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published in
Molecular Nutrition and Food Research
2012

DOI (link to publisher)
10.1002/mnfr.201200093

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)

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Furan in heat-treated foods: Formation, exposure, toxicity, and aspects of risk assessment

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Furan is formed in a variety of heat-treated foods through thermal degradation of natural food constituents. Relatively high levels of furan contamination are found in ground roasted coffee, instant coffee, and processed baby foods. European exposure estimates suggest that mean dietary exposure to furan may be as high as 1.23 and 1.01 μg/kg bw/day for adults and 3- to 12-month-old infants, respectively. Furan is a potent hepatotoxin and hepatocarcinogen in rodents, causing hepatocellular adenomas and carcinomas in rats and mice, and high incidences of cholangiocarcinomas in rats at doses ≥ 2 mg/kg bw. There is therefore a relatively low margin of exposure between estimated human exposure and doses that cause a high tumor incidence in rodents. Since a genotoxic mode of action cannot be excluded for furan-induced tumor formation, the present exposures may indicate a risk to human health and need for mitigation. This review summarizes the current knowledge on mechanisms of furan formation in food, human dietary exposure to furan, and furan toxicity, and highlights the need to establish the risk resulting from the genotoxic and carcinogenic properties of furan at doses lower than 2 mg/kg bw.

Keywords:
Carcinogen / Food contaminant / Furan / Genotoxicity / Liver

1 Introduction

Furan, a heterocyclic aromatic organic compound, is a colorless, inflammable, and volatile liquid widely used in various industrial processes, e.g. as an intermediate in the production process of tetrahydrofuran, pyrrole, and thiophene, in the manufacturing of lacquers and resins [1], and for the production of pharmaceuticals, agricultural chemicals (insecticides), and stabilizers. Furan also occurs in the environment as a constituent of cigarette smoke, wood smoke, and exhaust gas from diesel and gasoline engines [1]. Moreover, furan was shown to be present in a variety of food items that undergo heating processes [2], suggesting that humans may be exposed to furan via food. Furan is a potent hepatotoxic and hepatocarcinogenic in rodents, and has been classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (group 2B) [1]. However, the mechanisms of carcinogenesis are not clear and the relevance of the effects to other related compounds such as dimethylfuran needs to be established.

2 Formation of furan in food

Heat-induced formation of furan in food can occur through a variety of pathways. The most important food constituents serving as precursors for furan production appear to be ascorbic acid, sugars, amino acids, and unsaturated fatty acids [3]. Model reactions with individual compounds or mixtures of precursors provided important insight as to how furan may be formed during heating processes (Fig. 1). These experiments revealed that the most efficient precursor for the formation of furan is L-ascorbic acid, followed by dehydroascorbic acid, glycolaldehyde/L-alanine, and D-erythrose [4].
Formation of furan from polyunsaturated fatty acids (PUFAs) is initiated by oxidative degradation of PUFAs by reactive oxygen species, resulting in the generation of a range of lipid peroxidation products such as 2-alkenals, 4-oxo-alkenals, and 4-hydroxy-2-alkenals, including 4-hydroxy-2-butenal (Fig. 1). 4-Hydroxy-2-butenal can form furan through cyclization and dehydration [4].

The mechanism of furan formation from amino acids involves the production of two key molecules acetaldehyde and glycolaldehyde [4]. Both aldehydes occur as important intermediates in the thermal degradation of amino acids and are able to undergo aldol addition. The resulting 2-deoxyaldotetrose can then further react to yield furan (Fig. 1) [4].

Thermal degradation of the amino acids serine and cysteine can produce both acetaldehyde and glycolaldehyde, whereas aspartic acid, α-alanine, and threonine can give rise to acetaldehyde only. Furan formation thus depends on the presence of reducing sugars as a source for glycolaldehyde [4]. This mechanism is consistent with data demonstrating that heating of L-serine or L-cysteine leads to small amounts of furan, whereas heating of L-aspartic acid, L-alanine, and L-threonine alone does not result in detectable furan formation [4]. However, furan was formed when D-glucose (a source of glycolaldehyde) was added to the single amino acids.

Reactions of glycolaldehyde and L-alanine resulted in high amounts of furan [4].

The formation of furan through thermal degradation of sugars appears to involve formation of the reactive intermediates 1-deoxyosone and 3-deoxyosone, which further react to aldotetrose derivatives, such as aldolotetrose itself, 2-deoxyaldotetrose, and 2-deoxy-3-ketoaldotetrose (Fig. 1). These molecules occur during degradation of hexoses and pentoses. Aldotetrose derivatives as intermediates are also involved in the formation of furan from the degradation of ascorbic acid and dehydroascorbic acid.

Reaction conditions such as temperature, time, and pH can significantly affect furan formation [5, 6]. For instance, Fan et al. demonstrated a time- and temperature-dependent increase in the formation of furan in fresh apple cider. Heat treatment at 90°C for 10 min produced no measurable furan, whereas heating to 120°C resulted in a time-dependent increase in furan production [6]. Importantly, the effect of pH on furan formation may depend on the precursor from which furan is formed. In a study investigating the influence of pH on furan formation from sugars, ascorbic acid, and fatty acids, highest amount of furan was formed from linoleic acid at pH 6. In contrast, thermally induced furan formation in a solution of ascorbic acid was significantly higher at pH 3 than at pH 6 [6].
### 3 Furan content in food and human exposure

Since FDA scientists first identified furan in a number of heat-treated food items [7], a large number of samples from a wide variety of food categories that undergo heat processing have been analyzed worldwide. Results from these analyses have been published as individual reports or assembled in online databases on furan content in food [2, 7–13]. Representative data on furan in various food categories reported by The European Food Safety Authority (EFSA) are shown in Table 1. For most food categories, the levels of furan vary over a wide range. By far the highest contents are found in ground roasted coffee and instant coffee [12, 13]. Scholl et al. [14] found up to 1912 μg/kg in powdered ground coffee. Moreover, maximal furan contents of more than 100 μg furan/kg food were found in certain processed baby food, soups, cereals products, meat products, and sauces, all prepared by temperature treatments [12, 13]. Unexpectedly high concentrations of furan or contradicting levels of contamination were also reported for certain food categories such as fruit and vegetables juices, nutrition drinks, and bakery products [7, 13]. Thus, recent headspace gas chromatography analyses conducted within the frame of an EU-funded project focused on the analysis of furan in these food categories. High furan levels were found in sterilized baby carrot juices, in prune juices, and in different flavors of a particular type of pharmaceutical nutrition drink [15]. Moreover, bakery products, especially rye and wholegrain-based products, showed high but highly varying furan levels [15]. These data have been collected in a database that has been made public (http://www.furan-ra.toxi.uni-wuerzburg.de/furan_ra_2007_public_area/deliverablesresults_with_dissemination_level_public/deliverable_82/), together with data on furan levels in food from a large number of national, state, or federal food safety agencies and universities.

Human exposure to furan was estimated based on food consumption data and the content of furan in various food items. In Europe, the estimated mean exposure of adults to furan in food ranged from 0.34 to 1.23 μg/kg bw/day in different member states, with a median of 0.78 μg/kg bw/day [12]. For infants at 3–12 months of age, an estimated mean exposure between 0.27 and 1.01 μg/kg bw/day was calculated [12]. Minorczyk et al. [16] measured furan concentrations in infant foods and estimated an exposure range of 0.23–1.77 μg/kg bw/day. In the case of adults, coffee was identified as the main source of furan from food, while exposure of infants to furan is considered to be predominantly caused through intake of infant formulas and jarred baby food [12]. The relatively high level in hot coffee is intriguing in light of the volatility of furan and there is the possibility of sequestration within such products. Results from the recent EU-funded project suggest that furan in canned or bottled drinks may be another significant source of furan exposure, particularly in children [15] (http://www.furan-ra.toxi.uni-wuerzburg.de). Based on the determination of furan in juices and drinks, a number of exposure scenarios can be defined (Table 2) using average body weights and food intake assumptions. When compared to furan intake estimates by EFSA for adult and 3–12 months babies, furan intakes from some of the analyzed food items approach or even exceed the EFSA values. US exposure estimates for furan are similar to those made in Europe [17] for children, but are somewhat lower for adults. The lower intake estimates for adults may be due to a relatively low coffee consumption in the United States.

### 4 Toxicology of furan

#### 4.1 Toxicokinetics and biotransformation of furan

Studies on furan toxicokinetics in rats indicate that furan is rapidly absorbed from the gastrointestinal tract, extensively...
metabolized, and eliminated via expired air, urine, and feces. Within 24 h of administration of a single oral dose of [2,5-¹⁴C]-furan (8 mg/kg bw) to male F344 rats, more than 80% of the administered radioactivity was eliminated, with 14% exhaled as unchanged furan, 26% exhaled as CO₂, 20% excreted via urine, and 22% via feces [18]. Most of the radioactivity still present in rats after 24 h was recovered in the liver [18]. Repeated administration of [2,5-¹⁴C]-furan at daily doses of 8 mg/kg bw resulted in accumulation of radioactivity in the liver [18].

Physiologically-based pharmacokinetic model simulations of furan biotransformation after a single oral dose of 8 mg/kg bw predicted 14% of the administered dose exhaled unchanged and 84% metabolized [19].

Furan is metabolized by cytochrome P450 (CYP) enzymes, predominantly CYP2E1, to its major metabolite cis-2-butene-1,4-dial (BDA, maleic dialdehyde) [19, 20], a highly reactive electrophile identified as the key mediator of furan toxicity and carcinogenicity (Fig. 2). The activity of CYP2E1 toward furan is similar between human and rat liver microsomes [21]. Cytochrome P450 mediated bioactivation of furan is supported by studies in rat hepatocytes, which demonstrate that furan-mediated effects on glutathione (GSH) levels and cell viability can be suppressed by the CYP inhibitor 1-phenylimidazole and increased by pretreatment of rats with acetylcysteine—BDA–GSH conjugate and a BDA cross-link between GSH and glutamic acid, which may result from cleavage of the GSH group linked to the pyrrole ring by its thiol group, or by dipeptidase-dependent cleavage of the bis-GSH conjugates formed through the reaction of thiol-BDA with the amino groups of glutathione (GSH) levels and cell viability can be suppressed by the CYP inhibitor 1-phenylimidazole and increased by pretreatment of rats with acetylcysteine—BDA–GSH conjugate and a BDA cross-link between GSH and glutamic acid, which may result from cleavage of the GSH glutamyl residue to form bis-GSH adducts or cyclic mono-GSH adducts [30] (Fig. 3). In vivo, the cyclic mono-GSH conjugates were detected in both urine and bile of furan-treated rats [26, 28], whereas several metabolites consistent with enzymatic processing of the bis-GSH conjugates by γ-glutamyltransferase located at the canalicular membrane of hepatocytes were recently identified in rat bile [28]. These include a cysteinylglycine–BDA–GSH conjugate and a cystine–BDA–GSH conjugate, which may be formed by γ-glutamyltransferase- and dipeptidase-dependent cleavage of the GSH group linked to the pyrrole ring by its thiol group, and a BDA cross-link between GSH and glutamic acid, which may result from cleavage of the γ-glutamyl bond within the GSH moiety contributing the amine in the pyrrole or by direct reaction of thiol-BDA with the amino groups of glutamate. In addition, several metabolites derived from BDA-derived cross-links between cysteine and lysine, e.g. an N-acetylcysteine-N-acetyllysine conjugate and the corresponding sulfoxide, present biliary and/or urinary metabolites of furan (Fig. 3) [28, 29]. These may be formed by conjugation of BDA with GSH or cysteine and subsequent reaction with (free or protein-bound) lysine, but may also represent degradation products of protein adducts formed through the reaction of BDA with protein-bound cysteine and lysine residues. Indeed, a recent study suggests that a significant proportion of lysine-derived furan metabolites stems from degradation of protein adducts in which lysine is cross-linked by BDA to GSH or protein thiols [25]. Interestingly, a putative oxidoreductase (ucpA) offers resistance to furan in Escherichia coli [31].
Figure 3. Proposed metabolic pathways of furan based on metabolites identified in hepatocyte cultures (h) and/or urine (u) or bile (b) of rats exposed to furan. Note that conjugation of cis-butene-1,4-dial (BDA) with glutathione (GSH) or cysteine can occur in position 2 and 3 of the resulting pyrrol ring. For reasons of clarity, only 3-substituted metabolites are shown.

and, once this gene product has been characterized, it may offer leads to relationships between metabolism and toxicity.

4.2 Oral toxicity of furan as the most relevant route of human exposure via food

4.2.1 Rats

Due to the relatively high activity of CYP2E1 to bioactivate furan to its reactive intermediate, the liver is the main target organ of furan toxicity [32]. Male F344 rats given a single dose of 30 mg/kg bw furan by oral gavage showed hepatocellular necrosis, inflammation, and elevated liver enzyme activities in the serum within 24 h of furan administration, followed by regenerative cell proliferation (48 h) and residual inflammation after 8 days [33]. Similar to the single dose, exposure of Sprague-Dawley rats to furan at 40 mg/kg bw for up to 14 days resulted in decreased body weights, increased relative liver weights, and significant increases in serum transaminases, alkaline phosphatase, cholesterol, triglycerides, and total bilirubin, along with increased blood urea nitrogen and serum creatinine indicative of impaired hepatic and renal function [34]. Microscopic treatment-related changes in rat liver included hepatocellular degeneration, hepatic inflammation, and compensatory processes including hepatocellular cytomegaly, mitosis, and regenerative hyperplasia in response to hepatocyte loss [34]. Proliferative lesions involving the biliary tract consisted of biliary hyperplasia characterized by increased numbers of bile ducts, cholangiofibrosis, and oval cell hyperplasia [34]. In the kidney, furan treatment resulted in renal cortical tubular necrosis and regeneration [34].

In a 13-week oral toxicity study in male and female F344/N rats conducted at doses of 0, 4, 8, 15, 30, and 60 mg/kg bw, nine of ten male and four of ten female rats treated with 60 mg/kg bw died before the end of the study [32]. Relative liver weights were significantly increased in males given 15 and 30 mg/kg bw, and in females in the 15, 30, and
60 mg/kg bw dose groups [32]. Toxic lesions of the liver consisted of bile duct hyperplasia and cholangiofibrosis at doses ≥4 mg/kg bw, and hepatocyte cytomegaly, degeneration, and nodular hyperplasia at doses ≥8 mg/kg bw [32]. In contrast, histopathological changes involving the renal tubule epithelium were recorded in the high-dose group only. From these studies, a no observed adverse effect level (NOAEL) for furan hepatotoxicity could not be established. To address furan toxicity in the low-dose range closer to potential human exposures, Gill et al. recently investigated macroscopic and histological changes in livers of male and female F344 rats after 90 days of oral treatment with furan doses of 0.0, 0.03, 0.12, 0.5, 2.0, and 8.0 mg/kg bw [35]. Gross morphologic changes characterized by nodular structures were observed in livers of all high-dose animals, with the caudate and left lateral lobe being most affected [35]. This is consistent with previous reports indicating that the subcapsular visceral surfaces of the left lateral, caudate, and right posterior liver lobes are particularly susceptible to furan toxicity [33, 34, 36, 37]. Mild histopathological changes consisting of hepatocyte apoptosis, Kupffer cell pigmentation, and foci of inflammatory cells were evident at doses of ≥0.12 mg/kg bw [35]. At these dose levels, the changes were restricted to the subcapsular visceral surface of the left lateral and caudate lobes. However, with increasing doses, the lesions became more pronounced and extended deeper into the liver lobe. At the highest dose of 8 mg/kg bw, subcapsular inflammation, hyperplasia of biliary epithelial cells, and cholangiofibrosis with fibrotic tissue replacing the liver parenchyma were reported [35]. Based on these findings, 0.03 mg/kg bw was established as a NOAEL for furan hepatotoxicity in rats.

4.2.2 Mice

Studies in mice indicate that furan induces similar hepatic lesions in mice as in rats, although mice appear to be less susceptible to furan hepatotoxicity. Treatment of male B6C3F1 mice with a single oral furan dose of 50 mg/kg bw induced hepatocellular degeneration, necrosis, inflammation, and increased liver enzymes in plasma after 12 h, showing the most extensive response at 24 h postdose [33]. By 48 h, inflammation and a strong increase in cell proliferation were observed [33]. In a subacute toxicity study, oral treatment of male B6C3F1 mice with 15 mg/kg bw furan for up to 6 weeks caused necrosis and inflammation within the subcapsular parenchyma but did not induce bile duct proliferation, which contrasts the bile duct hyperplasia and peribiliary fibrosis observed in rats [33]. In a 13-week oral toxicity study, B6C3F1 mice were administered 0, 4, 8, 15, 30, or 60 mg/kg bw (females) and 0, 2, 4, 8, 15, or 30 mg/kg bw (males) [32]. Mice of all dose groups and both sexes showed increased relative liver weights and toxic lesions of the liver consisting of hepatocellular cytomegaly, degeneration, and necrosis [32]. In contrast, bile duct hyperplasia and cholangiofibrosis were only observed at 30 or 60 mg/kg bw furan.

To obtain dose-response data in the lower dose range closer to estimated human exposures, male and female B6C3F1 mice were treated with furan at 0.0, 0.03, 0.12, 0.5, 2.0, and 8.0 mg/kg bw by gavage for 90 days [38]. Increased absolute and relative liver weights were observed in the females at the highest dose, while no changes in these parameters were detected in males. In both sexes, serum alanine aminotransferase levels were found to be significantly increased at the highest dose of 8 mg/kg bw [38]. In the caudate lobes, light microscopic examination of liver sections revealed mild to moderate subcapsular lesions including hepatocellular apoptosis, altered Kupffer cells, oval cell hyperplasia, and inflammation at doses of ≥2 mg/kg bw in both sexes and in a single male at 0.5 mg/kg bw. In addition, some animals treated with the highest dose of 8 mg/kg bw showed slight hyperplasia of bile ducts. Similar changes were observed in the left lobes at doses of ≥2 mg/kg bw in both sexes, although no biliary hyperplasia was found in these lobes. In the right and median lobes, histopathological alterations were only rarely seen in singular animals. Based on this study, a NOAEL of 0.12 mg/kg bw was derived.

4.3 Carcinogenicity of furan

4.3.1 Rats

Chronic toxicity and carcinogenicity of furan was investigated in F344/N rats administered furan at doses of 0, 2, 4, and 8 mg/kg bw by oral gavage [32]. In this study, various non-neoplastic hepatic lesions similar to those seen after subchronic treatment at higher doses were reported in rats of both sexes [32]. Furan significantly increased the combined incidence of hepatocellular adenomas and carcinomas in male F344/N rats (Table 3). Furthermore, furan was reported to cause high incidences of cholangiocarcinomas in male and female F344/N rats after 2 years of furan administration at all three dose levels (Table 3) [32]. Cholangiocarcinomas accompanied by cholangiohepatitis were also recorded at the 9- and 15-month interim sacrifices in all treatment groups. In a stop-exposure study, 3-month treatment with 30 mg furan/kg bw was sufficient to produce 100% incidence of cholangiocarcinoma within 9–15 months without further treatment [32,37]. Cholangiocarcinomas in furan-treated rats were characterized by “a connective tissue stroma . . . interspersed between multiple biliary glands and cysts (markedly dilated glands) lined by biliary epithelial cells that varied considerably in morphologic features” [37]. Epithelium lining dilated glands consisted of mitotically active flattened or cuboidal columnar epithelial cells, accompanied by mucous-secreting epithelial cells and inflammatory cell infiltrates in the stroma and cystic lumen [37]. Although there appears to be some debate with regard to the malignant nature of such lesions, the morphological features and growth characteristics of furan-induced cholangial lesions, including cellular dysplasia, infiltrating
growth into the adjacent hepatic parenchyma, progressive growth after cessation of treatment, metastatic potential, and progressive growth and metastasis of 4 of 21 primary tumors transplanted into recipient F344 rats, were seen as convincing evidence for the malignancy of the tumors [37].

4.3.2 Mice

In contrast to rats, cholangiocarcinomas were not observed in male or female B6C3F1 mice treated with furan at 8 and 15 mg/kg bw for 2 years [32]. However, the combined incidence of hepatocellular adenomas and carcinomas was significantly increased to up to 100% in mice of both sexes dosed with furan at 8 and 15 mg/kg bw (Table 3) [32]. In a recent carcinogenicity study conducted at lower doses than in the original National Toxicology Program (NTP) bioassay, female B6C3F1 mice received 0.5, 1, 2, 4, or 8 mg/kg bw furan by gavage for 2 years [39]. At doses of 4 and 8 mg/kg bw furan, the incidences of liver nodules, foci of altered hepatocytes, and hepatocellular adenomas or carcinomas were found to be significantly increased.

5 Mechanisms of furan carcinogenicity

5.1 Genotoxicity of furan

Results of in vitro and in vivo studies on furan genotoxicity are largely inconsistent (Table 4). It is possible that some of the in vivo tests were hindered by loss of furan concentrations through volatility. Furan was not mutagenic in Salmonella typhimurium strains TA100, TA1535, TA1537, and TA98 with and without S9 metabolic activation [32], and no genotoxic effects were observed in L5178Y tk(+/-) mouse lymphoma cells (strand breaks, micronuclei, and tk(+/-) mutations) [40] and human lymphocytes (micronuclei) with or without metabolic activation [41]. However, furan reduced the percentage of DNA in the comet tail in turkey liver fetal hepatocytes indicating the presence of DNA–protein cross-links and to increase the percentage of DNA in the comet tail after proteinase K treatment [42]. Furans was also shown to induce chromosomal aberrations and sister chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) cells [32]. Consistent with these findings, a small increase in SCEs was also observed in a Chinese hamster V79 derived cell line stably expressing human cytochrome P450 (CYP) 2E1 (V79-hCYP2E1-hSULT1A1 cells) required for bioactivation of furan to BDA [43]. In vivo, chromosomal aberrations, but no SCEs, were found in mouse bone marrow cells after intraperitoneal administration of furan [32]. Furans did not induce unscheduled DNA synthesis (UDS) in mouse or rat hepatocytes in vivo and in vitro [32,33]. Furan was also negative in the micronucleus test in mouse peripheral blood erythrocytes [41]. Similarly, no γ-H2AX foci, DNA strand breaks, or cross-links were detected in livers of mice under bioassay conditions [44]. However, a statistically significant increase of micronucleated cells was recently reported in the spleen of furan-treated mice [45]. Importantly, a recent study in the transgenic Big Blue rat model found no evidence for furan mutagenicity in vivo by a range of assays (Pig-a and Hprt gene mutation and liver cII transgene mutation assay), although liver DNA damage was observed by the Comet assay at doses higher than the cancer bioassay doses [46]. In contrast, a reduction of percentage of DNA in comet tail in liver cells was observed following treatment of turkey fetuses in ovo [42]. Since DNA strand breaks were then increased by treatment of hepatocytes with proteinase K, it was suggested that DNA–protein cross-links had been formed by furan.

In contrast to furan, its reactive metabolite BDA was reported to be mutagenic in the Ames test in a strain sensitive to aldehydes (TA 104) [47], although these results could not be reproduced by an independent group [40]. In one study, BDA was shown to cause DNA single-strand breaks and cross-links in CHO cells [48]. In L5178Y tk(+/−) mouse lymphoma cells, BDA induced DNA strand breaks and tk(+/−) gene mutations in a narrow concentration range but did not cause micronuclei and cross-links [40].

While Byrns et al. demonstrated that BDA readily reacts with 2′-deoxyribonucleosides in vitro to form substituted 1,N6-etheno-2′-deoxyadenosine and 1,N2-etheno-2′-deoxyguanosine adducts [49] (Fig. 4), formation of these adducts in rat liver in vivo and their contribution to furan carcinogenicity remain to be established. A study...
Table 4. Results of assays on mutagenicity and genotoxicity of furan and its metabolite BDA in vitro and in vivo

<table>
<thead>
<tr>
<th>In vitro</th>
<th>Test system</th>
<th>Test item</th>
<th>Concentration</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutagenicity in bacteria</td>
<td>S. typhimurium TA100, TA1535, TA1537, and TA98</td>
<td>Furan</td>
<td>0, 33, 100, 333, 1000, 3330, 10,000 pg/plate</td>
<td>Negative (+/- metabolic activation)</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>S. typhimurium TA97, TA98, TA100, and TA102</td>
<td>BDA</td>
<td>1.4, 1.7, 2.1, 2.9, 4.3 μmol/plate</td>
<td>Negative</td>
<td>[47]</td>
</tr>
<tr>
<td></td>
<td>S. typhimurium TA104</td>
<td>BDA</td>
<td>1.4, 1.7, 2.1, 2.9, 4.3 μmol/plate</td>
<td>Positive</td>
<td>[47]</td>
</tr>
<tr>
<td>Mutagenicity in mammalian cells</td>
<td>L5178Y tk(-/-) mouse lymphoma cells</td>
<td>Furan</td>
<td>0, 0.25, 0.5, 1, 2, 4 g/L, 4 h</td>
<td>Negative</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>L5178Y tk(-/-) mouse lymphoma cells</td>
<td>BDA</td>
<td>0, 6.3, 12.5, 25, 50 μM, 4 h</td>
<td>Positive</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Chinese hamster ovary cells</td>
<td>BDA</td>
<td>0.17, 0.5, 1.5 mM</td>
<td>Positive</td>
<td>[48]</td>
</tr>
<tr>
<td>DNA strand breaks</td>
<td>L5178Y tk(-/-) mouse lymphoma cells</td>
<td>Furan</td>
<td>0, 0.25, 0.5, 1, 2, 4 g/L, 4 h</td>
<td>Negative</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>L5178Y tk(-/-) mouse lymphoma cells</td>
<td>BDA</td>
<td>0, 6.3, 12.5, 25, 50 μM, 4 h</td>
<td>Positive</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Chinese hamster ovary cells</td>
<td>BDA</td>
<td>0.17, 0.5, 1.5 mM</td>
<td>Positive</td>
<td>[48]</td>
</tr>
<tr>
<td>Sister chromatid exchange</td>
<td>Chinese hamster ovary cells</td>
<td>Furan</td>
<td>0, 1.6, 5, 16, 50, 160, (500) μg/mL</td>
<td>Positive</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>V79-hCYP2E1-hSULT1A1 cells</td>
<td>Furan</td>
<td>3–16 000 μM</td>
<td>Positive</td>
<td>[43]</td>
</tr>
<tr>
<td>Chromosomal aberration</td>
<td>Chinese hamster ovary cells</td>
<td>Furan</td>
<td>0, 100, 160, 300, 500, 1000 μg/mL</td>
<td>Positive</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>Chinese hamster ovary cells</td>
<td>Furan</td>
<td>up to ~200 mM</td>
<td>Positive</td>
<td>[64]</td>
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<table>
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<tr>
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<th>Test item</th>
<th>Dose</th>
<th>Results</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Unscheduled DNA synthesis</td>
<td>Mouse liver</td>
<td>Furan</td>
<td>200 mg/kg bw, (oral)</td>
<td>Negative</td>
<td>[32]</td>
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<td></td>
<td>Rat liver</td>
<td>Furan</td>
<td>100 mg/kg bw (oral)</td>
<td>Negative</td>
<td>[32]</td>
</tr>
<tr>
<td>DNA strand breaks</td>
<td>Mouse liver</td>
<td>Furan</td>
<td>10, 50, 100, 200 mg/kg bw (oral)</td>
<td>Negative</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>Rat liver</td>
<td>Furan</td>
<td>5, 30, 100 mg/kg bw (oral)</td>
<td>Negative</td>
<td>[33]</td>
</tr>
<tr>
<td>Micronuclei test</td>
<td>Mouse peripheral blood erythrocytes</td>
<td>Furan</td>
<td>0–300 mg/kg bw (i.p.)</td>
<td>Negative</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>Mouse splenocytes</td>
<td>Furan</td>
<td>0.2–275 mg/kg bw (s.c.)</td>
<td>Positive</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>Rat peripheral blood reticulocytes</td>
<td>Furan</td>
<td>0, 2, 4, 8, 15 mg/kg bw (oral gavage for 4 weeks, 5 days/week)</td>
<td>Positive at ≥16 mg/kg bw</td>
<td>[46]</td>
</tr>
<tr>
<td>Sister chromatid exchange</td>
<td>Mouse bone marrow cells</td>
<td>Furan</td>
<td>2, 4, 8, 16, 30 mg/kg bw (oral gavage for 8 weeks, 5 days/week)</td>
<td>Negative</td>
<td>[46]</td>
</tr>
</tbody>
</table>
Table 4. Continued

<table>
<thead>
<tr>
<th>Test system</th>
<th>Test item</th>
<th>Dose</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chromosomal aberration</strong></td>
<td>Mouse bone marrow cells</td>
<td>Furan 87.5, 175, 350 mg/kg bw (i.p.)</td>
<td>Positive</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5, 125, 250 mg/kg bw (i.p.)</td>
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<td></td>
</tr>
<tr>
<td><strong>Mutations</strong></td>
<td>Rat red blood cells (Pig-a mutations)</td>
<td>Furan 2, 4, 8, 16 30 mg/kg bw (oral gavage for 8 weeks, 5 days/week)</td>
<td>Negative</td>
<td>[46]</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td>Rat spleen lymphocytes (Pig-a and Hprt mutations)</td>
<td>Furan 2, 4, 8, 16 30 mg/kg bw (oral gavage for 8 weeks, 5 days/week)</td>
<td>Negative</td>
<td>[46]</td>
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<td></td>
<td>Rat liver (cII mutant frequency)</td>
<td>Furan 2, 4, 8, 16 30 mg/kg bw (oral gavage for 8 weeks, 5 days/week)</td>
<td>Negative</td>
<td>[46]</td>
</tr>
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Figure 4. Reactivity of cis-2-butene-1,4-dial toward DNA bases.

Irrespective of furan genotoxicity, results from a range of studies suggest that furan carcinogenicity may at least in part be mediated through nongenotoxic mechanisms or through indirect DNA damage through reactive oxygen formation. In particular, it is becoming increasingly clear that exposure to furan at doses associated with increased tumor incidences initially causes hepatocellular necrosis, accompanied by inflammation and sustained regenerative proliferation of hepatocytes, which may present key events in furan-induced hepatocellular carcinogenicity. Persistent proliferation of biliary and mesenchymal cells extending into the injured liver parenchyma—presumably as part of a repair response to replace the necrotic tissue—then leads to the development of cholangiofibrosis and intestinal type metaplasia as precursors for cholangiocarcinoma. It is feasible that different mechanisms contribute to carcinogenicity at different dose levels.

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5.2.1 Hepatocyte degeneration due to bioactivation of furan to a reactive metabolite

Several independent studies in rats and mice given a single high dose of furan (≥30 mg/kg bw) demonstrate that subcapsular and centrilobular necroses accompanied by markedly increased liver enzymes is the primary response to furan treatment [32, 33, 36]. Upon repeated administration of furan to rats and mice at carcinogenicity bioassay doses, hepatocyte degeneration and associated inflammatory cell infiltrates were initially recorded at the subcapsular visceral surface of furan target lobes, from where they extended into the liver parenchyma [33, 35, 38, 39].

Although various hypotheses have been put forward, the reason for the differential lobe and site susceptibility to furan toxicity evident from toxicity and gene expression [34] studies remains to be established. Considering the close anatomical proximity of susceptible areas to the stomach, Wilson et al. speculated that orally administered furan may directly diffuse from the stomach into the subcapsular area of target lobes [33]. Alternatively, intra- and interlobular differences in furan metabolism [54] or blood flow and thus furan delivery may contribute to the regional differences in susceptibility to furan [34, 55]. Finally, it is possible that vascular lesions may constrict the blood flow in specific areas and thus induce hypoxia and subsequent necrosis [56]. Although histopathological evaluation suggests that the endothelium is unaffected by furan [36].

While millimolar concentrations of furan may disrupt membrane integrity through a solvent effect, furan cytotoxicity at concentrations that reflect in vivo dosimetry after hepatotoxic doses (2–100 μM) [22] depends on cytochrome P450 2E1 mediated bioactivation of furan to its reactive intermediate cis-2-butene-1,4-dial [22]. cis-2-butene-1,4-dial readily reacts with amino- and sulphhydril groups of free or protein-bound amino acids. Consistent with the chemical reactivity, 80% of the radioactivity present in livers of rats 24 h after administration of 14C-labeled furan was found to be associated with proteins [18]. These data suggest that disruption of protein function through covalent binding to proteins critical to cell fate may present a key step in furan cytotoxicity. However, despite the established link between protein binding of reactive intermediates and cytotoxicity, the cellular and molecular consequences of toxic electrophile insult are still poorly understood. Although protein interactions may be implicated in cytotoxicity, the relevance to carcinogenicity is not known.

There is some evidence to suggest that mitochondria may present preferential cellular targets of furan. Mugford et al. found that furan cytotoxicity in isolated hepatocyte suspensions was preceded by an irreversible loss of ATP due to uncoupling of oxidative phosphorylation [57]. Toxicogenomics data showing altered expression of genes involved in mitochondrial function and hepatic metabolism in livers of rats treated with hepatotoxic doses of furan also indicate that furan may interfere with mitochondrial energy production [34, 58]. A recent proteomics analysis demonstrating covalent binding of [14C]-furan to a large number of mitochondrial and cytosolic proteins participating in glucose, amino acid, and fatty acid metabolism supports a mechanistic link between protein adduct formation and perturbation of hepatic energy metabolism [59].

5.2.2 Inflammation and oxidative stress may aggravate tissue injury

Kupffer cells filled with pigment and accumulation of mononuclear cells and neutrophils indicative of an inflammatory response are consistently recorded within areas of subcapsular and centrilobular necrosis induced by furan [33–36]. The involvement of inflammatory processes in furan toxicity is also reflected by increased expression of cytokines and other inflammation-associated genes [34, 52, 58], such as IFN-γ, IL-1β, IL-6, IL-10, and components of the complement system [58], which may, however, also derive from lesions involving the biliary tract. Irrespective, it is well established that activation of resident macrophages and recruitment of mononuclear cells/neutrophils may not only contribute to liver regeneration but can also exacerbate tissue injury and promote carcinogenesis through release of reactive oxygen and nitrogen intermediates. Indeed, increased production of reactive oxygen species in response to furan is suggested by immunohistochemical detection of 8-oxo-dG within nuclei of hepatocytes of centrilobular areas following high-dose exposure [52] and changes in the expression of genes responsive to oxidative stress in rats and/or mice [44, 52, 58]. In addition to inflammation-mediated effects, cytochrome P450 2E1-dependent release of reactive oxygen species has been associated with metabolism of CYP2E1 substrates [60]. Moreover, several of the recently identified target proteins of furan reactive metabolites such as thioredoxin and peroxiredoxin participate in maintenance of redox homeostasis, suggesting that increased production of reactive oxygen species coupled with impaired antioxidant defense may contribute to furan toxicity and carcinogenicity through DNA oxidation [59].

5.2.3 Regenerative hepatocellular proliferation in response to tissue injury

Compensatory hepatocyte proliferation in response to furan toxicity is documented by a wide range of studies in rats and mice. Wilson et al. demonstrated that midzonal and subcapsular necrosis induced in male B6C3F1 mice and F344 rats by treatment with a single high dose of furan (mice: 50 mg/kg bw; rats: 30 mg/kg bw) was followed by a marked increase in hepatocyte proliferation as assessed by incorporation of 3H-thymidine [33]. After repeated administration of the highest carcinogenicity bioassay dose (mice: 15 mg/kg bw; rats: 8 mg/kg bw) for 6 weeks, the hepatocyte-labeling index was significantly increased throughout the course of the study, suggesting that sustained cell proliferation subsequent to
cytotoxicity presents an early event during tumor development in rodents exposed to furan [33].

Similar results were obtained in male and female B6C3F1 mice treated with furan in the same dose range [23, 44]. In a recent study in female B6C3F1 mice exposed to 0.5, 1, 2, 4, or 8 mg/kg bw furan by gavage for 3 weeks or 2 years, evidence for hepatotoxicity and enhanced hepatic cell proliferation was observed at doses of ≥1 mg/kg bw furan, while the incidence of hepatocellular adenomas or carcinomas was increased only at doses of ≥4 mg/kg bw furan [39]. Based on these findings, the authors concluded that there is a threshold for furan hepatocarcinogenicity in mice, with a low level of cytotoxicity being not sufficient to produce tumors [39]. This has important implications for risk assessment since a recent investigation of furan toxicity in mice derived a NOAEL of 0.12 mg/kg bw/day for furan hepatotoxicity.

In the rat, there is also evidence to show that furan at doses lower than the ones applied in the 2-year bioassay may induce hepatotoxicity and compensatory cell replication. Assessment of hepatocyte proliferation in subcapsular areas of the left and caudate lobes of male F344 rats treated with 0, 0.1, 0.5, and 2 mg/kg bw furan for 4 weeks revealed a dose-related increase in the BrdU-labeling index. Although statistical significance was only reached at 2 mg/kg bw [56], changes in the rate of cell proliferation were accompanied by a statistically significant increase in the expression of genes involved in the processes of apoptosis and cell cycle even at the lowest dose of 0.1 mg/kg bw [51]. These findings are in line with a recent 90-day toxicity study in F344 rats orally treated with furan in the same dose range [32]. Whether the degree of tissue damage and associated regenerative cell proliferation at these low doses within 100-fold of human exposure will ultimately result in formation of hepatocellular adenomas and carcinomas in rats following long-term exposure remains to be established. Based on our current understanding, however, the minimal cytotoxicity and hepatocyte proliferation restricted to subcapsular regions at low doses would not explain the development of cholangiocarcinomas that appear to require extension of tissue injury into the liver parenchyma (see below).

### 5.2.4 Early and persistent biliary cell proliferation as a repair process to replace necrotic tissue

In a study conducted to elucidate the sequential events involved in the development of cholangiocarcinomas in furan-treated rats, furan administered at daily doses of 30 mg/kg bw was shown to cause subcapsular and centrilobular necrosis and inflammation within 24 h after the first dose [36]. By day 3 of the study, centrilobular lesions were largely repaired through proliferation of hepatocytes and inflammation was absent in these areas. In focal regions with more severe injury extending into portal areas, however, hepatocyte proliferation appeared to be insufficient for tissue regeneration and was accompanied by bile duct proliferation and sustained inflammation. Cells within bile ducts extending into the liver parenchyma differentiated into hepatocytes in what appeared to present an ordered biliary to hepatocyte repair process to replace the necrotic tissue. Ordered conversion into hepatocytes was evidenced by a loss of biliary markers such as OV-6, BD1, and Cx43 at the transitional zone between cell phenotypes. Importantly, these areas, in which normal sinusoidal structure was maintained, were not associated with significant inflammation and matrix deposition. However, in some regions where the initial injury had been severe, the hepatocyte stem cell repair response was perturbed and biliary ducts continued to extend into the parenchyma, with some ducts acquiring an intestinal phenotype with a columnar appearance and prominent brush border characteristic of the intestinal-type cholangiocarcinoma that later develops in furan-treated rats. Successively, the hepatic parenchyma was replaced by an abnormally expanding ductal system with mitotically active cells, vascular remodeling of sinusoids, and deposition of connective tissue, leading to cholangiofibrosis and eventually cholangiocarcinomas [36].

These investigations suggest that one mechanism of induction of cholangiocarcinomas in furan-treated rats may be dependent on an overwhelming of the capacity for adaptive repair following irretrievable hepatocyte loss associated with proliferative metaplasia and cholangiofibrosis. In addition to acute treatment with high doses of furan (30 mg/kg bw), early hepatic changes implicated in the pathogenesis of furan-induced cholangiocarcinoma, i.e. biliary hyperplasia and cholangiofibrosis, were evident following repeated administration of bioassay doses (≥2 mg/kg bw) [33, 35], thus correlating with tumor outcome. In contrast, furan doses ≤0.5 mg/kg bw did not produce lesions involving the biliary tract, potentially indicating a threshold for induction of cholangiocarcinoma in rats through the above proposed mechanism. However, whether or not doses below the previously tested bioassay doses cause cholangiocarcinoma following chronic administration, potentially through a different mechanism, remains to be seen. If the irreversible hepatocyte loss at high dose contributes to carcinogenesis, a nonlinear dose response relationship would be expected. In this regard, a large-scale 2-year carcinogenicity study in male F344 rats at furan doses of 20–2000 μg/kg bw/day presently conducted by the National Center for Toxicological Research will provide critical information on the shape of the dose response in the low-dose range.

In a recent 90-day study in mice, mild focal bile duct hyperplasia was recorded in individual animals treated with the bioassay dose of 8 mg/kg bw [38], consistent with non-neoplastic lesions of the biliary tract characterized by bile duct hyperplasia, inflammation, and fibrosis, seen after chronic exposure of mice to furan in the same dose range [32]. Similarly, hyperplastic bile ducts, sometimes surrounded by fibrous tissue, were observed following administration of furan at 30 and 60 mg/kg bw for 90 days [32]. Thus, it appears that furan induces essentially the same type of non-
neoplastic lesions involving the biliary tract with associated increases in plasma bile acids [23, 56] in mice as in rats, although with lesser severity. In contrast to rats, however, these lesions do not appear to progress to the tumor stage. A further response relevant to carcinogenesis is that of modulation of DNA methylation. Although effects on DNA methylation (along with modulation of expression of genes involved in DNA damage response) were observed at 30 mg/kg bw repeat dosing [53], a lower dose of 2 mg/kg bw led to gene expression changes in the liver of rats without detectable modulation of DNA methylation [51], again suggesting dose-specific mechanisms.

6 Risk assessment

Based on the presently available data, it appears that both genotoxicity and chronic cytotoxicity may contribute to furan-induced tumor formation. However, different mechanisms may be involved in the formation of hepatocellular carcinoma in rats/mice and cholangiocarcinoma in rats, and the contribution of DNA binding to the development of these different tumor types as well as human relevance of cholangiocarcinoma remain to be established.

In a recent opinion, the Scientific Committees of the European Commission evaluated the currently used methodologies for risk assessment of genotoxic carcinogens. These methodologies include: linear extrapolation from high doses in repeated dose animal studies to low-dose human exposure, the margin of exposure (MoE) approach, and threshold of toxicological concern (TTC) [61].

While the TTC concept is applied for compounds that lack toxicity data, linear extrapolation of the animal data on furan to calculate possible human tumor risks is uncertain because of the high incidence of tumors induced by furan even at the lowest applied dose. A systematic application of the MoE concept is also difficult since benchmark doses or T25 cannot be calculated with sufficient certainty based on the available tumor data. However, acknowledging the uncertainties in deriving a BMDL from the previous NTP study, the MoE approach was recently applied to furan-induced hepatocellular carcinoma incidences [62], resulting in MoEs for adults of 750–4300 for the BMDL10 (Table 5), and from 9000 to 5300 for the T25, depending on the exposure scenario applied (US or Europe; average or high-level exposure). For children, the estimated margins were relatively similar, from 1000 to 4300 (Table 5) and 1600 to 5300, respectively, for BMDL10 and T25. While an MoE of ≥10 000 is suggested to present a low concern for a genotoxic carcinogen, these estimated margins for both adults and children well below 10 000 and thus indicate a need for risk reduction. Similarly, the Joint FAO/WHO Expert Committee on Food Additives calculated MoEs of 480 and 960 for high and mean dietary exposures based on hepatocellular adenoma and carcinoma in female mice (Table 5) and concluded that these “indicate a human health concern for a carcinogenic compound that might act via a deoxyribonucleic acid (DNA) reactive genotoxic metabolite” [63]. While coffee consumption was a major contributor to intake, average exposure estimates for adult consumers in this study (380 and 494 ng/kg bw/day for the deterministic and probabilistic approach, respectively) were lower than those reported by Carthew et al. [62] and JECFA [65].

With regard to cholangiocarcinoma, the most sensitive endpoint for furan carcinogenicity, it is important to note that the average doses received from diet in humans are only 2000-fold below furan doses causing high incidences of cholangiocarcinoma in rats, and modeling of tumor data resulted in an even lower MoE (Table 5) [62]. However, the presently available tumor data for cholangiocarcinoma are inadequate to derive a point of departure (e.g. BMDL10) with sufficient certainty, highlighting the need to assess formation of cholangiocarcinoma in rodents at doses <2 mg/kg bw.

Considering the uncertainties with regard to the mode of action of tumor formation by furan and human relevance of furan-induced cholangiocarcinoma in the rat, and the absence of tumor data at doses lower than previously studied,
a need for risk reduction measures is indicated and furan exposures should be reduced as requested by the ALARA (as-low-as-reasonably-achievable) concept.

The authors would like to thank all members of the Furan-RA consortium for excellent collaboration (http://www.furan-ra.toxi.uni-wuerzburg.de/furan_ra_2007_public_area/). The authors’ work was supported by grants by the FP6 of the European Union (Furan-RA, SSPE-CT-2006-44393) and the DFG (MA 3323/3-1).

The authors have declared no conflict of interest.

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