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Low dose assessment of the carcinogenicity of furan in male F344/N Nctr rats in a 2-year gavage study

Linda S. Von Tungeln^a, Nigel J. Walker^b, Greg R. Olson^c, Maria C.B. Mendoza^d, Robert P. Felton^d, Brett T. Thorn^{d,f}, M. Matilde Marques^e, Igor P. Pogribny^a, Daniel R. Doerge^a, and Frederick A. Beland^{a,*}

^aDivision of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, AR 72079, United States

^bDivision of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, United States

^cToxicologic Pathology Associates, Jefferson, AR 72079, United States

^dDivision of Bioinformatics and Biostatistics, National Center for Toxicological Research, Jefferson, AR 72079, United States

^eCentro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal

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1. Introduction

Furan (Figure 1) is a volatile organic chemical used as a synthetic intermediate and in the production of pesticides, stabilizers, and pharmaceuticals (International Agency for Research on Cancer, 1995; Hazardous Substances Data Bank, 2011). The major sources of exposure for the general public to furan are tobacco products and food. Mainstream cigarette smoke is estimated to contain up to 65 µg furan/cigarette (Smith *et al.*, 2000; International Agency for Research on Cancer, 2004). Furan is produced during the cooking of many common foods, including coffee, baked or fried cereal products, canned and jarred foods, baby food, and infant formula (Hasnip *et al.*, 2006; Nyman *et al.*, 2006; Zoller *et al.*, 2007; Morehouse *et al.*, 2008). The mean daily dietary consumption of furan in the U.S. has been estimated to be 0.25 µg/kg body weight (BW) in adults (Morehouse *et al.*, 2008), 0.41 µg/kg BW in children during their first year of life, and 0.9 µg/kg BW in infants consuming only

*Correspondence to: Division of Biochemical Toxicology (HFT-110), National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079, U.S.A.; Telephone: (870)543-7205; FAX: (870)543-7136; frederick.beland@fda.hhs.gov.

^fDeceased.

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formula (DiNovi and Mihalov, 2007). Adults in Europe have been estimated to have a median dietary exposure to furan of 0.78 µg/kg BW (European Food Safety Authority, 2009). Coffee contributes approximately 50% of the total dietary exposure of adults to furan in the U.S. (Morehouse *et al.*, 2008).

Furan is rapidly absorbed from the gastro-intestinal tract and extensively metabolized, primarily by hepatic cytochrome P450 2E1, to *cis*-2-butene-1,4-dial (Figure 1; Kedderis *et al.*, 1993; Chen *et al.*, 1995), a highly reactive metabolite that can react with thiol and amino groups in glutathione and other peptides (Chen *et al.*, 1997; Peterson *et al.*, 2006; Kellert *et al.*, 2008a; Lu and Peterson, 2010). In addition to forming amino acid adducts, *cis*-2-butene-1,4-dial can react with the exocyclic nitrogens of deoxycytidine, deoxyadenosine, and deoxyguanosine to form bicyclic hemiketal derivatives (Figure 1; Gingipalli and Dedon, 2001; Byrns *et al.*, 2002; Bohnert *et al.*, 2004). The deoxyadenosine and deoxyguanosine adducts are relatively unstable and undergo ring-opening and dehydration to form secondary etheno-type DNA adducts (Byrns *et al.*, 2004). By using *O*-benzylhydroxylamine as a trapping agent, the deoxyadenosine and deoxycytidine adducts were detected in DNA reacted with *cis*-2-butene-1,4-dial (Byrns *et al.*, 2006). Accelerator mass spectrometry was used to assess the formation of *cis*-2-butene-1,4-dial deoxynucleoside adducts in liver DNA from rats administered 0, 0.1, or 2 mg [3,4-¹⁴C]furan/kg BW. There was a dose-related increase in ¹⁴C associated with liver DNA; however, upon hydrolysis and chromatography of the DNA, only very limited amounts of the radioactivity corresponded to the previously characterized *cis*-2-butene-1,4-dial deoxynucleoside adducts (Neuwirth *et al.*, 2012). More recently, the presence of deoxycytidine *cis*-2-butene-1,4-dial, the major and most stable of the DNA adducts obtained from furan, was examined in the livers of F344 rats treated 5 days/week by gavage for up to 360 days with 4.4 mg furan/kg BW (Churchwell *et al.*, 2015). Formation of DNA adducts was not detected above the background level of 1 – 2 adducts/10⁸ nucleotides.

The carcinogenicity of furan has been assessed in mice and rats. In male and female B6C3F₁ mice treated by gavage 5 days/week with 0, 8, or 15 mg furan/kg BW for 2 years, there was a dose-dependent increase in hepatocellular adenoma, carcinoma, and combined adenoma or carcinoma. With the exception of hepatocellular carcinoma in female mice, the incidence of each of these neoplasms was significantly increased at both doses of furan (National Toxicology Program, 1993). In a subsequent study, female B6C3F₁ mice were administered 0, 0.5, 1, 2, 4, or 8 mg furan/kg BW 5 days/week for 2 years. There was a dose-dependent increase in hepatocellular adenoma, carcinoma, and combined adenoma or carcinoma, with the incidence of adenoma and combined adenoma or carcinoma being significant at 4 and 8 mg furan/kg BW and the incidence of carcinoma being significant at 8 mg furan/kg BW (Moser *et al.*, 2009). The carcinogenicity of furan has also been assessed in mice treated as newborns. Male B6C3F₁ mice given a single intraperitoneal injection of 400 mg furan/kg BW on postnatal day 15 had a significant increase in liver adenoma. Likewise, male B6C3F₁ mice given intraperitoneal injections of 200 mg furan/kg BW on postnatal days 3, 6, 9, 12, 15, and 18 had a significant increase in liver adenoma and combined liver adenoma or carcinoma (Johansson *et al.*, 1997).

In male and female F344/N rats treated by gavage with 0, 2, 4, or 8 mg furan/kg BW 5 days/week for 2 years there was a dose-dependent increase in hepatocellular adenoma, carcinoma, and combined adenoma or carcinoma, liver cholangiocarcinoma, and mononuclear cell leukemia in both sexes. The incidence of each of the neoplasms, with the exception of hepatocellular carcinoma in female rats, was significantly increased at 4 and 8 mg furan/kg BW; liver cholangiocarcinoma was also significantly increased at 2 mg furan/kg BW. Likewise, the incidence of liver cholangiocarcinoma was significantly elevated in male and female rats treated by gavage with 2, 4, or 8 mg furan/kg BW 5 days/week for 9 or 15 months. In a subsequent experiment, male F344/N rats were administered by gavage 30 mg furan/kg BW 5 days/week for 13 weeks. When subgroups were assessed at the end of dosing and at 9 and 15 months after the initiation of dosing, the incidence of liver cholangiocarcinoma was 0, 100, and 100%, respectively (National Toxicology Program, 1993).

Although furan is carcinogenic in mice and rats (with rats being more sensitive than mice), the risk to humans from dietary exposure to furan cannot be estimated accurately because the lowest tested dose of furan (2 mg/kg BW) in the 2-year bioassay conducted by the National Toxicology Program (NTP) in rats gave nearly a 100% incidence of cholangiocarcinoma (86% in female F344/N rats and 98% in male F344/N rats; National Toxicology Program, 1993) leading to highly uncertain estimates of the cancer risk at lower doses. To provide bioassay data that can be used in preparing meaningful risk assessments, we determined the carcinogenicity of furan in F344/N Nctr rats at doses projected to give a wide range of carcinogenic responses. The bioassay was restricted to male rats because they appeared to be slightly more sensitive than female rats to the induction of cholangiocarcinoma (National Toxicology Program, 1993).

2. Materials and methods

2.1 Chemicals

Furan (CAS 110-00-9) was purchased as a single lot from Sigma-Aldrich (Milwaukee, WI). The acquired furan was stabilized with 0.025% butylated hydroxytoluene, a substance not present in the furan used in the previous NTP bioassay (National Toxicology Program, 1993). To prepare material that corresponded more closely to that used in the previous bioassay, the furan was distilled at atmospheric pressure to remove the butylated hydroxytoluene and stored at -20°C under nitrogen in sealed vials.

The identity and purity of the furan were assessed by gas chromatography coupled with electron impact mass spectrometry, NMR spectroscopy, and gas chromatography using flame ionization detection. These analyses indicated a purity of 99.5%, a value greater than the purity of the furan used in the previous NTP bioassay (National Toxicology Program, 1993). No butylated hydroxytoluene was detected, with a limit of detection of 0.003%.

Corn oil (CAS 8001-30-7), the same vehicle used in the previous NTP bioassay with furan, was obtained in 8 lots from Sigma Life Science (St. Louis, MO). The presence of peroxides was assessed by iodometric titration analysis in each lot of corn oil and values of < 2.9 mEq hydrogen peroxide/kg were obtained. These values were lower than the stated permissible

value of < 10 mEq hydrogen peroxide/kg used in the previous NTP bioassay (National Toxicology Program, 1993).

2.2 Dose selection

Male F344/N rats administered 2, 4, or 8 mg furan/kg BW 5 days/week for 2 years (24 months) had an 86–98% incidence of liver cholangiocarcinoma (National Toxicology Program, 1993). Male F344/N rats treated with 2 or 4 mg furan/kg BW for 9 months had a liver cholangiocarcinoma incidence of 50 and 70%, respectively, while those given 2 mg furan/kg BW for 15 months had a liver cholangiocarcinoma incidence of 78% (National Toxicology Program, 1993). Based upon these data, a high dose of 2 mg furan/kg BW was selected for the current study.

In order to design a study that would allow the best characterization of the dose response, calculations were conducted to predict the cholangiocarcinoma incidence as a function of dose. Peto *et al.* (1984) derived an empirical relationship between tumor latency and dose that allows an estimation of the dose that would give a specific tumor incidence after 2 years of dosing based upon the tumor incidence obtained from shorter dosing periods. When this relationship was applied to the 50% tumor incidence observed after 9 months of dosing with 2 mg furan/kg BW (National Toxicology Program, 1993), it was estimated that a dose of 0.11 – 0.28 mg furan/kg BW would result in a 50% incidence of liver cholangiocarcinoma after 2 years of dosing. Similarly, it was estimated that a 70% tumor incidence would be associated with a dose of 0.21 – 0.56 mg furan/kg BW and a 78% tumor incidence would be associated with a dose of 0.49 – 0.78 mg furan/kg BW. Linear extrapolation of these values allowed an estimation of the 24-month liver cholangiocarcinoma incidences from specific doses of furan. The results of this extrapolation indicated that a dose of 0.02 mg furan/kg BW would be associated with a cholangiocarcinoma incidence of 0 – 23% (Table 1), whereas a dose of 1 mg furan/kg BW would be associated with a cholangiocarcinoma incidence of nearly 100%. Based upon these considerations, doses of 0, 0.02, 0.044, 0.092, 0.2, 0.44, 0.92 and 2.0 mg furan/kg BW were selected for the 2-year bioassay (Table 1).

2.3 Study design

This study was conducted under the auspices of the NTP in accordance with NTP specifications (http://ntp.niehs.nih.gov/ntp/test_info/finalntp_toxcarspecsjan2011.pdf) and in compliance with Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations (CFR 21, Part 58). The approved GLP report (NCTR Study E2168.02) is available from the National Center for Toxicological Research (NCTR) on request. The Institutional Animal Care and Use Committee at the NCTR reviewed and approved the protocol for this bioassay. The study was conducted at NCTR in accordance with all relevant FDA, National Institutes of Health (NIH), and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines. The animal facility at NCTR is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Male F344/N Nctr rats were obtained from the NCTR breeding colony. While the NCTR sub-strain differs from the Charles River sub-strain used in the previous furan bioassay

(National Toxicology Program, 1993), the NCTR F344/N sub-strain has given tumorigenic responses comparable to those of other F344/N sub-strains in previously reported carcinogenicity bioassays (*e.g.*, acrylamide; Beland *et al.*, 2013). The treatment was initiated when the rats were 7 weeks of age. The rats were housed 2/cage in polycarbonate cages with hardwood chip bedding. Irradiated Purina 5K0L Diet (also designated NIH-41 Irradiated Rodent Diet) and Millipore-filtered tap water were provided *ad libitum*. The level of furan in feed was < 15 parts/billion as assessed by headspace gas chromatography mass spectral analysis. The animal rooms were maintained on a 12-hour light-dark cycle, with 10 – 15 air changes/hour. Environmental controls were set to maintain the temperature at 20 – 26°C, with a relative humidity of 30 – 70%.

The stability of furan in the corn oil vehicle was determined by capillary gas chromatography with flame ionization detection over a 14-day period and the concentrations were found to be within $\pm 10\%$ of the target values. Furan dosing solutions were prepared weekly. Dose certification analyses were conducted on all doses, typically on each weekly preparation, and all were within $\pm 10\%$ of the specified concentrations. Furan was not detected in the control corn oil, with the limit of quantitation being approximately 0.1 μg furan/mL of corn oil.

The furan was administered by gavage in corn oil 5 days/week for 2 years (104 weeks), with interim sacrifices conducted at 9 (36 weeks) and 15 (60 weeks) months. The dosing volume was 5.0 mL/kg BW. The number of rats in each group is given in Table 1. The group sizes were unbalanced, with more rats being assigned to the lower dose groups. The specific number of rats in each group was based upon power calculations to detect a significant difference between the projected tumor incidences in each treated group and the control group.

2.4 Necropsy and histopathology

Complete necropsies were performed on all animals, including those that died naturally or that were submitted moribund prior to the scheduled terminal sacrifice. Tissues were examined grossly, removed, and preserved in 10% neutral buffered formalin, except the eyes and testes, which were placed in modified Davidson's fixative. The tissues were trimmed, processed, and embedded in Formula R[®] infiltrating medium (Leica Micro Biosystems Division, Richmond, IL), sectioned at approximately 5 microns, and stained with hematoxylin and eosin for microscopic evaluation. In a few cases, special staining procedures were applied to selected lesions to aid in characterizing the tissue changes.

A quality assessment pathologist evaluated slides of all proliferative lesions. Histopathology slides containing the diagnoses made by the study pathologist and quality assessment pathologist were reviewed by a Pathology Working Group. Final diagnoses of the reviewed lesions represented a consensus of the study pathologist, the reviewing pathologist, and the Pathology Working Group.

2.5 Statistical analyses

The effect of furan dose on body weight was investigated using a repeated measures, mixed models analysis of variance, with main effects of dose and week, and a dose x week

interaction effect. Least squares estimates of mean body weight were obtained for each dose group in 4-week intervals starting at week 4 and continuing until the end of scheduled sacrifices. Dunnett's adjusted pair-wise comparisons (Dunnett, 1955) of dose group to control group body weight means were performed to determine if there was a difference between the control and the respective dose group means.

Mean survival times and plots of rodent survival functions were obtained using the Kaplan-Meier estimation (Kaplan and Meier, 1958). Cox proportional hazards regression analyses (Cox, 1972) were conducted to compare the hazard function of each dose group to that of the control group and to test for a linear trend between the hazard and furan dose.

Continuity-corrected Poly-3 tests (Bailer and Portier, 1988), as modified by Bieler and Williams (1993), were used to assess prevalence of neoplasms and non-neoplastic lesions. For the interim sacrifices at 36 and 60 weeks, an exact Cochran-Armitage linear trend test was run, as well as Fisher's Exact test, to compare the treatment groups to the control group. These results are referred to as CAFE, rather than Poly-3. The CAFE tests did not adjust for competing risks but there were generally very few early deaths before the interim sacrifices.

Benchmark doses (BMD) and the lower 95% confidence limits of the benchmark dose (BMDL) were calculated using Environmental Protection Agency Benchmark Dose software (version 2.1.1; <http://www.epa.gov/ncea/bmds>). The calculations were conducted using gamma, logistic, log-logistic, log-probit, multistage, probit, and Weibull models to fit the neoplastic incidences and administered dose of furan. The BMD₁₀ was defined as the dose that caused a 10% excess risk of the specified adverse effect over that observed in the appropriate control group.

3. Results

Male F344/N Nctr rats were administered 0, 0.02, 0.044, 0.092, 0.2, 0.44, 0.92, and 2.0 mg furan/kg BW by gavage in corn oil 5 days/week for 2 years (104 weeks), with interim sacrifices conducted at 9 (36 weeks) and 15 (60 weeks) months (Table 1).

3.1 Body weights

Administering furan to male F344/N Nctr rats by gavage caused only sporadic changes in body weight in the interim sacrifice groups (36 or 60 weeks) or in the groups treated for 2 years (104 weeks, Figure 2).

3.2 Survival

All rats in the 36-week interim sacrifice groups survived until the end of the treatment period. Survival in the 60-week interim sacrifice groups was 85%, with moribund and dead animals being distributed across the entire dose range. In the groups treated for 2 years, there was a significant ($P = 0.021$) dose-related decrease in survival (Figure 3); however, compared to the control group (0 mg furan/kg BW), none of the treatment groups had a significant decrease in survival.

3.3 Neoplasms

There were no treatment-related neoplastic findings in male F344/N Nctr rats treated with furan by gavage for 36 or 60 weeks. In the 36-week interim sacrifice, 8 spontaneous tumors (adrenal cortex adenoma; benign adrenal medulla pheochromocytoma; preputial gland adenoma, carcinoma, and papilloma; subcutaneous skin lipoma; benign neural crest ear tumor; and testicular interstitial cell adenoma) were evident across various treatment groups, including the control group. In the 60-week interim sacrifice, spontaneous neoplasms were observed in all treatment groups, including the control group.

The gavage administration of furan to male F344/N Nctr rats for 2 years was associated with the development of malignant mesothelioma on membranes surrounding the epididymis and on the testicular tunics (Table 2). The survival adjusted incidence of malignant mesothelioma was significantly increased in the combined testes or epididymis of rats administered 2 mg furan/kg BW. Microscopically, malignant mesotheliomas in the epididymis and testicular tunics were characterized by complex papillary surface growths of one to several layers of polyhedral-to-cuboidal mesothelial cells on pedunculated fibrovascular stalks. The neoplastic cells had either abundant weakly eosinophilic cytoplasm and ovoid nuclei with one or more nucleoli or scanty cytoplasm and numerous small basophilic nuclei. In some rats, malignant mesothelioma was associated with other organs or tissues including the large and small intestine, liver, mesentery, pancreas, stomach, adrenal gland, peritoneum, prostate, seminal vesicle, mesenteric lymph node, spleen, scrotum, skeletal muscle, and urinary bladder.

Male F344/N Nctr rats had dose-related increases in the incidence of mononuclear cell leukemia, with the increase in incidence being significant at 0.092, 0.2, 0.92, and 2 mg furan/kg BW (Table 2). There was a dose-related increasing trend in the incidence of hepatocellular adenoma; however, none of the treatment groups had a significant increase compared to the control group (0 mg furan/kg BW; Table 2). Likewise, the incidence of hepatocellular carcinoma and combined hepatocellular adenoma or carcinoma was not affected by treatment with furan (Table 2). Based upon the International Harmonization of Nomenclature and Diagnostic Criteria, as described by Thoolen *et al.* (2010), cholangiocarcinoma was not detected in any dose group.

3.4 Non-neoplastic lesions

The gavage administration of furan to male F344/N Nctr rats for 36 weeks was associated with dose-related increasing trends in liver lesions including cholangiofibrosis, mixed cell foci, biliary tract hyperplasia, bile duct subcapsular hyperplasia, oval cell hyperplasia, hepatocyte hypertrophy, periportal cytoplasmic alteration, and subcapsular fibrosis, chronic inflammation, and pigmentation (Table 3). Significant increases in cholangiofibrosis, bile duct subcapsular hyperplasia, and subcapsular fibrosis, chronic inflammation, and pigmentation were observed at 0.44 mg furan/kg BW. The average severity of each of these lesions increased from minimal to mild as the dose increased.

The subcapsular changes, usually noted along the margins of the left and caudate lobes, were focal to segmentally continuous and featured subcapsular bile duct hyperplasia,

inflammatory cell infiltrates, pigmentation, and fibrosis, along with periportal proliferation of oval cells and bile ducts. In many rats, hypertrophy was noted along the bordering layers of hepatocytes and these usually displayed a basophilic cytoplasm. A diagnosis of cholangiofibrosis was made only when the normal hepatic parenchyma was replaced by a combination of the features noted above, without markedly disturbing the lobe outline.

Rats treated for 60 weeks had a spectrum of liver lesions similar to that found after dosing for 36 weeks (Table 3), but the incidences tended to become significant at lower doses. For example, in the 60-week rats, the incidence of subcapsular fibrosis, chronic inflammation, and pigmentation became significant at 0.2 mg furan/kg BW (as compared to 0.44 mg furan/kg BW at 36 weeks).

Rats administered furan for 2 years had dose-related increasing trends in liver cholangiofibrosis, mixed cell foci, basophilic foci, biliary tract hyperplasia, oval cell hyperplasia, regenerative hyperplasia, and cytoplasmic vacuolization (Table 3). The incidence of cholangiofibrosis was significantly increased at 0.2 mg furan/kg BW, and a single occurrence was observed at 0.092 mg furan/kg BW. At 2 years, the cholangiofibrosis was characterized by dilated to cystic bile ducts, often irregular, filled with mucinous and cellular debris, surrounded by dense collagenous connective tissue, with a prominent inflammatory cell infiltrate (Figure 4). The epithelium varied from low cuboidal to tall columnar, with hyperbasophilic and pleomorphic cells, along with goblet and Paneth cells. Large sclerotic lesions, displaying only epithelial remnants or crescent-shaped structures, were observed in the higher dose groups. Regenerative hepatocellular hyperplasia was sometimes associated with these extensive lesions.

The severity of the cholangiofibrosis increased with dose, with the severity being minimal-to-mild at 0.2 mg furan/kg BW and moderate-to-marked at 2 mg furan/kg BW. Significant oval cell hyperplasia, regenerative hyperplasia, and cytoplasmic vacuolization were noted in the higher dose groups. At the highest dose of 2 mg furan/kg BW, there was an increase in biliary tract hyperplasia, basophilic foci, and mixed cell foci. Lesions with diagnostic features of cholangiocarcinoma (*e.g.* invasion into adjacent liver tissue; Thoolen *et al.*, 2010) were not evident in any of the treatment groups.

In addition to non-neoplastic lesions of the liver, rats treated with furan for 2 years had dose-related increasing trends in bone marrow hyperplasia, cataracts of the eye, and forestomach edema, epithelium hyperplasia, inflammation, and ulceration (Supplementary Table 1). The incidence of these lesions was significantly increased at 2 mg furan/kg BW and the severity tended to be mild-to-moderate. Slight, but statistically significant, increases in non-neoplastic lesions were also observed in other tissues and organs (Supplementary Table 1).

4. Discussion

Furan is produced during the cooking of many common foods, including coffee, baked or fried cereal products, canned and jarred foods, baby food, and infant formula. Previous bioassays have demonstrated furan to be hepatocarcinogenic in mice and rats (Maronpot *et al.*, 1991; Elmore and Sirica, 1993; National Toxicology Program, 1993; Johansson *et al.*,

1997; Moser *et al.*, 2009), with rats appearing to be more sensitive than mice due to the formation of cholangiocarcinoma. For example, when B6C3F₁ mice (Moser *et al.*, 2009) were administered furan for 2 years, the incidence of combined hepatocellular adenoma or carcinoma became significantly increased at a dose of 4 mg furan/kg BW. F344/N rats treated in a similar manner also had a significant increase in hepatocellular adenoma or carcinoma at 4 mg furan/kg BW; however, they also had nearly a 100% incidence of cholangiocarcinoma at the lowest dose tested, which was 2 mg furan/kg BW (National Toxicology Program, 1993). In order to provide bioassay data for use in risk assessments of dietary exposures to furan, we have investigated the carcinogenicity of furan in male F344/N Nctr rats at doses anticipated to give a wide range of carcinogenic responses (Table 1).

Furan at doses of up to 2 mg/kg BW for 2 years had no effect upon body weight in male F344/N Nctr rats (Figure 2), and while there was a dose-related decrease in survival (Figure 3), none of the furan treatment groups differed significantly from the control group. This is similar to what was observed in the previous NTP bioassay with furan: 2 mg furan/kg BW did not affect the body weight or survival of the F344/N rats; these parameters were only affected at 8 mg furan/kg BW (National Toxicology Program, 1993).

The administration of furan caused a dose-related increase in malignant mesothelioma of the epididymis or testes. This neoplasm was not reported in the previous NTP bioassay with furan, even at doses up to 8 mg furan/kg BW (National Toxicology Program, 1993). In the current bioassay, a statistically significant increase in malignant mesothelioma was observed at 2 mg furan/kg BW, with the incidence being 12% compared to 4% in the control group (Table 2). The incidence in the 2 mg furan/kg BW group exceeded the historical control range (mean, 4.2% (31/742); range, 0 – 6.4% for 10 studies) observed for malignant mesothelioma in male F344/N Nctr rats in bioassays conducted at the NCTR.

The mechanism for the induction of malignant mesothelioma of the epididymis or testes is currently not known. This neoplasm has been reported in F344 rats administered the dietary contaminant acrylamide (Johnson *et al.*, 1986; Friedman *et al.*, 1995; National Toxicology Program, 2012; Beland *et al.*, 2013), and benchmark dose modeling indicates that furan (Table 4 and Supplementary Figure 1) and acrylamide (BMD₁₀, 29.90 – 31.04 μmol/kg BW; BMDL₁₀ 18.08 – 23.71 μmol/kg BW; Beland *et al.*, 2013) have similar potencies for the induction of this neoplasm. DNA adducts derived from the electrophilic metabolite glycidamide have been detected in DNA from the testes and other tumor target tissues (*i.e.*, thyroid and mammary gland) of F344 rats administered acrylamide (Doerge *et al.*, 2005), which suggests a genotoxic mechanism for tumor induction by acrylamide; in contrast, evidence for the formation of DNA adducts derived from *cis*-2-butene-1,4-dial, the presumed electrophilic metabolite of furan, is minimal in tissues from rats treated with furan, even at high doses and for extended exposure times (Neuwirth *et al.*, 2012; Churchwell *et al.*, 2015). Although glycidamide-DNA adducts have been detected in testicular DNA of acrylamide-treated rats (Doerge *et al.*, 2005), Big Blue rats administered acrylamide did not show an increased mutant frequency in the testes (Mei *et al.*, 2010); likewise, increased mutant frequencies were not observed in various tissues of Big Blue rats administered furan (McDaniel *et al.*, 2012).

As an alternative to a genotoxic mechanism, malignant mesothelioma of the epididymis or testes has been proposed to be a consequence of Leydig cell tumor progression resulting from carcinogen-induced decreases in testosterone and increases in luteinizing hormone (Shipp *et al.*, 2006; Maronpot *et al.*, 2009; Maronpot *et al.*, 2016). Maronpot *et al.* (2015) have recently evoked such an explanation when assessing the induction of malignant mesothelioma in male Wistar Han rats treated with acrylamide. F344 rats administered relatively high doses of acrylamide (10 mg acrylamide/kg BW) do have decreased serum levels of testosterone and increased serum levels of luteinizing hormone; nonetheless, Leydig cell proliferation was not evident (Camacho *et al.*, 2012). In contrast, F344 rats treated with furan had increased serum levels of testosterone and decreased serum levels of luteinizing hormone, but again, Leydig cell proliferation was not evident (Gill *et al.*, 2010). Thus, while the induction of malignant mesothelioma is clearly related to treatment with furan, the mechanism for the induction of these tumors is still uncertain.

Mononuclear cell leukemia was observed in all dose groups of male F344/N Nctr rats, including the control group. Although statistically significant increases in prevalence were observed in the higher dose groups (0.092, 0.2, 0.92, and 2 mg furan/kg BW; Table 2), the incidences were within the historical control range (mean, 46.9% (348/742); range, 31.3 – 64.6% for 10 studies) observed for mononuclear cell leukemia in male F344/N Nctr rats in bioassays conducted at the NCTR. An increased incidence of mononuclear cell leukemia was observed in the previous NTP furan bioassay, with the increase in male F344/N rats being significant at 4 and 8 mg furan/kg BW (National Toxicology Program, 1993). Carthew *et al.* (2010) performed benchmark dose modeling on the mononuclear cell leukemia in male F344/N rats from the NTP bioassay and obtained a BMD₁₀ of 34.21 μmol furan/kg BW and a BMDL₁₀ of 16.88 μmol furan/kg BW. These values are quite close to those calculated for malignant mesothelioma of the epididymis or testes in the current furan bioassay (Table 4 and Supplementary Figure 1). Attempts to model the mononuclear cell leukemia data from the current furan bioassay were not successful (data not shown).

A major goal of this project was to define the dose response for the formation of cholangiocarcinoma upon the administration of furan. The criteria used to characterize cholangiocarcinoma were those recently specified by Thoolen *et al.* (2010) as part of the International Harmonization of Toxicologic Pathology Nomenclature initiative (Mann *et al.*, 2012). As a consequence, the diagnostic criteria used to identify cholangial lesions in the present study were not the same as those used in the previous study of furan by the NTP (National Toxicology Program, 1993). Based upon these updated criteria, there was no evidence for cholangiocarcinoma in any of the male F344/N Nctr rats treated with furan; instead, the major hepatic lesion detected was cholangiofibrosis, a precursor lesion to cholangiocarcinoma.

In the previous NTP furan bioassay, 43 of 50 male rats treated with 2 mg furan/kg BW were diagnosed with cholangiocarcinoma (National Toxicology Program, 1993). A re-assessment of 23 livers diagnosed with cholangiocarcinoma from the 2 mg furan/kg BW dose group in the previous NTP furan bioassay, based upon the updated criteria specified by Thoolen *et al.* (2010), resulted in 20 of the lesions being reclassified as cholangiofibrosis, while only 3 retained the designation of cholangiocarcinoma. By contrast, when livers from the 8 mg

furan/kg BW group were re-examined, all retained the classification of cholangiocarcinoma (Malarkey *et al.*, 2014). From these data, it appears that, in both bioassays, male F344/N rats administered 2 mg furan/kg BW developed a very high incidence of cholangiofibrosis, with cholangiocarcinoma becoming prevalent at higher doses.

The induction of cholangiofibrosis in the male F344/N Nctr rats in the current 2-year bioassay was markedly non-linear. The lesion was not detected in the control rats or in rats administered 0.02 or 0.044 mg furan/kg BW for 2 years (Table 3). A very low incidence (1%) occurred in rats given 0.092 mg furan/kg BW, whereas a very high prevalence (76%) was detected at higher doses (0.2, 0.44, 0.92, and 2 mg furan/kg BW). Furthermore, as the dose increased, so did the severity of the lesion, from minimal at 0.092 mg furan/kg BW to marked at 2 mg furan/kg BW.

Cholangiofibrosis was also present in the livers of the F344/N Nctr rats examined after 36 and 60 weeks of dosing. As with the rats treated for 2 years, the dose-response was markedly non-linear: cholangiofibrosis was not detected at doses 0.2 mg furan/kg BW, while at higher doses, the incidence increased dramatically (Table 3). The severity of the lesion also increased with the length of dosing, being minimal after 36 weeks of dosing and minimal-to-mild after 60 weeks of treatment.

The induction of hepatobiliary lesions in F344/N rats treated with furan has been the subject of intense investigations (reviewed by Bakhiya and Appel, 2010; Moro *et al.*, 2012a). A substantial number of these investigations have been conducted at doses that far exceed those used in the current study, which makes extrapolation to lower doses problematic. Two mechanisms have been considered to account for the hepatobiliary lesions: a genotoxic pathway, as a consequence of DNA adduct formation, and a non-genotoxic pathway involving furan-induced necrosis, inflammation, and proliferative regeneration. These mechanisms are not necessarily mutually exclusive.

Furan is known to undergo hepatic cytochrome P450 2E1-catalyzed oxidation to *cis*-butene-1,4-dial, a reactive dialdehyde (Kedderis *et al.*, 1993; Chen *et al.*, 1995). Inhibition of this metabolic oxidation through the use of cytochrome P450 inhibitors suppresses the hepatotoxicities associated with furan, thus reinforcing the importance of *cis*-butene-1,4-dial in the toxic manifestations of the compound (Fransson-Steen *et al.*, 1997). *cis*-Butene-1,4-dial will react with DNA and a number of DNA adducts resulting from the reaction have been characterized *in vitro* (Figure 1; Gingipalli and Dedon, 2001; Byrns *et al.*, 2002; Bohnert *et al.*, 2004; Byrns *et al.*, 2004; Byrns *et al.*, 2006). *cis*-Butene-1,4-dial was mutagenic in *S. typhimurium* TA104 (Peterson *et al.*, 2000) and increased the mutant frequency in L5158Y *tk*^{+/-} mouse lymphoma cells (Kellert *et al.*, 2008b), which supports the concept of a genotoxic pathway. Nonetheless, as noted above, there is minimal evidence for the formation of *cis*-butene-1,4-dial adducts in hepatic DNA from rats treated with furan (Neuwirth *et al.*, 2012; Churchwell *et al.*, 2015). Likewise, there was no increase in the mutant frequency in the *cII* transgene in DNA isolated from the livers of Big Blue rats treated with up to 8 mg furan/kg BW (McDaniel *et al.*, 2012).

cis-Butene-1,4-dial bears a C=C double bond conjugated with two aldehyde groups; as such, it will readily react with nucleophilic sites (thiols and amino groups) in proteins, which may play a role in furan toxicities. Extensive binding of furan to proteins has been demonstrated in rats treated with furan, with the highest levels being detected in the liver (Burka *et al.*, 1991). Substantial hepatic protein binding has been detected at doses as low as 0.1 mg furan/kg BW, with the binding increasing 10-fold at 2 mg/kg BW (Moro *et al.*, 2012b). Mass spectral analyses indicated that the targeted proteins were localized primarily in the cytosol and mitochondria, with fewer furan-modified nuclear proteins being detected. The targeting of mitochondrial proteins by *cis*-butene-1,4-dial could contribute to the impairment of oxidative phosphorylation that has been observed in F344 rats treated with furan (Mugford *et al.*, 1997). This interpretation is supported by the observation that the depletion of ATP could be prevented by prior treatment with the cytochrome P450 inhibitor 1-phenylimidazole. Other low-dose effects that have been observed upon administration of furan to F344/N rats include an increase in cell proliferation, as indicated by an increase in bromodeoxyuridine incorporation in the subcapsular area of the caudate liver lobe (Mally *et al.*, 2010), and an increased expression of hepatic genes associated with cell cycle and apoptosis (Chen *et al.*, 2010).

More recently, dose-dependent and time-dependent epigenetic changes have been examined in the livers of male F344 rats exposed to 0, 0.92, 2, and 4 mg furan/kg BW for up to 360 days (de Conti *et al.*, 2014). Promoter region hypermethylation was observed in the tumor suppressor genes *p16^{INK4a}* and *Rassf1a*, and this was accompanied by a decreased expression of *Rassf1a*. Subsequently, the persistence of the epigenetic changes was investigated in the livers of male F344 rats treated for 90 days with 8 mg furan/kg BW and then monitored for up to an additional 360 days (de Conti *et al.*, 2015). Upon the discontinuation of the treatment, most of the furan-associated changes were reversible; nonetheless, there was a sustained decrease in the acetylation of histone H3 lysines 9 and 56, and this was associated with the formation of heterochromatin and decreased gene expression. In further work, cholangiofibrotic lesions were examined in the livers of male F344 rats exposed to 0.92 and 2 mg furan/kg BW for 2 years (Tryndyak *et al.*, 2016). A number of genes involved in key pathways associated with different aspects of liver pathology showed alterations in promoter-region DNA methylation and gene expression. For example, decreased promoter methylation and increased gene expression were observed with *Areg* and *Jag1*, key components of the Hippo and Notch signaling pathways. Over-expression of these genes has been involved in the pathogenesis of liver fibrosis, activation of hepatic progenitor cells, and the development of liver cancer. In contrast to *Areg* and *Jag1*, hypermethylation of the promoter region and decreased gene expression was found with *Foxe1*, which encodes a thyroid-specific forkhead transcription factor that represses transcription of several rat genes, including fibrogenesis-associated *Duox2* and *Adamts1* genes. Therefore, hypermethylation-associated inhibition of *Foxe1* expression may cause activation of these genes and stimulation of liver fibrogenesis. These results indicate that gene-specific changes in DNA methylation have functional consequences that may be important for the induction of cholangiofibrosis and subsequently cholangiocarcinoma.

Non-neoplastic lesions were also detected in non-hepatic tissues, including the kidney, eye, forestomach, and bone marrow (Supplementary Table 1). The incidences of these lesions

tended to become significant at only the highest dose of 2 mg furan/kg BW, which is probably a reflection of the fact that much higher levels of furan-protein binding are detected in the liver as compared to other organs (Burka *et al.*, 1991; Moro *et al.*, 2012b).

Using data from the previous NTP bioassay on furan (National Toxicology Program, 1993), Carthew *et al.* (2010) applied a margin of exposure (MOE) approach to assess the risk associated with dietary exposures to furan. Their assessment was based upon furan-induced combined hepatocellular adenoma or carcinoma in male F344/N rats, for which benchmark dose modeling gave a BMDL₁₀ of 1.23 mg furan/kg BW (18.13 μ mole furan/kg BW). Applying this BMDL₁₀ led to MOEs of 750 – 4,300, depending upon age and geographic location. Benchmark dose modeling of the furan-induced malignant mesothelioma of the epididymis or testes in the currently bioassay gave BMDL₁₀ values of 1.44 – 1.53 mg furan/kg BW (21.13 – 22.48 μ mole furan/kg BW; Table 4; Supplementary Figure 1), which would afford MOEs similar to those calculated by Carthew *et al.* (2010). The application of benchmark dose modeling to the furan-induced cholangiofibrosis led to BMDL₁₀ values of 0.11 – 0.12 mg furan/kg BW (1.59 – 1.79 μ mole furan/kg BW; Table 4; Supplementary Figure 2), which would result in an approximately 10-fold reduction in the MOEs compared to the values obtained based upon hepatocellular neoplasms or malignant mesothelioma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BMD	benchmark dose
BMDL	lower limit of benchmark dose
BW	body weight
FDA	Food and Drug Administration
GLP	Good Laboratory Practice
MOE	margin of exposure
NCTR	National Center for Toxicological Research
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health

NTP National Toxicology Program**References**

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Highlights

Furan is a contaminant in many common foods

The carcinogenicity of furan was assessed in male F344/N rats

Exposure to furan induced malignant mesothelioma and mononuclear cell leukemia

The most sensitive non-neoplastic lesion was cholangiofibrosis

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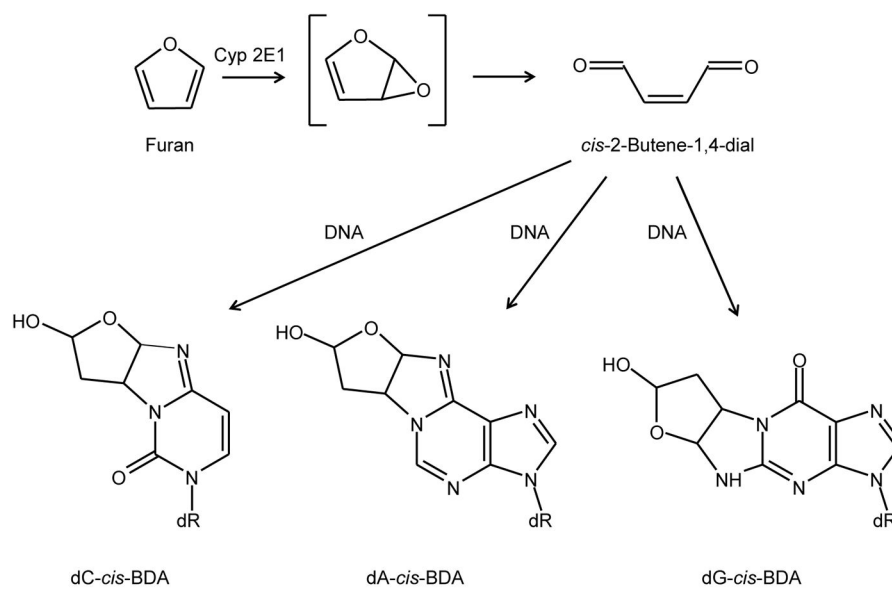


Figure 1. Structures of furan, *cis*-2-butene-1,4-dial, and the DNA adducts resulting from reaction of *cis*-2-butene-1,4-dial with DNA. The abbreviations used are: Cyp2E1, cytochrome P450 2E1; dC, deoxycytidine; dA, deoxyadenosine; dG, deoxyguanosine; dR, deoxyribose; *cis*-BDA, *cis*-2-butene-1,4-dial.

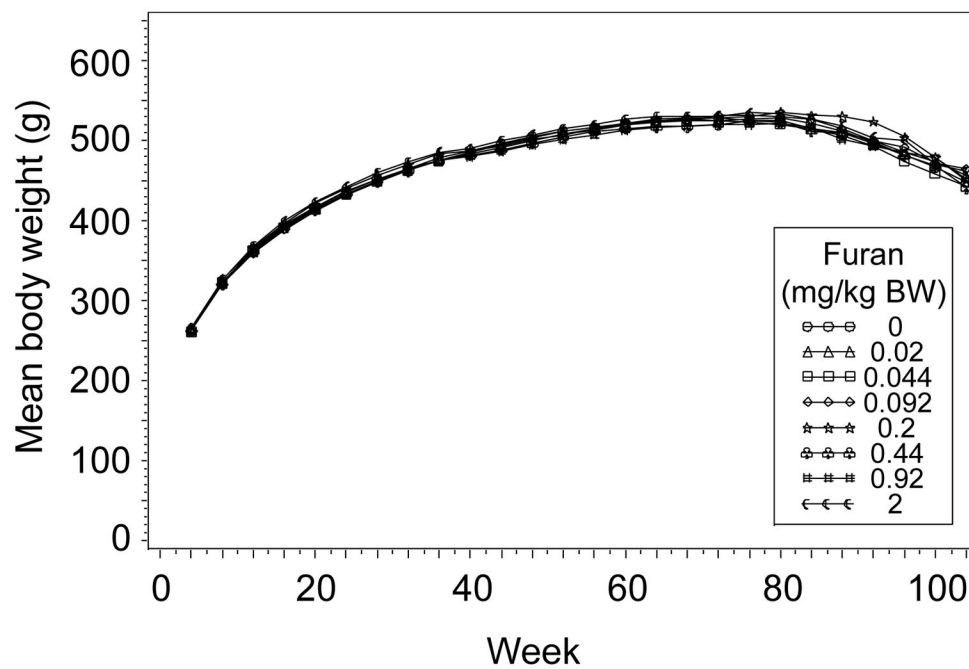


Figure 2. Body weights of male F344/N Nctr rats administered 0, 0.02, 0.044, 0.092, 0.2, 0.44, 0.92, or 2.0 mg furan/kg BW for 2 years (104 weeks), as a function of the number of weeks on treatment.

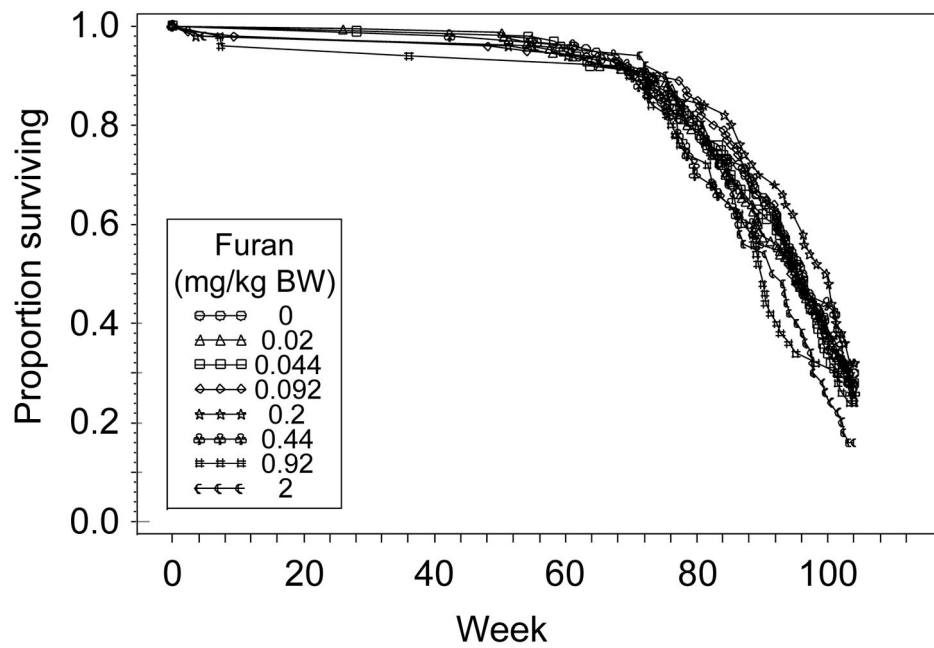


Figure 3. Survival of male F344/N Nctr rats administered 0, 0.02, 0.044, 0.092, 0.2, 0.44, 0.92, or 2 mg furan/kg BW for 2 years (104 weeks), as a function of the number of weeks on treatment. There was a significant ($P = 0.021$) dose-related decrease in survival; however, compared to the control group (0 mg furan/kg BW), none of the treatment groups had a significant decrease in survival. The Kaplan-Meier estimates for the percent probability of survival at the end of the study were 30, 28, 28, 28, 32, 26, 24, and 16% for 0, 0.02, 0.044, 0.092, 0.2, 0.44, 0.92, and 2 mg furan/kg BW, respectively.

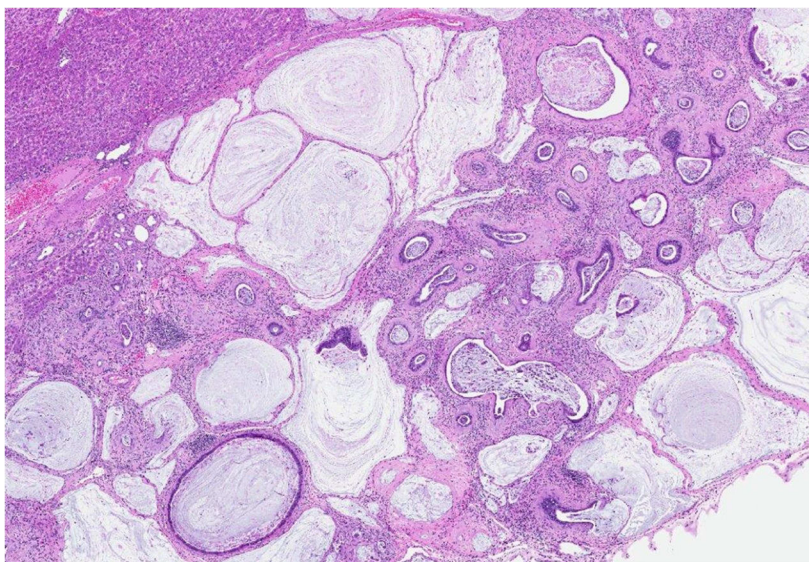


Figure 4. Cholangiofibrosis observed in a male rat treated with 2 mg furan/kg BW for 2 years. The lesion is characterized by dilated and cystic bile ducts filled with mucinous and cellular debris surrounded by dense collagenous connective tissue with a prominent inflammatory cell infiltrate. The epithelium varies from low cuboidal to tall columnar, with hyperbasophilic and pleomorphic cells along with goblet cells and Paneth cells.

Table 1

Doses of furan and group sizes for each dose administered to male F344/N Nctr rats for 2 years and the predicted incidence of cholangiocarcinoma at 2 years^a

Doses of furan (mg/kg BW)	Total number of rats/group	Number of rats examined at the 36-week interim sacrifice	Number of rats examined at the 60-week interim sacrifice	Number of rats remaining for the entire 2-year bioassay	Predicted cholangiocarcinoma incidence at 2 years ^b
0	190	20	20	150	0%
0.02	180	20	10	150	0 – 23%
0.044	130	20	10	100	0 – 37%
0.092	130	20	10	100	19 – 50%
0.2	80	20	10	50	41 – 64%
0.44	80	20	10	50	63 – 78%
0.92	80	20	10	50	83 – 92%
2	80	20	10	50	100%

^aFuran was administered by gavage in corn oil vehicle 5 days/week for 2 years (104 weeks), with interim sacrifices conducted at 36 and 60 weeks.

^bThe predicted cholangiocarcinoma incidences were derived with the empirical relationship of Peto *et al.* (1984), using exponents of 2 and 3.

Table 2

Incidences of malignant mesothelioma, mononuclear cell leukemia, and hepatocellular neoplasms in male F344/N Nctr rats administered 0, 0.02, 0.044, 0.092, 0.2, 0.44, 0.92, or 2 mg furan/kg BW for 2 years.

Neoplasm	Furan (mg/kg BW)							
	0	0.02	0.044	0.092	0.2	0.44	0.92	2
Malignant mesothelioma, epididymis	Overall rate ^a 6/149 (4%) P = 0.018	8/149 (5%) P = 0.371	1/98 (1%) P = 0.163N	2/100 (2%) P = 0.301N	0/50 (0%) P = 0.155N	2/50 (4%) P = 0.647	2/50 (4%) P = 0.617	5/50 (10%) P = 0.087
Malignant mesothelioma, testes	Overall rate 5/150 (3%) P = 0.032	7/149 (5%) P = 0.362	1/98 (1%) P = 0.235N	1/100 (1%) P = 0.222N	0/50 (0%) P = 0.202N	2/49 (4%) P = 0.569	2/50 (4%) P = 0.544	4/50 (8%) P = 0.142
Malignant mesothelioma, epididymis or testes	Overall rate 6/150 (4%) P = 0.003	8/150 (5%) P = 0.373	1/98 (1%) P = 0.164N	2/100 (2%) P = 0.303N	0/50 (0%) P = 0.156N	2/50 (4%) P = 0.645	2/50 (4%) P = 0.616	6/50 (12%) P = 0.033
Malignant mesothelioma, all organs	Overall rate 6/150 (4%) P = 0.005	9/150 (6%) P = 0.283	1/100 (1%) P = 0.161N	2/100 (2%) P = 0.303N	0/50 (0%) P = 0.156N	3/50 ^c (6%) P = 0.402	2/50 (4%) P = 0.616	6/50 (12%) P = 0.033
Mononuclear cell leukemia	Overall rate 47/150 (31%) P < 0.001	56/150 (37%) P = 0.182	36/100 (36%) P = 0.275	44/100 (44%) P = 0.037	29/50 (58%) P = 0.001	18/50 (36%) P = 0.341	27/50 (54%) P < 0.001	28/50 (56%) P = 0.001
Hepatocellular adenoma	Overall rate 2/149 (1%) P = 0.017	2/150 (1%) P = 0.690	1/99 (1%) P = 0.639N	2/100 (2%) P = 0.549	0/50 (0%) P = 0.485N	1/49 (2%) P = 0.624	1/50 (2%) P = 0.603	3/49 (6%) P = 0.086
Hepatocellular carcinoma	Overall rate 3/149 (2%) P = 0.392N	0/150 (0%) P = 0.125N	0/99 (0%) P = 0.206N	0/100 (0%) P = 0.200N	0/50 (0%) P = 0.352N	0/49 (0%) P = 0.377N	0/50 (0%) P = 0.398N	0/49 (0%) P = 0.386N
Hepatocellular adenoma or carcinoma	Overall rate 5/149 (3%) P = 0.066	2/150 (1%) P = 0.226N	1/99 (1%) P = 0.224N	2/100 (2%) P = 0.399N	0/50 (0%) P = 0.199N	1/49 (2%) P = 0.514N	1/50 (2%) P = 0.544N	3/49 (6%) P = 0.318

^aNumber of animals with neoplasm/number of animals examined microscopically.

^bBeneath the 0 mg furan/kg BW incidences are the P-values associated with the trend test. Beneath the treated group incidences are the P-values corresponding to pair-wise comparisons between the 0 mg furan/kg BW group and each treated group. The Poly-3 test accounts for differential mortality in animals that do not reach the terminal sacrifice. P-values < 0.05 were considered significant and are bolded. An N indicates a lower incidence compared to the 0 mg furan/kg BW group.

^cIncludes a mesothelioma (not otherwise specified) of the heart atrium.

Incidences of non-neoplastic lesions in the liver of male F344/N Nctr rats administered 0, 0.02, 0.044, 0.092, 0.2, 0.44, 0.92, or 2 mg furan/kg BW for 36 or 60 weeks or 2 years.

Table 3

Non-neoplastic liver lesion	Treatment duration	Furan (mg/kg BW)									
		0	0.02	0.044	0.092	0.2	0.44	0.92	2		
Cholangio-fibrosis	36 weeks	Overall rate ^a	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	6/20 (30%)	17/20 (85%)	19/20 (95%)	
		CAFE ^b	P < 0.001					P = 0.010	P < 0.001	P < 0.001	P < 0.001
		Average severity ^c						1.0	1.1	1.2	
	60 weeks	Overall rate ^a	0/20 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (100%)	10/10 (100%)	8/10 (80%)		
		CAFE ^b	P < 0.001					P < 0.001	P < 0.001	P < 0.001	
		Average severity ^c						1.1	1.6	2.1	
Mixed cell foci	2 years	Overall rate ^a	0/149 (0%)	0/150 (0%)	0/99 (0%)	1/100 (1%)	38/50 (76%)	49/49 (100%)	49/49 (100%)		
		Poly-3 test ^d	P < 0.001			P = 0.422	P < 0.001	P < 0.001	P < 0.001	P < 0.001	
		Average severity ^c				1.0	1.7	2.6	3.4	3.9	
	36 weeks	Overall rate	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	1/20 (5%)	0/20 (0%)	1/20 (5%)	5/20 (25%)	
		CAFE	P < 0.001				P = 0.500	P = 0.500	P = 0.500	P = 0.024	
		Average severity ^c	- ^e	-	-	-	-	-	-	-	
	60 weeks	Overall rate	1/20 (5%)	0/10 (0%)	1/10 (10%)	0/10 (0%)	1/10 (10%)	1/10 (10%)	3/10 (30%)	6/10 (60%)	
		CAFE	P < 0.001	P = 0.667N	P = 0.563	P = 0.667N	P = 0.563	P = 0.563	P = 0.095	P = 0.002	
		Average severity ^c	- ^e	-	-	-	-	-	-	-	
	2 years	Overall rate	7/149 (5%)	7/150 (5%)	6/99 (6%)	3/100 (3%)	5/50 (10%)	3/49 (6%)	6/50 (12%)	13/49 (27%)	
		Poly-3 test	P < 0.001	P = 0.588	P = 0.418	P = 0.364N	P = 0.170	P = 0.485	P = 0.056	P < 0.001	
		Average severity ^c	- ^e	-	-	-	-	-	-	-	
Biliary tract hyperplasia	36 weeks	Overall rate	1/20 (5%)	1/20 (5%)	0/20 (0%)	1/20 (5%)	0/20 (0%)	0/20 (0%)	6/20 (30%)	19/20 (95%)	
		CAFE	P < 0.001	P = 0.756	P = 0.500N	P = 0.756	P = 0.500N	P = 0.500N	P = 0.046	P < 0.001	
		Average severity ^c	1.0	1.0		1.0		1.3	1.8		
	60 weeks	Overall rate	7/20 (35%)	3/10 (30%)	1/10 (10%)	2/10 (20%)	1/10 (10%)	2/10 (20%)	3/10 (30%)	8/10 (80%)	
		CAFE	P < 0.001	P = 0.560N	P = 0.154N	P = 0.344N	P = 0.154N	P = 0.344N	P = 0.560N	P = 0.025	
		Average severity ^c	1.0	1.0	2.0	1.0	1.0	1.0	1.3	2.5	

Non-neoplastic liver lesion	Treatment duration	Furan (mg/kg BW)							
		0	0.02	0.044	0.092	0.2	0.44	0.92	2
Oval cell hyperplasia	2 years	89/149 (60%) P < 0.001	86/150 (57%) P = 0.308N	59/99 (60%) P = 0.440N	56/100 (56%) P = 0.303N	29/50 (58%) P = 0.419N	25/49 (51%) P = 0.195N	32/50 (64%) P = 0.341	43/49 (88%) P = 0.002
	36 weeks	1.4 0/20 (0%) P < 0.001	1.6 0/20 (0%)	1.5 0/20 (0%)	1.6 0/20 (0%)	2.0 0/20 (0%)	2.9 0/20 (0%)	3.2 1/20 (5%)	3.6 16/20 (80%) P < 0.001
	60 weeks	0/20 (0%) P < 0.001	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	3/10 (30%)	7/10 (70%) P < 0.001
Bile duct subcapsular hyperplasia	2 years	14/149 (9%) P < 0.001	15/150 (10%) P = 0.484	10/99 (10%) P = 0.500	8/100 (8%) P = 0.434N	7/50 (14%) P = 0.284	6/49 (12%) P = 0.360	14/50 (28%) P < 0.001	33/49 (67%) P < 0.001
	36 weeks	2.2 0/20 (0%) P < 0.001	2.5 0/20 (0%)	2.0 0/20 (0%)	2.1 0/20 (0%)	2.1 1/20 (5%)	2.7 12/20 (60%)	3.1 19/20 (95%)	3.1 20/20 (100%) P < 0.001
	60 weeks	0/20 (0%) P < 0.001	0/10 (0%)	0/10 (0%)	0/10 (0%)	1/10 (10%)	9/10 (90%)	10/10 (100%)	7/10 (70%) P < 0.001
Hepatocyte hypertrophy	36 weeks	0/20 (0%) P < 0.001	0/20 (0%)	0/20 (0%)	0/20 (0%)	1.0 1/20 (5%)	1.1 0/20 (0%)	1.5 2/20 (10%)	1.9 12/20 (60%) P < 0.001
	60 weeks	0/20 (0%) P < 0.001	0/10 (0%)	0/10 (0%)	0/10 (0%)	2.0 0/10 (0%)	1.5 4/10 (40%)	1.5 8/10 (80%)	2.1 19/20 (95%) P < 0.001
	36 weeks	0/20 (0%) P < 0.001	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	2.2 0/20 (0%)	1.5 7/20 (35%)	2.0 19/20 (95%) P < 0.001
Periportal cytoplasmic alteration	60 weeks	0/20 (0%) P < 0.001	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/20 (0%)	1/10 (10%)	1.1 7/10 (70%)	2.0 8/10 (80%) P < 0.001
	36 weeks	0/20 (0%) P < 0.001	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	1.1 7/10 (70%)	2.0 8/10 (80%) P < 0.001
	60 weeks	0/20 (0%) P < 0.001	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/20 (0%)	1/10 (10%)	1.1 7/10 (70%)	2.0 8/10 (80%) P < 0.001

Non-neoplastic liver lesion	Treatment duration	Furan (mg/kg BW)								
		0	0.02	0.044	0.092	0.2	0.44	0.92	2	
Subcapsular fibrosis	36 weeks	CAFE	P < 0.001			P = 0.333		0.44	P < 0.001	2
		Average severity			2.0		1.0		P < 0.001	2.0
Subcapsular fibrosis	60 weeks	Overall rate	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	3/20 (15%)	14/20 (70%)	20/20 (100%)	20/20 (100%)
		CAFE	P < 0.001				P = 0.115	P < 0.001	P < 0.001	P < 0.001
Subcapsular fibrosis	60 weeks	Average severity					1.3	1.1	1.3	1.8
		Overall rate	0/20 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	4/10 (40%)	9/10 (90%)	10/10 (100%)	7/10 (70%)
Subcapsular chronic inflammation	36 weeks	CAFE	P < 0.001				P = 0.008	P < 0.001	P < 0.001	P < 0.001
		Average severity					1.0	1.1	1.5	1.9
Subcapsular chronic inflammation	60 weeks	Overall rate	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	2/20 (10%)	14/20 (70%)	20/20 (100%)	20/20 (100%)
		CAFE	P < 0.001				P = 0.244	P < 0.001	P < 0.001	P < 0.001
Subcapsular chronic inflammation	60 weeks	Average severity					1.0	1.1	1.3	1.8
		Overall rate	0/20 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	8/10 (80%)	9/10 (90%)	10/10 (100%)	7/10 (70%)
Subcapsular pigmentation	36 weeks	CAFE	P < 0.001				P < 0.001	P < 0.001	P < 0.001	P < 0.001
		Average severity					1.0	1.1	1.5	1.9
Subcapsular pigmentation	60 weeks	Overall rate	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	2/20 (10%)	14/20 (70%)	20/20 (100%)	20/20 (100%)
		CAFE	P < 0.001				P = 0.244	P < 0.001	P < 0.001	P < 0.001
Subcapsular pigmentation	60 weeks	Average severity					1.0	1.1	1.3	1.8
		Overall rate	0/20 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	8/10 (80%)	9/10 (90%)	10/10 (100%)	7/10 (70%)
Basophilic foci	2 years	CAFE	P < 0.001				P < 0.001	P < 0.001	P < 0.001	P < 0.001
		Average severity					1.0	1.1	1.5	1.9
Basophilic foci	2 years	Overall rate	28/149 (19%)	16/150 (11%)	19/99 (19%)	18/100 (18%)	11/50 (22%)	13/49 (27%)	10/50 (20%)	21/49 (43%)
		Poly-3 test	P < 0.001	P = 0.036N	P = 0.536	P = 0.445N	P = 0.485	P = 0.164	P = 0.390	P < 0.001
Regenerative hyperplasia	2 years	Average severity	- ^e	-	-	-	-	-	-	-
		Overall rate	0/149 (0%)	1/150 (1%)	1/99 (1%)	2/100 (2%)	1/50 (2%)	1/49 (2%)	7/50 (14%)	12/49 (24%)
Regenerative hyperplasia	2 years	Poly-3 test	P < 0.001	P = 0.498	P = 0.419	P = 0.158	P = 0.290	P = 0.276	P < 0.001	P < 0.001
		Average severity		4.0	4.0	2.5	3.0	2.0	3.6	3.9
Cytoplasmic vacuolization	2 years	Overall rate	23/149 (15%)	23/150 (15%)	14/99 (14%)	19/100 (19%)	12/50 (24%)	18/49 (37%)	23/50 (46%)	37/49 (76%)
		Poly-3 test	P < 0.001	P = 0.548	P = 0.493N	P = 0.280	P = 0.153	P = 0.001	P < 0.001	P < 0.001

Non-neoplastic liver lesion	Treatment duration	Average severity	Furan (mg/kg BW)							
			0	0.02	0.044	0.092	0.2	0.44	0.92	2
			2.3	2.2	2.1	2.3	3.0	3.0	2.8	3.3

^aNumber of animals with lesion/number of animals examined microscopically.

^bBeneath the 0 mg furan/kg BW incidences are the P-values associated with a Cochran-Armitage linear trend test. Beneath the treated group incidences are the P-values corresponding to pair-wise comparisons, as assessed by a Fisher's Exact test, between the 0 mg furan/kg BW group and each treated group. CAFE refers to Cochran-Armitage linear trend test/Fisher's Exact test. P-values < 0.05 were considered significant and are bolded. An N indicates a lower incidence compared to the 0 mg furan/kg BW group.

^cAverage severity of the observed lesions. The severity of the lesions was graded as 1, minimal; 2, mild; 3, moderate; and 4, marked.

^dBeneath the 0 mg furan/kg BW incidences are the P-values associated with the trend test. Beneath the treated group incidences are the P-values corresponding to pair-wise comparisons between the 0 mg furan/kg BW group and each treated group. The Poly-3 test accounts for differential mortality in animals that do not reach the terminal sacrifice. P-values < 0.05 were considered significant and are bolded. An N indicates a lower incidence compared to the 0 mg furan/kg BW group.

^eSeverity score not recorded; the lesion was indicated as present or absent.

Table 4

Benchmark dose modeling of incidences of epididymis or testes malignant mesothelioma and cholangiofibrosis in male F344/N Nctr rats administered 0, 0.02, 0.044, 0.092, 0.2, 0.44, 0.92, or 2 mg furan/kg BW for 2 years.

Neoplasm/non-neoplastic lesion	Model	AIC ^a	Fitted model ^b	GOF ^c	BMD ₁₀ ^d	BMDL ₁₀ ^e
Malignant mesothelioma	Gamma	227.9	0.154	0.279	30.47	21.86
	Logistic	226.8	0.182	0.306	33.10	22.48
	Log-Logistic	227.9	0.154	0.279	30.38	21.93
	Log-Probit	227.9	0.154	0.279	30.62	21.13
	Multistage	226.1	0.225	0.368	31.70	22.05
	Probit	226.9	0.175	0.298	34.20	22.08
Cholangiofibrosis ^f	Weibull	227.9	0.154	0.279	30.34	22.12
	Gamma	70.3	1.000	1.000	1.81	1.60
	Logistic	70.5	0.995	0.998	2.04	1.79
	Log-Logistic	70.4	0.999	1.000	1.88	1.65
	Log-Probit	70.3	1.000	1.000	1.76	1.59
	Multistage	-g				
	Probit	70.3	1.000	1.000	1.90	1.69
	Weibull	70.3	1.000	1.000	1.95	1.70

^a AIC, Akaike information criterion.

^b P-value of fitted model compared to the full model.

^c GOF, Goodness-of-fit P-value.

^d BMD₁₀, benchmark dose (μmol furan/kg BW) that caused a 10% excess risk of the specified adverse effect over that observed in the appropriate control group.

^e BMDL₁₀, lower 95% confidence limit of benchmark dose (μmol furan/kg BW).

^f The models were constructed using the cholangiofibrosis incidence in male rats administered 0, 0.02, 0.044, 0.092, 0.2, or 0.44 mg furan/kg BW for 2 years. Satisfactory fits could not be obtained when the 2 highest doses (0.92 and 2 mg furan/kg BW) were included in the calculations.

^g Model rejected.