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Influence of *Helicobacter hepaticus* infection on the chronic toxicity and carcinogenicity of triethanolamine in B6C3F1 mice

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Abstract

Helicobacter hepaticus (*H. hepaticus*) infection causes hepatitis and increased hepatocellular neoplasms in male mice; although females are also infected, liver lesions are not typically expressed. In the 1990s, B6C3F1 mice from some chronic National Toxicology Program (NTP) studies were found to be infected with *H. hepaticus*. In these studies, there was hepatitis in many of the males, and there were more hepatocellular neoplasms in control males compared to studies with uninfected mice. In one of these studies, increased hepatocellular neoplasms at the high doses in male and female mice exposed topically to triethanolamine (TEA) provided the only evidence of carcinogenic activity. This study was repeated in mice free of *H. hepaticus*. However, the NTP mouse production colony and the diet differed between studies; these differences were the result of NTP programmatic decisions. In repeat study males, although control incidences were similar between studies, exposure did not result in increased hepatocellular neoplasms. In repeat study females, the control incidence of hepatocellular neoplasms was half that observed in the initial study, and these neoplasms were increased over controls at all doses. These data suggest that in the initial study, *H. hepaticus* influenced the induction of hepatocellular neoplasms in males, but not females.

Keywords

National Toxicology Program; triethanolamine; *Helicobacter hepaticus*, mice; hepatocellular neoplasms

Introduction

Helicobacter hepaticus (*H. hepaticus*) infection was discovered in the early 1990s in mice used in a carcinogenesis study (Fox, et al., 1994; Ward, et al., 1994a). Although males and females may both become infected, hepatitis and hepatocellular neoplasms associated with the infection occur predominantly in males (Ward, et al., 1994a; Ward, et al., 1994b). *H. hepaticus* commonly colonizes the cecum and colon, but results in hepatic effects upon invasion of the liver through the bile canaliculi. The progression of *H. hepaticus* induced lesions from hepatitis to neoplasms has been described previously (Fox, et al, 1996; Ward, 1994a). A detailed investigation of preneoplastic lesions resulting from *H. hepaticus* infection is also available (Rogers et al., 2004). It has been suggested that a non-genotoxic mode of action is involved in the induction of hepatocellular tumors by *H. hepaticus*. Increased liver cell proliferation has been observed in infected mice (Fox, et al. 1996; Ward, et al., 1994b). In addition, *H.*

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hepaticus infection promoted hepatocellular tumors initiated by n-nitrosodimethylamine and colon tumors initiated by azoxymethane (Diwan, et al., 1997; Nagamine, et al., 2007). Immune mediated mechanisms may contribute to the tissue damaging and proliferative effects observed in infected mice (Ward, et al., 1996; Canella, et al., 1996). Although *H. hepaticus* infection has been associated with increased production of reactive oxygen species and increased 8-oxo-dG (Sipowicz, et al., 1997), studies to determine if DNA alkylation or mutations were increased in infected animals, as a result of the production of genotoxic compounds by the bacteria or bacterial enzymes, or through the inflammatory process, were negative (Canella, et al. 1996).

A retrospective analysis revealed that male and female B6C3F1 mice from 12 National Toxicology Program (NTP) 2-year studies started in the late 1980s or early 1990s were infected with *H. hepaticus* (Hailey, et al., 1998). Hepatitis was observed microscopically in many (16 – 78%; average 48%) male mice from 9 of these studies; incidences of hepatitis in females were much lower (0 – 8%; average 2%). In male mice, increases in the incidences of hepatocellular neoplasms, hemangiosarcomas, total malignant neoplasms and total neoplasms were associated with *H. hepaticus* infection when accompanied by hepatitis (Hailey, et al. 1998). In studies where infection was present and there was hepatitis in some of the male mice, increased hepatocyte proliferation in the males with hepatitis, and to a lesser extent in males without hepatitis, were observed, when compared to mice from studies without either hepatitis or infection and hepatitis (Nyska, et al., 1997).

In 8 of the 9 NTP studies with *H. hepaticus* infection, accompanied by hepatitis in many male mice, biologically significant increases in neoplasms were not observed or were observed at sites other than the liver (Hailey et al., 1998). Because *H. hepaticus* is thought to only adversely affect the liver, the carcinogenic activity of the test compound could be assessed in these studies. However, following topical exposure to triethanolamine (TEA), increases in hepatocellular tumors provided the only evidence of carcinogenic activity in male and female mice (NTP, 1999; Hailey, et al, 1998).

TEA is widely used in cosmetics, where it is present at concentrations of up to 5% (CIR, 1983). In both rats and mice, TEA is absorbed following topical application (NTP, 2004). The available data suggest that TEA is not genotoxic (IARC, 2000). Concurrent with the initial TEA study in mice, male and female F344/N rats were also exposed topically to TEA (NTP, 1999); the rats were not infected with *H. hepaticus*. There were increases in non-neoplastic lesions at the site of application in both male and female rats and increases in renal tubule hyperplasia and adenoma in males, following exposure to TEA. The NTP concluded that the increase in renal tubule adenomas may have been related to TEA exposure.

Because increased incidences of hepatocellular neoplasms in male mice have been shown to be associated with *H. hepaticus* infection when hepatitis is also present (Ward et al., 1994a; Fox et al., 1996; Hailey et al., 1998), and because of the uncertainty of the role, if any, that *H. hepaticus* plays in hepatocarcinogenesis in female mice, the 2-year studies of TEA were considered inadequate in both male and female mice. To characterize potential toxic and carcinogenic effects in mice free of *H. hepaticus*, the 2-year study of TEA was repeated in males and females, following rederivation of the B6C3F1 mouse colony and determination that these animals were free of *H. hepaticus* infection (NTP, 2004).

The doses used in the initial 2-year mouse study of TEA were based on the results of a 3-month toxicity study in male and female mice exposed topically to doses of 0, 250, 500, 1000, 2000 or 4,000 mg/kg (NTP, 1999). The primary non-neoplastic lesions observed in this study were acanthosis and inflammation at the site of application. Selection of doses of 0, 200, 630 or 2,000 mg/kg for male mice and 0, 100, 300, or 1000 for female mice for the 2-year study were

based on these local effects. Doses used in the repeat study were the same as those used in the initial study.

After determining that a repeat 2-year study of TEA was necessary in male and female mice, every attempt was made at preserving the design of the initial study. The primary differences in the protocol of the repeat study were animal source and diet. Initial study mice were obtained from Simonsen and were fed the NIH-07 diet, while repeat study mice were obtained from Taconic and were fed the NTP-2000 diet. The change in diet occurred because prior to the conduct of the repeat study, the NTP made a programmatic decision to change the diet used in all rodent toxicity and carcinogenicity studies from NIH-07 to NTP-2000. A comparison of these diets is available (Rao, 1997). Although the mice used in the repeat study came from a different NTP production colony (source) from those used in the initial study, the mice used in both studies were derived from a single cryopreserved embryo stock.

Widespread *Helicobacter* infection has been found in research mice in the United States and worldwide (Taylor, et al., 2007; Shames, et al., 1995). A large proportion of *Helicobacter* infected mice are infected with *H. hepaticus*. Despite the prevalence of *H. hepaticus* infection and the effects on hepatic carcinogenicity, the NTP 2-year studies of TEA in mice, to our knowledge, represent the only effort to characterize the carcinogenic potential of any chemical in the presence and absence of *H. hepaticus* infection. The objectives of this report were to compare the results of the initial (NTP, 1999) and repeat (NTP, 2004) 2-year bioassays of TEA in male and female B6C3F1 mice and to examine the influence of *H. hepaticus* infection on the chronic toxicity and carcinogenicity of TEA.

Materials and Methods

Chemical

TEA, a clear, colorless, viscous liquid, was obtained from Texaco Chemical Company (Division of Texaco, Inc., Bellaire, TX). The same lot was used in both the initial and repeat studies. Prior to both studies, several methods were used to identify the chemical as TEA and determine the purity. Methods common to both studies were infrared, ultraviolet/visible, and proton nuclear magnetic resonance spectroscopy for identity and gas chromatography for purity. In both cases, the purity was $\geq 99\%$, indicating that degradation did not occur between studies.

Study Design

Both the initial and repeat studies were conducted at Battelle Columbus Laboratories (Columbus, OH). Male and female B6C3F₁ mice used in the initial study were obtained from Simonsen Laboratories, Inc. (Gilroy, CA); mice used in the repeat study were obtained from Taconic Laboratory Animals and Services (Germantown, NY). Mice were quarantined for up to 2 weeks prior to the initiation of dosing, were approximately 6 weeks of age at the initiation of dosing and were housed individually throughout the dosing period. Feed and tap water were available *ad libitum*. Mice used in the initial study were fed NIH-07 open formula pelleted diet, while mice used in the repeat study were fed NTP-2000 open formula pelleted diet; both diets were obtained from Zeigler Brothers, Inc. (Gardners, PA). Cages and bedding were changed every week, and cages and racks were rotated every 2 weeks. Study animals were randomly distributed into dose groups of approximately equal initial mean body weights and identified by tail tattoo.

In both studies, groups of 50 core study male and female mice received dermal applications of 0, 200, 630, or 2,000 mg/kg (males) and 0, 100, 300, or 1,000 mg/kg (females) TEA in acetone (2 mL/kg), 5 days per week for two years. Doses were applied to an area extending from the

mid-back to the intrascapular region, which was clipped approximately once per week during the study. Moribund and terminal sacrifice animals were humanely euthanized by carbon dioxide asphyxiation.

All animals were observed twice daily. Clinical findings were recorded monthly and body weights were recorded at the beginning of the study, weekly for 13 weeks, and monthly thereafter. Complete necropsies and microscopic examinations were performed on all mice. At necropsy, all organs and tissues were examined for grossly visible lesions, and all tissues, including major organs, were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4–6 μm , and stained with hematoxylin and eosin for microscopic examination. All neoplasms and non-neoplastic lesions were diagnosed according to previously published criteria (Maronpot, 1999). All neoplasms and target organ lesions were reviewed using the NTP pathology peer review process and represent a consensus of a group of pathologists (Boorman, et al. 1985; Maronpot and Boorman, 1982).

Animal use was in accordance with the United States Public Health Service policy on humane care and use of laboratory animals and the Guide for the Care and Use of Laboratory Animals. These studies were conducted in compliance with the Food and Drug Administration Good Laboratory Practice Regulations (21CFR, Part 58).

Statistical Methods

The first set of statistical analyses compared survival, body weight and tumor incidences among dose groups within the initial or repeat study. The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958). 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. Body weight data, which has an approximately normal distribution, was analyzed using the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (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). 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. Unless otherwise specified, a value of $k=3$ was used in the analysis of site specific lesions. Tests of significance included pairwise comparisons of each exposed group with controls and a test for an overall exposure-related trend. Continuity-corrected Poly-3 tests were used in the analysis of lesion incidence, and reported P values are one sided.

The second set of statistical analyses compared survival, body weight and tumor incidences between initial and repeat studies, controlling for dose; tumors from all sites were considered. Survival was compared between the initial and repeat studies at each dose level using Cox's method for testing equality of two groups. Body weights were compared using two-sample t-tests for each dose group because body weights are approximately normally distributed. Comparisons of tumor incidences at each dose level between initial and repeat studies involved chi-square tests (or Fisher's exact tests if incidences were low) using the Poly-3 weighting as described above.

Logistic regression models, based on the methods of Haseman *et al.* (1997), were generated using the historical control database for each study, which contained animals fed the NIH-07 diet (initial study) or the NTP-2000 diet (repeat study), and were used to predict the incidence

of liver tumors in control mice in each study. As observed by Haseman *et al.* (1997), liver tumor incidences in control mice are related to body weight, age, housing (single vs. multiple), route of exposure, and separate models are required for males and females; these factors were incorporated into the models to predict control tumor incidences. Furthermore, animals in earlier NTP studies were obtained from a variety of sources, while animals in later studies were obtained from a single source. Animals in the initial TEA study were obtained from Simonsen Labs. Therefore, source of the animals (as an indicator variable, Simonsen or other) was included as a predictor variable in models for the initial study to control for differences in tumor rates between Simonsen Labs and other sources; all historical control animals in the repeat study were from the same source. The parameter estimates used to calculate the predicted incidence of each tumor type are presented in Table 1 (males) and Table 2 (females). Using these estimated logistic regression models, predicted incidences of the common tumor types for control animals in the initial and repeat TEA studies are presented in Table 10. The observed control incidences in Table 10 were compared to their predicted incidences using chi-square tests. Predicted control incidences were compared between initial and repeat studies using z-tests.

Results

Survival and Body Weight

Survival and body weight data are shown in Table 3. In both the initial and repeat studies, survival rates of all dosed groups of males and females were similar to those of the vehicle controls. When comparing survival between studies, there was no difference in survival in females, while mean survival of male mice was significantly longer in the initial study than in the repeat study at all doses.

Body weights of dosed male and female mice were statistically similar to controls within both studies; however, at the end of both studies, the mean body weights of males administered 2,000 mg/kg were approximately 10 % less than controls. When comparing between studies, the one year body weights of repeat study males were significantly higher than those of initial study males in all dose groups; however, by two years, body weights of males in the repeat study were similar to those in the initial study, with the exception of the low dose group. Body weights of repeat study females were significantly higher at one year than those of the initial study females; however, by two years, differences between the repeat and initial studies remained only in the control and low dose groups.

Histopathology of the Skin

In both studies, lesions were observed at the site of application in males and females, with males generally more severely affected than females. Histologically, TEA induced increased incidences of chronic inflammation of the skin and epidermal hyperplasia (acanthosis), and ulcers often accompanied by suppurative inflammation. The incidences and severities of these lesions were greater in the repeat study (Table 4 and Table 5).

In the initial study, the incidence of acanthosis (epidermal hyperplasia) and segmental atrophy of hair follicles and associated sebaceous glands was significantly increased only in males at 2,000 mg/kg. The acanthosis was mild (thickness two to three times normal) and had a focal to segmental distribution. Chronic inflammation was increased in both males and females the high doses.

In the repeat study, acanthosis, suppurative inflammation, ulcers and dermal chronic inflammation were commonly observed, particularly in dosed mice. In males, these lesions were increased at all doses, with the exception of ulcers which were increased at only the two

highest dose groups. At 200 and 630 mg/kg, the incidences of acanthosis and dermal chronic inflammation were generally higher than those of ulcers and suppurative inflammation, while at 2,000, the incidences of all lesions were similar. In females, acanthosis and dermal chronic inflammation were increased at all doses. There were significant increases in suppurative inflammation at 300 and 1,000 mg/kg and in ulcers at 1,000 mg/kg; the incidences of these lesions were lower compared to acanthosis and dermal chronic inflammation. In general, the incidences and severities of these lesions increased with increasing dose and the severities were higher in males than in females at the high dose.

Histopathology of the Liver

Males—Non-neoplastic and neoplastic liver lesion incidences in male mice are presented in Table 6.

Neoplastic Lesions: In the initial study, there were significant increases in hepatocellular adenomas and in multiple hepatocellular adenomas over controls at 2,000 mg/kg. Although there was no increase in hepatocellular carcinomas, hepatoblastomas, which rarely occur spontaneously, were observed in the livers of three males in the 2,000 mg/kg group. Hepatoblastomas arise from other proliferative lesions, most often from hepatocellular carcinomas. When observed arising from an hepatocellular neoplasm, hepatoblastoma was the only diagnosis given for the mass. The NTP considers hepatoblastomas to be part of the spectrum of hepatocellular neoplasms that occurs spontaneously and as a result of chemical treatment. When benign and malignant tumor incidences were combined, there were increases in adenoma, carcinoma, or hepatoblastoma and in all liver neoplasms over controls at 2000 mg/kg.

In the repeat study, single or combined hepatocellular neoplasms were not significantly increased over controls. The incidence of hemangioma in 2,000 mg/kg males was higher than observed in controls, and the incidence of hemangiosarcoma was significantly increased in 630 mg/kg males; these incidences exceeded the historical control ranges. Two 630 mg/kg males had multiple hemangiosarcomas of the liver (data not shown). Hemangiomas are not known to progress to malignancy.

In both the initial and repeat studies, concurrent control liver neoplasms were within the historical control ranges for all routes of exposure. Generally, the observed control incidences of individual or combined liver neoplasms were similar between studies. The greatest disparity was for hepatocellular adenomas, which were observed in 54% of mice in the initial study and in 38% of mice in the repeat study.

Non-neoplastic Lesions: In the initial study, karyomegaly and oval cell hyperplasia were observed in all dose groups, including controls. These lesions were indicative of hepatitis resulting from *H. hepaticus* infection and were not observed in the repeat study. Eosinophilic foci of altered hepatocytes (eosinophilic foci) were significantly increased in males at 2,000 mg/kg in the initial study, and in all dosed groups in the repeat study; control incidences were similar between studies.

Females—Selected non-neoplastic and all neoplastic liver lesion incidences in female mice are presented in Table 7.

Neoplastic Lesions: Significant increases in hepatocellular adenomas over controls were observed in both studies, at 1,000 mg/kg in the initial study and at all doses in the repeat study. There were also significant increases in multiple adenomas over controls in both studies, at 300 mg/kg in the repeat study and at 1,000 in both the initial and repeat studies. Carcinomas

were significantly increased at 300 mg/kg in the initial study, but not in the repeat study at any dose. Hepatoblastomas were not observed in either study. There were hemangiosarcomas at the low dose in both studies; neither incidence was significantly increased over controls. In both studies, dose responses of benign and malignant tumors (combined) were similar to that of adenomas, with significant increases over controls at 1,000 mg/kg in the initial study and at all dose levels in the repeat study.

In both the initial and repeat studies, incidences of control liver neoplasms were within the historical control ranges for all routes of exposure. Control incidences of single or combined hepatocellular neoplasms in the repeat study were typically half of those observed in the repeat study. There was an even greater disparity in the incidence of hepatocellular adenoma between the initial (44%) and repeat (18%) studies. In contrast to other control hepatocellular neoplasm incidences, carcinomas were higher in the repeat study (12%) than in the initial study (2%).

Non-neoplastic Lesions: Non-neoplastic lesions indicative of hepatitis were not observed in either study, except for karyomegaly in one mouse in the initial study at 100 mg/kg. In females, eosinophilic foci were significantly increased at 300 mg/kg in the initial study, and at 300 and 1000 mg/kg in the repeat study. In all dose groups, incidences in the repeat study were 1.5–2 fold higher than at the corresponding dose level in the initial study.

Comparison of lesion incidences between studies

Incidences of neoplasms at all sites observed in both the initial and repeat studies were compared at each dose level to determine if there were differences between studies. Neoplasms for which there were significant differences between studies at one or more dose levels are presented in Table 8 and Table 9.

Males—In males, neoplasms with significant different incidences in at least one dose between studies are summarized in Table 8. There were significantly different incidences of neoplasms in at least one dose between studies at multiple sites; however, the liver was the only site with increased neoplasms over controls within either study. At 2000 mg/kg, hepatocellular adenoma, adenoma or carcinoma (combined) or adenoma, carcinoma, or hepatoblastoma (combined) were significantly higher in the initial study compared to the repeat study; these lesions were significantly increased over controls at this dose in the initial study. In all organs, both hemangiosarcoma and hemangioma or hemangiosarcoma (combined) were significantly higher at the mid dose in the repeat study, and were significantly increased over controls within the study. There were significantly higher incidences of both benign tumors and benign or malignant tumors (combined) at all sites in the initial study at both the low and high doses. The incidence of benign or malignant tumors (combined) was significantly increased over controls in the initial study at 2000 mg/kg.

Females—In females, neoplasms with significantly different incidences in at least one dose between studies are summarized in Table 9. There were significant differences in control benign and malignant tumors in all organs and in control liver tumors between studies. The incidences of adenoma, adenoma or carcinoma (combined) and adenoma, carcinoma, or hepatoblastoma (combined) were significantly higher in controls in the initial study; there were significant increases in hepatocellular tumors over controls in both studies. The incidences of benign tumors were significantly increased over controls in both the initial and repeat studies at 1,000 mg/kg, while the incidence of malignant tumors was increased over controls at all doses in the initial study.

Logistic Regression Modeling of Historical Control Liver Tumor Incidences

The results of the logistic regression modeling of control liver tumor incidences using the historical control data for the initial and repeat studies are presented in Table 10; these data were generated using the modeling approach presented in Haseman, et al., 1997, the parameter estimates presented in Table 1 and Table 2 and the body weight and survival data presented in Table 3. Because mice in the historical control database for the initial study (NIH-07 diet) and repeat study (NTP-2000 diet) were fed different diets, separate models were generated for each study; body weight, age, housing (single vs. multiple), route of exposure, and source (Simonsen vs. other; initial study only) were included in the models. For control males in the initial and repeat studies and for control females in the repeat study, observed liver neoplasm incidences were not significantly different from those expected. For control females in the initial study, significantly more adenomas and significantly fewer carcinomas were observed than expected; however, the combined incidences of adenoma and carcinoma or all liver neoplasms were not significantly different from expected differences. When comparing the expected incidences of neoplasms between the initial and repeat studies, the expected incidences of adenoma in the initial study were higher in males, while the expected incidences of adenoma, adenoma or carcinoma, or all liver neoplasms in the initial study were higher in females.

Discussion

Although previous studies suggest that *H. hepaticus* infection is associated with hepatitis and increased liver neoplasms in male mice (Ward, et al., 1994a; Fox, et al., 1996; Hailey, et al., 1998), to our knowledge, the present studies of TEA provide the only example of a chemical tested for carcinogenic activity in both infected mice and in mice free of infection. In *H. hepaticus* infected mice exposed to TEA (NTP, 1999), the absence of increased neoplasms at sites other than the liver made the interpretation of the carcinogenic activity of TEA difficult. As a result, the study was repeated in animals free of infection. The objectives of this report were to compare the results of the initial (NTP, 1999) and repeat (NTP, 2004) NTP chronic bioassays of TEA in B6C3F1 mice and to examine the impact of *H. hepaticus* infection on study outcome and interpretation.

The primary treatment-related effect in males and females in both studies was an increase in both neoplastic and non-neoplastic lesions of the liver. Several approaches were used to determine the effects of *H. hepaticus* or differences in protocol between studies on the TEA bioassay in mice. A comprehensive examination of individual and combined liver neoplasm incidences in each study was undertaken. The incidences of liver neoplasms were then compared at each dose between studies. Finally, control liver tumor incidences in both studies were compared to those predicted by logistic regression modeling; these models took animal source and diet, the two primary differences in protocol between studies, into account. These analyses revealed that the observed differences in the incidences of liver lesions between studies were likely influenced by infection in males or differences in diet and animal source in females. Alternatively, these differences may have been the result of normal variability or the rederivation of the mouse colony and weak carcinogenic activity of TEA. There were also differences in neoplasm incidences at other sites between studies at various doses; however, these lesions were not increased over controls in either study.

In males, the neoplastic response in the liver was greater in mice infected with *H. hepaticus*. In initial study males, there were lesions indicative of hepatitis (Ward, 1994a; Fox, et al., 1996; Hailey, et al., 1998), which occurred at similar incidences across dose groups, including controls. At 2,000 mg/kg TEA, there were also increases in hepatocellular neoplasms. In contrast, neither increases in hepatocellular neoplasms nor hepatitis were observed in repeat study males. Although liver hemangiosarcomas were increased at 630 mg/kg, this increase was marginal, there was no increase in the next and highest dose group (2,000 mg/kg), or in any of

the dosed groups of repeat study females, or in initial study mice of either sex. Comparison of neoplasm incidences between studies revealed that although there were fewer adenomas in the repeat study, the incidences of control liver neoplasms were not statistically different between studies, and that there were significantly fewer hepatocellular neoplasms in the repeat study at 2,000 mg/kg. Logistic regression modeling of control liver tumor incidences did not reveal any differences between observed and expected tumor incidences in either study; however, fewer control hepatocellular adenomas were expected in the repeat study compared to the initial study. Collectively, these observations suggest that the increase in hepatocellular neoplasms at 2,000 mg/kg in the initial study resulted from the combined stimulus of TEA and *H. hepaticus*. The ability of *H. hepaticus* to increase liver neoplasms in male mice has been demonstrated previously, both in untreated controls (Ward, et al., 1994a; Fox, et al., 1996; Hailey, et al., 1998) and following treatment with genotoxic carcinogens (Diwan, et al., 1997; Nagamine, et al., 2007), and likely involves increased cell proliferation (Fox, et al., 1996; Ward, et al., 1994b; Nyska, et al., 1997).

In females, the dose-response of hepatocellular neoplasms was strengthened in the absence of *H. hepaticus* infection. Hepatocellular neoplasms were increased at all dose levels in the repeat study, while these neoplasms were increased over controls only at the high dose in the initial study. Statistical comparison of tumor incidences between studies revealed that the control incidences of liver neoplasms were lower in the repeat study. Comparison of observed control liver lesions with those predicted by the logistic regression model revealed that in both studies, combined incidences of control liver neoplasms were similar to those expected. In addition, the expected incidences of these control neoplasms were higher in the initial study compared to the repeat study. Collectively, these observations suggest that *H. hepaticus* did not influence the neoplastic response in the initial study and that differences in both diet and animal source may have contributed to the lower control liver neoplasm incidence in the repeat study, providing more sensitivity to the statistical tests. The absence of an effect of *H. hepaticus* on the induction of hepatocellular neoplasms in TEA exposed females is consistent with the general absence of hepatitis in infected females. The NTP-2000 diet was designed in part to reduce tumor burden. The results of one study suggest that liver neoplasm incidences are lower in control mice from NTP feed and inhalation studies that were fed the NTP-2000 compared to control mice fed the NIH-07 diet (Rao and Crockett, 2003); however, the studies used for the NIH-07 were more recent than the studies included in the historical control database used for the initial study of TEA. Furthermore, examination of the historical databases (all routes) for each study revealed that in *H. hepaticus* free mice, control hepatocellular neoplasms incidences were lower in mice obtained from Taconic Labs and fed the NTP-2000 diet compared to mice obtained from Simonsen Labs and fed the NIH-07 diet; however, because control incidences of neoplasms vary with exposure route and TEA study was the only dermal study that fit these source and diet conditions, a further analysis of these differences was not possible.

The ability of TEA to induce changes in survival or body weight was not modified by *H. hepaticus* or differences in protocol between studies, as survival was not affected in either study and treatment-related reductions in body weight gain were observed in males at 2,000 mg/kg in both studies. Although in both studies there were increases in non-neoplastic lesions of the skin at the site of application, which were more apparent in males, the extent of this damage was greater in the repeat study. The greater extent of skin damage in males, compared to females, in both studies may have been a result of the higher doses administered to males. The increased skin damage in the repeat study relative to the initial study in both males and females could not be explained by differences in protocol or failure to observe lesions in the initial study.

Because of the *H. hepaticus* infection, the initial study of TEA in B6C3F1 mice was considered inadequate. As a result, the NTP used the data from the repeat study to assess the carcinogenic activity of TEA and concluded that TEA was hepatocarcinogenic in female mice, based on increased hepatocellular neoplasms at all doses and may have been hepatocarcinogenic in male mice, based on an increase in hemangiosarcomas at the mid dose.

In conclusion, the data suggest that the increase in hepatocellular tumors in initial study male B6C3F1 mice resulted from the combined effects of *H. hepaticus* and TEA, while the increase in hepatocellular neoplasms at lower doses in repeat study female mice than in initial study females resulted from the reduction in control liver neoplasms and the effect of TEA without influence of *H. hepaticus*.

Abbreviations

H. hepaticus, *Helicobacter hepaticus*; NTP, National Toxicology Program; TEA, Triethanolamine; Eosinophilic foci, Eosinophilic foci of altered hepatocytes.

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Table 1
Logistic regression model parameter estimates for the prediction of liver tumors in male mice. ^{a,b}

	Intercept (b ₀)	Weight (b ₁)	Weight ² (b ₂)	Age (b ₃)	Housing ^c (b ₄)	Route (b ₅)	Source of animals ^d (b ₆)
Liver tumor							
Initial Study							
Adenoma	3.0748	0.1318	-0.00280	-0.0041	-0.1415	0.6577	0.0300
Carcinoma	4.2548	-0.2169	0.00204	0.0037	-0.0525	0.2249	-0.0329
Hepatoblastoma ^e	--	--	--	--	--	--	--
Adenoma or Carcinoma	0.7706	0.0601	-0.00179	0.0001	-0.1450	0.4974	0.0042
Adenoma, Carcinoma or Hepatoblastoma	0.7422	0.0611	-0.00180	0.0001	-0.1450	0.5086	0.0044
Carcinoma or Hepatoblastoma	3.4451	-0.1785	0.00158	0.0037	-0.0601	0.2487	-0.0273
Hemangiosarcoma	8.1216	-0.2535	0.00248	0.0016	0.5174	0.5764	0.1306
All liver tumors	1.1707	0.0210	-0.00131	0.0004	-0.0930	0.5391	0.0229
Repeat Study							
Adenoma	15.6581	-0.4017	0.00316	-0.0040	--	0.1249	--
Carcinoma	1.5657	-0.2758	0.00241	0.0103	--	0.2061	--
Hepatoblastoma	148.4554	-5.0969	0.04520	-0.0035	--	1.1101	--
Adenoma or Carcinoma	7.8858	-0.3752	0.00293	0.0047	--	0.2565	--
Adenoma, Carcinoma or Hepatoblastoma	7.5600	-0.3584	0.00273	0.0046	--	0.2954	--
Carcinoma or Hepatoblastoma	1.2678	-0.2464	0.00202	0.0098	--	0.2814	--
Hemangiosarcoma	2.9351	0.0250	-0.00090	0.0030	--	-0.3594	--
All liver tumors	6.9464	-0.3651	0.00280	0.0056	--	0.2642	--

^a Logistic regression model is $\text{Prob}(\text{Tumor}) = 1/(1 + \exp(bZ))$ where $bZ = b_0 + b_1(\text{Weight}) + b_2(\text{Weight})^2 + b_3(\text{Age}) + b_4(\text{Housing}) + b_5(\text{Route}) + b_6(\text{Source})$; Weight = weight (g) at 1 year, Age = days on study, Housing = 0 if group-housed and = 1 if individually housed, Route = 0 if chemical was administered by skin or water and = 1 otherwise, Source = 1 if animals were from Simonsen Labs and = 0 otherwise;

^b Parameter estimates were determined using the historical control databases for each diet;

^c All male mice on the NTP 2000 diet were individually housed;

^d All male mice on the NTP 2000 diet were from the same source;

^e Only 8 hepatoblastomas were observed in control male mice on the NIH-07 diet and the model estimates were unreliable.

Table 2
Logistic regression model parameter estimates used in the prediction of liver tumors in female mice.^{a,b}

	Intercept (b ₀)	Weight (b ₁)	Weight ² (b ₂)	Age (b ₃)	Housing (b ₄)	Route (b ₅)	Source of animals ^c (b ₆)
Liver tumor							
Initial Study							
Adenoma	11.9493	-0.0779	-0.00020	-0.0097	-0.4724	0.7711	-0.1412
Carcinoma	1.9354	0.1021	-0.00173	-0.0003	-1.1511	0.4120	-0.2587
Hepatoblastoma ^d	--	--	--	--	--	--	--
Adenoma or Carcinoma	6.4544	0.0018	-0.00096	-0.0051	-0.7670	0.6456	-0.1489
Adenoma, Carcinoma or Hepatoblastoma	6.4544	0.0018	-0.00096	-0.0051	-0.7670	0.6456	-0.1489
Carcinoma or Hepatoblastoma	6.4544	0.0018	-0.00096	-0.0051	-0.7670	0.6456	-0.1489
Hemangiosarcoma	-2.9069	0.2715	-0.00316	0.0015	0.4194	1.0115	-0.1564
All liver tumors	5.8122	0.0082	-0.00100	-0.0047	-0.7018	0.6767	-0.1690
Repeat Study							
Adenoma	8.0468	-0.0398	-0.00011	-0.0055	-0.1560	-0.0093	--
Carcinoma	2.1422	0.0337	-0.00064	0.0006	-0.3831	0.1625	--
Hepatoblastoma ^e	--	--	--	--	--	--	--
Adenoma or Carcinoma	4.9813	-0.0064	-0.00043	-0.0028	-0.2742	-0.0561	--
Adenoma, Carcinoma or Hepatoblastoma	4.9813	-0.0064	-0.00043	-0.0028	-0.2742	-0.0561	--
Carcinoma or Hepatoblastoma	4.9813	-0.0064	-0.00043	-0.0028	-0.2742	-0.0561	--
Hemangiosarcoma	133.9554	-3.5232	0.02690	-0.0216	0.9389	0.5480	--
All liver tumors	5.3510	-0.0380	-0.00015	-0.0022	-0.3974	-0.1088	--

^a Logistic regression model is $\text{Prob}(\text{Tumor}) = 1/(1 + \exp(bZ))$ where $bZ = b_0 + b_1 (\text{Weight}) + b_2 (\text{Weight})^2 + b_3 (\text{Age}) + b_4 (\text{Housing}) + b_5 (\text{Route}) + b_6 (\text{Source})$; Weight = weight (g) at 1 year, Age = days on study, Housing = 0 if group-housed and = 1 if individually housed, Route = 0 if chemical was administered by skin or water and = 1 otherwise, Source = 1 if animals were from Simonsen Labs and = 0 otherwise;

^b Parameter estimates were determined using the historical control databases for each diet;

^c All female mice on the NTP 2000 diet were from the same source;

^d Only 1 hepatoblastoma was observed in control female mice on the NIH-07 diet and the model estimates were unreliable.

^eNo hepatoblastomas were observed in control female mice on the NTP 2000 diet.

Table 3

Survival and body weight data for male and female mice.^a

		Control	200 mg/kg	630 mg/kg	2000 mg/kg
Male Mice					
Mean Survival					
Initial	718 ^b (92) ^c	712 (80)	700 (78)	715 (82)	
Repeat	710 (74)	705 (86)	683 (68)	701 (80)	
p-value ^d	<0.001	<0.001	<0.001	<0.001	
One Year (week 53) Mean Body Weight (g)					
Initial	48.4 ^e (48.4) ^f	48.2 (45.0)	48.2 (47.0)	48.9 (44.0)	
Repeat	50.6 (51.0)	50.0 (50.9)	51.3 (51.2)	50.8 (46.5)	
p-value	0.016 (0.31)	0.035 (0.023)	0.001 (0.40)	0.006 (0.28)	
		Control	100 mg/kg	300 mg/kg	1000 mg/kg
Female Mice					
Mean Survival					
Initial	669 ^b (78) ^c	695 (80)	697 (76)	694 (74)	
Repeat	692 (70)	682 (68)	686 (82)	687 (65)	
p-value	0.37	0.49	0.69	0.14	
One Year (week 53) Mean Body Weight (g)					
Initial	46.4 ^d (49.3) ^e	45.2 (47.8)	46.3 (48.8)	46.1 (48.3)	
Repeat	57.3 (57.1)	56.1 (56.8)	56.5 (55.8)	57.1 (54.1)	
p-value	<0.001 (0.015)	<0.001 (0.001)	<0.001 (0.09)	<0.001 (0.07)	

^a n = 50 per group;

^b survival in days;

^c % survival;

^d p-values are based on log-rank tests for survival and tow-sample t-tests for body weight

^e one-year body weight (50–54 weeks);

$f_{\text{two-year body weight (102–104 weeks)}}$

Table 4
Incidences (%) of skin lesions in male and female mice in the initial study.^a

	Control	200 mg/kg	630 mg/kg	2000 mg/kg
Male Mice				
Acanthosis (epidermal hyperplasia)	4 ^b (2.0) ^c	2 (1.0)	12 (1.3)	22 ^{**} (1.9)
Inflammation, Chronic	4 (1.5)	0	14 (1.1)	22 ^{**} (1.6)
Ulcer	2 (1.0)	0	4 (2.0)	4 (2.5)
Hair Follicle, Sebaceous Gland, Atrophy	0	0	2 (1.0)	30 ^{**} (1.7)
Female Mice				
Acanthosis (epidermal hyperplasia)	0	4 (2.0)	2 (1.0)	6 (1.3)
Inflammation, Chronic	0	4 (1.5)	4 (1.0)	10 [*] (1.4)

^a n = 50 per group;

^b lesion incidence (%);

^c average severity grade of lesion in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked;

* significantly increased over the control group ($P \leq 0.05$) by the poly-3 test;

** $P \leq 0.01$

Table 5
Incidences (%) of skin lesions in male and female mice in the repeat study.^a

	Control	200 mg/kg	630 mg/kg	2000 mg/kg
Male Mice				
Acanthosis (epidermal hyperplasia)	10 ^b (1.2) ^c	88 ^{**} (1.5)	90 ^{**} (2.0)	98 ^{**} (2.7)
Epidermis, Inflammation, Suppurative	2 (1.0)	22 ^{**} (1.5)	66 ^{**} (1.7)	84 ^{**} (2.8)
Epidermis, Ulcer	0	6 (1.0)	40 ^{**} (1.4)	94 ^{**} (2.6)
Dermis, Inflammation, Chronic	2 (1.0)	30 ^{**} (1.1)	80 ^{**} (1.6)	98 ^{**} (2.5)
	Control	100 mg/kg	300 mg/kg	1000 mg/kg
Female Mice				
Acanthosis (epidermal hyperplasia)	28 (1.3)	100 ^{**} (1.9)	92 ^{**} (2.0)	100 ^{**} (2.0)
Epidermis, Inflammation, Suppurative	2 (2.0)	4 (2.0)	40 ^{**} (1.5)	64 ^{**} (2.2)
Epidermis, Ulcer	2 (3.0)	2 (3.0)	12 (1.5)	34 ^{**} (1.6)
Dermis, Inflammation, Chronic	8 (1.8)	54 ^{**} (1.1)	62 ^{**} (1.6)	88 ^{**} (1.8)

^a n = 50 per group;

^b lesion incidence (%);

^c average severity grade of lesion in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked;

^{**} significantly increased over the control group (P≤0.01) by the poly-3 test

Table 6
Incidences (%) of non-neoplastic and neoplastic liver lesions in male mice^a.

	Historical Controls ^b Mean % ± S.D. (Min %, Max %)	Overall Incidence (Survival Adjusted Incidence)			
		Control	200 mg/kg	630 mg/kg	2000 mg/kg
Karyomegaly					
Initial	N/A ^c	22	34	18	32
Repeat	N/A	0	0	0	0
Oval Cell, Hyperplasia					
Initial	N/A	20	28	18	32
Repeat	N/A	0	0	0	0
Eosinophilic Foci					
Initial	N/A	20 ^d	34	22	46 ^{**}
Repeat	N/A	18	40 [*]	62 ^{**}	60 ^{**}
Hepatocellular Adenoma					
Initial	27.6 ± 15.6 (4.0, 67.8)	54 ^d (54.0) ^e	54 (56.7)	58 (62.5)	74 (75.2) [*]
Repeat	32.2 ± 10.6 (12.0, 46.0)	38 (40.0)	36 (38.4)	46 (52.4)	40 (42.8)
Multiple Hepatocellular Adenoma					
Initial	N/A	34	36	34	58 [*]
Repeat	N/A	12	8	22	10
Hepatocellular Carcinoma					
Initial	17.5 ± 7.3 (2.9, 42.4)	30 (30.8)	40 (40.9)	30 (31.7)	28 (28.6)
Repeat	22.3 ± 8.8 (8.0, 46.9)	34 (34.4)	28 (29.1)	28 (29.6)	22 (23.6)
Hepatoblastoma					
Initial	0.2 ± 0.7 (0.0, 3.4)	0 (0.0)	0 (0.0)	0 (0.0)	6 (6.3)
Repeat	1.5 ± 2.6 (0.0, 10.0)	2 (2.1)	2 (2.1)	4 (4.6)	6 (6.5)
Hepatocellular Adenoma or Carcinoma (Combined)					
Initial	40.5 ± 16.0 (10.0, 79.7)	62 (62.0)	68 (69.4)	66 (69.8)	84 (84.3)
Repeat	48.9 ± 14.7 (20.0, 73.5)	66 (66.6)	54 (56.1)	66 (69.2)	50 (52.7)
Hepatocellular Carcinoma or Hepatoblastoma (Combined)					
Initial	17.6 ± 7.5 (2.9, 45.8)	30 (30.8)	40 (40.9)	30 (31.7)	32 (32.6)
Repeat	23.4 ± 9.1 (8.0, 49.0)	36 (36.4)	30 (31.1)	30 (31.8)	26 (27.6)
Hepatocellular Adenoma, Carcinoma, or Hepatoblastoma (Combined)					

	Historical Controls ^b Mean % ± S.D. (Min %, Max %)	Overall Incidence (Survival Adjusted Incidence)			
		Control	200 mg/kg	630 mg/kg	2000 mg/kg
Liver Hemangioma	Initial	62 (62.0)	68 (69.4)	66 (69.8)	84 (84.3) *
	Repeat	66 (66.6)	54 (56.1)	68 (71.3)	52 (54.8)
	Initial	0 (0.0)	2 (2.1)	0 (0.0)	2 (2.1)
	Repeat	0 (0.0)	2 (2.1)	0 (0.0)	4 (4.4)
Liver Hemangiosarcoma	Initial	4 (4.1)	6 (6.4)	2 (2.2)	2 (2.1)
	Repeat ^e	2 (2.1)	0 (0.0)	12 (13.5) *	2 (2.2)
Liver Hemangioma or Hemangiosarcoma (Combined)					
Initial	2.7 ± 2.5 (0.0, 10.0)	4 (4.1)	8 (8.4)	2 (2.2)	4 (4.2)
Repeat	2.7 ± 1.2 (0.0, 4.1)	2 (2.1)	2 (2.1)	12 (13.5) *	6 (6.5)
All Liver Neoplasms (Combined)					
Initial	42.5 ± 16.0 (11.4, 84.7)	62 (62.0)	72 (73.2)	68 (71.9)	88 (88.3) **
Repeat	51.2 ± 13.9 (22.0, 73.5)	66 (66.6)	54 (56.1)	70 (72.8)	56 (58.4)

^a n = 50 per group;

^b Historical control tumor rates from 70 studies for the initial triethanolamine study, excluding those affected by *H. hepaticus*, and 20 studies for the repeat triethanolamine study (all routes of exposure);

^c Not available;

^d overall percent incidence;

^e survival adjusted percent incidence;

* significantly increased over the control group ($P \leq 0.05$) by the poly-3 test;

** ($P \leq 0.01$)

Table 7
Incidences (%) of non-neoplastic and neoplastic liver lesions in female mice^a.

		Historical Control ^b Mean % ± S.D. (Min %, Max %)	Overall Incidence (Survival Adjusted Incidence)			
			Control	100 mg/kg	300 mg/kg	1000 mg/kg
Karyomegaly						
Initial	N/A ^c	0	2	0	0	
Repeat	N/A	0	0	0	0	
Oval Cell, Hyperplasia						
Initial	N/A	0	0	0	0	
Repeat	N/A	0	0	0	0	
Eosinophilic Foci						
Initial	N/A ^c	18 ^d	20	36 [*]	32	
Repeat	N/A	32	44	56 [*]	64 ^{**}	
Hepatocellular Adenoma						
Initial	14.5 ± 12.6 (0.0, 61.3)	44 ^d (50.6) ^e	44 (47.6)	48 (52.0)	80 (87.5) ^{**}	
Repeat	16.4 ± 6.6 (6.0, 28.6)	18 (19.9)	36 (41.0) [*]	40 (43.5) [*]	66 (72.5) ^{**}	
Multiple Hepatocellular Adenoma						
Initial	N/A	22	18	26	58 ^{**}	
Repeat	N/A	0	6	14 [*]	34 ^{**}	
Hepatocellular Carcinoma						
Initial	7.8 ± 7.5 (0.0, 43.1)	2 (2.3)	8 (8.6)	14 (14.9) [*]	10 (11.0)	
Repeat	8.1 ± 4.5 (2.0, 17.8)	12 (13.3)	16 (17.9)	8 (8.9)	10 (11.3)	
Hepatoblastoma						
Initial	0.0 ± 0.0 (0.0, 2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Repeat	0.0 ± 0.0 (0.0, 0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Hepatocellular Adenoma or Carcinoma (Combined)						
Initial	20.3 ± 15.0 (1.7, 70.7)	46 (52.9)	52 (55.2)	56 (58.9)	82 (88.8) ^{**}	
Repeat	22.9 ± 9.7 (8.0, 40.0)	24 (26.3)	46 (51.0) [*]	48 (51.7) ^{**}	68 (74.6) ^{**}	
Hepatocellular Carcinoma or Hepatoblastoma (Combined)						
Initial	7.8 ± 7.5 (0.0, 43.1)	2 (2.3)	8 (8.6)	14 (14.9) [*]	10 (11.0)	
Repeat	8.0 ± 4.5 (2.0, 17.8)	12 (13.3)	16 (17.9)	8 (8.8)	10 (11.2)	

	Historical Controls ^b Mean % ± S.D. (Min %, Max %)	Overall Incidence (Survival Adjusted Incidence)			
		Control	100 mg/kg	300 mg/kg	1000 mg/kg
Hepatocellular Adenoma, Carcinoma, or Hepatoblastoma (Combined)					
Initial	20.3 ± 15.0 (1.7, 70.7)	46 (52.9)	52 (55.2)	56 (58.9)	82 (88.8) ^{**}
Repeat	22.9 ± 9.7 (8.0, 40.0)	24 (26.3)	46 (50.9) [*]	48 (51.6) ^{**}	68 (74.5) ^{**}
Liver Hemangioma					
Initial	0.3 ± 0.7 (0.0, 2.1)	0 (0.0)	0 (0.0)	2 (2.2)	0 (0.0)
Repeat	0.1 ± 0.5 (0.0, 2.0)	2 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)
Liver Hemangiosarcoma					
Initial	0.7 ± 1.0 (0.0, 3.4)	0 (0.0)	6 (6.5)	0 (0.0)	0 (0.0)
Repeat	0.6 ± 1.3 (0.0, 4.0)	0 (0.0)	2 (2.3)	0 (0.0)	0 (0.0)
Liver Hemangioma or Hemangiosarcoma (Combined)					
Initial	1.0 ± 1.3 (0.0, 4.2)	0 (0.0)	6 (6.5)	2 (2.2)	0 (0.0)
Repeat	0.7 ± 1.6 (0.0, 6.0)	2 (2.3)	2 (2.3)	0 (0.0)	0 (0.0)
All Liver Neoplasms (Combined)					
Initial	21.3 ± 15.2 (2.9, 72.4)	46 (52.9)	52 (55.2)	56 (58.9)	82 (88.8) ^{**}
Repeat	24.4 ± 10.4 (8.0, 42.0)	24 (26.3)	46 (50.9) [*]	48 (51.6) ^{**}	68 (74.5) ^{**}

^a n = 50 per group;

^b Historical control tumor rates from 70 studies for the initial triethanolamine study, excluding those affected by *H. hepaticus*, and 20 studies for the repeat triethanolamine study (all routes of exposure);

^c Not available;

^d overall percent incidence;

^e survival adjusted percent incidence;

* significantly increased over the control group ($P \leq 0.05$) by the poly-3 test;

** ($P \leq 0.01$)

Table 8
Statistically significant differences in tumor incidences between the initial and repeat studies in male mice.

Tumor Location and Morphology	Initial Study Incidence (%)				Repeat Study Incidence (%)				Dose(s) with Difference Between Studies
	0	200	630	2,000	0	200	630	2,000	
Dose (mg/kg)									
Adrenal Cortex									
Adenoma	2	2	0	0	6	6	12	10	630*, 2,000**
All Organs									
benign tumors	70	76	70	82	60	50	64	64	200*, 2,000*
hemangiosarcoma	4	8	2	2	6	4	18*	4	630**
hemangiosarcoma or hemangioma	6	10	2	6	8	6	18	8	630**
malignant lymphoma	8	2	18	2	0	0	2	2	630**
malignant and benign tumors	80*	90	84	96	90	70	86	82	200*, 2,000*
Liver									
hepatocellular adenoma	54*	54	58	74*	38	36	46	40	2,000**
hepatocellular adenoma or carcinoma	62*	68	66	84*	66	54	66	50	2,000**
hepatocellular adenoma, carcinoma or hepatoblastoma	62*	68	66	84*	66	54	68	52	2,000**
Lung									
alveolar/bronchiolar adenoma	30	30	28	20	12	14	24	18	0*, 200*
alveolar/bronchiolar carcinoma	4	10	2	2	14	12	16	8	630*

* Significantly different between studies ($P \leq 0.05$) using chi-square tests (or Fisher's exact tests if incidences were low) using Poly-3 weighting;

** $P \leq 0.01$

Table 9 Statistically significant differences in tumor incidences between the initial and repeat studies in female mice.

Tumor Location and Morphology	Initial Study Incidence (%)				Repeat Study Incidence (%)				Dose with Difference Between Studies
	0	100	300	1,000	0	100	300	1,000	
All Organs									
benign tumors	68*	68	64	88*	48*	56	54	76*	0*
malignant tumors	22*	44*	42*	50*	54	52	32	42	0**
Liver									
hepatocellular adenoma	44*	44	48	80*	18*	36*	40*	66*	0**
hepatocellular adenoma or carcinoma	46*	52	56	82*	24*	46*	48*	68*	0*
hepatocellular adenoma, carcinoma or hepatoblastoma	46*	52	56	82*	24*	46*	48*	68*	0*

* Significantly different between studies ($P \leq 0.05$) using chi-square tests (or Fisher's exact tests if incidences were low) using Poly-3 weighting;

** $P \leq 0.01$

Table 10
Logistic regression modeling of control liver tumors in male and female mice

	Initial			Repeat		
	Obs ^a (%)	Exp ^b (%)	Obs-Exp ^c (%)	Obs (%)	Exp (%)	Obs-Exp (%)
Males						
Adenoma	54	53.1 ^{§§}	0.9	38	35.3	2.7
Carcinoma	30	24.8	5.2	34	25.5	8.5
Adenoma or Carcinoma	62	64.2	-2.2	66	56.3	9.7
All liver tumors	62	65.8	-3.8	66	59.0	7
Females						
Adenoma	44	31.5 [§]	12.5 [*]	18	18.9	-0.9
Carcinoma	2	21.0	-19.0 ^{**}	12	12.0	0.0
Adenoma or Carcinoma	46	45.6 ^{§§}	0.4	24	27.8	-3.8
All liver tumors	46	48.5 ^{§§}	-2.5	24	32.0	-8.0

^a Obs = Observed percentage;

^b Exp = Expected percentage, as modeled by logistic regression;

^c Obs-Exp = Difference between Observed and Expected percentages;

* Significantly different, using chi-square tests on tumor frequencies ($p \leq 0.05$);

** ($p \leq 0.01$);

[§] Exp significantly higher than repeat study, using z test on tumor frequencies ($p \leq 0.05$);

^{§§} ($p \leq 0.01$)