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Furan-induced dose–response relationships for liver cytotoxicity, cell proliferation, and tumorigenicity (furan-induced liver tumorigenicity)

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Abstract

Rodent studies of furan are associated with liver cell necrosis, release of liver-associated enzymes, increased hepatocyte proliferation, and hepatocarcinogenesis. For carcinogens whose proposed mode of action is cytolethality, it is hypothesized that the dose–response curve for tumor development would parallel the dose–response curve for cell death with compensatory proliferation in the target organ. To prospectively test this hypothesis, female $B6C3F_1$ mice were exposed to furan at carcinogenic doses and lower for 3 weeks or 2 years. At 3 weeks and in the 2-year study, there were dose-dependent and significant increases in hepatic cytotoxicity at 1.0, 2.0, 4.0, and 8.0 mg furan/kg. For cell proliferation as measured by 5-bromo-2′-deoxyuridine (BrdU) labeling index (LI), there was a statistically significant trend with increasing dose levels of furan and increased LI at 8.0 mg/kg. There was an increased incidence of foci of altered hepatocytes, hepatocellular adenomas, and adenomas or carcinomas at 4.0 and 8.0 mg/kg and carcinomas at 8.0 mg/kg. The multiplicity of microscopic tumors was increased and latency was decreased in mice exposed to 8.0 mg/kg. Prevalence of hepatic nodules at necropsy was increased in mice exposed to 4.0 and 8.0 mg/kg. Data demonstrate an association among furan-induced hepatic cytotoxicity, compensatory cell replication, and liver tumor formation in mice; at high doses $\geqslant$4.0 mg/kg, furan induced hepatotoxicity, compensatory cell replication and tumorigenesis in a dose-related manner, while furan did not produce tumors at cytotoxic doses of 1.0 and 2.0 mg/kg.
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Keywords: Furan; Mouse liver carcinogenesis; Cytotoxic; Proliferation; Inflammation

Introduction

Furan is a highly toxic chemical used in the synthesis of nylon, lacquers, insecticides, pharmaceuticals, and stabilizers. It is also found in baby food, cigarette smoke, and air pollutants (IARC, 1995; Maga, 1979; US FDA, 2004). Furan is hepatocarcinogenic based upon an increased incidence of hepatocellular adenomas and carcinomas in male and female mice (NTP, 1993; Table 1) and rats. When administered by gavage in a 2-year study, furan increased the incidence of hepatocellular adenomas in male and female F344/N rats and $B6C3F_1$ mice and hepatocellular carcinomas in male F344/N rats and male and female $B6C3F_1$ mice; increased hepatic cholangiocarcinomas also were found in male and female F344/N rats dosed at 2.0, 4.0, and

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Table 1. Incidence of liver tumors in B6C3F$_1$ mice exposed to furan in the National Toxicology Program Bioassay[a]

	Dose of furan		
	0 mg/kg	8 mg/kg	15 mg/kg
Female mice			
Hepatocellular adenomas (%)	10	62[b]	96[b]
Hepatocellular carcinomas (%)	4	14	54[b]
Hepatocellular adenomas or carcinomas (%)	14	68[b]	100[b]
Male mice			
Hepatocellular adenomas (%)	40	66[b]	84[b]
Hepatocellular carcinomas (%)	14	64[b]	68[b]
Hepatocellular adenomas or carcinomas (%)	52	88[b]	100[b]

[a]NTP (1993).
[b]Statistically significant increase as compared to controls ($p<0.05$).

8.0 mg furan/kg bwt 5 days/week for 9, 15, or 24 months (NTP, 1993).

Despite several negative tests for genotoxicity, positive results in some prokaryotic, mammalian, and cell culture systems and mice *in vivo* plus mutational analysis of furan-induced liver tumors (NTP, 1993) indicate that furan has genotoxic potential. Consequently, the carcinogenicity of furan may be due in part to its genotoxicity potential.

Under bioassay conditions, furan induces compensatory cell proliferation secondary to hepatic cytotoxicity (Fransson-Steen et al., 1997; Wilson et al., 1992). Thus, at high exposure concentrations, cytotoxicity may be involved in the carcinogenic response. Reparative cell proliferation has been demonstrated in a variety of *in vivo* studies in mice and rats in which single and multiple doses of furan induced liver cell necrosis and/or release of liver-associated enzymes (Butterworth et al., 1994; Fransson-Steen et al., 1997; Goldsworthy et al., 1996; Wilson et al., 1992). These hepatotoxic effects are followed by an elevation in the percentage of hepatic nuclei in the S phase of the cell cycle. For carcinogens whose proposed mode of action involves cytolethality, it is hypothesized that the dose–response curve for tumor development would closely parallel the dose–response curve for cell death with compensatory proliferation in the target organ. We examined this hypothesis by evaluating furan-induced hepatocyte toxicity and liver cell proliferation in a multidose 3-week study and liver neoplasia in a 2-year carcinogenicity study in female mice. Data from these studies demonstrate an association among the furan-induced dose–response curve for hepatotoxicity, cell death, compensatory cell replication, and liver tumor formation in female mice. No observed effect levels of cytotoxicity below 1.0 mg furan/kg and hepatocarcinogenicity below 4.0 mg furan/kg bwt were identified.

Materials and methods

Test compound and dosing solutions

Furan (CAS No. 110-00-9) used in the 3-week and 2-year carcinogenicity studies (>99% pure) was obtained from Sigma-Aldrich Chemical Co., (Milwaukee, WI). The appropriate quantity of the test article was mixed with Mazola® corn oil to a concentration that delivered the required dose levels. Each dose level was prepared separately on a volume-to-weight (v:w) ratio. Chilled corn oil was weighed to the nearest milligram in a conical flask. Chilled furan was drawn up in a Hamilton syringe, measured to the nearest microliter, injected into corn oil, and mixed using a magnetic stir bar. Dosing solutions were dispensed into de-aerated (with inert gas) 8 mL brown glass vials and capped and sealed with plastic closures adapted with silicon septa. Dosing solutions were stored in a refrigerator until use, but for no longer than 14 days. Previous studies have demonstrated the stability of furan dosing formulations for up to 14 days under these conditions (NTP, 1993).

Animals

Female specific-pathogen-free (SPF) B6C3F$_1$ mice were received from Charles River Breeding Laboratories (Portage, ME) at 5–6 weeks of age and observed for approximately 7 days before studies began. B6C3F$_1$ mice were chosen in these studies since they were used in other short-term studies with furan and the cancer bioassay (Butterworth et al., 1994; IARC, 1995; NTP, 1993; Wilson et al., 1992). Male and female B6C3F$_1$ mice appear to respond similarly to furan-induced liver biochemical, proliferative, and cytotoxic effects (Fransson-Steen et al., 1997; Wilson et al., 1992). Female mice were chosen for this hepatocarcinogenicity study because they have a lower incidence of spontaneous liver tumors (14%) relative to male mice (52%) (Table 1 and Haseman et al., 1998). A lower background incidence should permit improved detection of slight increases in liver tumor formation among dose groups. The animals were housed in cages in a SPF and AAALAC-accredited facility. All procedures were conducted in compliance with the Animal Welfare Act Regulations (9 CFR 1–4). Animals were handled and treated according to the guidelines provided in the National Institute of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (ILAR, 1996). Feed (NIH-07; Zeigler Brothers, Inc., Gardners, PA) and tap water were available *ad libitum*. Mice were housed five per cage in polycarbonate cages

(17 cm wide, 28 cm long [476 cm^2 area] and 13 cm height) with stainless steel wire-bar lids. As deaths occurred during the course of studies, animals were not consolidated to maintain five mice in each cage. If tail tattoos faded in the carcinogenicity study, the mice were ear punched for supplemental identification. The temperature and humidity of the room were targeted for 18–26 °C and 30–70%, respectively, and monitored continuously; occasional excursions outside the targeted range were deemed not to have adversely affected study results. Mice were observed for signs of illness or death twice daily during the week and once daily on weekends and holidays. Sentinel animals for health surveillance were housed in the same animal room as study animals and remained disease-free throughout the study.

Experimental design

Animal husbandry activities and dosing in these studies were similar to those employed in the NTP bioassay so that data could be correlated among studies (Tables 2 and 3). For the 3-week study, groups of 15 female B6C3F$_1$ mice received 0.0, 0.5, 1.0, 2.0, 4.0, and 8.0 mg furan/kg bwt at a dosing volume of 5 mL/kg bwt. For the carcinogenicity study, groups of 50 female mice were exposed to the highest doses of furan (2.0, 4.0, and 8.0 mg furan/kg bwt) and vehicle control, while 100 and 75 mice were exposed to 0.5 and 1.0 mg furan/kg bwt, respectively. The number of mice in the low dose groups was increased to facilitate potential detection of small differences in tumor incidence or multiplicity.

Table 2. Serum alanine aminotransferase (ALT) levels and labeling index (LI) in female B6C3F$_1$ mice exposed to furan by gavage for three weeks[a]

Group (mg/kg)	ALT (IU)[c]	LI (%)[b,c]
0.0 Furan	18.0±2.14	1.1±0.50
0.5 Furan	19.6±3.91	1.2±0.79
1.0 Furan	20.4±1.33[d]	1.1±0.41
2.0 Furan	28.6±2.70[d]	1.7±0.95
4.0 Furan	52.3±16.23[d]	1.9±0.74
8.0 Furan	85.3±39.06[d]	3.4±0.76[d]

[a]Female B6C3F$_1$ mice were exposed to furan in corn oil by gavage (5 mL/kg) 5 days/week for three weeks. For quantification of LI, mice were administered 0.02% BrdU in the drinking water for 5 days. Values reported are the mean±SD (for ALT, $N = 6, 5, 5, 5, 5$ and 8 for mice exposed to 0.0, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/kg, respectively; for LI, $N = 10$ for all groups). All indices were quantified from the left lobe of the liver.

[b]Quantified by dividing the number of BrdU immunoreactive hepatic nuclei by the total number of nuclei evaluated and multiplying by 100.

[c]There was a dose-related trend in ALT and LI.

[d]Significantly different from control ($p < 0.050$).

Blood collection and necropsy

Mice were anesthetized by CO_2 inhalation prior to blood collection. For the 3-week study, serum was harvested from the blood, frozen, and stored at −70 °C until evaluated for alanine aminotransferase (ALT) activity (Table 2). Unscheduled necropsies were performed as needed for mice found dead or euthanized when moribund. Complete gross examinations were performed at necropsy. Livers were removed, rinsed in saline, blotted dry, and weighed. Relative liver weights (% body weight) were determined by dividing absolute liver weight by terminal body weight and multiplying by 100. After weighing, the liver was separated into lobes. Representative sections from the left, median, right posterior, right anterior, and caudate lobes of the liver and sections of macroscopic lesions >0.5 cm in size along with accompanying normal tissue were fixed in 10% (neutral buffered formalin) NBF. All tissues collected in 10% NBF were transferred to 70% ethanol within 18–24 h. Liver samples and visible lesions were processed and embedded in paraffin blocks. Sections were cut to 4–6 μm and stained with hematoxylin and eosin (H&E) for histological evaluation. The location and size of liver lesions were recorded at necropsy. Macroscopic liver lesions with three measurable dimensions were counted as nodules. The incidence of animals with liver nodules was quantified.

Serum enzyme

ALT levels were analyzed using a Roche Cobas Mira Chemistry Analyzer (Roche Diagnostic Systems, Inc., Montclair, NJ). Reagents were obtained from Sigma Diagnostics (St. Louis, MO). ALT is a relatively specific membrane-bound liver enzyme.

Hepatic cytotoxicity

For the 3-week and 2-year carcinogenicity studies, H&E-stained sections were evaluated for the presence of liver cytotoxicity without knowledge of animal identity. Hepatotoxicity, which included the presence of subcapsular or parenchymal inflammation or cytological degeneration, and/or infiltration with lymphocytes or mixed inflammatory cells, was graded as normal, mild, moderate, or marked. Hepatotoxicity was defined as an increased incidence or severity of microscopic cytotoxicity and/or increased levels of ALT.

In the 2-year carcinogenicity study, H&E-stained liver sections were evaluated without knowledge of animal identity for the presence of preneoplastic and neoplastic lesions. Liver lesions were characterized as foci of altered hepatocytes (presumptive preneoplastic lesions) or hepatocellular adenomas or carcinomas based on published nomenclature and criteria (Maronpot et al., 1989), and

Table 3. Liver preneoplastic and neoplastic lesions in female mice exposed to furan

	Control	0.5	1.0	2.0	4.0	8.0
Number examined microscopically	36	72	53	41	36	39
Focus of altered hepatocytes						
Overall rate[a]	3/36 (8%)	5/72 (7%)	4/53 (8%)	5/41 (12%)	10/36 (28%)	19/39 (49%)
Adjusted rate[b] (%)	10	7	9	14	32	55
Terminal rate[c]	3/25 (12%)	5/55 (9%)	4/36 (11%)	5/31 (16%)	10/25 (40%)	13/22 (59%)
Latency	725	725	725	725	725	590
Poly-3 test[d]		$p = 0.511$ N	$p = 0.620$ N	$p = 0.445$ N	$p = 0.030$[g]	$p < 0.001$[g]
Dose trend[e]	$p < 0.001$					
Hepatocellular adenoma						
Overall rate	3/36 (8%)	4/72 (6%)	4/53 (8%)	4/41 (10%)	11/36 (31%)	25/39 (64%)
Adjusted rate (%)	10	6	9	11	34	72
Terminal rate	3/25 (12%)	3/55 (5%)	4/36 (11%)	3/31 (10%)	8/25 (32%)	18/22 (82%)
Latency	725	713	725	525	511	489
Poly-3 test		$p = 0.407$ N	$p = 0.620$ N	$p = 0.596$	$p = 0.018$[g]	$p < 0.001$[g]
Dose trend	$p < 0.001$					
Hepatocellular carcinoma						
Overall rate	0/36 (0%)	4/72 (6%)	2/53 (4%)	1/41 (2%)	2/36 (6%)	11/39 (28%)
Adjusted rate (%)	0	6	4	3	6	33
Terminal rate	0/25 (0%)	2/55 (4%)	1/36 (3%)	1/31 (3%)	1/25 (4%)	6/22 (27%)
Latency	NA[f]	439	566	726	511	652
Poly-3 test		$p = 0.208$	$p = 0.322$	$p = 0.532$	$p = 0.244$	$p < 0.001$[g]
Dose trend	$p < 0.001$					
Hepatocellular adenoma or carcinoma						
Overall rate	3/36 (8%)	8/72 (11%)	6/53 (11%)	5/41 (12%)	12/36 (33%)	29/39 (74%)
Adjusted rate (%)	10	12	13	14	35	63
Terminal rate	3/25 (12%)	5/55 (9%)	5/36 (14%)	4/31 (13%)	9/25 (36%)	14/22 (64%)
Latency	725	439	566	525	511	489
Poly-3 test		$p = 0.516$	$p = 0.449$	$p = 0.455$	$p = 0.015$[g]	$p < 0.001$[g]
Dose trend	$p < 0.001$					

[a]Number of tumor-bearing animals/total number of animals evaluated microscopically.
[b]Number of tumor-bearing animals/effective number of animals i.e., number of animals alive at first occurrence of this tumor type in any of the groups.
[c]Observed incidence at terminal kill.
[d]Beneath the control incidence are the p-values associated with the trend test. Beneath the dosed group incidence are the p-values corresponding to pair wise comparisons between the controls and that dosed group. The logistic regression tests regard tumors in animals dying prior to terminal kill as nonfatal.
[e]Dose-related trend as calculated by a modified Cochran–Armitage linear trend test.
[f]Not applicable; no tumors in animal group.
[g]Significant increase as compared to control mice ($p < 0.05$).

foci were further characterized as basophilic, eosinophilic, or clear-cell with and without inflammation. The number of lesions documented histologically was limited to those present in the representative sections from the five lobes plus up to five nodules >0.5 cm in diameter. Consequently, histologic evaluation would detect microscopic lesions not detected grossly, but did not necessarily include all grossly visible lesions.

In some animals, microscopic evaluation of the normal liver and lesions taken at necropsy indicated the presence of histiocytic sarcomas, lymphomas, or hemangiosarcomas only; animals with these neoplastic lesions were excluded in calculating the incidence of liver nodules at necropsy and incidence and number of microscopic preneoplastic and neoplastic lesions. There were 4 animals with both histiocytic sarcomas in the liver and foci of altered hepatocytes and 5 animals with lymphomas in the liver and hepatocellular foci, adenomas, or carcinomas; these animals were included when quantifying incidence and number of liver lesions.

Cell proliferation analysis

For the 3-week study, ten mice at each dose level received 0.02% 5-bromo-2′-deoxyuridine (BrdU; Sigma

Chemical Co., St. Louis, MO) in the drinking water for 5 days immediately prior to sacrifice. For cell proliferation analysis, an additional 5 μm section of liver was placed on Probe-on-Plus slides to be processed immunohistochemically for BrdU incorporation. Paraffin sections of the liver were stained as previously described by Nyska et al., 2002. BrdU staining in the duodenum was used as a positive control to confirm delivery and adequacy of BrdU immunohistochemical staining. Random fields in the left lobe of the liver were scored without knowledge of animal identity for BrdU incorporation by light microscopy. At least 1000 hepatic nuclei were scored per animal. As a measure of cell proliferation, the labeling index (LI) was calculated by dividing the number of BrdU immunoreactive hepatic nuclei by the total number of nuclei scored and multiplying by 100. Additionally, a semi-quantitative assessment of liver cell proliferation based on increases in mitoses was done at the same time H&E-stained slides were evaluated for cytotoxicity.

Statistical analysis

For all quantitative data (e.g., body weights, clinical chemistry, and liver weights), means and standard deviations (SD) were calculated using Microsoft Excel 97 (PC). Mice whose deaths were attributed to gavage accidents, mice for which no necropsy was performed because of autolysis, and an escaped mouse were censored. The probability of survival was estimated using the Kaplan–Meier product-limit procedure (Kaplan and Meier, 1958). Dose-related effects on survival were tested using Tarone's method for dose-related trends (Tarone, 1975), and pair wise comparisons of each dose group with the control were made using Cox's method for testing equality of two groups (Cox, 1972). The p-values for survival analyses are two-sided. One-way ANOVA and Student's t-test comparisons were done using StatMost 32 (PC) for all continuous data.

The continuity-corrected Poly-3 procedure was used to assess microscopic liver lesion prevalence (Bailer and Portier, 1988; Bieler and Williams, 1993; Piegorsch and Bailer, 1997; Portier and Bailer, 1989). This is a survival-adjusted quantal-response procedure that modifies the Cochran–Armitage linear trend test to account for survival differences. The procedure involves a test for an overall dose-related trend and follow-up tests for pair wise comparisons of each dose group with the control group. p-Values for the tests of lesion prevalence are all one-sided. F-tests were used to compare the mean numbers of tumors of each dose group with the control group (Kanji, 1999). p-Values from this test were two-sided.

Incidence of macroscopic liver nodules was evaluated by Fisher's Exact Test using StatMost 32. Hepatotoxicity data were also tested for normality; the data were not normally distributed and were evaluated for group differences and trend by non-parametric means (Mann–Whitney U-test; Cochran–Armitage Trend Test). The analyses were one-sided.

Since the LI was not normally distributed, statistical significance of group LI was determined by a one-tailed pair wise comparison using the Mann–Whitney U-test. A dose-related trend in percent cell replication was tested with Kendall's tau correlation coefficient, tau-β. In the tables and figures, notations indicate groups with means that were significantly different ($p \leqslant 0.050$) from the controls or other treatment groups.

Results

Three-week study in female mice

Survival percentage, body weight, clinical observations

All animals survived to terminal sacrifice, and there were no treatment-related clinical observations recorded relevant to the liver. No changes in body weight or body weight gain were found in groups of mice exposed to furan relative to control mice (data not shown).

Liver findings at necropsy

There were no treatment-related gross lesions noted at the 3-week necropsy. A statistically significant increase ($p = 0.005$) in relative liver weight as compared to control mice was found in mice exposed to the top dose of 8.0 mg furan/kg bwt (data not shown).

Hepatic cytotoxicity

Consistent with previous observations in furan studies, hepatic cytotoxicity was characterized by hepatic parenchymal degeneration and inflammation and/or subcapsular hepatocyte necrosis and inflammation localized on visceral surfaces of the liver in contact with the forestomach (Maronpot et al., 1991). Two of 10 mice dosed with 0.5 mg furan/kg bwt had evidence of hepatic cytotoxicity and higher doses had a dose-related increased severity and/or incidence of hepatic cytotoxicity (Fig. 1). A statistically significant increased incidence of hepatic cytotoxicity was found at the 1.0 mg ($p = 0.002$) and 2.0, 4.0, and 8.0 mg furan/kg bwt dose levels ($p < 0.001$ for all). There was a dose-dependent increase in serum ALT levels with group mean ALT levels significantly increased in mice exposed to 1.0 mg/kg bwt and higher dose levels as compared to control mice (Table 2).

Hepatocyte proliferation

The BrdU LI was significantly increased in female mice exposed to 8.0 mg furan/kg bwt (3.1-fold) as

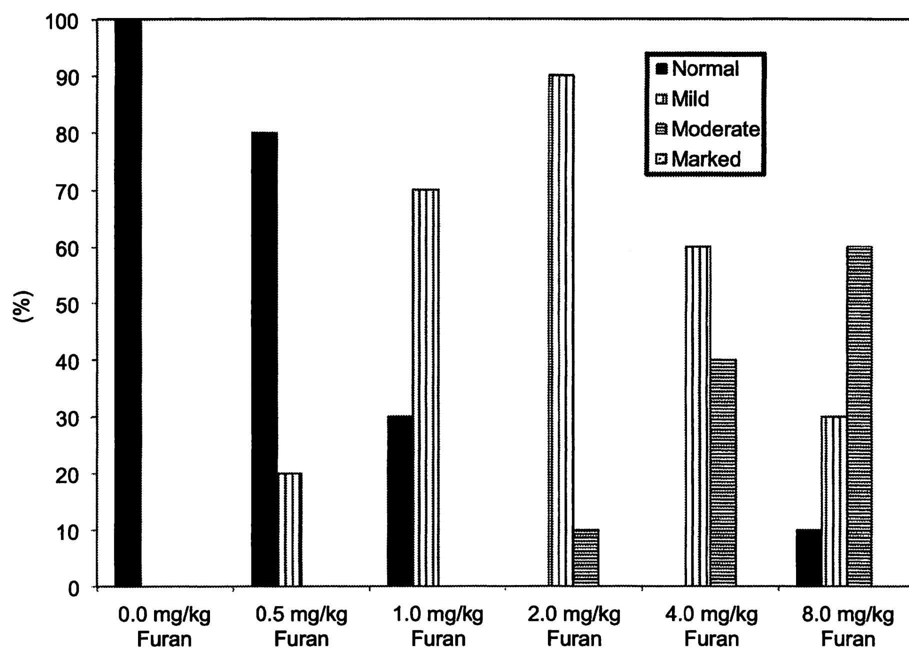

Fig. 1. Furan-induced liver subcapsular inflammation at 3 weeks in female B6C3F1 mice. H&E-stained liver sections were evaluated by light microscopy. Hepatotoxicity was graded semi-quantitatively as normal, mild, moderate or marked. There was a significant increase in subcapsular inflammation at 1.0, 2.0, 4.0, and 8.0 mg furan/kg bwt.

compared to control mice ($p = 0.006$) (Table 2), and there was a dose-related trend in cell proliferation ($p = 0.005$). On H&E-stained slides, a qualitative assessment of hepatocyte proliferation was performed based on increases in mitotic figures. At dose levels of 1.0, 2.0, 4.0, and 8.0 mg furan/kg bwt, increased mitotic figures were found in 1/10, 3/10, 4/10, and 5/10 mice, respectively (data not shown).

Two-year carcinogenicity study in female mice

Mortality

Although mortality was highest in the group of mice exposed to 8.0 mg furan/kg bwt, there were no significant differences in the percentage of mice that survived to the scheduled terminal necropsy in any group exposed to furan relative to control mice (Fig. 2). At the terminal necropsy, the percent mortality in control mice was 34% compared to 31%, 40%, 19%, 36%, and 46% in mice exposed to 0.5, 1.0, 2.0, 4.0, and 8.0 mg furan/kg bwt, respectively.

Body weights

At study start, group mean body weight was similar in all groups (19.1–19.6 g). Up to week 89, body weight was comparable in control animals and mice exposed to furan. At weeks 89 and 101, there was a significant decrease in mean group body weight in mice exposed to 8.0 mg furan/kg bwt as compared to the mean body weight of control mice. At the terminal necropsy, mean group body weight was 42.5 g in control mice and 39.8, 39.7, 38.5, 42.4, and 38.6 g in mice exposed to 0.5, 1.0, 2.0, 4.0, and 8.0 mg furan/kg bwt, respectively. In mice exposed to 2.0 and 8.0 mg furan/kg bwt, there were significant decreases in body weight gain from study initiation to the terminal necropsy; the decrease in body weight gain was not dose-dependent (data not shown).

Liver findings at necropsy

At the terminal necropsy, mean group absolute and relative liver weight of groups of mice exposed to 0.5, 1.0, and 2.0 were comparable to that of control mice. In mice exposed to 4.0 and 8.0 mg furan/kg bwt, absolute and relative liver weights were significantly increased ($p = 0.007$ and 0.004, respectively) (data not shown).

The incidence of liver nodules at the terminal necropsy was significantly increased to an incidence of 60% (15/25) and 100% (22/22) in mice exposed to 4.0 and 8.0 mg furan/kg bwt ($p < 0.001$), respectively. The incidence of gross liver nodules was 8% (2/25) in control mice and 17–20% in remaining groups of treated mice. In animals that were necropsied before the terminal necropsy date, a significant increase in the incidence of visible liver nodules was found in mice exposed to 4.0 (54%; 6/11) and 8.0 mg furan/kg bwt (71%; 12/17) ($p = 0.032$ and 0.002, respectively). The incidence of gross liver nodules in control mice was 9% (1/11). To glean further insight into furan-induced liver tumorigenesis, grossly observed nodules at the terminal necropsy

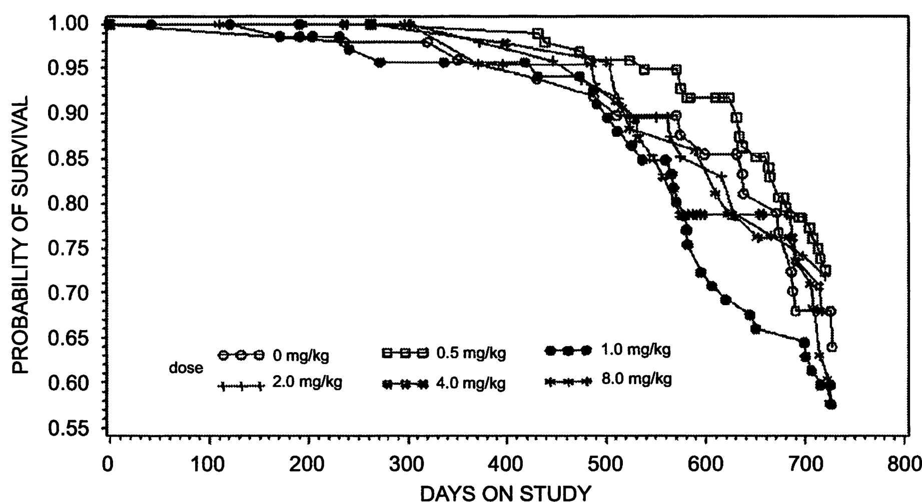

Fig. 2. Survival curve for female B6C3F1 mice exposed to furan for 24 months. Beginning at approximately 7 weeks of age, female B6C3F1 mice were exposed to furan in corn oil by gavage (5 mL/kg) 5 days/week.

were grouped by size. The largest nodules ($\geqslant$0.5 mm) were detected in mice exposed to 8.0 mg furan/kg bwt (data not shown).

Histopathology

Hepatic cytotoxicity

Two of 25 control mice had histologic evidence of hepatic cytotoxicity. At the terminal necropsy, there was a dose-dependent increase in the incidence and severity of hepatic cytotoxicity (Fig. 3). At 0.5 mg furan/kg bwt, 7 of 55 mice showed mild hepatic cytotoxicity. Similar to the 3-week study, a statistically significant increase in mild hepatic cytotoxicity was found at 1.0 mg furan/kg bwt ($p = 0.004$) and higher doses ($p < 0.001$ for all). In mice exposed to 4.0 and 8.0 mg furan/kg bwt, there was a significant increase in the incidence of moderate liver cytotoxicity ($p = 0.009$ and < 0.001, respectively). Only in mice exposed to 8.0 mg furan/kg bwt was an increased incidence of marked cytotoxicity ($p = 0.037$) found.

Proliferative liver lesions

There was a dose-dependent trend in the incidence of foci of altered hepatocytes (foci), adenomas, carcinomas, and adenomas or carcinomas ($p < 0.001$ for all) (Table 3). A statistically significant increase was found in the incidence of hepatocellular foci in mice exposed to 4.0 and 8.0 mg furan/kg bwt ($p = 0.030$ and < 0.001, respectively) as compared to control mice. There was no difference in the percentage of focal lesions that were classified as basophilic, eosinophilic, or clear-cell foci. In mice exposed to 8.0 mg furan/kg bwt, foci with and without inflammation were common findings; in other exposure groups, foci without inflammation were more common than foci with inflammation. Significant increases were found in the prevalence of hepatocellular adenomas in groups exposed to 4.0 and 8.0 mg furan/kg bwt ($p = 0.018$ and < 0.001, respectively), carcinomas in mice exposed to 8.0 mg furan/kg bwt ($p < 0.001$), and adenomas or carcinomas in those exposed to 4.0 and 8.0 mg furan/kg bwt ($p = 0.015$ and < 0.001, respectively) (Table 3). Only animals exposed to 4.0 and 8.0 mg furan/kg bwt had both adenomas and carcinomas [1/36 (3%) and 7/39 (18%), $p = 0.007$, respectively]. There was a significant increase in the mean number of adenomas in mice administered the highest dose levels of 4.0 and 8.0 mg furan/kg bwt ($p = 0.015$ and < 0.001, respectively) and adenomas or carcinomas at 8.0 mg furan/kg bwt ($p = 0.002$) (data not shown). Many mice exposed to 8.0 mg furan/kg bwt had multiple grossly visible nodules and microscopic preneoplastic and neoplastic liver lesions. Two carcinomas metastasized to the lung in mice administered the highest dose level of furan. In general, latency period or time to first tumor was decreased with increasing dose of furan.

Among mice only at the terminal necropsy at 24 months, the incidence of foci, adenomas, carcinomas and adenomas or carcinomas was similar in controls and animals exposed to 0.5, 1.0, and 2.0 mg furan/kg bwt. Significant increases at 4.0 mg furan/kg bwt in the prevalence of foci ($p = 0.030$), adenomas ($p = 0.018$), and adenomas or carcinomas ($p = 0.015$), and at 8.0 mg furan/kg bwt in the prevalence of foci, adenomas, carcinomas and adenomas and carcinomas ($p < 0.001$ for all) were observed (Table 3).

Animals with gross and microscopic findings of lymphomas, histiocytic sarcomas, and hemangiosarcomas were not included when quantifying incidence,

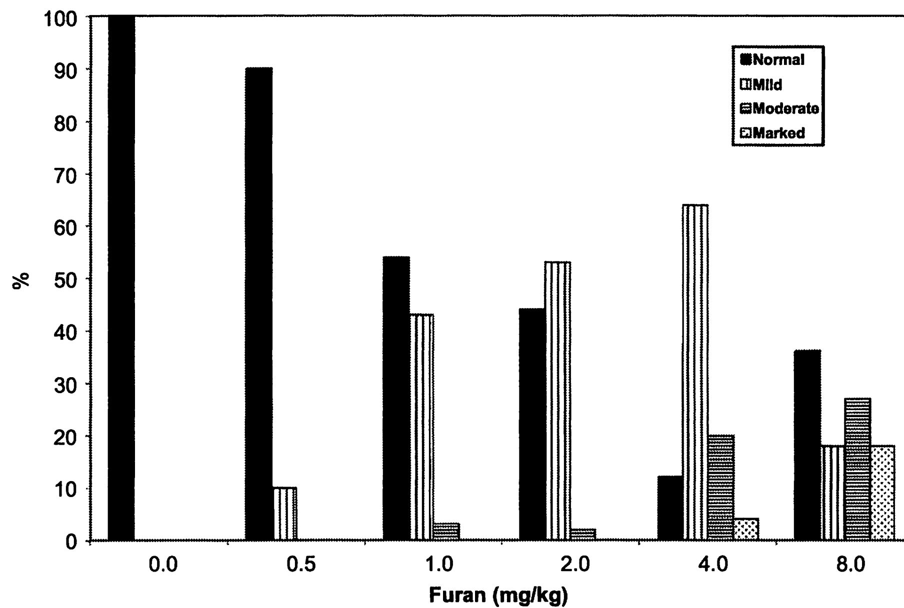

Fig. 3. Hepatic subcapsular inflammation in female B6C3F1 mice exposed to furan for 24 months. Beginning at approximately 7 weeks of age, female B6C3F1 mice were exposed to furan in corn oil by gavage (5 mL/kg) 5 days/week. H&E-stained liver sections were evaluated by light microscopy. Hepatotoxicity was graded semi-quantitatively as normal, mild, moderate or marked. There was a significant increase in subcapsular inflammation at 1.0, 2.0, 4.0, and 8.0 mg furan/kg bwt.

multiplicity, and latency period of furan-induced hepatocarcinogenicity. However, there was a dose-dependent decrease in the incidence of lymphomas in the liver as a function of exposure to furan ($p = 0.004$). Prevalence of lymphomas was 22, 16, 13, 9, 5, and 3% in mice administered 0.0, 0.5, 1.0, 2.0, 4.0, and 8.0 mg furan/kg bwt, respectively, with a significant decrease in the percentage of mice with lymphomas in animals administered 8.0 mg furan/kg bwt ($p = 0.033$). The latency period for the development of lymphomas in the liver was 431, 439, 272, 575, 575, and 722 days for control mice and mice exposed to 0.5, 1.0, 2.0, 4.0, and 8.0 mg furan/kg bwt, respectively. For most animals, the microscopic identification of lymphoma in the liver was supported by the presence of lymphomas in other organ systems (spleen, thymus, or lymph nodes). There were no trends or significant differences among groups for the presence of histocytic sarcomas or hemangiosarcomas.

Discussion

The purpose of this study was to prospectively test the hypothesis that hepatotoxicity followed by compensatory hepatocyte replication is a driving factor in induction of murine hepatocarcinogenicity, while doses of a toxicant that do not induce sufficient hepatocyte death and regenerative cell proliferation are not hepatocarcinogenic. To test this hypothesis, we utilized furan, a known liver cytotoxicant and carcinogen (Fransson-Steen et al., 1997; NTP, 1993; Wilson et al., 1992). Our studies, conducted under conditions similar to those used in the NTP bioassay but utilizing dose levels not anticipated to be overtly cytotoxic, demonstrated that furan caused some evidence of hepatotoxicity even at low doses, but the lower level of cytotoxicity at doses below 4.0 mg/kg was not associated with increased hepatocarcinogenesis. We also showed there is a threshold for furan induced liver tumors with doses $<$4.0 mg furan/kg bwt not hepatocarcinogenic.

Dose selection was based on results of an unpublished 13-week study (Pilot Study Foley, personal communication) with a 4-week recovery in male mice exposed to 0.5, 2.0, 4.0, 8.0 and 15.0 mg furan/kg bwt. In the 13-week Foley study, there was histologic evidence of cytotoxicity at 8.0 and 15.0 mg furan/kg bwt, transitory enhanced hepatocyte proliferation at dose levels as low as 2.0 mg/kg, and persistent enhanced hepatocyte proliferation at 8.0 and 15.0 mg/kg by week 13. There was no evidence of either hepatic cytotoxicity or enhanced hepatocyte proliferation following a 4-week recovery indicating a reversible phenomenon at all dose levels at 13 weeks of exposure. Thus, there was an increased incidence of liver tumors at doses of furan that showed reversibility in liver cytotoxicity at 13 weeks. Because male $B6C3F_1$ mice have a relatively high (52%) spontaneous incidence of hepatocellular neoplasia, we elected to conduct our study in female $B6C3F_1$ mice where the spontaneous incidence (14%) is appreciably lower, and thus the ability to detect minor changes is increased.

To confirm that female mice are as susceptible as male mice to furan exposure, we conducted a 3-week cell proliferation study in female B6C3F$_1$ mice utilizing furan doses of 0.0, 0.5, 1.0, 2.0, 4.0, and 8.0 mg furan/kg bwt. In females, we found focal evidence of cytotoxicity involving subcapsular hepatocytes at all dose levels (Fig. 1) with only minimal effects in 2 of 10 mice at 0.5 mg furan/kg bwt. This observation is taken as evidence that all dose levels used resulted in hepatocytes being exposed to furan or its metabolite. In female mice, statistically significant elevated ALT levels and liver subcapsular inflammation and cytological degeneration were present histologically at 1.0, 2.0, 4.0, and 8.0 mg furan/kg bwt. Additionally, there was a statistically significant increased BrdU LI at 8.0 mg furan/kg bwt.

Our data demonstrated dose-dependent hepatic cytotoxicity at 3 weeks and 2 years using increased ALT levels and LI at 3 weeks, and increases in grossly visible and microscopic liver lesions at 2 years. Overall, these data support a strong association among furan-induced hepatic cytotoxicity, compensatory hepatocyte proliferation, and hepatocarcinogenicity. There was a high correlation between subcapsular hepatocyte necrosis and inflammation and elevated serum ALT levels. Preneoplastic foci of cellular alteration and neoplastic liver lesions were significantly increased only at 4.0 and 8.0 mg furan/kg bwt. Dose levels of 1.0 and 2.0 mg furan/kg bwt produced hepatic cytotoxicity without detectable compensatory hepatocyte proliferation at 3 weeks. A furan dose level of 4.0 mg/kg bwt produced an increased LI, although not statistically significant, and increased the incidence of hepatocellular foci and adenomas, but not carcinomas. The dose level of 8.0 mg furan/kg bwt produced a highly significant increase in the incidence of benign and malignant liver tumors, increased multiplicity, decreased latency of preneoplastic foci of cellular alteration and benign tumors, and increased hepatotoxicity and compensatory cell proliferation.

Although a sufficient dose of furan or its cytochrome P450-induced metabolite *cis*-2-butene-1,4-dialdehyde (Burka et al., 1991) is clearly cytotoxic, genotoxicity assays provide a mixed positive and negative response. Given that carcinogenesis is a highly complex process, it is possible that furan-induced hepatocarcinogenicity is a result of both a genotoxic and chronic cytotoxicity mode of action, and the relative role of the two modes of action would vary with dose, treatment duration, species, sex, strain, and environmental conditions. With some genotoxic and non-genotoxic agents, induction of cell proliferation in the target tissue appears to be closely associated with the development of cancer (Cohen et al., 1991). DNA replication following a genotoxic-induced alteration can "fix" the alteration into a permanent heritable change. Chloroform is an example of a cytotoxic rodent carcinogen (Butterworth et al., 1995). After exposure to chloroform, liver tumors in rodents were observed under dosing conditions and at doses that also produced cell death. An expert panel convened to evaluate the US Environmental Protection Agency's "Proposed Guidelines for Carcinogen Risk Assessment" unanimously agreed that for chloroform a mode of action involving obligatory cytotoxicity as a precursor to cancer was plausible. The liver effects presented here and seen in other studies are similar to the chloroform-induced liver findings; exposure to 8.0 and/or 15.0 mg furan/kg bwt elevated the level of liver enzyme levels in serum in female mice and LI in male and female mice (Fransson-Steen et al., 1997; Wilson et al., 1992). Collectively, these previously reported data show that exposure to 8.0 mg furan/kg bwt or higher doses are cytotoxic to the liver, and hepatic cytotoxicity is followed by reparative hepatic proliferation. The degree of hepatic cytotoxicity and reparative proliferation are evidently of sufficient magnitude and adequate duration to be associated with hepatocarcinogenesis at furan doses of 8.0 and 15.0 mg/kg bwt. Our present study identified a significant tumorigenic response at a dose lower than previously reported (4.0 mg furan/kg bwt).

Although the mechanisms of cytolethality are unknown, several investigators have shown a link between carcinogenesis and activation of endonucleases, DNA double-strand breaks, cytolethality, cell proliferation, and induction of mutations (Butterworth et al., 1995; Elia et al., 1993; Mugford and Kedderis, 1997). Improper repair of DNA double-strand breaks may produce cell death or cells with chromatid exchanges, chromosomal aberrations, or mutations (Nicotera et al., 1990). The type and site of mutations in the *ras* genes were different in DNA derived from furan-induced tumors as compared to spontaneous liver tumors (Reynolds et al., 1987). The unique mutations in the *Ha-ras* gene may be secondary to errors in DNA repair or errors concurrent with increases in the number of DNA replications.

In addition to inducing necrosis, cytotoxicants like furan and acetaminophen may also induce cytolethality through DNA fragmentation and apoptosis (Ledda-Columbano and Columbano, 1991; Fransson-Steen et al., 1997; Ray et al., 1993) or inflammation in conjunction with apoptosis (Bursch et al., 1986). The apoptotic index was significantly increased in female B6C3F$_1$ mice treated with 8.0 and 15.0 mg furan/kg bwt, but not 4.0 mg furan/kg bwt (Fransson-Steen et al., 1997).

Similar to the previous findings (NTP, 1993), a negative correlation of the incidence of lymphomas and furan dose was observed in this study. Since the greatest percentage of lymphomas was found in control mice which exhibited no liver cytotoxicity and the lowest LI, there is no apparent relationship among the presence of hepatic cytotoxicity, compensatory cell proliferation, and lymphomas. Although it is possible that lymphomas

inhibited the development of furan-induced hepatocellular tumors, this seems unlikely since there were animals that had lymphomas and hepatocellular foci, adenomas, and/or carcinomas. The significance of a decreased incidence of lymphomas with increasing dose of furan is unknown.

The effects of a variety of chemicals in a number of rodent models and in humans with chronic liver disease support a mode of action of carcinogenicity involving persistent cytotoxicity and sustained cell proliferation. A proposed mode of action of the hepatocarcinogenicity of chloroform, carbon tetrachloride, thioacetamide, and acetaminophen, nephrocarcinogenicity of chloroform, and thyroid carcinogenicity of phenobarbital is also sustained cytotoxicity and chronic cell turnover (Ledda-Columbano and Columbano, 1991; Becker, 1983; Hard et al., 2000; McClain, 1989; Tsukamoto et al., 1990). Induction of cholangiocarcinomas in rats by dose levels of furan as low as 2.0 mg/kg bwt is also accompanied by increased hyperplasia and/or cell proliferation (Maronpot et al., 1991). Liver chronically damaged by infectious agents or alcohol also shows increased incidence of liver cancer in humans (Cohen et al., 1991; IARC, 1988, 1994). There is also evidence that human endometrial, breast, ovary, bladder, biliary tract, oral cavity, lung, and skin neoplasms are attributable in part to drugs, viruses, chemicals or physical-induced irritation, all of which can produce cell death that is accompanied by subsequent increased cell turnover (Preston-Martin et al., 1990). Thus, elevated LI data may be a useful indicator of potential tumor formation and cancer risk assessment in rodents and humans in a variety of tissues after exposure to certain cytotoxicants. As with other potential biomakers identified for a complex biological process such as cancer, extreme care must be taken in interpretation of the utility of the biomarker with the realization that multiple factors influence disease outcome.

Risk models extrapolate the toxic and carcinogenic effects seen in rodents to estimate the potential human risk at low exposure concentrations typically encountered in the environment or workplace. Data from studies with the carcinogens chloroform, carbon tetrachloride, and acetaminophen demonstrate tissue, species, and strain differences in susceptibility to chemically induced cytolethality, cell replication, and liver cancer. The significance and relevance of liver tumors in mice for human risk assessment remain controversial; approximately half of agents tested by the NTP and NCI were tumorigenic in the liver of $B6C3F_1$ mice. $B6C3F_1$ mice have hepatocarcinogen sensitivity genes (Drinkwater, 1994) and are inherently sensitive to liver carcinogenesis. The presence of genes that predispose some strains of mice to liver tumors demonstrate that genetic background can greatly influence target organ specificity and tumor outcome and needs to be considered in extrapolating rodent data to human risk assessment. In considering furan cancer risk assessment, all data and tumor types must be considered. Our studies focused solely on the hepatocellular adenomas and carcinomas seen in mice. Little is known about the origin, dose–response characteristics, and potential mechanisms of furan-induced cholangiocarcinomas in rats.

The high incidence of liver tumors often seen only at high doses in chemical rodent carcinogenicity studies is problematic for human health risk assessments. High doses may produce effects that do not occur at the low levels to which humans are exposed. Humans are generally exposed to low doses of furan in food, smog, and cigarette smoke. Occupational exposure also occurs. (IARC, 1995; Maga, 1979). Exposure to doses of furan that do not cause significant hepatic cytotoxicity may not pose a cancer risk to humans. *In vitro* studies have shown similar bioactivation and dosimetry in human and rat hepatocytes (Kedderis and Held, 1996).

In conclusion, our studies demonstrated an association between hepatotoxicity, regenerative cell proliferation and liver tumorigenicity in mice following administration of furan, a known hepatic toxicant and carcinogen.

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