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GENOTOXICITY AND CARCINOGENICITY

Toxicity and carcinogenicity of furan in human diet

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Abstract Furan is formed during commercial or domestic thermal treatment of food. The initial surveys of furan concentrations in heat-treated foods, published by European and US authorities, revealed the presence of relatively high furan levels in coffee, sauces, and soups. Importantly, furan is consistently found in commercial ready-to-eat baby foods. Furan induces hepatocellular tumors in rats and mice and bile duct tumors in rats with a high incidence. Epidemiological studies are not available. It is assumed that *cis*-2-butene-1,4-dial, the reactive metabolite of furan, is the causative agent leading to toxicity and carcinogenicity. Based on this data, furan is classified as a possible human carcinogen. The initial exposure estimates revealed a relatively small margin (~2,000) between human exposure and those furan doses, which induce liver tumors in experimental animals. As this may give rise for concern, in this review, the currently available toxicological and mechanistic data of furan are summarized and discussed with regard to its applicability in assessing the risk of furan in human diet.

Keywords Furan · Dietary exposure · Carcinogenicity · Mechanism of action · Risk assessment

Introduction

Furan (C_4H_4O) (CAS-Nr. 110-00-9) is an organic compound with high volatility and lipophilicity, which serves as an intermediate in the chemical synthesis and preparation of numerous polymers (NTP 1993). Furan is also known as a by-product of high-energy radiation and thermal treatments of foods and is found in a variety of solid foods and beverages that undergo heat treatment (Bolger et al. 2009).

Although the occurrence of furan and its derivatives in heat-treated foods has long been recorded (Maga 1979), it became a potential concern first in the middle nineties, when, based on studies in laboratory animals at high exposures, furan was considered as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC 1995). Since then, monitoring furan levels in foods was initiated by US and EU authorities. The US Food and Drug Administration (FDA) published the results on furan measurements in food in May 2004. The European Food and Safety Authority's (EFSA) Scientific Panel on Contaminants in the Food Chain (CONTAM) published an initial report on this issue in December 2004, and in June 2009, the results on furan monitoring in food from different EU countries were reported (EFSA 2009a). These reports outlined the occurrence of furan in a broad variety of foods, such as coffee, baked products, canned, or jarred foods, including baby foods. The presence of furan in baby foods is of particular attention, as those may comprise an important part of diet for many infants. The initial exposure estimates, made in these reports, revealed the comparatively small margin between human exposure and the furan doses, which induce liver tumors in experimental animals (~2,000). Although a number of studies have been conducted on furan toxicity, the knowledge about the mechanism(s) of tumor induction by furan in laboratory animals remains uncertain. Up to now epidemiological studies investigating a possible association between furan exposure and human cancer have not been reported.

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Therefore, concern still exists, whether furan may also cause cancer in humans through long-term exposure to low levels of furan in foods. The purpose of the present paper was to summarize the currently available toxicological data on furan and to discuss its applicability in human risk assessment.

Furan in human diet

Occurrence of furan in foods

The original monitoring of furan levels in a number of foods that have undergone heat treatment was initiated by the US-FDA. The obtained data were posted on the FDA website (FDA 2004) and is continuously updated. Results were recently summarized by Morehouse et al. (2008). The European data were collected by EFSA from 14 Member States and described in the recent EFSA scientific report (EFSA 2009a). Moreover, the Swiss Federal Office of Public Health have recently published a survey on furan measurements in a wide selection of food items (Zoller et al. 2007).

The major analytical method used in these surveys for furan quantification in foods is headspace gas chromatography/mass spectrometry with the use of an internal standard (isotopically labeled furan). The limit of detection for this method is less than 1 μg/kg for major matrices (Nyman et al. 2006). The detailed protocol of the method is available on the FDA website (US-FDA 2005). An alternative method is a head space/solid phase micro-extraction/gas chromatography/mass spectrometry, with the limit of detection in the low ng/kg range (Bianchi et al. 2006; Goldmann et al. 2005).

According to US and European data, high furan concentrations are found in roasted coffee, in foods that underwent a heat treatment in sealed containers, for example canned and jarred products, as well as in crusty and dry products such as toasted bread or bread crust (Table 1).

Coffee powders contain very high concentrations of furan—up to 5,938 μg/kg were reported by FDA for ground coffee (Morehouse et al. 2008) and up to 6,500 μg/kg by EFSA for 'roasted coffee' (EFSA 2009a). In contrast, the prepared coffee beverages have furan concentrations in the range of 18–88 μg/kg. As reported by Kuballa et al. (2005), the furan concentration in coffee drink depends on the furan content of coffee powder, as well as on the type of coffee brewing procedure. Coffee brews from espresso-type machines have considerably higher amounts of furan than other coffee brews, reaching concentrations of 88 μg/l. Much lower levels were reported in coffee produced by standard home coffee-making machines (18 μg/l) or by manual brewing (20 μg/l, Kuballa et al. 2005). Similar results were reported by Zoller et al. (2007), with the highest furan concentration (199 μg/kg) found in an espresso-type coffee brewed with ground roasted coffee containing almost 6,000 μg/kg furan. According to US-FDA data, the average furan concentration of 52 μg/kg was found in ground coffee, brewed using automatic drip coffee makers (Morehouse et al. 2008).

Table 1 Processed foods with high furan levels

Food type	Furan (μg/kg)	
	Average	Maximum
Baby foods	25[c]	215[c]
Baby foods, containing vegetables	40[b]	93[a], 153[b]
Baby foods, containing fruits	4[b]	16[b]
Infant formula	19[c]	56[c]
Coffee roasted (beans or ground)	1,691[c]	6,500[c]
Coffee roasted (beans)	2,272[c]	4,895[c]
Coffee roasted (ground)	1,113[c]	5,938[b], 5,749[c]
Coffee soluble (powder)	589[c]	2,150[b], 2,200[c]
Meat products	17[a], 22[c]	39[a], 115[c]
Soy sauce	51[a], 25[c]	76[a], 91[b], 78[c]
Sauces	39[a], 12[c]	43[a], 39[b], 120[c]
Soups	24[c]	225[c]
Soups containing meat	88[a]	125[a], 43[b]
Cereal products	14[c]	168[c]
Bread	0.2[a]	1[a], 30[b]
Bread crust	83[b]	193[b]
Toasted bread		18[b]
Vegetables	12[c]	74[c]
Baked beans	84[a], 27[c]	122[a], 80[c]
Nutritional/diet drinks	29[a]	174[a]

[a] Morehouse et al. (2004)—US-FDA data; [b]Zoller et al. (2007)—data from Swiss Federal Office of Public Health; [c]EFSA (2009a)—data summary from 14 EU Member States

Jarred baby foods consistently contain furan at concentrations of 24–28 μg/kg (average values), with mixtures of meat and vegetables showing the highest furan content (up to 153 μg/kg). Self-prepared baby foods, in contrast, do not appear to contain furan (Bianchi et al. 2006; Lachenmeier et al. 2009; Zoller et al. 2007).

At present, the mechanism of furan formation in foods is not well understood, and various precursors have been postulated. There appears to be no correlation between food composition and furan formation, suggesting that furan could be generated from multiple precursors via multiple formation routes, as has been shown in several studies (Becalski and Seaman 2005; Limacher et al. 2007; Perez and Yaylayan 2004; Yaylayan 2009). Potential precursors include carbohydrates, amino acids, lipids, ascorbic acid, and citric acid. The currently available data on furan formation pathways have been presented in detail in recent

reviews (Crews and Castle 2007; Vranova and Ciesarova 2009).

During heating of foods in closed systems, furan is found to accumulate in jars and cans. Due to its volatility, however, heating of these products in open containers would be expected to result in a lower furan exposure from the food as consumed. The effects of domestic cooking on furan content in foods are not sufficiently characterized yet, although this is of particular importance for exposure estimation. Some studies show that furan persists during normal heating procedures, which precede consumption (Hasnip et al. 2006; Lachenmeier et al. 2009), whereas other researchers reported that furan content decreases (reduction to about half the initial content) during warming procedures such as heating in a pot or in microwave oven (Zoller et al. 2007; EFSA 2009b). Large declines of furan in baby foods (>90% loss) were determined when standing for 10 min under continuous stirring in opened containers after heating (Roberts et al. 2008). A recent study shows that food constituents/matrix could play an important role in the retention or release of furan (Van Lancker et al. 2009).

Dietary exposure estimates

Estimates of dietary furan exposure, calculated up to now, are only preliminary since a limited set of data is available, and the real furan concentrations in foods as consumed are not known (the effect of domestic cooking on furan content in the different foods prior to consumption).

The US-FDA assessed the exposure by using the furan-concentration data posted through spring 2007 and the known consumption factors for the various foods and reported a mean and 90th percentile of 0.3 and 0.6 furan/kg bwt and day for the 2+-year-olds, and 0.4 and 1.0 μg furan/kg bwt and day for the 0- to 1-year-olds from the consumption of adult and infant foods, respectively (DiNovi and Mihalov 2007). The brewed coffee was concluded to be the major source of furan in the adult diet (mean 0.15 μg/kg bwt and day). Recently, EFSA estimated the exposure to furan by using analytical data collected by 14 Member States from 2004 to 2009. The mean furan intake for infants (3–12 months old) in Europe was 0.3–1.0 μg furan/kg bwt and day with the highest at 6 and 9 months of age, and the 95th percentile for 6- to 9-month-old infants was between 1.1 and 1.3 μg/kg bwt and day depending on the scenario used. For adults in Europe, the daily average intake and the 95th percentile were estimated to be 0.8 and 1.8 μg/kg bwt, respectively, with coffee as the major dietary furan source.

Based on the data on furan levels in foods, derived from the German national food monitoring, the Federal Institute for Risk Assessment (BfR) estimated the dietary furan exposure for the German population. The average intake for 9-month-old infants, based on the national consumption data for commercial baby foods (DONALD study 1985), was 0.8 μg/kg bwt per day with the 95th percentile of 1.4 μg/kg bwt per day. Due to incomplete data, the daily intake for adults could be estimated only from coffee consumption, and averaged 0.1 μg/kg bwt and day with the 95th percentile of 0.4 μg/kg bwt per day. Thus, the exposure estimates made by BfR for infants were similar to those made by EFSA, while the estimated adult exposure was much below those calculated by EFSA for Germany: mean and 95th percentile of around 1 and 2 μg/kg bwt and day, respectively (EFSA 2009a). Notably, 75% of adult furan exposure in EFSA estimations was attributed to uptake from coffee/tea/cocoa with the average furan level of 80 μg/kg for this food group, which is far higher than the average level of furan in coffee beverage from the German monitoring (24.1 μg/kg) and approximates the furan levels found in espresso-type coffee prepared in automatic machines (Kuballa et al. 2005). An attempt to correct for an overestimation of coffee consumption resulted in a reduced median exposure of between 0.5 and 0.7 μg/kg bwt per day and a mid-range 95th percentile between 1.2 and 1.5 μg/kg bwt per day (EFSA 2009a).

It was also emphasized that further research is needed on the effects of the domestic cooking on the furan content in the different foods prior to consumption and on the impact of food handling past preparation (EFSA 2009a).

Toxicology of furan

The toxicity database of furan is incomplete. The critical toxicological effect of furan is carcinogenicity, and liver is the main target organ of furan-induced toxicity in rodents. In studies of the National Toxicology Program (NTP 1993), furan administrated by gavage in corn oil induced liver tumors in mice and cholangiocarcinomas, hepatocellular tumors, and mononuclear cell leukemia in rats. However, the mechanism of furan-induced carcinogenicity in rodents has not been fully clarified yet. Both genotoxic and non-genotoxic mechanisms have been proposed. There is no data available on reproductive and developmental toxicity, and there is also no scientifically sound data from human studies (see "Human data").

Kinetics and metabolism

Furan has a low polarity and thus can easily pass through biological membranes. As shown in rats, orally administered [2,5-^{14}C]-labeled furan (8 mg/kg bwt in corn oil) was rapidly and extensively absorbed from intestine (Burka et al. 1991). About 80% of radioactivity was eliminated in 24-h post-dosing: 40% in the expired air (14% as

unchanged furan, 26% as CO_2), 22% in feces, and 20% in the urine. HPLC analysis of 24-h urine revealed extensive metabolism: at least 10 metabolites were detected, none of which, however, have been identified at that time. From the radioactivity remaining in the tissues 24 h post-dosing (19% of the dose), the main portion was found in liver (13% of the dose) followed by kidney and gastrointestinal tract (together <1%). The tissue-bound radioactivity in the liver was found to be associated with protein, but not with DNA. In a multiple-dose study (8 mg furan/kg bwt per day for 8 days by gavage), the part of total administrated radioactivity eliminated in urine increased to 33%, and amount of tissue-bound radioactivity increased in the liver and kidneys four- and sixfold, respectively (Burka et al. 1991).

The liver possess high capacity to eliminate furan from the bloodstream by first-pass metabolism (Kedderis and Held 1996). Furan is rapidly metabolized by cytochrome P-450 (CYP) enzymes to the α,β-unsaturated dialdehyde, *cis*-2-butene-1,4-dial, identified as the major primary metabolite (Chen et al. 1995; Peterson et al. 2005) (Fig. 1). Biotransformation of furan was studied in freshly isolated rat hepatocytes in vitro (Kedderis et al. 1993). This study indicated that the furan metabolism, determined by measuring the disappearance of furan from the incubation medium, was a single saturable process with a K_m of 0.4 μM and V_{max} of 0.02 μmol/h/10^6 cells in rat hepatocytes. Subsequent studies with hepatocytes yielded a K_m of 1.0 μM and V_{max} of 0.05 μmol/h/10^6 cells for mouse and a K_m in the range of 2.1–3.3 μM and V_{max} in the range of 0.02–0.04 μmol/h/10^6 cells for human hepatocytes, demonstrating that metabolism of furan is a rapid process in rodents as well as in humans (Kedderis and Held 1996). These kinetic data and physiological parameters from the literature were used to develop a human dosimetry model for furan. Using PBPK modeling (simulation of inhalational exposure to 10 ppm furan for 4 h) leading to an absorbed dose of approximately 1 mg/kg bwt, Kedderis and Held (1996) demonstrated rates of furan oxidation in human liver to be almost 40-fold greater than the rate of blood flow delivery of furan to the liver. This indicates that the rate-limiting step in metabolic clearance of furan is the delivery of furan to the liver rather than the metabolic capacity.

Modulation of CYP activity in isolated rat hepatocytes with ethanol, 1-phenylimidazole, and 1-aminobenzotriazole demonstrated that this enzyme system is a catalyst of furan oxidation. Furthermore, a fivefold increase in the rate of the hepatocytes oxidation of furan following acetone pre-treatment of the rats suggested an important role of CYP2E1 (Kedderis et al. 1993). Moreover, cytotoxic effects of furan in rat hepatocytes, such as reduction of cell viability and glutathione (GSH) depletion as well as ATP depletion and uncoupling of oxidative phosphorylation, were inhibited by CYP inhibitors such as 1-phenylimidazole and induced by acetone pre-treatment of animals (Carfagna et al. 1993; Mugford et al. 1997).

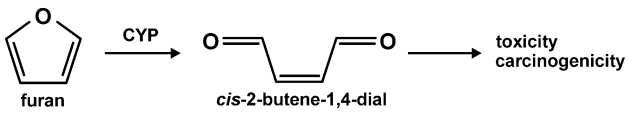

Fig. 1 Bioactivation of furan

Due to its reactivity, the furan oxidation product *cis*-2-butene-1,4-dial is difficult to detect directly in microsomal incubations, but could be trapped either as *bis*-semicarbazone derivative or as GSH reaction product (Chen et al. 1995, 1997; Peterson et al. 2005). Recent in vitro studies with rat liver microsomes (from both untreated and acetone-pretreated Fisher-344 rats) as well as human CYP2E1 supersomes indicated that CYP2E1 is an efficient catalyst for the furan oxidation, but other CYPs may also be involved in the metabolic activation of this compound (Peterson et al. 2005). In this study, the kinetic measurements of furan oxidation yielded a K_m of 37.6 μM, which was at least an order of magnitude higher than that reported from the hepatocytes experiments by Kedderis et al. (1993). The authors suppose that one of the possible reasons for such discrepancy could be the difference in methods used: measuring the metabolic formation of cis-2-butene-1,4-dial—GSH conjugate in their study compared to measuring the disappearance of furan in hepatocytes studies by Kedderis et al. (1993). They argue that, while the oxidation of furan to *cis*-2-butene-1,4-dial is the actual rate-determining step in the formation of the GSH conjugate, a large variety of rate-determining steps comprise the kinetics observed in hepatocytes studies (Peterson et al. 2005).

Cis-2-Butene-1,4-dial is a highly reactive compound and is considered to be the ultimate (geno)toxic intermediate in furan-treated animals. It can bind covalently to cellular nucleophiles including proteins and nucleosides. The biotransformation of furan to a protein-binding intermediate was observed in earlier studies both in vivo and in vitro (Burka et al. 1991; Parmar and Burka 1993). Compounds such as GSH were able to inhibit this binding (Parmar and Burka 1993). Studying protein alkylation by incubation of *cis*-2-butene-1,4-dial with model protein nucleophiles (*N*-acetyl-L-lysine, *N*-acetyl-L-cysteine and GSH) turned out to be complex and multistep reactions, including formation of pyrrole and pyrrolin-2-one derivatives (Chen et al. 1997). Moreover, *cis*-2-butene-1,4-dial was found to cross-link amino acids like lysine (amino group) and cysteine (thiol group), suggesting that it is a good candidate for the protein reactive microsomal metabolite of furan (Chen et al. 1997). When *cis*-2-butene-1,4-dial reacts with thiols, either via 1,2- or 1,4-addition, the resulting product still retains reactivity toward nucleophiles. The subsequent reaction with an

amino group yields stable pyrrole adducts. In the presence of excess GSH, bis-GSH conjugates were the major reaction products. Mono-GSH conjugates were also formed, resulting from an intramolecular cross-link between the cysteinyl thiol and glutamate amino group of the same GSH molecule (Chen et al. 1997; Peterson et al. 2005).

Recently, several urinary furan metabolites have been identified in furan-treated rats. Peterson et al. (2006) reported the presence of the mono-GSH conjugate of *cis*-2-butene-1,4-dial among 19 furan-derived metabolites, detected in the 24-h urine of rats treated with furan (8 mg/kg in corn oil, gavage). No bis-GSH conjugates or their further metabolites were found in the urine of furan-treated animals, but given their larger molecular size, they are expected to be preferentially excreted via the bile (Peterson et al. 2005). Four additional rat urinary metabolites of furan were characterized by Kellert et al. (2008a, b): one as a downstream reaction product of mono-GSH conjugate of *cis*-2-butene-1,4-dial, and other three were most likely derived from degraded protein adducts. One metabolite was supposed to result from the reaction of *cis*-2-butene-1,4-dial with lysine, and two other metabolites to result from the cross-linking of cysteine and lysine by *cis*-2-butene-1,4-dial (Kellert et al. 2008b). Further in vitro investigations confirmed the ability of *cis*-2-butene-1,4-dial to cross-link GSH to protein lysinyl residues in hepatocytes (Lu et al. 2009). Moreover, immunoblotting analysis of liver and hepatocyte protein extracts using anti-GSH antibodies confirmed the covalent binding of GSH to cellular proteins by *cis*-2-butene-1,4-dial in tissues or cells exposed to furan (Lu et al. 2009). Altogether, these findings demonstrate that *cis*-2-butene-1,4-dial conjugates with GSH to yield an intermediate, which is able to react further with cellular nucleophiles.

The fact that carbon dioxide is a major (26% of the dose) in vivo metabolite of furan (Burka et al. 1991), indicates that *cis*-2-butene-1,4-dial is the subject of extensive further metabolism in vivo. Subsequent oxidation of *cis*-2-butene-1,4-dial would produce maleic acids (*cis*), which might be converted to the citric acid cycle intermediate fumarate (*trans*) that would be oxidized to carbon dioxide or will be a substrate incorporated in other metabolic pathways. Notably, both the possible intermediates have toxicological properties—they are mitochondrial toxins and bind to proteins—and thus could contribute to the overall toxic properties of furan (Burka et al. 1991; Peterson 2006).

Single- and repeated-dose toxicity

Administration of high doses of furan induces severe damage in the liver and kidney of animals, with the liver as the primary target organ. Application of a single intraperitoneal furan dose of 300 mg/kg bwt to adult male Swiss albino mice resulted in centrilobular hepatic necrosis and coagulative necrosis of the proximal convoluted tubules of the outer renal cortex. Pretreatment of mice with CYP inhibitor piperonyl butoxide decreased the incidence and severity of hepatic and renal necrosis (McMurtry and Mitchell 1977). A single furan dose of ~350 mg/kg (i.p. application) caused serious damage to the liver and produced somewhat lesser effects on the kidney of male ICR mice (Wiley et al. 1984).

Liver toxicity and hepatocyte proliferation was investigated by Wilson et al. (1992) following single and repeated oral furan administration to Fischer 344 rats and B6C3F1 mice. In the single-dose study, furan induced hepatotoxicity (manifested as hepatocellular necrosis and marked increase in the activities of plasma liver enzymes—aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase) in the first 24 h following administration in F344 rats (30 mg/kg bwt) and B6C3F1 mice (50 mg/kg bwt). This was followed by an increase in compensatory cell proliferation (measured as increase in labeling indices) 48 h after the treatment. In the repeated-dose studies (furan gavage, 8 mg/kg bwt and day to rats and 15 mg/kg bwt and day to mice, 5 days/week), male and female rats, but not mice, developed cholangiofibrosis with signs of metaplasia in subcapsular areas of the left or caudate lobes of the liver following 6 weeks of furan treatment. Hepatocyte proliferation (measured at weeks 1, 3, and 6) was increased in furan-treated mice and rats. Authors concluded that non-genotoxic mechanisms involving hepatotoxicity and regenerative hepatocytes proliferation could play an important role in furan-induced hepatocarcinogenesis (Wilson et al. 1992).

Exposure of male Fischer 344 rats for 2 or 3 weeks to furan (0, 15, 30, 45 or 60 mg/kg bwt and day, gavage for 5 days/week) resulted in a rapid development of cholangiofibrosis in the liver of animals receiving the higher doses (30 mg/kg bwt and more). Notably, these effects were localized primary to the middle and caudate liver lobes (Elmore and Sirica 1991).

As preliminary tests for a 2-year carcinogenicity study with F344/N rats and B6C3F1 mice, the repeated-dose studies were performed with furan administration by gavage in corn oil for 16 days and 13 weeks (NTP 1993). In the 16-day study, increased mortality in both species and both sexes were observed after furan doses of 80 mg/kg bwt per day and higher. In rats, mottled and enlarged livers were observed at necropsy in males that received 20, 40, or 80 mg/kg bwt and in females that received 40, 80, or 160 mg/kg bwt furan. No lesions related to furan exposure were observed in mice at any dose. However, no histopathological examinations were conducted in this study. After the 13-week exposure of rats to 0, 4, 8, 15, 30, or 60 mg furan/kg bwt, 9 of 10 male and four of 10 female animals in the highest dosage group died before the end of the studies.

A dose-dependent increase in relative liver- and kidney weights was observed in rats that received 15 mg/kg bwt or more. Lesions of the liver (bile duct hyperplasia and cholangiofibrosis, cytomegaly, degeneration, and necrosis of hepatocytes) were associated with furan administration in all dose groups of rats. The severity of the lesions increased with dose. In addition, kidney lesions (tubule dilatation and necrosis of tubule epithelium) and atrophy of thymus and testis/ovarian were found in the high-dose group of rats (60 mg/kg). No chemical-related deaths were observed in mice exposed for 13 weeks to furan (0, 2, 4, 8, 15, 30, or 60 mg/kg bwt). Relative and absolute liver weights in mice of both sexes were dose-dependently increased in male mice that received 15 or 30 mg/kg bwt and in females that received 30 or 60 mg/kg bwt. Similar liver findings, as were seen in rats, were observed in mice exposed to 8 mg/kg bwt furan and higher. Thus, the NOAEL in this study was 4 mg/kg and day for mice, and adverse effects were observed at all dose levels tested in the rat (NTP 1993; MAK 2006). Clinical chemistry was not examined in this study.

In a 3-week study with female B6C3F1 mice, hepatocyte proliferation and apoptosis were analyzed following furan exposure (gavage, 0, 4, 8, and 15 mg/kg bwt and day, 5 days/week). Furan treatment resulted in a two- to three-fold increase in serum levels of liver-related enzymes (alanine aminotransferase and sorbitol dehydrogenase) and bile acids compared with control animals. These changes were accompanied by minor subcapsular inflammation and minimal necrosis at 8 and 15 mg furan/kg bwt. The apoptotic index, determined by morphological examination of stained liver sections, was increased 6- to 15-fold compared to control in animals received 8 and 15 mg furan/kg bwt, respectively. In addition, a dose-dependent increase in labeling index (LI) was observed in all groups of furan-treated mice. Co-treatment of mice with the CYP inhibitor aminobenzotriazole prevented the observed hepatotoxic effects induced by furan (Fransson-Steen et al. 1997).

Furan-induced hepatotoxicity was thoroughly evaluated in a recent 3-week study on female B6C3F1 mice with doses much lower than those used in the NTP study (Moser et al. 2009). Following furan exposure (0, 0.5, 1, 2, and 8 mg/kg bwt and day, gavage, 5 days/week for 3 weeks), various toxicity parameters and cell proliferation (measured by BrdU LI) were examined in the liver of the animals. Hepatotoxicity was defined as an increased incidence or severity of histologic parameters (subcapsular or parenchymal inflammation or cytological degeneration, and/or infiltration with lymphocytes or mixed inflammatory cells) and/or increased serum levels of alanine aminotransferase (ALT). No chemical-related deaths were observed during the study, and no treatment-related gross lesions were noted at the 3-week necropsy. A statistically significant increase in relative liver weight was found only in mice exposed to the highest furan dose (8 mg/kg bwt). Furthermore, a statistically significantly increased incidence of hepatic cytotoxicity and serum ALT levels were observed in mice exposed to 1 mg/kg bwt and higher dose levels when compared to control animals. Hepatocyte proliferation (BrdU LI) was found to be significantly elevated only in mice exposed to 8 mg/kg bwt furan. Thus, the dose of 0.5 mg furan/kg bwt and day could be considered as NOAEL for hepatotoxicity.

Furan hepatotoxicity: mechanistic studies

Incubation of isolated hepatocytes from Fischer 344 rats in suspension with furan (2–100 μM) for 1–4 h with subsequent culturing for 24 h resulted in cytolethality (5–70%) and glutathione (GSH) depletion in an exposure time- and concentration-dependent manner (Carfagna et al. 1993). Both effects were prevented by addition of CYP inhibitor 1-phenylimidazole to the cultures and were enhanced in hepatocytes from rats that had been pretreated with acetone to induce CYP2E1. In another in vitro study with isolated Fischer 344 rat hepatocytes conducted by the same working group (Mugford et al. 1997), furan was found to produce concentration- and incubation-time-dependent irreversible ATP depletion, which occurred prior to cell death and was prevented by including 1-phenylimidazole in the suspensions. In the same study, rats were treated by gavage with furan (0, 8, 15, or 30 mg/kg bwt) and killed 24 h later to prepare hepatic mitochondria to assess for respiratory activity. Furan was seen to produce an uncoupling of hepatic mitochondrial oxidative phosphorylation that was prevented by pre-treating the rats with 1-phenylimidazole. The same results were also found in mitochondria from hepatocytes treated with furan in vitro (Mugford et al. 1997). Further studies of this laboratory have found that incubating isolated rat hepatocytes with toxic furan concentrations (100 μM) produced DNA double-strand breaks, which were observed already after 30-min exposure and were repaired in the surviving cells by 24 h in culture. Addition of 1-phenylimidazole as well as endonuclease inhibitor aurintricarboxylic acid to the incubations reduced the formation of furan-induced DNA double-strand breaks. Unfortunately, further information is not available, as these data were published only as abstracts (Mugford and Kedderis 1996, 1997; Ploch et al. 1999). The results were, however, summarized in a review by Kedderis and Ploch (1999). The authors conclude that metabolically activated furan rapidly deplete hepatic ATP via irreversible uncoupling of oxidative phosphorylation, leading to the activation of endonucleases that produce double-strand breaks in DNA prior to cell death. Authors hypothesized that erroneous repair of some of the DNA double-strand breaks in the surviving hepatocytes leads to mutational events involved in furan-induced tumor development (Kedderis and Ploch

1999). In another abstract, Ploch and Kedderis (2001) reported that furan induced a 1.8-fold increase in DNA double-strand breaks in liver cells following in vivo exposure of male Fischer 344 rats (5 mg furan/kg bwt, liver removed after 4 h).

Genotoxicity

Genotoxicity of furan in vitro

Furan was not mutagenic in the Ames *Salmonella typhimurium* assay with and without S9 in TA98, TA100, TA1535, and TA1537 (NTP 1993), but was weakly positive in TA100 in another study by Lee et al. (1994) with and without S9 activation. Furan was reported to be positive in the thymidine kinase gene mutation assay with L5178 mouse lymphoma $tk^{\pm}$ cells in the absence of S9 activation (McGregor et al. 1988, NTP 1993). However, analysis of this data reveals a large difference between the three trials. A negative response was seen in the first trial, with furan concentrations up to 2,000 μg/ml. In a second trial, a dose-related mutagenic response was observed at furan 2,600 μg/ml and higher, which, however, was accompanied by reduction of relative total growth (50% and lower). In a third trial a mutagenic response was seen already at furan concentration of 1,139 μg/ml. Nevertheless, authors concluded that furan was mutagenic in this assay. In a recent study in L5178 mouse lymphoma $tk^{\pm}$ cells by Kellert et al. (2008a, b), no genotoxic effects were observed for furan concentrations up to 3,100 μM, when tested in thymidine kinase gene mutation assay, micronucleus test as well as comet assay.

Furan was reported positive in several in vitro tests for clastogenicity, such as induction of chromosomal aberrations (CA) and sister chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells (NTP 1993). In one of the three trials reported for the SCE-test, furan (26-h exposure) induced a positive SCE response at concentrations of 1.6–160 μg/ml in the absence of an activation system. In the second trial, conducted likewise in the absence of S9, a weak treatment-related increase in SCE was observed only at 160 μg/ml furan. In the third trial, the cells were exposed to furan in the presence of S9 activation system for 2 h, and a weak positive response was observed only at the highest furan concentration tested (500 μg/ml). In chromosomal aberrations test, furan was positive at concentrations of 100–500 μg/ml in two trials conducted in the absence of S9 (10-h incubation with furan). In the only one trial of chromosomal aberrations test, conducted in the presence of S9, the positive response was observed at concentrations of 500 and 1,000 μg/ml (2-h exposure to furan). No detailed information on cytotoxicity in the presence of furan was reported for these studies. Moreover, the frequencies of SCE and chromosomal aberrations were higher in the absence of S9 activation system, than in its presence. One possible reason for this observation could be the rapid reaction of the furan metabolite with the proteins of the S9 mix (see "Kinetics and metabolism"). However, in another study on CHO cells, furan caused a dose-dependent increase in chromosome aberrations only in the presence of S9 metabolic activation at a concentration range of 25–250 mM (Stich et al. 1981). The results in this report are presented only in graphical form, and no details on furan cytotoxicity are provided.

Genotoxicity of furan in vivo

Furan was not mutagenic in the sex-linked recessive lethal assay in germ cells of male *Drosophila melanogaster* (Foureman et al. 1994). Furan did not induce unscheduled DNA synthesis in mouse or rat hepatocytes after single oral application at doses up to 200 mg/kg bwt in male Fischer 344 rats and 100 mg/kg bwt in male B6C3F1 mice (Wilson et al. 1992). No induction of SCE was observed in bone marrow cells of male B6C3F1 mice, following intraperitoneal application of 25–350 mg/kg bwt (NTP 1993). Furan was reported to induce structural chromosomal aberrations in bone marrow cells of male B6C3F1 mice. However, the positive results were observed only at the highest dose tested (250 mg/kg bwt, i.p.), and no information on toxicity in the experimental animals was reported in this study (NTP 1993). In another in vivo study (Wilson et al. 1992), the single oral dose of 250 mg/kg to male B6C3F1 mice resulted in extensive liver necrosis and was reported to be at the limit of lethality to the animals. Thus, these positive in vivo genotoxic effects in bone marrow assays were obtained at dose levels associated with high toxicity and may therefore not to be of special relevance.

Negative results were obtained from the in vivo micronuclei-tests: no increase of micronucleated erythrocytes was detected after single intraperitoneal or subcutaneous furan administration to male Balb/C mice (up to 300 and 275 mg/kg bwt, respectively) or intraperitoneal injection of furan to male CBA mice (up to 225 mg/kg bwt) (Durling et al. 2007). In a recently published study, the genotoxic potential of furan was assessed in splenocytes of B6C3F1 mice, treated for 4 weeks with 2, 4, 8, and 15 mg furan/kg bwt by gavage (Leopardi et al. 2009). The authors' expectation was that differently from the proliferating bone marrow, in which furan produced negative results, quiescent spleen lymphocytes may accumulate unrepaired DNA damage during subchronic exposure of animals. Indeed, a statistically significant increase of micronucleated cells was detected in the mitogen-stimulated splenocytes of mice following 4-week administration of 4, 8, and 15 mg furan/kg bwt. Furthermore, a significant increase in foci of phosphorylated histone γ-H2AX, a marker for DNA double-strand

breaks generation in the cell, was observed in mitogen-stimulated splenocytes of animals treated with the two highest furan doses. Concurrently, no effect of the in vivo furan exposure was detected when γ-H2AX foci were scored in freshly isolated quiescent splenocytes. The researchers hypothesized that the clastogenic effect of furan, observed in the micronucleus test, could result from induction of DNA–DNA or DNA–protein cross-links by furan metabolite *cis*-2-butene-1,4-dial (see "Genotoxicity of furan metabolite *cis*-2-butene-1,4-dial"), which in turn could lead to the arrest of the DNA replication and subsequent formation of double-strand breaks. However, analysis for induction of cross-links in freshly isolated splenocytes by alkaline comet assay yielded negative results, with both standard and radiation-modified protocols. In this regard, authors mentioned that also other researchers (Kellert et al. 2008a) failed to show an induction of cross-links by *cis*-2-butene-1,4-dial, when testing for reduction of comet tail length in irradiated mammalian cells. Accordingly to studies of Speit and colleagues, the sensitivity of both standard and modified protocols of comet assay could be considerably low for detection of certain kinds of cross-links (Merk and Speit 1999; Speit et al. 2008).

Genotoxicity of furan metabolite cis-2-butene-1,4-dial

In contrast to furan, its metabolite *cis*-2-butene-1,4-dial was directly mutagenic at non-toxic concentrations in the *Salmonella typhimurium* TA104, a strain sensitive to aldehydes (Peterson et al. 2000). It was negative in strains TA97, TA98, TA100, and TA102. When compared to other reactive aldehydes, the mutagenic activity of *cis*-2-butene-1,4-dial was similar to that of crotonaldehyde, acrolein and glyoxal. Furthermore, the incubation of *cis*-2-butene-1,4-dial with glutathione prior to addition of bacteria inhibited the genotoxic as well as cytotoxic activity of the metabolite.

In cell-free systems, *cis*-2-butene-1,4-dial has been shown to react with deoxyribonucleosides 2′-deoxycytidine (dCyd), 2′-deoxyriboguanosine (dGuo), and 2′-deoxyadenosine (dAdo) to form adducts (Byrns et al. 2002; Gingipalli and Dedon 2001). Thymidine did not react with *cis*-2-butene-1,4-dial in these studies. While the initially formed dCyd adducts were relatively stable, dGuo and dAdo reaction products were unstable and decomposed to secondary products. The authors also mentioned that the relative reactivity and the rate of reaction of *cis*-2-butene-1,4-dial with deoxyribonucleosides paralleled those observed for formaldehyde (Byrns et al. 2002).

Further analysis on the basis of UV absorbance, fluorescence, 1H NMR, and mass spectral data indicated that primary dAdo and dGuo adducts undergo dehydration to form substituted etheno products (Byrns et al. 2004). It was emphasized that these secondary products retain a reactive aldehyde and, therefore, can be expected to generate DNA–DNA and/or DNA–protein cross-links. In line with these suggestions are the findings of Marinari et al. (1984), where induction of DNA cross-links and DNA single-strand breaks were detected via alkaline elution assay in CHO cells after exposure to millimolar *cis*-2-butene-1,4-dial concentrations (Marinari et al. 1984).

Recently, a sensitive detection assay was developed for *cis*-2-butene-1,4-dial-derived adducts in DNA, which includes their derivatization with *O*-benzylhydroxylamine and subsequent analysis by liquid chromatography electrospray tandem mass spectrometry (Byrns et al. 2006). Using this assay, the dCyd and dAdo adducts were detected in bacterial DNA isolated from *Salmonella typhimurium* TA104 after exposure to mutagenic *cis*-2-butene-1,4-dial concentrations. However, the efficiency of the *cis*-2-butene-1,4-dial reaction with DNA was much lower in bacteria than with isolated calf thymus DNA in vitro. The latter observation would point out the important protective role of cellular nucleophiles in the bacterial cell, which compete with DNA for the reaction with *cis*-2-butene-1,4-dial.

The genotoxic potential of *cis*-2-butene-1,4-dial was assayed in a recent in vitro study with L5178Y mouse lymphoma cells (Kellert et al. 2008a). Due to remarkable cytotoxicity of the compound, the tested dose range was limited to 25 μM. At this concentration, *cis*-2-butene-1,4-dial was positive in comet assay (1.6-fold increase in comet tail length above control) and thymidine kinase gene mutation assay (2.4-fold increase in mutation frequency above control). No significant effects were observed in the micronucleus test. Test for DNA-cross-linking activity by determining the reduction of the γ-ray-induced DNA mitigation in the modified comet assay was likewise negative (Kellert et al. 2008a). However, it should be stressed that according to the studies with other cross-linking agents, such as mitomycin C and cisplatin, the modified comet assay is not optimally suited for the evaluation of certain types of cross-linkers (Merk and Speit 1999; Speit et al. 2008).

Carcinogenicity studies

The carcinogenicity of furan was examined in the NTP 2-year gavage study with Fischer-344 rats and B6C3F1 mice of each sex (NTP 1993). Furan was administered in corn oil 5 days per week at doses of 0, 2, 4, or 8 mg/kg bwt and day to rats, and 0, 8 or 15 mg furan/kg bwt and day to mice, respectively. As summarized in Tables 2 and 3, furan treatment resulted in neoplastic lesions localized primarily in the liver. Significantly increased incidence of hepatocellular adenomas and carcinomas were found in both genders

Table 2 Incidence of neoplasmic lesions in the 2-year carcinogenicity study in Fischer 344 rats

Furan dose, mg/kg bwt	0		2		4		8	
	♂	♀	♂	♀	♂	♀	♂	♀
Liver neoplasms, %								
Cholangiocarcinomas	0	0	86*	98*	96*	100*	98*	96*
Hepatocellular adenomas	2	0	8	4	36*	8*	54*	14*
Hepatocellular carcinomas	0	0	2	0	12*	0	36*	2
Hepatocellular adenomas and carcinomas	1	0	10	4	44*	8*	70*	16*
Mononuclear leukemia, %	16	16	22	18	34*	34*	50*	42*

Substance application in corn oil by gavage, 5 days/week for 2 years; 50 animals were examined per group of each sex. Historical incidence of hepatocellular adenoma or carcinoma: 2.5 ± 2.8%, range 0–10% (males), 1.2 ± 2.7%, range 0–10% (females); historical incidence of mononuclear leukemia: 21.3 ± 8.9%, range 4–38% (males), 26.8 ± 7.0%, range 16–38% (females)

Reference: NTP (1993)

* Statistically significant increase when compared to control ($P < 0.05$) by the Fisher's exact test

Table 3 Incidence of neoplasmic lesions in the 2-year carcinogenicity study in B6C3F1 mice

Furan dose, mg/kg bwt	0		8		15	
	♂	♀	♂	♀	♂	♀
Liver neoplasms, %						
Hepatocellular adenomas	40	10	66*	62*	84*	96*
Hepatocellular carcinomas	14	4	64*	14	68*	54*
Hepatocellular adenomas and carcinomas	52	14	88*	68*	100*	100*
Benign pheochromocytoma of adrenal medulla, %	2	4	12*	2	20*	12*

Substance application in corn oil by gavage, 5 days/week for 2 years; 50 animals were examined per group of each sex. Historical incidence of hepatocellular adenoma or carcinoma: 35.1 ± 11.0%, range 14–52% (males), 10.1 ± 4.3%, range 2–16% (females); historical incidence of benign pheochromocytoma: 2.7 ± 1.6%, range 0–4% (males), 1.5 ± 2.4%, range 0–38% (females)

Reference: NTP (1993)

* Statistically significant increase when compared to control ($P < 0.05$) by the Fisher's exact test

of mice and in male rats, as well as a dose-related increase in the incidence of hepatocellular adenomas in female rats. Additionally, cholangiocarcinoma of the liver occurred in all groups of dosed rats (almost 100% incidence already at the lowest furan dose, Table 2). Furthermore, the incidence of mononuclear leukemia was increased in rats at 4 and 8 mg/kg dose levels, and the incidence of benign pheochromocytomas of the adrenal gland was increased in high-dosed mice. Simultaneously, high incidences of numerous non-neoplastic liver lesions were observed in all groups of furan-treated rats and mice. These lesions included biliary tract hyperplasia, which were usually accompanied by inflammation, fibrosis and cysts, liver cytomegaly, degeneration, necrosis, nodular hyperplasia, and vacuolization. Moreover, inflammation and hyperplasias of the forestomach as well as nephropathy (severity grade increased with the dose) were observed in treated rats and mice (NTP 1993).

Thus, in the 2-year NTP carcinogenicity studies, furan induced cytotoxic and carcinogenic effects at all doses tested in rats and mice, and no statements can be made about no-observed-adverse-effect-levels (NOAEL). Nevertheless, it can be concluded that rats react more sensitive to furan than mice.

Additionally, a separate "stop-exposure" carcinogenicity assay was conducted within the NTP study, where male rats (50 animals) were dosed with 30 mg furan/kg bwt in corn oil by gavage, 5 days/week, for 13 weeks and then maintained for the remainder of the 2 years without additional furan administration (NTP 1993). Groups of 10 animals were evaluated at the end of the furan administration period, and at 9 and 15 months. Cholangiocarcinoma and hepatocellular carcinoma occurred with an overall incidence of 100% (40/40) and 15% (6/40), respectively, in all rats that survived at least 9 months.

Based on the results from the NTP studies, IARC has classified furan as "possibly carcinogenic to humans" (group 2B, IARC 1995). The German MAK Commission classified furan in carcinogenicity category 2 (substances, considered to be carcinogenic to humans, MAK 2006).

The histopathology of liver injury and carcinogenesis induced by hepatotoxic furan doses was intensively studied in male Fischer 344 rats. Whereas short-term furan exposure (gavage for 2–3 weeks, 15–60 mg/kg bwt and day, 5 days/week) yielded induction of small intestinal metaplasia with subsequent cholangiofibrosis, which were essentially localized to the caudate and right liver lobes, the longer-term chronic furan administration (gavage for 9–19 weeks, 30 mg/kg bwt and day, 5 days/week) resulted in the preferential development of primary liver tumors (cholangiocarcinomas) which arose at 70–100% incidence from right/caudate liver lobes and were histologically characterized by small intestinal-like mucosal cell differentiation. The incidences of primary hepatocellular carcinomas ranged from 0 to 20% in the chronic exposure study. The authors concluded that the small intestinal metaplasia and subsequent cholangiofibrosis developing in rats after short-term furan exposure represent the early changes relevant to the development of intestinal-type cholangiocarcinomas which occur with high incidences in rats after long-term furan exposure (Elmore and Sirica 1991, 1993).

In a recently published 2-year carcinogenicity bioassay (Moser et al. 2009), female B6C3F1 mice were exposed to

Table 4 Incidence of liver neoplasms in the 2-year carcinogenicity study in female B6C3F1 mice

Furan dose, mg/kg bwt	0	0.5	1.0	2.0	4.0	8.0
Animals, examined microscopically, *n*	36	72	53	41	36	39
Liver neoplasms						
Hepatocellular adenomas	3/36 (8%)	4/72 (6%)	4/53 (8%)	4/41 (10%)	11/36 (31%)*	25/39 (64%)*
Hepatocellular carcinomas	0/36 (0%)	4/72 (6%)	2/53 (4%)	1/41 (2%)	2/36 (6%)	11/39 (28%)*
Hepatocellular adenomas or carcinomas	3/36 (8%)	8/72 (11%)	6/53 (11%)	5/41 (12%)	12/36 (33%)*	29/39 (74%)*

Furan was applied in corn oil by gavage, 5 days/week for 2 years

Reference: Moser et al. 2009

* Statistically significant increase when compared to control ($P < 0.05$), by the *F*-test

furan under conditions similar to those used in the NTP bioassay, but utilizing lower furan concentrations (0, 0.5, 1, 2, 4 or 8 mg furan/kg bwt and day, by gavage in corn oil, 5 days/week). Male B6C3F1 mice were not used in this study, because they have a high spontaneous incidence of liver tumors (52%), when compared to female mice (14%, see Table 3). As summarized in Table 4, a statistically significant increase in the incidence of hepatocellular adenomas and carcinomas occurred in animals given 4 and 8 mg furan/bwt and day, but not in mice dosed with 0.5, 1.0, or 2.0 mg furan/bwt and day. Concurrently with hepatocarcinogenicity, induction of hepatic toxicity and cell proliferation by furan were examined in the livers of treated animals. Hepatic toxicity, characterized by parenchymal degeneration and inflammation, hepatocyte necrosis and inflammation localized on visceral surfaces of the liver in contact with the forestomach, was seen at 1.0 mg/kg bwt and higher furan doses. Cell proliferation, measured by BrdU labeling index, was significantly increased at 8.0 mg/kg. From this study, a NOAEL for the tumorigenic effect of 2 mg furan/kg bwt and day and a NOAEL for hepatotoxicity of 0.5 mg furan/kg bwt and day for female mice can be derived. The authors conclude that their data give an evidence for a dose–response relationship between furan-induced hepatic cytotoxicity, regenerative cell proliferation, and subsequent liver tumor formation in furan-exposed female mice (Moser et al. 2009).

Mutations of H-*ras* oncogene in furan-induced tumors

To clarify the mechanisms of tumor induction by furan, Reynolds et al. (1987) transfected DNA from hepatocellular adenomas and carcinomas from control and furan-treated mice from the NTP 2-year study into NIH3T3 cells and examined resulting foci for the presence of activated oncogenes and activating mutations. While the frequencies of activated H-*ras* and K-*ras* oncogenes were similar in liver tumors from furan-treated mice (12/29) and vehicle controls (15/27), the spectrum of mutations in the H-*ras* gene differed. In addition to five commonly occurring point mutations at codon 61 of H-*ras*, eight novel mutations were found in furan-induced tumors, including point mutations at codon 117 of H-*ras* as well as activation of K-*ras* oncogene. The different spectrum of mutations in the furan-induced tumors compared with that seen in the spontaneous tumors was interpreted by the authors as genotoxic activity of furan (Reynolds et al. 1987).

In a later study on this issue, Johansson et al. (1997) have tested whether short-term exposure of infant mice to furan is sufficient to induce liver tumors with *ras* gene mutations similar to those found in the 2-year carcinogenicity study. Pre-weaning male B6C3F1 mice were treated by intraperitoneal administration of furan in tricaprylin as a single dose of 400 mg/kg bwt or six daily doses of 200 mg/kg bwt, and killed between 28 and 95 weeks after dosing (Johansson et al. 1997). In the single-dose group, a trend for an increase in the overall frequency of hepatocytic neoplasia and overall liver tumor multiplicity was observed, compared with the vehicle control, which was, however, statistically non-significant. In the multiple-dose group, there was a statistically significant increased incidence of hepatocellular neoplasms and tumor multiplicity compared with control group. No treatment-related histopathological lesions other than development of liver tumors were reported in this study. The relative frequency of H-*ras* activation (mutations in codon 61) was 82% in tumors analyzed from the single-dose group, and 32% in tumors analyzed from the multiple-dose group. The frequency of H-*ras* activation in the respective control animals was 33 and 58%. Noting that the 82% relative frequency of H-*ras* activation in the tumors from the single-dose study was greater than that of the controls or historical control frequencies (54%), the authors postulated that furan-induced hepatocarcinogenicity may be at least partially attributable to genotoxicity. The *Hras1* mutation in codon 117 was found only in one from 40 furan-induced tumors from the multiple-dose furan animal group (78 animals in this group) (Johansson et al. 1997).

A comparison of the types of mutations observed in the mouse liver tumors that developed in furan-treated animals

Table 5 Patterns of H-*ras* base substitutions in furan-exposure-associated and spontaneous mouse liver tumors

	H-*ras* codon 61 mutations (wild-type CAA)			H-*ras* codon 117 mutations (wild-type AAG)	
	AAA	CGA	CTA	AAC	AAT
Reynolds et al. (furan 2-years)	4	1	0	2	2
Johannson et al. (furan 1x)	17	5	1	0	0
Johannson et al. (furan 6x)	2	5	1	0	1
Σ (total 36 mutations)	23 (**64%**)	11 (**30.5%**)	2 (**5.5%**)		
Jackson et al. (spontaneous; total 290 mutations)	**59%**	**26%**	**14%**		

in the studies of Reynolds et al. (1987) and Johansson et al. (1997) with the types of mutations observed in spontaneously occurring mouse liver tumors (summarized in Jackson et al. 2006) is also of interest. For a summary, see Table 5. In total, 36 H-*ras* codon 61 base substitutions were observed in the studies performed by the two groups. 64% of these were of the type CAA>AAA (CG>AT), 30.5% of the type CAA>CGA (AT>GC), and 5.5% of the type CAA>CTA (AT>TA). This distribution of base substitutions in codon 61 is very similar to the one found in the spontaneously occurring liver tumors (see Table 5).

H-*ras* mutations in codon 117 were only described to occur in the furan-associated liver tumors but not in spontaneous tumors (Reynolds et al. 1987). However, one has to consider that base substitutions outside of codon 61 of H-*ras* have not been routinely screened for, after it became obvious that mutations in H-*ras* in liver tumors occur almost exclusively in codon 61. Therefore, it is well feasible that a very minor subpopulation of spontaneous mouse liver tumors might also harbor a mutation in codon 117.

As mentioned earlier ("Genotoxicity of furan metabolite *cis*-2-butene-1,4-dial"), the furan metabolite *cis*-2-butene-1,4-dial is known to react with guanine, adenine and cytosine, but not with thymine in DNA (Burns et al. 2002). In the case of adenine and guanine, the respective etheno-adducts are formed (Byrns et al. 2004). Moreover, *cis*-2-butene-1,4-dial was mutagenic in the Ames test (Peterson et al. 2000) as well as in mammalian cells (Kellert et al. 2008a, b). Unfortunately, the types of base substitutions caused by the furan metabolite are not known. However, the closely related malondialdehyde is also known to form etheno-adducts in both guanine and cytosine (Moriya et al. 1994) and also covalently reacts with the exocyclic amino groups from both adenine and guanine (Benamira et al. 1995). The major types of malondialdehyde-induced base substitutions observed in bacterial cells are GC>TA transversions, CG>TA transitions, and AT>GC transitions (Benamira et al. 1995). Since the H-*ras* codon 61 wild-type sequence is CAA, mutations could result from adducts to C or the opposite G, and from adducts to either of the two A, but not from the opposite T. The mutated sequence AAA, which is also the most frequent spontaneous lesion (see Table 5), would be compatible with the etheno-adduct of the furan metabolite with the G opposite to C. The "C-adduct-derived" sequence TAA (UAA in the RNA), however, is a stop codon and therefore not expected to be seen in tumors. Finally, the rarely seen CTA sequence would be compatible with the formation of an A adduct at the second position of codon 61.

In summary, it cannot be excluded that part of the H-*ras* codon 61 mutations that have been observed in liver tumors from furan-treated mice were directly induced by adducts of the reactive metabolites to DNA. However, the H-*ras* codon 61 mutational spectra are also compatible with a spontaneous occurrence of the base substitutions. The codon 117 mutations which were only observed in the liver tumors from furan-treated mice may not be a characteristic footprint of this agent for reasons outlined earlier. Mutations at this codon may also occur spontaneously, but may have remained undetected because of their low frequency. There is unpublished evidence that mutations in codon 117 may cause constitutive activation of Ras like the mutations in the well documented ras hot spot positions (codons 12, 13, and 61). Activating K-*ras* codon 117 mutations were observed in human colorectal cancers at a very low frequency (G. Smith, personal communication). The etiology of these mutations is not known. They may have been produced by some unknown exogenous agent or occurred spontaneously.

Human data

Human studies investigating an association between furan exposure and cancer have not been reported yet. In this connection, it should be pointed out that epidemiological studies on coffee consumption—the major dietary source of furan for adults (Morehouse et al. 2008; Zoller et al. 2007)—give no evidence of a possible carcinogenic effects in humans. Surprisingly, a recent meta-analysis of published studies on relationship between coffee drinking and the risk of primary liver cancer revealed an inverse association between coffee consumption and hepatocellular

carcinoma (Bravi et al. 2007). The authors reported a 41% reduction of hepatocellular carcinoma among coffee drinkers compared to those who never drank coffee, with similar results obtained from case–control and prospective studies. Moreover, this apparent protective effect of coffee was found both in studies from southern Europe, where coffee is widely consumed, and from Japan, where coffee consumption is less frequent, as well as in studies with subjects with chronic liver diseases. However, it should be mentioned that coffee is a complex mixture of chemicals, including those having antioxidant and anticarcinogenic properties (Cavin et al. 2002; Higdon and Frei 2006), which may have an influence on cancer development.

In a recent study, Jun et al. (2008) reported about an association between γ-glutamyl transferase (γ-GT) activity and furan concentrations in urine of 100 healthy volunteers. Following a regular Korean diet, furan was detected in the morning urine of 56 subjects with the highest value of 3.14 µg/l. Various clinical laboratory parameters were concurrently studied in fasting blood of test persons, and a highly significant correlation between furan concentrations in urine and γ-GT activity in blood plasma was reported. The authors interpreted this result as an indication of a hepatotoxic effect of furan. However, this conclusion is not convincing, because the study has several shortcomings. First, in the case of a hepatotoxic effect of furan, not only increase of serum γ-GT, but particularly ALT (and AST) would be expected, as was shown in animal studies with hepatotoxic furan doses (Fransson-Steen et al. 1997; Moser et al. 2009; Wilson et al. 1992). No evidence has been presented that the furan levels detected in spontaneous morning urine (no 24-h urine) are a parameter/biomarker for dietary furan exposure. Furan metabolites were not investigated in urine. Though all volunteers were reported to be healthy, test persons with pathological γ-GT values (up to 620 U/L) as well as those with elevated fasting blood glucose values (a mean of 198.8 ± 24.8 mg/dl for the six male test persons in the 70 s age group) were not excluded from this study. Moreover, no information was given about alcohol consumption, which could also influence γ-GT values.

Mechanistic aspects and discussion

Furan is both cytotoxic and carcinogenic in rats and mice, and liver is the main target organ. A dose-dependent increase in hepatocellular adenomas and carcinomas was observed in both genders. Furthermore, in rats furan induces a very high incidence of intrahepatic bile duct carcinomas and a dose-dependent increase in mononuclear leukemias. The mechanisms of furan-induced carcinogenesis are not well understood. Particular uncertainty exists concerning the possible effects in the low dose range, which is of major importance for the risk assessment.

Both in vitro and in vivo studies show that metabolic activation is involved in furan-induced toxic effects. CYP enzymes in liver parenchymal cells but probably also in biliary epithelial cells (Shen et al. 1998) mediate the formation of the highly reactive, electrophilic furan metabolite *cis*-2-butene-1,4-dial, which is capable to bind to both proteins and DNA. The in vivo binding to DNA seems to be low and products have not been identified yet. Therefore, currently discussed are both—direct genotoxic (based on binding of reactive metabolites to DNA) as well as indirect non-genotoxic (based on protein modification with induction of cytotoxicity, chronic inflammation, and compensatory cell proliferation) mechanisms of furan carcinogenicity.

The currently available data on genotoxicity of furan are highly inconsistent and controversial. In the in vitro systems, this could be due to the high volatility of the compound. On the other hand, the genotoxic in vivo effects were often first observed in the cytotoxic range.

In contrast to furan, its metabolite *cis*-2-buten-1,4-dial shows definite genotoxicity in vitro and reacts with DNA. Thus, in relevant dose ranges the existing genotoxic potential of furan results from its metabolite *cis*-2-buten-1,4-dial which forms adducts with the deoxyribonucleosides of cytosine, guanine and adenine in vitro. Interestingly, the reaction rate and the sequence of *cis*-2-butene-1,4-dial binding to the deoxyribonucleosides (dCyt ≫ dGuo ∼ dAdo at pH 6.5) parallels that observed for formaldehyde (Byrns et al. 2002). Furthermore, *cis*-2-butene-1,4-dial showed a direct mutagenic effect in the aldehyde-sensitive Salmonella strain TA104 (Peterson et al. 2000). Notably, the mutagenic activity of furan in this strain is similar to that of the structurally related α,β-unsaturated carbonyl compounds like acrolein and crotonaldehyde. Acrolein reacts directly with DNA, predominantly with guanine residues, forming stereoisomeric exocyclic 1,N^2-dG adducts. However, the experimental data suggest that these adducts are not strongly miscoding and the mutagenic affect of acrolein is manifested only when the extent of DNA damage is substantial. The acrolein-induced DNA lesions are effectively repaired and cells are generally accurate in replicating DNAs containing these lesions, with mutations frequencies being remarkably low (Minko et al. 2009). Finally, *cis*-2-butene-1,4-dial was positive in the genotoxicity studies in L5178Y mouse lymphoma cells and induced DNA double-strand breaks and cross-links in CHO cells (Marinari et al. 1984). The EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM) came in its report to the conclusion, that furan-induced carcinogenicity is probably linked to a genotoxic mechanism of action (EFSA 2004; Heppner and Schlatter 2007).

With regard to risk assessment, it is of particular importance to clarify from which furan dose an adduct formation

of *cis*-butene-1,4-dial with DNA in the target cells and hence a genotoxic effect will play a relevant role with regard to a contribution to significant cancer risks. In the target cell, the concentration of furan is influenced by the type of its intake. The furan doses administered by gavage (bolus administration once daily) in the animal experiment are certainly linked with high peak furan concentrations (C_{max}) in the target tissue. In contrast, a dietary human intake of the same daily furan dose—spread over several meals—will lead to a longer duration of exposure of the target cells with a lower C_{max}. It is therefore possible that under these more realistic exposure conditions, the generated reactive metabolite will be preferentially bound to GSH and proteins.

Binding of furan metabolites to proteins and GSH was shown in vitro as well as in vivo (Burka et al. 1991; Chen et al. 1997; Parmar and Burka 1993). The large scale of this reaction should certainly offer DNA protection. In the in vivo study with [2,5-^{14}C]-furan by Burka et al. (1991) (discussed in "Kinetics and metabolism"), 80% of tissue-bound radioactivity in the liver was associated with proteins, while no radioactivity was found to be associated with DNA. However, the results of this study were considered inconclusive by EFSA (European Food Safety Authority) (2004), due to the low sensitivity of the radioactivity determinations (low specific activity of [2,5-^{14}C] furan used as well as possible lability of the labeled carbon atoms at position 2 and 5). In the recent study, performed in the scope of a currently ongoing EU-project (FURAN-RA http://www.furan-ra.toxi.uni-wuerzburg.de), DNA and protein binding of furan was examined in rats by oral administration of ^{14}C-furan labeled on carbons 3 and 4 using Accelerator Mass Spectrometry as analytical technique. A very low, yet dose-dependent increase in ^{14}C-DNA associated binding was detected. The structure of a possible DNA adduct has not been clarified up to now. Notably, 500- to 1,000-fold more radioactivity was associated with proteins (Professor Dekant, Würzburg, personal communication; Hamberger et al. 2009).

On the other hand, damage of cellular proteins may play an important role in furan-induced carcinogenicity. Under bioassay conditions, furan induces compensatory cell proliferation secondary to hepatic cytotoxicity (Fransson-Steen et al. 1997; Wilson et al. 1992). Thus, the furan-induced hepatocarcinogenicity may be primarily the consequence of strong cytotoxic effects of furan or rather its metabolites. Furan was found to uncouple oxidative phosphorylation and to deplete cellular ATP in vitro as well as in vivo (Mugford et al. 1997). Furan-induced cytolethality was proposed to trigger a cascade of events which activate DNA-degrading endonucleases, which in the turn induce DNA double-strand breaks. Kedderis and Ploch (1999) suggested that the defective repair of DNA double-strand breaks in surviving hepatocytes can lead to mutations and, possibly in this way, to furan-induced liver tumors.

In addition, furan concentrations leading to tumor formation in the experimental animals were associated with a high hepatic toxicity and chronic inflammation. Chronic inflammation is known to play an important role in carcinogenesis, inducing genomic instability, alteration in epigenetic events and subsequent inappropriate gene expression (Coussens and Werb 2002; Kundu and Surh 2008). A number of changes in gene expression occur, following furan exposure, leading to altered cell proliferation. Irreversible changes in expression of apoptosis- and cell cycle-related genes were recently reported in the livers of rats after three-month furan treatment (Chen et al. 2009).

Based on these findings, one may conclude that furan-induced tumors are the result of both—genotoxic and chronic cytotoxic—mechanisms of action. The relative importance of these mechanisms would dependent on the dose level and exposure pattern. In the case of lower furan exposure levels, it is likely that reactive furan metabolites are bound by proteins/GSH and/or further metabolized, that would lead to extremely low DNA binding. At higher doses, the observed cytotoxic-related elevated proliferation rate would lead to higher risks for the formation of tumors and a non-monotonous dose–response curve would be expected. In its flat course at low doses, the carcinogenic risk would be extremely low. A curve of this kind was determined for female B6C3F1 mice: furan-induced liver tumorigenicity may occur at doses at and above the cytotoxic levels of furan (Moser et al. 2009). However, for the more sensitive species—the rat—such a dose–response curve is not available for the lower relevant dose range. Moreover, it is still unclear whether a non-hepatotoxic furan concentration can still lead to a significant incidence of bile duct tumors observed in the rat. Thus, the available toxicological data are at present not sufficient to draw any concrete conclusions concerning a reliable risk assessment.

Conclusions

Furan is present in a range of heat-treated foods. Initial exposure estimates identified coffee as the major contributor to the adult and jarred baby food to the infant furan exposure. For infants and young children—the possibly sensitive population group—the estimated exposure ranges between 0.3 and 1.4 μg furan/kg bwt and day.

Up to now, relevant experience with regard to toxic effects associated with human exposure is not available. Furan was found to induce liver tumors in rats and mice at lowest doses of 2 and 4 mg/kg bwt and day, respectively. Cholangiocarcinomas in rats were induced with a high incidence. The reaction of *cis*-butene-1,4-dial as the genotoxic and cytotoxic metabolite with DNA in vivo is low and has not been characterized so far. The CONTAM of EFSA concluded that "the

weight of evidence indicates that furan-induced carcinogenicity is probably attributable to a genotoxic mechanism" but did not make any risk considerations (EFSA (European Food Safety Authority) 2004). In the case of a genotoxic mechanism with clearly irreversible and initiating characteristics, risk characterization should be based on the Margin of Exposure (MoE) assessment concept proposed by EFSA (EFSA 2005). If one would apply this concept, then the MoEs assessed are relatively low and the estimated furan exposures might therefore be a matter of concern.

At the present state of knowledge, however, it appears not possible to elucidate the risk associated with the furan exposure with certainty. In particular, more information is required about effects and mechanisms in the relevant low dose range. In the recent study with female mice (Moser et al. 2009), no elevated tumor incidence was observed for the furan doses below 2 mg/kg bwt and day. However, for the more sensitive species—the rat—no studies have been conducted so far on the dose–response relationships in this range. Furthermore, there are questions concerning the relevance of the genotoxic properties of *cis*-butene-1,4-dial, and how they should be assessed with respect to the carcinogenic action of furan. It should be kept in mind that we have to deal with a reactive aldehyde for which its genotoxicity may play only a minor role in the lower relevant dose range, if we would refer to results obtained for other aldehydes, for example formaldehyde or even acrolein (Appel et al. 2006; Minko et al. 2009; Schulte et al. 2006). Also it is rather certain that the observed cytotoxicity with consecutive cell proliferation in the higher dose range may indirectly amplify the tumor response. The recent study in female mice may reveal such a threshold phenomenon for the induction of hepatic tumors (Moser et al. 2009).

Thus, investigating both the relevance of genotoxicity and the course of the dose–response curve in rats below 2 mg furan/kg bwt per day would be of the major priority to enable extrapolation of toxicological results from animal experiments to the human situation at least with some certainty.

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