Comparative Metabolism of Furan in Rodent and Human Cryopreserved Hepatocytes[§]

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ABSTRACT

Furan is a liver toxicant and carcinogen in rodents. Although humans are most likely exposed to furan through a variety of sources, the effect of furan exposure on human health is still unknown. In rodents, furan requires metabolism to exert its toxic effects. The initial product of the cytochrome P450 2E1-catalyzed oxidation is a reactive α,β -unsaturated dialdehyde, *cis*-2-butene-1,4-dial (BDA). BDA is toxic and mutagenic and consequently is considered responsible for the toxic effects of furan. The urinary metabolites of furan in rats are derived from the reaction of BDA with cellular nucleophiles, and precursors to these metabolites are detected in furan-exposed hepatocytes. Many of these precursors are 2-(S-glutathionyl) butanedial-amine cross-links in which the amines are amino acids

and polyamines. Because these metabolites are derived from the reaction of BDA with cellular nucleophiles, their levels are a measure of the internal dose of this reactive metabolite. To compare the ability of human hepatocytes to convert furan to the same metabolites as rodent hepatocytes, furan was incubated with cryopreserved human and rodent hepatocytes. A semiquantitative liquid chromatography with tandem mass spectrometry assay was developed for a number of the previously characterized furan metabolites. Qualitative and semiquantitative analysis of the metabolites demonstrated that furan is metabolized in a similar manner in all three species. These results indicate that humans may be susceptible to the toxic effects of furan.

Introduction

Humans are exposed to furan through a variety of environmental sources, including food, smog, engine exhaust, and tobacco and wood smoke (Capurro, 1973; International Agency for Research on Cancer, 1995; Wang et al., 2009; Barboni et al., 2010; Eschner et al., 2011; Simpson et al., 2011; Smits et al., 2012). Many of these mixtures are associated with adverse health outcomes such as respiratory illnesses and cancer at multiple sites (Hu and Ran, 2009; Barboni et al., 2010; United States Department of Health and Human Services, 2014). Furan is highly toxic to laboratory animals following either inhalation or oral exposure (Egle, Jr. and Gochberg, 1979; Elmore and Sirica, 1993; National Toxicology Program, 1993), and it is a liver carcinogen when given orally (National Toxicology Program, 1993). The contribution of furan to the adverse health effects of harmful mixtures containing furan in humans is unknown.

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Furan's toxic effects in model systems require metabolism (Burka et al., 1991; Carfagna et al., 1993; Kedderis et al., 1993; Parmar and Burka, 1993; Fransson-Steen et al., 1997; Mugford et al., 1997). The initial product of CYP-catalyzed oxidation of furan is *cis*-2-butene-1,4-dial (BDA; Fig. 1), with CYP2E1 as the major catalyst of this reaction in both rodents and humans (Kedderis et al., 1993; Peterson et al., 2005; Lu et al., 2009; Gates et al., 2012). BDA is toxic and mutagenic (Peterson et al., 2000; Kellert et al., 2008a) and widely considered to be central to the mechanism of action for furan's toxic and carcinogenic activity. It is very reactive, readily alkylating cellular nucleophiles such as proteins and DNA (Chen et al., 1997; Gingipalli and Dedon, 2001; Byrns et al., 2002, 2004, 2006; Lu et al., 2009; Phillips et al., 2014). Chemical characterization of the urinary furan metabolites in rats demonstrate that they are all derived from the reaction of BDA with cellular nucleophiles (Kellert et al., 2008b; Lu et al., 2009; Lu and Peterson, 2010; Peterson et al., 2011).

Freshly isolated rat hepatocytes generate a variety of metabolites that result from the reaction of BDA with cellular nucleophiles (Fig. 1) (Lu et al., 2009; Peterson et al., 2011). BDA reacts directly with lysine to form pyrrolinone adducts (Chen et al., 1997). Acetylation of this reaction product generates a *N*-acetyl-lysine (NAL) metabolite, BDA-NAL (1; Fig. 1), which has been detected as a hepatocellular and urinary metabolite of furan (Kellert et al., 2008b; Lu et al., 2009). The other identified furan metabolites result from reaction of BDA with glutathione (GSH) to form 2-(S-glutathionyl)butanedial (GSH-BDA), which then reacts with a variety of cellular amines to form GSH-BDA-amine cross-links (Fig. 1) (Lu et al., 2009; Peterson et al., 2011). Collectively, these metabolites serve as biomarkers for BDA-derived adducts.

ABBREVIATIONS: BDA, *cis*-2-butene-1,4-dial; GSH, glutathione; GSH-BDA, 2-(S-glutathionyl)butanedial; LC-MS/MS, liquid chromatography with tandem mass spectrometry; NAL, *N*-acetyl-L-lysine; RT, retention time.

Fig. 1. Identified pathways of furan metabolism in hepatocytes.

Our hypothesis is that the reaction of BDA with cellular nucleophiles is responsible for furan's harmful effects. If human hepatocytes convert furan to the same BDA-derived reaction products as rat hepatocytes, it would provide strong evidence that humans are susceptible to its harmful effects. Rates of furan oxidation to BDA in human liver microsomes were comparable to those observed in rat and mouse liver microsomes (Gates et al., 2012). Similarly, CYP2E1-dependent metabolism of furan as measured by the disappearance of furan in human hepatocytes was comparable to that observed in rat and mouse hepatocytes (Kedderis and Held, 1996). However, this latter study did not characterize the products of furan oxidation. In this report, we compared the relative amount of BDA-derived metabolite formation in cryopreserved human hepatocytes to that generated by cryopreserved hepatocytes from B6C3F1 mice and F344 rats, two species susceptible to the harmful effects of furan.

Materials and Methods

Chemicals and Reagents. Aqueous solutions of BDA were prepared as previously described (Byrns et al., 2004). [$^{13}\text{C}_6^{15}\text{N}_2$]Lysine and [glycine- $^{13}\text{C}_2^{15}\text{N}$] glutathione ([$^{13}\text{C}_2^{15}\text{N}$]GSH) were purchased from Cambridge Isotope Laboratories (Andover, MA). Putrescine was purchased from MP Biomedicals (Solon, OH). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Furan was distilled prior to use and stored at -20°C in an amber vial with Teflon septa. Cryopreserved hepatocytes from male B6C3F1 mice (Lot 1010004), male F344 rats (Lot 0810456), and humans (Lot HC3-5A, HC5-6, HC5-8, and 780); hepatocyte isolation kits; and hepatocyte media were purchased from XenoTech (Lenexa, KS). The synthesis of [$^{13}\text{C}_6^{15}\text{N}_2$](R)-2-acetylaminopyrrolidin-6-(2,5-dihydro-2-oxo-1H-pyrrol-1-yl)-1-hexanoic acid ([$^{13}\text{C}_6^{15}\text{N}_2$]1) and the [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-amine standards are reported in Supplemental Material.

Hepatocyte Incubations. Rodent and human cryopreserved hepatocytes from XenoTech were thawed and isolated, according to the manufacturer's instructions. Cells were suspended in XenoTech's hepatocyte incubation media at a concentration of 1×10^6 cells/ml in sealed screw-capped 10-ml Erlenmeyer flasks to prevent furan evaporation, as previously reported (Carfagna et al., 1993; Lu et al., 2009; Peterson et al., 2011). After a 10-minute preincubation period at 37°C in an atmosphere of 5% CO_2 , the flasks were capped tightly and a solution of furan was added with a syringe through a septum to a final concentration of $100 \mu\text{M}$ (total volume = 1 ml) to mimic our previous studies with freshly isolated hepatocytes (Lu et al., 2009). Controls were performed in the absence of furan or in the presence of $200 \mu\text{M}$ 1-phenylimidazole (an inhibitor of CYP2E1) (Carfagna et al., 1993). The mixtures were then incubated at 37°C with gentle shaking. After 4 hours, the incubation mixtures were centrifuged at $6000g$ for 5 minutes. In both cases, the supernatant was removed and stored at -80°C for metabolite analysis. The experiments were repeated on another day with different vials of the same hepatocyte preparations to provide replicate experiments for furan-exposed hepatocytes. For each hepatocyte lot, one flask of cells was incubated with $125 \mu\text{M}$ *p*-nitrophenol to assess CYP2E1 activity under our incubation conditions. After treatment with β -glucuronidase ($28 \text{ U}/\mu\text{l}$ supernatant) at 37°C for 24 hours, levels of *p*-nitrophenol were determined according to published methods (Chang et al., 2006; Gates et al., 2012).

LC-MS/MS. The hepatocyte supernatants were diluted 1:8 in water prior to the addition of labeled standards. Two different sample preparations were performed, as follows: $2 \mu\text{l}$ [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-NAL (4) and $5 \mu\text{l}$ [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-amine mixture were added to one aliquot of supernatant, and 20 pmol [$^{13}\text{C}_6^{15}\text{N}_2$]NAL-pyrrolinone was added to another (total volume = $40 \mu\text{l}$). Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis was performed on a Synergi Hydro RP ($250 \times 0.5 \text{ mm}$, $4 \mu\text{m}$) column (Phenomenex, Torrance, CA) coupled to a Thermo Fisher TSQ Vantage or Quantum Ultra AM mass spectrometer (Thermo Fisher Scientific, Waltham, MA).

The GSH-BDA-amine metabolites were measured with the following LC-MS/MS assay. Samples (8 μ l) were eluted at a flow rate of 10 μ l/min in 10 mM ammonium formate, pH 2.8 (solvent A), and solvent A containing 50% (v/v) acetonitrile (solvent B) employing the following gradient: after 1 minute at 100% A, a 22-minute linear gradient to 30% B was performed, followed by a 3-minute linear gradient to 70% B and finally a 6.5-minute linear gradient to 90% B. The analysis for GSH-BDA-spermidine (**8**, retention time (RT): 18.9 and 21.0 minutes), GSH-BDA-NAL (**4**, RT: 23.8 minutes), and GSH-BDA-spermine (**9**, RT: 17.2 minutes) was performed in positive ionization mode with the following selected reaction transitions monitored: GSH-BDA-spermidine (**8**), m/z 501 to 483; [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-spermidine, m/z 504 to 486; GSH-BDA-NAL (**4**), m/z 544 to 415; [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-NAL, m/z 547 to 418; GSH-BDA-spermine (**9**), m/z 558 to 429; and [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-spermine, m/z 561 to 432. The analysis for GSH-BDA (**2**, RT: 33.4 minutes), GSH-BDA-putrescine (**7**, RT: 24.5 minutes), GSH-BDA-ornithine (**6**, RT: 19.4 and 23.4 minutes), GSH-BDA-glutamine (**5**, RT: 18.6 minutes), and GSH-BDA-lysine (**3**, RT: 22.4 and 26.2 minutes) was performed in negative ionization mode with the following selected reaction transitions monitored: GSH-BDA (**2**), m/z 354 to 141; [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA, m/z 357 to 143; GSH-BDA-putrescine (**7**), m/z 442 to 272; [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-putrescine, m/z 445 to 275; GSH-BDA-ornithine (**6**), m/z 486 to 272; [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-ornithine, m/z 489 to 275; GSH-BDA-lysine (**3**) and GSH-BDA-glutamine (**5**), m/z 500 to 272; and [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-lysine and [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-glutamine, m/z 503 to 275.

BDA-NAL (**1**) was analyzed separately with the mass spectrometer in negative ion mode. In this case, a Synergi column was eluted in 15 mM ammonium acetate, pH 6.8 (solvent A), and methanol (solvent B) employing the following gradient (flow rate of 10 μ l/min): after 5 minutes at 1% B, a 13-minute linear gradient to 70% B was performed. BDA-NAL eluted at 17.5 minutes and was quantified by monitoring the following selected reactions: BDA-NAL, m/z 253 to 211 and [$^{13}\text{C}_6^{15}\text{N}_2$]BDA-NAL, m/z 261 to 219.

Hepatocellular GSH Levels. GSH levels in lysates of untreated hepatocytes were determined using a Promega GSH-GSSG Glo assay kit (Madison, WI). The assay was performed according to the manufacturer's protocol.

Results and Discussion

To compare the levels of furan metabolites between different hepatocyte preparations and to assist in the confirmation of metabolite structure, isotopically labeled standards were prepared. BDA-NAL (**1**) was quantified with BDA- $^{13}\text{C}_6^{15}\text{N}_2$ NAL. This standard was synthesized by reacting BDA with [$^{13}\text{C}_6^{15}\text{N}_2$]lysine, followed by acetylation of the N^α -amine of lysine with acetic anhydride. Levels of this metabolite were measured by monitoring the neutral loss of 42 Da. Standard curves were generated with known ratios of unlabeled to labeled standard and had a slope of 1.3 (Supplemental Fig. 1A).

A semiquantitative approach was taken to compare the levels of GSH-BDA-amine cross-links. BDA was combined with [$^{13}\text{C}_2^{15}\text{N}$]GSH in the presence of lysine, glutamate, glutamine, ornithine, putrescine, spermidine, and spermine in 150 mM sodium phosphate, pH 7.4, to generate a mixture of [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-amine cross-links. GSH-BDA cross-links to these amines had been reported as furan metabolites in rat hepatocytes (Lu et al., 2009; Peterson et al., 2011). All the expected [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-amine cross-links were present in the reaction mixture. To test the ability of this reaction mixture to evaluate the extent of metabolite formation in hepatocytes, increasing amounts of a similarly prepared reaction mixture with unlabeled GSH were combined with a constant amount of the [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-reaction mixture and then analyzed by LC-MS/MS selectively monitoring the neutral loss of 129 Da. There was a linear relationship between the amount of the unlabeled reaction mixture and the ratio of the unlabeled to labeled GSH-BDA-amine cross-link (Supplemental Fig. 1, B–J).

Cryopreserved human hepatocyte preparations were selected based on their reported CYP2E1 activity (Supplemental Table 1). To ensure that the hepatocytes were metabolically active under our incubation

conditions (closed container for 4 hours at 37°C), their ability to convert the CYP2E1 substrate *p*-nitrophenol into *p*-nitrocatechol was measured under these conditions. All hepatocyte preparations had measurable CYP2E1 activity (Supplemental Table 1).

The ability of the cryopreserved hepatocyte preparations to convert furan to BDA-derived metabolites was determined using conditions previously reported for freshly isolated rat hepatocytes (Lu et al., 2009). They were incubated with 0 or 100 μ M furan for 4 hours in a closed container. The resultant media was analyzed by LC-MS/MS for the presence of the previously characterized rat hepatocyte metabolites (Supplemental Fig. 2). The formation of these metabolites required the presence of furan and was inhibited by the inclusion of the CYP2E1 inhibitor, 1-phenylimidazole (Supplemental Table 2). These observations are consistent with previous reports on the involvement of CYP2E1 in furan metabolism (Kedderis et al., 1993; Lu et al., 2009; Peterson et al., 2011; Gates et al., 2012).

Most of the expected metabolites were produced by all of the hepatocyte preparations. Coelution of the metabolites with the stable isotopically labeled standards provides support for their chemical structure. The detection of these metabolites indicates that humans can convert to furan the reactive metabolite BDA and that this molecule is targeting the same cellular nucleophiles as was observed in rats.

Because the [$^{13}\text{C}_2^{15}\text{N}$]GSH-BDA-amine reaction mixture was composed to produce approximately equal levels of the GSH-BDA-amine reaction products (Peterson et al., 2011), the ratio of metabolite to internal standard provides an approximate measure of the relative formation of each of the GSH-BDA-amine reaction products so some comparison between GSH-BDA-amine products can be made. GSH-BDA-lysine was the most abundant cross-link detected, particularly in the rodent hepatocytes. Levels of GSH-BDA-glutamine (**5**), GSH-BDA-ornithine (**6**), and GSH-BDA-spermidine (**8**) were less than or equal to those of GSH-BDA-lysine (**3**), depending on the hepatocyte source. Only low levels of GSH-BDA-putrescine (**7**) were detected.

In addition, the labeled standards were used to compare the amount of metabolism between the different preparations. Whereas the absolute concentration of each metabolite was not determined, this semiquantitative approach allows us to make interspecies comparisons for each of the metabolites. Based on this approach, it is clear that the rat and mouse hepatocytes produced similar levels of all of the furan metabolites, with the mouse preparation generally making more of the metabolites than rat hepatocytes. This is consistent with the approximately twofold higher level of CYP2E1 activity of the mouse hepatocytes relative to the rat cells (Supplemental Table 1) and matches the relative levels reported in previous studies (Kedderis and Held, 1996; Gates et al., 2012).

Human 1 hepatocytes were the most active preparation of the human cells in transforming furan to the BDA-derived metabolites and produced the highest levels of BDA-NAL (**1**; Fig. 2A). Measurable levels of mono-GSH-BDA (**2**) were detected in all six hepatocyte preparations (Fig. 2B). Rat and mouse hepatocytes were the most active at generating GSH-BDA-lysine (**3**), GSH-BDA-glutamine (**5**), and GSH-BDA-spermidine (**8**; Fig. 2, C, E, and H). GSH-BDA-NAL (**4**) was only detected in the rodent hepatocytes (Fig. 2D). Hepatocytes from Human 1 produced similar levels of GSH-BDA-ornithine (**6**) as the rat and mouse preparations (Fig. 2F). Preparations from Human 1 and 2 were the most active at generating GSH-BDA-putrescine (**7**; Fig. 2G). The detection of GSH-BDA-spermine proved unreliable in the hepatocyte media, so no conclusions could be drawn regarding the relative formation of this metabolite.

Although the human preparations had similar or greater CYP2E1 activity than rat and mouse hepatocytes, they generally produced lower levels of furan metabolites. Interestingly, the extent of furan

Fig. 2. Relative levels of furan metabolites in media from human, mouse, and rat hepatocytes incubated with 100 μ M for 4 hours at 37°C. (A) BDA-NAL, 1; (B) mono-GSH-BDA, 2; (C) GSH-BDA-lysine, 3 (solid bar: GSH-BDA- N^{α} -lysine, 3a; striped bar: GSH-BDA- N^{β} -lysine, 3b); (D) GSH-BDA-NAL, 4; (E) GSH-BDA-glutamine, 5; (F) GSH-BDA-ornithine, 6 (solid bar: GSH-BDA- N^{α} -ornithine, 6a; striped bar: GSH-BDA- N^{β} -ornithine, 6b); (G) GSH-BDA-putrescine, 7; (H) GSH-BDA-spermidine, 8 (solid bar: GSH-BDA- N^1 -spermidine, 8a; striped bar: GSH-BDA- N^8 -spermidine, 8b). Data are averages of two to four replicates. The data used to generate these charts are presented in Supplemental Table 3.

metabolite formation varied more than 10-fold for most of the metabolites despite the fact that all four human hepatocyte preparations had comparable levels of CYP2E1 activity (Supplemental Table 1). The liver cells from Human 1 were the most active at generating the metabolites detected in this study; hepatocytes from Humans 2–4 were less active and more similar to one another. One explanation of these observations is that some humans have alternative pathways of BDA biotransformation such that BDA is metabolized to other products, reducing its reaction with cellular nucleophiles. For example, certain CYP enzymes readily catalyze the reduction of α,β -unsaturated aldehydes (Amunom et al., 2011). Specific enzymes involved in this process are CYP2B6, CYP3A4, and CYP1A2. Interestingly, Human 4 had much higher levels of all three enzymes than Human 1, and Human 2 had 10 times the CYP1A2 activity than Human 1. It is possible that these enzymes are affecting the levels of BDA. Because Human 3 had similar levels of these enzymes as Human 1, it is likely that multiple factors influence the levels of these metabolites. Future studies will focus on the role of other enzymes to influence the levels of BDA-derived reaction products in hepatocytes.

Although the formation of BDA is catalyzed by CYP2E1, its reaction with GSH and other cellular nucleophiles is so rapid that it is most likely nonenzymatic (Peterson et al., 2005; Lu et al., 2009). Therefore, the relative distribution of the metabolites is driven in part by the relative concentrations of the cellular nucleophiles in the different hepatocyte preparations (Peterson et al., 2011). Consistent with this idea, the levels of the GSH-BDA-amine metabolites (GSH-BDA-lysine, GSH-BDA-glutamine, and GSH-BDA-spermidine) are much higher in the rodent hepatocytes than in the human ones, whereas the direct lysine reaction product, BDA-NAL, was more abundant in the human hepatocytes, particularly those from Human 1. These levels parallel the differences in the levels of GSH measured in cell lysates; the rodent cells have significantly higher levels of GSH than the human hepatocytes (Supplemental Table 1). Based on these observations, it is possible that there is a species difference in exposure to the two reactive metabolites of furan. Both BDA and GSH-BDA are known to alkylate protein nucleophiles, and both may contribute to furan's toxicity, with BDA being the more reactive of the two. Studies with 2-methylfuran suggest that, whereas GSH protects against DNA adduct formation, it

enhances the toxicity of 2-methylfuran and increases protein adduct formation in rats (Ravindranath and Boyd, 1991). The preferential formation of GSH-BDA-amine reaction products in the rat hepatocytes might explain the low levels of DNA adduct formation in furan-treated rats (Burka et al., 1991; Neuwirth et al., 2012).

The distribution of GSH-BDA-amine reaction products was also different in the three species. For example, Human 1 produced comparable levels of GSH-BDA-ornithine as the rodent hepatocytes but significantly lower levels of GSH-BDA-lysine (3), GSH-BDA-glutamine (5), and GSH-BDA-spermidine (8). Human 1 and 2 hepatocytes were the most active at generating GSH-BDA-putrescine (7); however, this metabolite was a minor metabolite detected in these incubation mixtures. The species and individual differences in GSH-BDA-amine metabolites are most likely a reflection of species and individual differences in the hepatocellular amine concentrations. In addition, these variations could also be driven by species differences with respect to further metabolism of the reaction product. For example, rat and mouse hepatocytes convert GSH-BDA-lysine (3) to its *N*-acetylated version, GSH-BDA-NAL (4), whereas this latter metabolite is not observed in any of the human liver cells.

In summary, human hepatocytes generate the same BDA-derived reaction products previously observed in rat hepatocytes in a CYP2E1-dependent manner. However, the levels of these metabolites were most likely influenced by the hepatocellular concentrations of GSH and the amines. In addition, the wide range of metabolite levels in hepatocytes from different people suggests that other factors influence the concentration of BDA available for reaction with nucleophiles in liver cells. Given that BDA is toxic and mutagenic, these studies provide further evidence that humans may be susceptible to the harmful effects of furan.

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Authorship Contributions

Participated in research design: Gates, Phillips, Peterson.

Conducted experiments: Gates, Phillips.

Contributed new reagents or analytic tools: Gates, Matter.

Performed data analysis: Gates, Phillips, Peterson.

Wrote or contributed to the writing of the manuscript: Gates, Phillips, Matter, Peterson.

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