Hepatobiliary Toxicity of Furan: Identification of Furan Metabolites in Bile of Male F344/N Rats

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ABSTRACT:

Furan, which occurs in a wide variety of heat-treated foods, is a potent hepatotoxicant and liver carcinogen in rodents. In a 2-year bioassay, furan caused hepatocellular adenomas and carcinomas in mice and rats but also high incidences of bile duct tumors in rats. Furan is bioactivated by cytochrome P450 enzymes to cis-2-butene-1,4-dial, an α,β-unsaturated dialdehyde, which readily reacts with tissue nucleophiles. The objective of this study was to structurally characterize furan metabolites excreted with bile to better understand the potential role of reactive furan intermediates in the biliary toxicity of furan. Bile duct-cannulated F344/N rats (n = 3) were administered a single oral dose of 5 mg/kg b.wt. [12C4]furan or stable isotope-labeled [3,4-13C]furan, and bile samples collected at 30-min intervals for 4 h were analyzed by liquid chromatography-tandem mass spectrometry. A total of eight furan metabolites derived from reaction of cis-2-butene-1,4-dial with GSH and/or amino acids and subsequent enzymatic degradation were detected in bile. The main metabolite was a cyclic monoglutathione conjugate of cis-2-butene-1,4-dial, which was previously detected in urine of furan-treated rats. Furthermore, a N-acetylcysteine-N-acetyllysine conjugate, previously observed in rat urine, and a cysteinylglycine-gluathione conjugate were identified as major metabolites. These data suggest that degraded protein adducts are in vivo metabolites of furan, consistent with the hypothesis that cytotoxicity mediated through binding of cis-2-butene-1,4-dial to critical target proteins is likely to play a key role in furan toxicity and carcinogenicity.

Introduction

Furan, which occurs in a variety of heated foods and beverages (European Food Safety Authority, 2004), is a potent hepatotoxicant and liver carcinogen in rodents (National Toxicology Program, 1993). A 2-year carcinogenesis study on furan conducted by the National Toxicology Program revealed significant increases in the combined incidence of hepatocellular adenomas and carcinomas in male and female B6C3F1 mice and in male F344/N rats. Moreover, high incidences of cholangiocarcinomas were reported to occur in F344/N rats of both sexes after 2 years of furan administration (males: control, 0/50; 2 mg/kg b.wt., 43/50; 4 mg/kg b.wt., 48/50; and 8 mg/kg b.wt., 50/50; females: control, 0/50; 2 mg/kg b.wt., 49/50; 4 mg/kg b.wt., 49/50; 4 mg/kg b.wt., 50/50; and 8 mg/kg b.wt., 48/50) and were also observed at the 9- or 15-month interim evaluations (National Toxicology Program, 1993). In addition to tumors, furan administration for 2 years resulted in numerous non-neoplastic hepatic lesions, including bile duct hyperplasia, cholangiofibrosis, necrosis, chronic inflammation, biliary cell proliferation and cytomegaly, degeneration, and nodular hyperplasia of hepatocytes. These toxic liver lesions were also present in rats given furan at 0, 4, 8, 15, 30, or 60 mg/kg b.wt. for 90 days, with increasing severity related to dose (National Toxicology Program, 1993). The molecular mechanism(s) underlying the hepatobiliary toxicity of furan are still poorly understood. A recent histological and immunocytochemical investigation provided evidence to suggest that cholangiofibrosis as a precursor lesion of cholangiocarcinoma arises from perturbation of a portal bile ductular hepatocyte repair process after irretrievable hepatocyte loss (Hickling et al., 2010). On the other hand, a study on the disposition of [14C]furan in male F344 rats demonstrated that biliary excretion is a major route of elimination of furan (Burka et al., 1991), suggesting that damage to the bile duct epithelium may occur as a result of high concentrations of toxic furan metabolites in bile.

Bioactivation of furan to the α,β-unsaturated dialdehyde, cis-2-butene-1,4-dial (BDA), by cytochrome P450 enzymes, mainly CYP2E1, is thought to be the initiating step in furan toxicity (Kedderis et al., 1993; Chen et al., 1995). In contrast to furan, BDA has been shown to be mutagenic in L5178Y tk(+/−) mouse lymphoma cells (Kellert et al., 2008a) and in the Ames test in a strain sensitive to aldehydes (Peterson et al., 2000) and to cause DNA single-strand...
breaks and cross-links in Chinese hamster ovary cells (Marinari et al., 1984). BDA has been demonstrated to covalently bind to nucleosides (Byrns et al., 2002), GSH, and amino acids in vitro (Chen et al., 1997; Peterson et al., 2005). Because of the bifunctionality of BDA, intermediates formed by conjugation of BDA with GSH or amino acids still remain chemically reactive and may rapidly alkylate free or protein-bound amino groups to form pyrrole derivatives.

Several metabolites derived from conjugation of BDA with GSH and amino acids and downstream processing have recently been identified in primary rat hepatocytes and in urine of rats treated with furan (Scheme 1). These include a cyclic mono-GSH conjugate, a glutamic acid methanethiol conjugate, a N-acetylysine conjugate, and a N-acetylcysteine-N-acetylysine conjugate and its sulfoxide, which were detected in urine of rats exposed to furan (Peterson et al., 2006; Kellert et al., 2008b; Lu et al., 2009). In addition, the mono-GSH conjugate, the N-acetylcysteine-N-acetylysine conjugate, a N-acetylcysteine-lysine conjugate, and metabolites in which the thiol group of GSH is cross-linked by BDA with an amino group of glutamine, lysine, or N-acetylysine were found in culture media of primary rat hepatocytes treated with furan in vitro (Lu et al., 2009). Moreover,
GSH and glutamic acid BDA conjugates with N-acetylcysteine were previously identified in in vitro reaction mixtures (Peterson et al., 2006).

The objective of this study was to structurally characterize furan metabolites excreted with bile after oral administration of furan to rats to better understand the potential role of reactive furan intermediates in the biliary toxicity of furan. Bile duct-cannulated rats were administered a single oral dose of 5 mg/kg b.wt. $^{13}$C-Furan or [3,4-13C]Furan and bile samples collected at 30-min intervals for 4 h were analyzed by LC-MS/MS. Chemical characterization of furan-derived metabolites was based on mass spectral data and coelution with reference compounds obtained by in vitro incubation of cis-2-butene-1,4-diol with GSH and amino acids.

Materials and Methods

**Chemicals.** Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). The BDA precursor 2,5-diacteyloxy-3-methylfuran (petroleum ≥99%) was prepared as described elsewhere (Holzapfel and Williams, 1995). [3,4-13C]Furan (chemical purity ≥99.6%; isotopic purity ≥96%) was purchased from Tjaden Biosciences (Burlington, IA). Isofuran was obtained from Abbott (Wiesbaden, Germany). Solvents for LC-MS/MS were purchased from Roth (Karlsruhe, Germany).

**Animals.** All animal experiments were performed according to national animal welfare regulations after authorization by the local authorities. Male F344/N rats (180–220 g) were purchased from Harlan Winkelmann (Borchern, Germany). Animals were housed in Macrolon cages with wire mesh tops and standard softwood bedding under standard conditions (22 ± 2°C, 30–70% humidity, 12–15 air exchanges/h, and a 12-h light/dark cycle) with food (SSN II, Soest, Germany) and tap water ad libitum. Bile duct cannulation was performed under isofuran anesthesia.

After laparotomy, a flexible polyethylene tube (i.d. 0.28–0.40 mm, o.d. 0.61–0.80 mm, Portex; Smiths Medical International, Ashford, Kent, UK) was inserted into the common bile duct and fixed by placing a ligature around it to prevent dislocation during the bile sampling period. Blank bile samples were collected on ice for 30 min before administration of 5 mg/kg b.wt. furan or [3,4-13C]furan in corn oil by oral gavage (n = 3/group). This dose was chosen to obtain sufficient amounts of furan metabolites in bile to facilitate metabolite identification but was not expected to result in liver damage or irritation of the gastrointestinal tract. The control group received corn oil only. After furan administration, bile was collected at 30-min intervals for 4 h, and aliquots of bile were stored at −20°C.

**Synthesis of Reference Compounds.** Reference compounds were prepared by in vitro incubations of BDA with the respective thiols and amines in 0.1 M potassium phosphate (pH 7.4) at 37°C. BDA and its mono- and bis-GSH conjugates, in which the thiol group of GSH is attached to either the 2- or 3-position of the pyrrole ring (Peterson et al., 2005), i.e., N-[4-carboxy-3-(3-mercaptop-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteinylglycine cyclic sulfide and N-[4-carboxy-4-(3-mercaptop-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteinylglycine cyclic sulfide (a) (cyclic GSH-BDA) and l-glutamyl-l-cysteinylglycine cyclic sulfide (N-[4-carboxy-4-(3-mercaptop-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteinylglycine cyclic sulfide (a) (cyclic GSH-BDA)) and l-glutamyl-l-cysteinylglycine cyclic sulfide (N-[4-carboxy-4-(3-mercaptop-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteinylglycine cyclic sulfide (a) (cyclic GSH-BDA)) were prepared as described previously (Chen et al., 1997; Byrns et al., 2004; Peterson et al., 2005). In addition to the bis-GSH conjugates, a further reaction product eluting at 15.6 min was formed when BDA was reacted with 10 equivalents of GSH, and this was identified as l-cysteinylglycine (2→1)-sulfide with N-[4-carboxy-4-(3-mercaptop-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteinylglycine or N-[4-carboxy-4-(3-mercaptop-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteinylglycine (d) based on mass spectral data. LC-MS/MS: m/z 523 ([M − H]+), 514 ([M − H − H2O]+), 498 ([M − H − H2S]+), 272 ([M − H − GlyCys-S-C3H7]+), 254 ([M − H − GlyCys-S-C3H7 − H2O]+), 225, 210 ([M − H − GlyCys-S-C3H7 − H2O − CO2]+), 179, 143 ([M − H − N-[4-carboxy-4-(3-mercaptop-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteinylglycine]+), 128.

Incubation of cis-2-Butene-1,4-diol with GSH and l-Cysteine. BDA (0.01 mmol) was incubated with 0.01 mmol of l-cysteine in 0.1 M potassium phosphate (pH 7.4) at 37°C for 15 min (total volume 1 ml). Then 0.01 mmol of GSH was added, and the mixture was incubated at 37°C for 24 h. In addition to the mono-GSH conjugate, LC-MS/MS analysis revealed a further reaction product eluting at 16.5 min, which was tentatively identified as l-cysteine (2→1)-sulfide with N-[4-carboxy-4-(3-mercaptop-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteinylglycine or l-cysteine (2→1)-sulfide with N-[4-carboxy-4-(3-mercaptop-1H-pyrrol-1-yl)-1-oxobutyl]-l-cysteine (Peterson et al., 2006). N-Acetyl-S-[1-[5-(acetylamino)-5-carboxypropyl]-1H-pyrrol-3-yl]-l-cysteine (g) was prepared by combining BDA with equimolar amounts of N-Acetyl-l-lysine and N-Acetyl-l-cysteine as described previously (Chen et al., 1997).

Sample Preparation. Bile samples were diluted 1:1 with distilled water and acidified with concentrated HCl to a final concentration of 1% (pH 1). Synthesized standards were diluted 1:10 with distilled water and acidified with concentrated HCl to a final concentration of 1% (pH 1). Dilutions were centrifuged at 23,100g at 4°C for 10 min to remove insoluble constituents. Supernatants were transferred into glass vials and measured by LC-MS/MS.

Instrumental Analysis. The LC-MS/MS system consisted of an autosampler (1100 series; Agilent, Waldbronn, Germany), two high-performance liquid chromatography pumps (1100 series; Agilent), and an automated switching valve linked to a Q TRAP 2000 LC-MS mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada). The solvents were acetonitrile (C) and 0.1% formic acid in water (D) for pump 1 and 0.1% formic acid in water (A) and acetonitrile (B) for pump 2, respectively. The analytes were
separated by a linear gradient (pump 1) from 5% C/95% D to 50% C/50% D within 25 min and to 90% C/10% D within 1 min at a flow rate of 200 μl/min. This solvent composition was held for 7 min before reconstitution of initial conditions. Bile samples (50 μl) of control rats and animals treated with [13C6]- or [3,4-13C]furan were analyzed separately. In addition, bile samples obtained after treatment with [13C6]- or [3,4-13C]furan were mixed at a ratio of 1:5 to confirm coelution of biliary metabolites derived from [13C6]- and [3,4-13C]furan. Samples were loaded onto a ReproSil-Pur C18-AQ precolumn (5 μm, 10 × 2 mm; Dr. Maisch Gmbh, Ammerbuch, Germany) before separation by the analytical column (ReproSil-Pur C18-AQ, 3 μm, 150 × 2 mm; Dr. Maisch Gmbh). After 20 min, the column-switching valve changed its position, allowing the solvent to directly enter the analytical column, thereby retaining nonpolar bile constituents on the precolumn to prevent contamination of the analytical column. The precolumn was then washed with 5% A/95% B for 15 min at a flow rate of 500 μl/min. Data acquisition was performed in negative ion mode with a source temperature of 400°C and an ion spray voltage of −4200 V. The declustering potential, entrance potential, and collision energy were set to −50 V, −10 V, or −30 V, respectively. Samples were initially analyzed in full scan mode in the range of 200 to 1500 amu with a scan rate of 4000 amu/s and subsequently in enhanced mass spectrometry with information-dependent acquisition of enhanced product ion (EMS-IDA-EPI) mode in the range of 200 to 1000 amu with a scan rate of 4000 amu/s. Because various background molecules are present in bile samples, peaks from undosed and furan-treated rats obtained in EMS-IDA-EPI mode were extracted with MarkerView software 1.2 (Applied Biosystems/MDS Sciex) as described previously (Kellert et al., 2008b). The extraction parameters were set to 10 counts/s for the noise threshold, 0.1 amu for the minimal spectral width, and 3 to 50 scans for the peak width. Retention time shifts between runs were defined to 1 min and mass shifts to 0.2 amu. The maximum number of extracted peaks was set to 1000, and results were only accepted when peaks were present in bile of at least two of the three furan-dosed animals and absent in controls. For each potential biliary furan metabolite, multiple reaction monitoring with information-dependent acquisition of enhanced product ion (MRM-IDA-EPI) spectra was performed. MRM transitions selected for LC-MS/MS analysis are listed in Table 1. EPI spectra were compared with those of synthesized standards or literature data as available. Biliary metabolites were further confirmed by mass spectral data of the corresponding metabolites present in bile of [3,4-13C]furan-treated animals, which showed a shift of 2 amu upward.

Results

Identification of Biliary Furan Metabolites. To identify biliary furan metabolites, bile duct-cannulated male F344 rats (n = 3 per group) were administered single oral doses of 5 mg/kg b.w.

Table 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>m/z</th>
<th>Selected MRM Transition</th>
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<tbody>
<tr>
<td>Cyclic GSH-BDA from [12C]furan</td>
<td>498</td>
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</tr>
<tr>
<td>Cyclic GSH-BDA from [12C]furan</td>
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<td>128</td>
</tr>
<tr>
<td>GSH-BDA-Glu from [12C]furan</td>
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<td>230</td>
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<tr>
<td>GSH-BDA-Glu from [12C]furan</td>
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<td>228</td>
</tr>
<tr>
<td>GSH-BDA-Glu from [12C]furan</td>
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<td>230</td>
</tr>
<tr>
<td>GSH-BDA-Gly from [12C]furan</td>
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<td>498</td>
</tr>
<tr>
<td>GSH-BDA-Gly from [12C]furan</td>
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<td>500</td>
</tr>
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<td>GSH-BDA-Gly from [12C]furan</td>
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<td>GSH-BDA-Gly from [12C]furan</td>
<td>429</td>
<td>300</td>
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<td>GSH-BDA-Gly from [12C]furan</td>
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<td>302</td>
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<td>N-Acetyllysine-Glu</td>
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<td>N-Acetyllysine-Glu</td>
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<td>271</td>
</tr>
<tr>
<td>N-Acetyllysine-Glu</td>
<td>414</td>
<td>285</td>
</tr>
<tr>
<td>N-Acetyllysine-Glu</td>
<td>416</td>
<td>287</td>
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</table>

[12C]furan- or [3,4-13C]furan. Bile samples were collected before furan application and at 30-min intervals for up to 4 h after dosing. A total of eight metabolites, which were not present in predose bile or bile samples of control rats, were detected by LC-MS in EMS-IDA-EPI mode and subsequent multiple reaction monitoring after furan administration (Fig. 1). EPI spectra and retention times of potential furan metabolites corresponded to those of the reference compounds synthesized by reaction of BDA with GSH and amino acids (Table 2; Supplemental Fig. 1). To further verify that these biliary excreted compounds represent metabolites of furan, the study was repeated using [3,4-13C]furan. As shown in Fig. 1, the metabolites previously observed were also found in bile after administration of [3,4-13C]furan, with an expected mass shift of 2 amu upward (Table 2).

Within 30 min of furan application, several furan-derived metabolites were excreted in bile, including the mono-GSH conjugate of BDA a (m/z 354), which has previously been identified in hepatocyte cultures and urine of rats exposed to furan (Peterson et al., 2006; Kellert et al., 2008b; Lu et al., 2009) and a GSH-BDA-glutamic acid adduct b (m/z 501) (Peterson et al., 2006). In addition, four not previously characterized metabolites c to f were detected (Fig. 1A).

Metabolite c (m/z 372), eluting at 9.6 min, showed the same characteristic fragment of m/z 228 [corresponding to 2-(mercaptopropyl)-pyrrol-1-yl]pentanedioic acid − H+] as the GSH-BDA-glutamic acid adduct b, suggesting that this metabolite also contains glutamic acid incorporated into the pyrrole ring via its amino group. Loss of 44 amu (CO2) and subsequent loss of 17 amu (NH3), resulting in fragments m/z 328 and m/z 311, indicate the presence of a free carboxylic and an amino group. On the basis of the same mass and fragmentation pattern, it therefore appears that this metabolite c (m/z 372) may represent a BDA cross-link between cysteinylglycine and glutamic acid, presumably formed by γ-glutamyltranspeptidase-dependent cleavage of the isopeptide bond between γ-glutamyl and cysteinyl residues within the GSH moiety of b or by cleavage of the cyclic GSH-BDA conjugate a.

EPI spectra of three further metabolites (m/z 532, 475, and 429) displayed fragments characteristic of GSH conjugates (m/z 272, 254, 210, 179, 143, and 128) (Dieckhaus et al., 2005). Retention times and mass spectral data of these compounds were consistent with synthesized reference compounds, i.e., a cysteinylglycine-BDA-GSH conjugate d (m/z 532), a cysteiny1-BDA-GSH conjugate e (m/z 475), and a GSH-BDA-glycine conjugate f (m/z 429). The structure of f is further supported by the characteristic neutral loss of 129, resulting from cleavage of the γ-glutamyl-cysteinyl-isopeptide bond within a GSH residue and loss of 273 (GSH cleavage at the SCH2 group) (Xu et al., 2008), which indicate that GSH is linked to the pyrrole ring by its thiol group and not by an amino group. The amount of metabolite f was below the limit of detection in bile samples from [3,4-13C]furan-treated rats.

Although the GSH-derived metabolites were rapidly excreted, with maximum concentrations occurring within 30 min and 1 h of furan administration, additional metabolites were only detected in bile samples collected at later time points (≥1.5 h) after furan administration (Figs. 1 and 2). These included a N-acetylcycteine-N-acetylcysine conjugate g (m/z 398) (Fig. 1C) and trace amounts of a metabolite, which was tentatively identified as the corresponding sulfoxide h based on retention time (14.4 min), mass (m/z 414), and transition to 285 (Fig. 1F) (Kellert et al., 2008b). Both the N-acetylcycteine-N-acetylcysine conjugate g and the sulfoxide h have previously been characterized and shown to be present in urine of rats treated with furan in addition to a pyrrolinone adduct of N-acetylcysteine, which we did not detect in rat bile (Kellert et al., 2008b; Lu et al., 2009). Unfortunately, further characterization of
the mercapturate-sulfoxide-N-acetyllysine conjugate h was not possible, because its concentration in bile was too low to record an EPI spectrum.

With the exception of f and the sulfoxide h, which is likely to be formed from g via cysteine conjugate S-oxidase, all biliary metabolites identified were confirmed by LC-MS/MS analysis in MRM-IDA-EPI mode after administration of [3,4-13C]-furan (Fig. 1). As expected, the molecular masses of the metabolites shifted by 2 amu upward in bile of [3,4-13C]furan-treated rats compared with samples obtained from rats treated with [12C4]furan. EPI spectra of biliary metabolites detected in samples from isotopically labeled and unlabeled furan-treated rats showed an identical fragmentation pattern (Table 2). To estimate elimination kinetics, peak areas of each metabolite excreted at different time intervals were integrated. In contrast to the rapid excretion of metabolites derived from GSH conjugation (Fig. 2, A–C), the N-acetylcysteine-N-acetyllysine conjugate g and its sulfoxide h were only evident 1.5 h after furan administration, and their concentrations in bile continued to rise during the collection period (Fig. 2D).

For some of the metabolites (b, c, d, f, and g), a second peak with an identical mass spectrum but different retention times from those of the synthesized reference compound was observed in rat bile. Although this result may be consistent with the ability of GSH to attack carbons 1 and 2 of BDA, leading to the formation of either 2- or 3-(S-glutathionyl)pyrroles, we cannot conclude whether these minor peaks represent regioisomers of these compounds.

Discussion

Because of its high capacity to bioactivate furan to the reactive intermediate cis-2-butene-1,4-dial, the liver is the main target organ of furan toxicity and carcinogenicity. Furan hepatotoxicity is characterized not only by hepatocellular alterations but also by damage to the bile duct epithelium, leading to cholangiofibrosis and subsequent development of cholangiocarcinoma. However, the mechanisms involved in the biliary toxicity of furan are poorly understood. Previous studies on the disposition and biotransformation of furan in rats have demonstrated 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-\(^{14}\)C]-furan (8 mg/kg b.w.) to male F344 rats, approximately 40% of the furan-derived radioactivity was expired as the parent compound (14%) and CO\(_2\) (26%). Approximately 20 and 22% of the administered dose was eliminated in urine and feces, respectively, whereas 15% of the total radioactivity was recovered in tissues, predominantly in the liver (Burka et al., 1991). Consistent with in vitro data showing that cis-2-buten-1,4-dial readily reacts with nucleophiles to form protein adducts and GSH conjugates (Chen et al., 1997; Peterson et al., 2005; Lu et al., 2009), a range of metabolites derived from binding of cis-2-buten-1,4-dial to GSH and amino acids have recently been identified in urine of rats treated with furan (Peterson et al., 2006; Kellert et al., 2008b; Lu et al., 2009). In contrast, furan metabolites excreted with bile, which may contribute to the biliary toxicity of furan, have not yet been characterized.

In this study, a total of eight metabolites were identified in bile after administration of [\(^{12}\)C]furan and [3,4-\(^{13}\)C]furan to male F344 rats. Consistent with the current understanding of furan biotransformation and previous findings, the cyclic mono-GSH conjugate \(a\), which results from intramolecular reaction of the \(\alpha\)-amino group of the GSH glutamyl residue, and a metabolite in which cis-2-buten-1,4-dial cross-links \(N\)-acetylcysteine and \(N\)-acetylcysteine \(g\) were found to be present in rat bile. In addition, our data provide evidence to suggest that the bis-GSH conjugate, which represents a major reaction product of cis-2-buten-1,4-dial and GSH in vitro but has not yet been demonstrated to be formed in vivo (Chen et al., 1997; Peterson et al., 2005), is also generated in rat liver. Although we did not detect the bis-GSH conjugate per se, metabolites consistent with enzymatic degradation of the bis-GSH-BDA conjugate by GSH conjugate-processing enzymes were present in rat bile. This includes cysteinylglycine-GSH conjugate \(d\) and cysteine-GSH conjugate \(e\), which may be formed by sequential hydrolysis of the bis-GSH-BDA reaction product by \(\gamma\)-glutamyltranspeptidase and dipeptidase (Scheme 2). However, it is also possible that \(e\) may originate from a cross-link between a protein-bound cysteine residue and the glutamate amino group of GSH.

Recent work by Lu et al. (2009) suggested that degradation of protein adducts of cis-2-buten-1,4-dial is a major source of lysine-derived metabolites of furan. Analysis of GSH-BDA-lysine cross-links formed in primary rat hepatocytes shows that, under physiological conditions, lysine is linked to cis-2-buten-1,4-dial predominantly via its \(\alpha\)-amino group, although formation of the \(\alpha\)-substituted regioisomer was also observed (Lu et al., 2009). Furthermore, relative levels of the \(\alpha\)-reaction product increased with time, presumably as a result of degradation of alkylated proteins (Lu et al., 2009). Experiments conducted in culture media supplemented with [4,4,5,5-\(^2\)H\(_4\)]-lysine provide further evidence that at least some of the lysine adducts are derived from degraded protein by demonstrating that a greater percentage of the \(\alpha\)-substituted but not the \(\alpha\)-substituted regioisomer was formed from unlabeled lysine (ratio [H\(_4\)]/[H\(_4\)] = 3 for the \(\alpha\)-substituted versus 1.2 for the \(\alpha\)-substituted regioisomer) (Lu et al., 2009). On the basis of the different ratios for the \(\alpha\)-substituted versus the \(\alpha\)-substituted products, which would be expected to be the same if the metabolites were exclusively derived from the reaction of GSH-BDA with free lysine, Lu et al. (2009) concluded that some of the \(\alpha\)-substituted regioisomers are probably derived from the reaction of GSH-BDA with lysine moieties in proteins.

In this respect, it is also interesting to note that metabolites derived from cysteine-BDA-lysine cross-links, i.e., the \(N\)-acetylcysteine-\(N\)-acetylcysteine conjugate \(g\) and its corresponding sulfoxide \(h\), were not detected in rat bile until 1.5 h after furan administration but continued to rise thereafter. The delayed biliary excretion of the lysine adducts

### Table 2

<table>
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<th>Metabolite</th>
<th>In Vivo</th>
<th>m/z (13C)</th>
<th>m/z (12C)</th>
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</tr>
<tr>
<td>Cyclic-GSH-BDA-Gly-Glu (f)</td>
<td>20.9</td>
<td>429, 411, 300, 272</td>
<td>254, 210, 179</td>
<td>20.9</td>
<td>429, 411, 300, 272</td>
<td>254, 210, 179</td>
</tr>
<tr>
<td>Cyclic-GSH-BDA-Gly-Glu (g)</td>
<td>21.5</td>
<td>475, 457, 441, 397</td>
<td>272, 254, 210, 179</td>
<td>21.5</td>
<td>475, 457, 441, 397</td>
<td>272, 254, 210, 179</td>
</tr>
<tr>
<td>Cyclic-GSH-BDA-Gly-Glu (h)</td>
<td>22.1</td>
<td>429, 411, 300, 272</td>
<td>254, 210, 179</td>
<td>22.1</td>
<td>429, 411, 300, 272</td>
<td>254, 210, 179</td>
</tr>
</tbody>
</table>

**RT, retention time; N.A., not applicable; LOD, limit of detection.**

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On the basis of the different ratios for the \(\alpha\)-substituted versus the \(\alpha\)-substituted products, which would be expected to be the same if the metabolites were exclusively derived from the reaction of GSH-BDA with free lysine, Lu et al. (2009) concluded that some of the \(\alpha\)-substituted regioisomers are probably derived from the reaction of GSH-BDA with lysine moieties in proteins.

In this respect, it is also interesting to note that metabolites derived from cysteine-BDA-lysine cross-links, i.e., the \(N\)-acetylcysteine-\(N\)-acetylcysteine conjugate \(g\) and its corresponding sulfoxide \(h\), were not detected in rat bile until 1.5 h after furan administration but continued to rise thereafter. The delayed biliary excretion of the lysine adducts
compared with that of the mono-GSH conjugate and metabolites to may provide further support that these metabolites may represent degraded protein adducts. However, even though formation of the N-acetylcysteine-N-acetylylsine conjugate is consistent with enzymatic processing of the GSH-BDA-lysine conjugate shown to be formed in rat hepatocytes in vitro (Lu et al., 2009) via the mercapturic acid pathway (Scheme 2), we cannot conclude from our data that the cysteine in g and is indeed derived from GSH as opposed to protein.

Similar to the GSH-BDA-lysine conjugate observed in rat hepatocyte cultures (Lu et al., 2009), we identified metabolites resulting from cross-links between the cysteinyl thiol of GSH and an amino group of glycine (f) or glutamate (b and c) (Scheme 2). Although b and c may present downstream metabolites of the GSH-BDA-GSH and/or the cyclic GSH-BDA conjugate resulting from cleavage of the γ-glutamyl bond in the GSH group contributing the amine in the pyrrole cross-link, both the glycine and glutamate adducts may be formed by reaction of the GSH-BDA conjugate with the free amino acids, which are both present in high concentrations in rat liver (1.7 and −14 mM for glycine and glutamate, respectively) (Gregus et al., 1993; Geerts et al., 1997) or N-terminal glycine or glutamate residues of proteins. In contrast to the lysine derivatives, however, the suggested glutamate adducts b and c and the GSH-BDA-glutamate conjugate are rapidly excreted in rat bile, with maximum concentrations occurring within 30 min and 1 h of furan administration, suggesting that these metabolites may be derived from free amino acids and/or GSH rather than from degraded protein adducts.

Consistent with the hypothesis that cytotoxicity mediated through binding of cis-2-butene-1,4-dial to critical target proteins is likely to play a key role in furan toxicity and carcinogenicity (Wilson et al., 1992), chemical characterization of furan metabolites in bile provided further evidence to suggest that degraded protein adducts are in vivo metabolites of furan. Based on our findings, however, we cannot conclude that the bile duct epithelium is a primary target of reactive furan metabolites, because biliary toxicity may also occur due to hepatocellular injury. Lu et al. (2009) speculated that the primary reaction product of cis-2-butene-1,4-dial and GSH may not be as short-lived as generally assumed and may even migrate across membranes. Although this speculation suggests that the BDA-GSH intermediate may directly react with proteins located within the bile duct epithelium after transport across the canalicular membrane, no experimental proof has been obtained. On the other hand, many xenobiotics known to cause injury to the biliary tract have been shown to selectively damage the canalicular membrane of hepatocytes, which forms the origin of the biliary tract, resulting in disruption of membrane integrity, interference with hepatobiliary transport mechanisms, and release of toxic compounds (e.g., lysosomal enzymes) into bile (Woodard and Moslen, 1998; Pauli-Magnus and Meier, 2006). This process frequently involves bioactivation to chemically reactive metabolites and formation of glutathione conjugates or acyl-gluconorides, which are substrates for hepatobiliary transporters (Jones et al., 2003). Therefore, it is possible that the BDA-GSH intermediate may preferentially cause damage to proteins involved in transport of GSH-conjugates across the canalicular membrane, thereby interfering with hepatobiliary function and leading to irritation of the biliary epithelium. In support of this possibility, a small rise in serum cholesterol accompanied by a significant increase in unconjugated bile acids indicative of impaired hepatocellular function was observed. This increase in cholesterol levels occurred after 2 h of furan administration and persisted throughout the remaining experimental period (7 days). This finding is consistent with the hypothesis that altered bile acid metabolism may contribute to the observed increase in serum cholesterol levels.

FIG. 2. Elimination kinetics of furan metabolites in bile. A, cyclic mono-GSH conjugate. B, cysteinylglycine-BDA-GSH and cysteine-BDA-GSH conjugate. C, GSH-BDA-glutamic acid, cysteinylglycine-BDA-glutamic acid, and GSH-BDA-glycine. D, N-acetylcysteine-BDA-N-acetylylsine conjugate and the sulfoxide of the N-acetylcysteine-BDA-N-acetylylsine conjugate. Data were obtained by integrating peak areas of each metabolite detected in bile after administration of [14C]-furan and are presented as mean area ± S.D. of three individual animals. Ac, acetyl.
biliary function was recently observed in F344 rats dosed with furan at 2 mg/kg b.wt. for up to 4 weeks (Mally et al., 2010). However, whether or not membrane transporters represent furan target proteins remains to be established. Alternatively, it has been suggested that proliferative signals to biliary epithelial cells may arise due to hepatocyte damage as part of a portal bile ductular hepatocyte repair process that is activated when the capacity for adaptive repair is overwhelmed by high doses or sustained exposure to furan (Hickling et al., 2010). In summary, further investigations will be required to understand the cellular and functional consequences associated with furan-mediated protein damage and current work in our laboratory is focused on identifying critical target proteins that may play a causal role in the pathogenesis of furan-associated liver toxicity.

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References

Scheme 2. Proposed metabolic pathway of furan by conjugation with GSH and/or amino acids in rat bile. Note that conjugation can also occur in position 2 instead of 3 in the pyrrol ring. Metabolites printed in bold were detected and verified in rat bile after administration of [3,4-13C]furan. N-AcCys, N-acetyl-L-cysteine; N-AcLys, N-acetyl-L-lysine; GGT, γ-glutamyltransferase.


National Toxicology Program (1993) Toxicology and carcinogenesis studies of furan (CAS No. 110-00-9) in F344 rats and B6C3F1 mice (gavage studies). Natl Toxicol Program Tech Rep Ser 402:1–286.


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Supplemental information Figure 1. Co-elution Data.

LC-MS MRM chromatograms of (A) bile from furan treated rats, (B) an in vitro cis-2-butene-1,4-dial (BDA) incubation product mixture (equal amounts of in vitro incubation products of 0.01 mmol BDA with 0.01 mmol or 0.1 mmol glutathione (GSH) and/or 0.01 mmol glutamic acid, 0.01 mmol cysteine, 0.01 mmol glycine, 0.01 mmol N-acetyllysine and 0.01 mmol N-acetylcysteine, respectively) and (C) bile from furan treated rats spiked with in vitro BDA incubation product mixture in a ratio of 5:1 show co-elution of in vivo generated furan metabolites and synthesized BDA conjugate standards.