM214	61 CAA → CTA	—	—	—
HM333	61 CAA → CTA	—	52 CCT → CAT	—
LM129	—	—	32 GAT → AAT	—
MM236	—	—	32 GAT → GGT	41 ACC → GCC
MM244	—	—	33 TCT → TTT	—
HM360	—	—	5 GCT → TCT	—
HM364	—	—	35 ATC → AGC	—
			—	19-46 deletion

^a No mutations detected