Stereoselective Flunoxaprofen-S-acyl-glutathione Thioester Formation Mediated by Acyl-CoA Formation in Rat Hepatocytes^S

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ABSTRACT:

Flunoxaprofen (FLX) is a chiral nonsteroidal anti-inflammatory drug that was withdrawn from clinical use because of concerns of potential hepatotoxicity. FLX undergoes highly stereoselective chiral inversion mediated through the FLX-S-acyl-CoA thioester (FLX-CoA) in favor of the (*R*)-(-)-isomer. Acyl-CoA thioester derivatives of acidic drugs are chemically reactive species that are known to transacylate protein nucleophiles and glutathione (GSH). In this study, we investigated the relationship between the stereoselective metabolism of (*R*)-(-)- and (*S*)-(+)-FLX to FLX-CoA and the subsequent transacylation of GSH forming FLX-S-acyl-glutathione (FLX-SG) in incubations with rat hepatocytes in suspension. Thus, when hepatocytes (2 million cells/ml) were treated with (*R*)-(-)- or (*S*)-(+)-FLX (100 μ M), both FLX-CoA and FLX-SG were detected by sensitive liquid chromatography-tandem mass spectrometry techniques. However, these derivatives were observed

primarily from (*R*)-(-)-FLX incubation extracts, for which the formation rates of FLX-CoA and FLX-SG were rapid, reaching maximum concentrations of 42 and 2.8 nM, respectively, after 6 min of incubation. Incubations with (*S*)-(+)-FLX over 60 min displayed 8.1 and 2.7% as much FLX-CoA and FLX-SG area under the concentration versus time curves, respectively, compared with corresponding incubations with (*R*)-(-)-FLX. Coincubation of lauric acid (1000 μ M) with (*R*)-(-)-FLX (10 μ M) led to the complete inhibition of FLX-CoA formation and a 98% inhibition of FLX-SG formation. Reaction of authentic (*R,S*)-FLX-CoA (2 μ M) with GSH (10 mM) in buffer (pH 7.4, 37°C) showed the quantitative formation of FLX-SG after 3 h of incubation. Together, these results demonstrate the stereoselective transacylation of GSH in hepatocyte incubations containing (*R*)-(-)-FLX, which is consistent with bioactivation by stereoselective (*R*)-FLX-CoA formation.

Flunoxaprofen, [(*S*)-(+)-2-[2-(4-fluorophenyl)-1,3-benzoxazol-5-yl]propanoic acid; FLX] (Fig. 1), is a nonsteroidal anti-inflammatory drug (NSAID) that belongs to the 2-phenylpropionic acid (profen) structural class of carboxylic acid-containing drugs used for the treatment of inflammatory diseases (Williams et al., 1993). Profen drugs

have a chiral center at the carbon alpha to the carboxylic acid, where the significant anti-inflammatory activity resides primarily in the (*S*)-(+)-isomer (Evans, 1992). FLX was marketed only in Italy and specifically as the (*S*)-(+)-isomer; however, it was withdrawn from clinical use because of concerns of potential hepatotoxicity based on its close-structural similarity to the previously withdrawn hepatotoxic NSAID, benoxaprofen [(*R,S*)-2-[2-(4-chlorophenyl)-1,3-benzoxazol-5-yl]propanoic acid, BNX] (Bakke et al., 1995). Other profen-type NSAIDs that have been withdrawn from clinical use include indoprofen, suprofen, and pirofen (Fung et al., 2001). The most frequent types of adverse reactions leading to their discontinuation were idiosyncratic allergic reactions including hepatotoxicity, nephrotoxicity, blood dyscrasias, photosensitivities, allergic skin reactions, and anaphylaxis, sometimes associated with fever, rash, and eosinophilia (Boelsterli et al., 1995).

A remarkable metabolic feature of profen-type drugs is that they can undergo stereoselective chiral inversion resulting in the (*R*)-isomer being converted to the (*S*)-isomer (Caldwell et al., 1988; Iwakawa et al., 1991). The profen chiral inversion process is known to be initiated by acyl-CoA ligase-mediated conjugation with CoA,

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ABBREVIATIONS: FLX, flunoxaprofen, (*S*)-(+)-2-[2-(4-fluorophenyl)-1,3-benzoxazol-5-yl]propanoic acid; NSAID, nonsteroidal anti-inflammatory drug; BNX, benoxaprofen, (*R,S*)-2-[2-(4-chlorophenyl)-1,3-benzoxazol-5-yl]propanoic acid; FLX-CoA, FLX-S-acyl-CoA thioester; FLX-1-O-G, FLX-1-O-acyl glucuronide; GSH, glutathione; FLX-SG, FLX-S-acyl-glutathione thioester; CBZ, carbamazepine; LC, liquid chromatography; MS/MS, tandem mass spectrometry; MS, mass spectrometry; CID, collision-induced dissociation; HPLC, high-performance liquid chromatography; MRM, multiple reaction monitoring; I-CoA, (*R,S*)-ibuprofen-acyl-CoA; I-SG, ibuprofen-S-acyl-glutathione; P450, cytochrome P-450; AUC, area under the curve.

FIG. 1. Proposed scheme for the metabolism of FLX by acyl glucuronidation and acyl-CoA formation, leading to the stereoselective transacylation of GSH to form FLX-SG.

followed by an epimerase-mediated chiral inversion of the acyl-CoA intermediate and finally by nonstereoselective hydrolysis to the chiral inverted free acid (Williams et al., 1993). The stereoselectivity of profen chiral inversion is believed to be driven by the stereoselectivity of profen-*S*-acyl-CoA thioester formation in favor of the (*R*)-isomer (Caldwell et al., 1988; Xiaotao and Hall, 1993). Indeed, for FLX, in vivo studies in rat with the (*R*)-(-)- and (*S*)-(+)-isomers showed that chiral inversion was highly stereoselective for the (*R*)-(-)-isomer (Iwakawa et al., 1991), and, therefore, FLX was proposed to have undergone highly stereoselective metabolism to (*R*)-FLX-*S*-acyl-CoA [(*R*)-FLX-CoA] (Fig. 1). However, studies on the direct identification of FLX-CoA formed in vitro have not been performed before the present work. In contrast to undergoing (*R*)-isomer selective chiral inversion, FLX undergoes stereoselective acyl glucuronidation to FLX-1-*O*-acyl glucuronide (FLX-1-*O*-G) in vivo in rat by approximately 2-fold in favor of the (*S*)-(+)-isomer (Iwakawa et al., 1991). The FLX-1-*O*-G metabolite is known to be unstable and chemically reactive and has been proposed to mediate the covalent binding of FLX to hepatic and plasma proteins shown to occur in vivo in rat and human (Dahms and Spahn-Langguth, 1996; Dong et al., 2005). Acyl glucuronide metabolites of acidic drugs can react with protein by the transacylation of nucleophilic amino acid residues with the 1-*O*-acyl glucuronide isomer and by glycation of lysine amino groups via the open-chain aldehyde forms of acyl migration glucuronide isomers (Faed, 1984; Spahn-Langguth and Benet, 1992). Acyl-CoA thioester derivatives of carboxylic acid-containing drugs are also chemically reactive species that can transacylate protein nucleophiles as well as the nucleophilic cysteinyl thiol of GSH (Grillo and Benet, 2002; Skonberg et al., 2008). A working hypothesis is that carboxylic acid drug-protein adducts, formed from the reaction of acyl glucuronides and/or acyl-CoA thioester intermediates with protein nucleophiles, are recognized by the immune system as foreign, resulting in an idiosyn-

cratic immune-mediated hepatotoxic reaction (Benet et al., 1993; Zia-Amirhosseini et al., 1995; Boelsterli, 2002; Dong et al., 2005; Skonberg et al., 2008).

In the present studies, we investigated the ability of the (*R*)-(-)- and (*S*)-(+)-isomers of FLX to be metabolized stereoselectively to FLX-CoA in incubations with freshly isolated rat hepatocytes and subsequently through the transacylation-type reaction of FLX-CoA with GSH to FLX-*S*-acyl-GSH (FLX-SG) (Fig. 1). We also performed experiments examining the chemical reactivity of authentic FLX-CoA with GSH forming FLX-SG in buffer under physiologic conditions.

In summary, these studies were focused on investigating the contribution of FLX metabolism by acyl-CoA formation in the subsequent transacylation of GSH and therefore potentially the transacylation of hepatocellular protein nucleophiles, in incubations with freshly isolated rat hepatocytes in suspension.

Materials and Methods

Materials. (*R,S*)-, (*R*)-(-)-, and (*S*)-(+)-FLX were obtained from Ravizza Laboratories (Milan, Italy) and are the same derivatives that were used in prior studies (Iwakawa et al., 1991). The (*R*)-(-)-isomer contained 9.3% of the (*S*)-(+)-antipode, and the (*S*)-(+)-isomer contained 3.8% of the (*R*)-(-)-antipode. Carbamazepine (CBZ), GSH, lauric acid sodium salt, (-)-borneol, and CoA were purchased from Sigma-Aldrich (St. Louis, MO). FLX-CoA and FLX-SG were synthesized as described below. Ibuprofen-*S*-acyl-CoA and ibuprofen-*S*-acyl-glutathione were available from previous studies (Grillo and Hua, 2008). All solvents used for liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis were of chromatographic grade. Stock solutions of (*R*)-(-)-FLX, (*S*)-(+)-FLX, FLX-CoA, and FLX-SG were prepared as solutions in acetonitrile-water (50:50, v/v; pH 5). All FLX-related solutions were stored frozen (-20°C) and kept away from light to avoid photo degradation.

Instrumentation and Analytical Methods. The FLX-CoA and FLX-SG derivatives were characterized by LC-MS/MS on a TSQ Quantum Discovery Max mass spectrometer (Thermo Fisher Scientific, Waltham, MA) linked to an Agilent 1100 high-performance liquid chromatograph (Agilent Technologies, Santa Clara, CA) and a CTC HTS PAL autosampler (Leap Technologies, Carrboro, NC). LC-MS/MS analysis of FLX-CoA, FLX-SG, and FLX-1-*O*-G derivatives was performed with a reverse-phase column [Luna 5 μ , C18(2), 100 Å, 150 $\times$ 2.00 mm; Phenomenex, Torrance, CA] and eluted with a mobile phase flow rate of 0.3 ml/min. The mobile phase used for the analysis of FLX-CoA consisted of ammonium acetate (10 mM, pH 6.0) in water (solvent A) and acetonitrile (solvent B). The mobile phase used for the analysis of FLX-SG and FLX-1-*O*-G derivatives consisted of 0.1% formic acid in water (solvent C) and 0.08% formic acid in acetonitrile (solvent D). For these LC-MS/MS analyses, gradient elution was achieved by increasing solvent B or solvent D from 0 to 95% over 13 min. Electrospray ionization was used with the needle potential held at 4.5 kV. The MS/MS conditions used were 2 mtorr argon collision gas and a collision potential of 25 eV. Positive ion mode full-scan (m/z 50 to m/z 1200) LC-MS analysis was conducted with a scan time of 0.73 s and source collision energy of 10 V. Xcalibur software (version 2.0; Thermo Fisher Scientific) was used to acquire all data. ^1H NMR spectra were recorded on an Avance II spectrometer (Bruker Deltronics, Billerica, MA) operating at 500 MHz and using a 5-mm, general-purpose, cryogenically cooled probe (QNP CryoProbe; Bruker BioSpin Corporation, Fremont, CA). Chemical shifts are reported in parts per million as referenced to the residual solvent peak (2.49 ppm for $^2\text{H}_6$ -dimethyl sulfoxide).

Synthesis of (*R,S*)-FLX-SG Thioester. (*R,S*)-FLX-SG was synthesized by a conventional method using ethyl chloroformate, analogous to a procedure reported previously for the synthesis of clofibryl-*S*-acyl-glutathione thioester (Grillo and Benet, 2002). When the synthetic (*R,S*)-FLX-SG derivative, which was obtained as a white solid (27% yield), was characterized by LC-MS with use of the gradient elution method described above, it eluted at a retention time of 7.0 min and showed no detectable impurities when analyzed by both positive and negative ion scan modes. LC-MS/MS analysis of (*R,S*)-FLX-SG: [collision-induced dissociation (CID) of MH^+ ion at m/z 575], m/z (%): m/z 500 ($[\text{M} + \text{H} - \text{Gly}]^+$, 32%), m/z 446 ($[\text{M} + \text{H} - \text{pyroglutamic acid}]^+$, 21%),

m/z 428 ($[M + H - \text{pyroglutamic acid} - \text{water}]^+$, 60%), m/z 371 ($[\text{FLX-S-CH}_2\text{CH}(\text{NH}_2)\text{C} = \text{O}]^+$, 100%), m/z 343 ($[\text{FLX-S-CH}_2\text{CH} = \text{NH}_2]^+$, 95%), m/z 308 ($[\text{glutathione} + \text{H}]^+$, 4%), m/z 240 ($[\text{2(4-fluorophenyl)-}\alpha\text{-methyl-5-ethylbenzoxazole}]^+$, 54%). ^1H NMR analysis of (*R,S*)-FLX-SG ($^2\text{H}_6$ -dimethyl sulfoxide): δ 1.49–1.50 (d, 3H, $\alpha\text{-CH}_3$), δ 1.75–1.95 (m, 2H, Glu- β , Glu- β'), δ 2.22–2.38 (m, 2H, Glu- γ , γ'), δ 2.85–2.95 (m, 2H, Cys- β , β'), δ 2.40–2.42, 2H, isopropyl- CH_2), δ 3.44–3.48 (t, 1H, Glu- α), δ 3.65–3.72 (m, 2H, Gly- α , α'), δ 4.18–4.22 (d, 1H, FLX $\alpha\text{-CH}$), δ 4.30–4.41 (dt, 1H, Cys- α), δ 7.35–7.39 (dd, 1H, benzoxazole, *para* to N), δ 7.45–7.51 (m, 2H, fluorobenzene, *meta* to F), δ 7.74–7.78 (d, 2H, benzoxazole, *meta* and *ortho* to N), 8.24–8.28 (m, 2H, fluorobenzene, *ortho* to F), δ 8.42–8.47 (d, 1H, Cys NH), δ 8.64–8.67 (d, 1H, Gly NH).

Synthesis of (*R,S*)-FLX-CoA Thioester. (*R,S*)-FLX-CoA thioester was obtained by a synthetic procedure using ethyl chloroformate and analogous to that previously reported for the synthesis and purification of clofibryl-S-acyl-CoA thioester (Grillo and Benet, 2002). The (*R,S*)-FLX-CoA thioester eluted at a retention time of 5.5 min and showed no detectable impurities when analyzed by both positive and negative ion LC-MS scan modes via reverse-phase gradient elution as described above. LC-MS/MS analysis of synthetic (*R,S*)-FLX-CoA by CID of the protonated molecular ion at $\text{MH}^+ m/z$ 1035 yielded a product ion mass spectrum, m/z (%): m/z 608 ($[M + H - 427]^+$, 13%), m/z 528 ($[M + H - 507]^+$, 100%), m/z 428 (adenosine diphosphate + 2H) $^+$, 5%), m/z 426 ($[M + H - 609]^+$, 5%), m/z 240 ($[\text{2(4-fluorophenyl)-}\alpha\text{-methyl-5-ethylbenzoxazole}]^+$, 45%).

In Vitro Studies with Rat Hepatocytes. Freshly isolated hepatocytes were prepared and incubated according to the method of Moldéus et al. (1978). Hepatocytes were isolated from Sprague-Dawley rats (250–300 g, male; Charles River Laboratories, Worcester, MA) and >95% viability was achieved as assessed by trypan blue exclusion testing. Hepatocytes were warmed to 37°C in a water bath under an atmosphere of 95% O_2 and 5% CO_2 for 15 min before the initiation of metabolism experiments. Incubations of hepatocytes (2 million viable cells/ml; 4–10 ml total volume; $n = 3$) with (*R*)-(-) and/or (*S*)-(+)-FLX isomers were performed in Krebs-Henseleit buffer (pH 7.4) in 20-ml glass vials and with continuous rotation under an atmosphere of 95% O_2 and 5% CO_2 at 37°C in a model 1927 humidified cell culture incubator (VWR, Willard, OH).

For time-dependent studies, freshly isolated hepatocytes were incubated with (*R*)-(-) or (*S*)-(+)-FLX (100 μM) and analyzed for FLX-CoA, FLX-SG, and FLX-1-*O*-G formation over a 60-min time period. For the analysis of FLX-SG and FLX-1-*O*-G derivatives, aliquots (200 μl) of the incubation mixture were taken at 0.2, 4, 6, 8, 10, 20, 30, and 60 min and added directly to microcentrifuge tubes (2 ml) containing a quench solution (200 μl) consisting of acetonitrile, 3% formic acid, and 2 μM CBZ internal standard. Samples then were centrifuged (14,000g, 5 min), and aliquots (300 μl) of the supernatants were transferred to HPLC autosampler vials (0.4-ml polypropylene; Sun International, Wilmington, NC) before LC-MS/MS analysis.

For the analysis of FLX-CoA formation, aliquots (200 μl) from the same incubations as described above were taken and added to microcentrifuge tubes (2 ml) containing a quench solution consisting of acetonitrile (without formic acid) and CBZ (2 μM , 400 μl), followed by the addition of hexane (600 μl). The samples then were vortex-mixed (1 min) and centrifuged (14,000g, 10 min), and aliquots (300 μl) of the aqueous layer were transferred to HPLC autosampler vials for LC-MS/MS analysis of FLX-CoA.

Concentration-dependent experiments were performed with increasing concentrations of (*R*)-(-)-FLX or (*S*)-(+)-FLX (3.90, 7.81, 15.6, 31.3, 62.5, 125, and 250 μM) incubated with rat hepatocytes (2 million cells/ml) for 6 min and processed as described above for LC-MS/MS analysis of FLX-SG and FLX-CoA derivatives.

An inhibition experiment was performed with (*R*)-(-)-FLX (10 μM) incubated with rat hepatocytes (2 million cells/ml) in the presence or absence of (-)-borneol (100 μM), for the inhibition of FLX-1-*O*-G formation, or lauric acid (1000 μM), for the inhibition of FLX-CoA formation. Hepatocyte incubations ($n = 3$) were performed for 6 min, and aliquots were taken and processed as described above for the analysis of FLX-SG, FLX-1-*O*-G, and FLX-CoA derivatives. A stock solution of (-)-borneol (100 mM) was prepared in ethanol, and control incubations included the same final concentration of ethanol (0.1%, v/v). A stock solution of lauric

acid sodium salt was prepared as a 100 mM solution in distilled water (pH 7).

An experiment was performed to assess the stability of (*R,S*)-FLX-SG thioester (1 μM) in incubations with a rat hepatocytes (2 million cells/ml). Aliquots (200 μl) from hepatocyte incubations (5-ml volume, $n = 2$ replicates) were taken at 0, 1, 2, 3, 4, 5, and 7 min, added to quench solution, and processed as described above for the LC-MS detection of FLX-SG. Analysis of the amount of FLX-SG remaining in the incubations was performed by LC-MS (positive ion scan mode) detection and quantified by a linear standard curve generated from (*R,S*)-FLX-SG ($\text{MH}^+ m/z$ 575)/CBZ ($\text{MH}^+ m/z$ 237) peak area ratios obtained from extracted ion chromatograms.

Identification and Quantification of FLX-SG. Extracts of (*R*)-(-) and (*S*)-(+)-FLX-treated rat hepatocyte incubations were analyzed by LC-MS/MS for FLX-SG and CBZ by using the multiple reaction monitoring (MRM) transitions $\text{MH}^+ m/z$ 575 to m/z 240, for FLX-SG detection, and $\text{MH}^+ m/z$ 237 to m/z 194, for CBZ detection, in the positive ion mode with use of the chromatographic method described above. Authentic FLX-SG standard eluted at a retention time of 7.0 min, whereas CBZ eluted at 7.6 min. The concentration of FLX-SG thioester was determined from a linear standard curve generated from FLX-SG/CBZ peak area ratios.

Identification and Quantification of FLX-CoA. Extracts of (*R*)-(-) and (*S*)-(+)-FLX-treated rat hepatocyte incubations were analyzed by LC-MS/MS for FLX-CoA and CBZ by using the MRM transitions $\text{MH}^+ m/z$ 1035 to m/z 528 and $\text{MH}^+ m/z$ 237 to m/z 194, respectively, in the positive ion mode and by using the chromatographic method described above. Authentic (*R,S*)-FLX-CoA standard eluted at a retention time of 5.5 min, whereas CBZ eluted at 6.2 min. The concentration of FLX-CoA thioester was determined from a linear standard curve generated from FLX-CoA/CBZ peak area ratios.

Identification of FLX-1-*O*-G. The (*R*)- and (*S*)-FLX-1-*O*-G derivatives were not obtained as authentic standards for these studies, but their formation in incubations with hepatocytes was confirmed by treatment of (*R*)-(-) and (*S*)-(+)-FLX (100 μM) incubation (2 million cells/ml, 10 min) extracts with β -glucuronidase. For carboxylic acid compounds, the β -glucuronidase enzyme is known to specifically cleave 1-*O*-acyl-linked acyl glucuronides and not the 2-, 3-, or 4-*O*-acyl glucuronide migration isomers (Faed, 1984). Thus, (*R*)-(-) and (*S*)-(+)-FLX hepatocyte incubation extracts (acetonitrile and formic acid, 1:1, 50 μl) were incubated with β -glucuronidase (1000 units/ml) for 0 and 30 min (2-ml total volume, pH 5.0, and 37°C, as per the manufacturer's instructions) and then quenched by the addition of a solution containing acetonitrile, 3% formic acid, and 0.2 μM CBZ, followed by centrifugation (14,000 rpm, 10 min). Results from HPLC analysis of the resulting supernatants (reverse-phase gradient elution as described above with UV analysis at 210 nm) of the 0- and 30-min incubation extracts showed that the HPLC peaks corresponding to (*R*)-FLX-1-*O*-G (retention time $\sim$ 10.6 min) and (*S*)-FLX-1-*O*-G (retention time $\sim$ 10.3 min) detected in the 0-min β -glucuronidase-treated extract, were completely absent in the 30-min β -glucuronidase-treated extract (data not shown). Therefore, these acyl glucuronides were designated as the FLX-1-*O*-G metabolites of (*R*)-(-) and (*S*)-(+)-FLX. LC-MS/MS analysis showed mass spectra that were nearly identical for the FLX-1-*O*-G derivatives obtained from incubations with (*R*)-(-) and (*S*)-(+)-FLX: (CID of MH^+ ion at m/z 462), m/z 240 ($[\text{2(4-fluorophenyl)-}\alpha\text{-methyl-5-ethylbenzoxazole}]^+$, 20%), m/z 286 ($\text{FLX} + \text{H}^+$, 100%) (Supplemental Fig. 3). Analysis for the formation of FLX-1-*O*-G in incubations of (*R*)-(-) or (*S*)-(+)-FLX with rat hepatocytes was performed by LC-MS/MS in the positive ion mode with MRM transitions $\text{MH}^+ m/z$ 462 to m/z 286 for FLX-1-*O*-G detection and $\text{MH}^+ m/z$ 237 to m/z 194 for CBZ detection and with the same LC-MS/MS chromatography method as described above for the analysis of FLX-SG.

Reactions of (*R,S*)-FLX-CoA with GSH in Buffer. Incubations (4 ml, $n = 3$) containing both (*R,S*)-FLX-CoA (2 μM) and (*R,S*)-ibuprofen-acyl-CoA (I-CoA, 2 μM , used as a direct comparator) thioesters were performed in phosphate buffer (0.1 M, pH 7.4) at 37°C with 10 mM GSH in 20-ml glass vials. Aliquots (200 μl) were removed from the incubations at 0, 5, 10, 20, 30, 60, 120, and 180 min and added to a quench solution (acetonitrile containing 3% formic acid and 2 μM CBZ, 200 μl) in a 96-well plate. The quenched mixtures then were analyzed for FLX-SG (as described above) and ibuprofen-S-acyl-glutathione (I-SG) concentrations by LC-MS/MS. The I-SG derivative was detected using the MRM transition $\text{MH}^+ m/z$ 496 to the major product ion m/z 349 (Grillo and Hua, 2008).

FIG. 2. Representative reverse-phase gradient LC-MS/MS MRM chromatograms of FLX-CoA from the analysis of extracts from untreated rat hepatocytes (A), rat hepatocytes incubated for 6 min with 100 μ M (*S*)-(+)-FLX (B), rat hepatocytes incubated for 6-min with 100 μ M (*R*)-(-)-FLX (C), and extract from rat hepatocytes spiked with (*R,S*)-FLX-CoA authentic standard (10 nM) (D). The LC-MS/MS MRM transition used for the analysis of FLX-CoA was MH^+ m/z 1035 to m/z 528. The relative abundances for each trace on the y-axes are equal.

Identification of Thioether-Linked FLX-GSH Adducts. Extracts of (*R*)-(-)- and (*S*)-(+)-FLX-treated rat hepatocyte incubations (60 min) were analyzed by LC-MS/MS for proposed GSH adducts potentially formed through common cytochrome P450 (P450)-mediated bioactivation reactions by searching for the respective mass changes of the drug moiety (Zheng et al., 2007). Therefore, we examined extracts by LC-MS, using the same chromatography methods as those used for the detection of FLX-SG, by positive ion scanning for MH^+ ions at m/z 589, m/z 591, and m/z 609, which represent GSH adduct compositions of FLX + GSH + oxygen - F, FLX + GSH, and FLX + GSH + oxygen, respectively. LC-MS/MS analysis of these extracts was performed on these ions as described above for the analysis of FLX-SG.

Results

Identification of FLX-CoA. Analysis of incubation extracts by LC-MS/MS MRM detection allowed identification of FLX-CoA

formed in rat hepatocyte incubations (Fig. 2). The transition used for this analysis was MH^+ m/z 1035 to m/z 528, which was chosen because it is a major fragmentation pathway for authentic (*R,S*)-FLX-CoA as assessed by positive ion LC-MS/MS CID of the MH^+ ion (Fig. 3A). Reverse-phase LC-MS/MS analysis showed the presence of FLX-CoA in incubations of (*R*)-(-)- and (*S*)-(+)-FLX (100 μ M) with rat hepatocytes, which coeluted with authentic (*R,S*)-FLX-CoA standard at a retention time of 5.5 min (Fig. 2). FLX-CoA was also detected in extracts from incubations with (*S*)-(+)-FLX (Fig. 3B), but at a level that was $\sim 8\%$ (relative LC-MS/MS peak area) as much as detected at the 6-min time point compared with that detected in extracts from incubations with (*R*)-(-)-FLX. LC-MS/MS analysis of FLX-CoA formed in hepatocytes provided a product ion spectrum from CID of the MH^+ ion at m/z 1035 that was identical to the

FIG. 3. Tandem mass spectra of authentic (*R,S*)-FLX-CoA standard (A) and FLX-CoA (B) formed in rat hepatocyte incubations with (*R*)-(-)-FLX (100 μ M, 6 min) obtained by CID of the protonated molecular ion at m/z 1035. The origins of the characteristic fragment ions are as shown.

FIG. 4. Representative reverse-phase gradient LC-MS/MS MRM chromatograms of FLX-SG from the analysis of untreated rat hepatocytes (A), extract from rat hepatocytes incubated for 6 min with 100 μ M (S)-(+)-FLX (B), extract from rat hepatocytes incubated for 6 min with 100 μ M (R)-(-)-FLX (C), and extract from rat hepatocytes spiked with (R,S)-FLX-SG authentic standard (2 nM) (D). The transition used for the LC-MS/MS MRM analysis of FLX-SG was MH^+ m/z 575 to m/z 240. The relative abundances for each trace on the y-axes are equal.

authentic (R,S)-FLX-CoA standard and consistent with its chemical structure (Fig. 3).

Identification of FLX-SG. Analysis of extracts by a sensitive LC-MS/MS MRM detection technique allowed identification of FLX-SG formed in rat hepatocyte incubations (Fig. 4). The transition used for this analysis was MH^+ m/z 575 to m/z 240, which was chosen because it is the major fragmentation pathway for FLX-SG as assessed by positive ion LC-MS/MS CID of the MH^+ ion of authentic (R,S)-FLX-SG (Fig. 5A). LC-MS/MS analysis showed the presence of FLX-SG in incubations of (R)-(-)-FLX (100 μ M) with rat hepato-

cytes, which coeluted with authentic (R,S)-FLX-SG standard at a retention time of 7.0 min (Fig. 4). FLX-SG was also detected in extracts from incubations with (S)-(+)-FLX (Fig. 4B) but at a level that was $\sim$ 3% (relative LC-MS/MS peak area) as much as detected at the 6-min time point compared with that detected in extracts from incubations with (R)-(-)-FLX. LC-MS/MS analysis of FLX-SG formed in hepatocytes provided a product ion spectrum that produced fragment ions identical to the authentic (R,S)-FLX-SG standard and also consistent with its chemical structure (Fig. 5) [see Baillie and Davis (1993) for characteristic GSH adduct product ions from positive ion electrospray LC-MS/MS analysis].

LC-MS/MS Detection of FLX-1-O-G. The 1-O-acyl glucuronides of (R)-(-)- and (S)-(+)-FLX were detected by positive ion LC-MS/MS analysis of (R)-(-)- and (S)-(+)-FLX rat hepatocyte (2 million cells/ml) incubation extracts. Results from these analyses showed FLX-1-O-G eluting at retention times 10.6 and 10.3 min from incubations with (R)-(-)- and (S)-(+)-FLX, respectively (Supplemental Figs. 1 and 2). The order of elution of the FLX-1-O-G isomers, for which the glucuronide formed from the (S)-(+)-isomer eluted earlier than the glucuronide of the (R)-(-)-isomer, is consistent with the elution order shown in previous reports for other profen-1-O-acyl glucuronides (el Mouelhi et al., 1987). LC-MS/MS analysis of FLX-1-O-G metabolites formed in hepatocytes incubated with (R)-(-)- or (S)-(+)-FLX provided product ion spectra containing fragment ions that were consistent with their chemical structures (Supplemental Fig. 3). The product ion spectrum for both derivatives showed that the major fragment ion (100% relative abundance) upon CID of the MH^+ ion at m/z 462 was m/z 286. Evidence of acyl migration isomers was detected during the analysis of both (R)-(-)- and (S)-(+)-FLX incubation extracts (30- and 60-min time points) (Supplemental Figs. 1 and 2). For the (R)-(-)-FLX incubation extracts, the formation of at least two acyl migration isomers was detected; they eluted before and after the 1-O-acyl isomer at 9.9 and 11.2 min, respectively (Supplemental Fig. 1). Acyl migration seemed to occur less for the (S)-FLX-1-O-acyl glucuronide, for which only one acyl migration isomer was detected, eluting after (S)-FLX-1-O-G at 11.3 min (Supplemental Fig. 2). The increased instability of the (R)-FLX-1-O-acyl isomer compared with that of the (S)-antipode is consistent with our chemical

FIG. 5. Tandem mass spectra of authentic (R,S)-FLX-SG standard (A) and FLX-SG formed in rat hepatocyte incubations with (R)-(-)-FLX (100 μ M, 6 min) obtained by CID of the protonated molecular ion at m/z 575 (B). The origins of the characteristic fragment ions are as shown.

stability reports on a range of profen 1-*O*-acyl glucuronides (Benet et al., 1993; Li et al., 2002a).

Time Course of FLX-CoA, FLX-SG, and FLX-1-*O*-G Formation in Incubations with Rat Hepatocytes. When (*R*)-(-)-FLX (100 μ M) was incubated with hepatocytes, the formation of FLX-CoA was rapid and reached a maximum concentration of 42 nM after only 6 min of incubation (Fig. 6A). The formation of FLX-CoA in incubations with (*S*)-(+)-FLX (100 μ M) was much lower, whereas area under the curve (AUC_{0→60 min}) was 8.1% compared with the formation of FLX-CoA occurring in corresponding incubations with (*R*)-(-)-FLX. Formation of FLX-SG in hepatocyte incubations with (*R*)-(-)-FLX was also rapid and reached a maximum concentration of ~2.8 nM at the 6-min time point (Fig. 6B). In contrast, incubations with (*S*)-(+)-FLX showed significantly less AUC_{0→60 min} of FLX-SG, forming ~2.7% of that produced in corresponding incubations with (*R*)-(-)-FLX. For both (*R*)-(-)- and (*S*)-(+)-FLX isomers in these hepatocyte incubations, no significant change in the initial substrate concentration was observed over the 60-min time period; however, we were not able to distinguish between the (*R*)-(-)- and (*S*)-(+)-FLX isomers and the potential for chiral inversion in the present work (Fig. 6D). The time course of FLX-1-*O*-G formation observed from the analysis of extracts from hepatocyte incubations with (*R*)-(-)- and (*S*)-(+)-FLX and based on FLX-1-*O*-G/CBZ peak area ratios showed that the FLX-1-*O*-G formation rates were linear and identical over the first 20 min of incubation for these two isomers (Fig. 6C). After 60 min of incubation, based on LC-MS/MS peak area ratios, the relative amount of (*R*)-FLX-1-*O*-G formed in incubations with (*R*)-(-)-FLX was ~60% of the (*S*)-FLX-1-*O*-G formed in incubations with (*S*)-(+)-FLX. In addition to these time course data, the time course of degradation of authentic (*R,S*)-FLX-SG (1 μ M) in incubations with rat hepatocytes showed the derivative to be degraded in a very rapid fashion ($t_{1/2}$ ~1.5 min) (Supplemental Fig. 4) and almost completely cleared from the incubation by the 7-min time point. The FLX-SG derivative was found to be hydrolyzed to FLX free acid in an almost quantitative fashion, similar to that determined

for diclofenac-*S*-acyl-glutathione (Grillo et al., 2003) and not due to γ -glutamyltranspeptidase-mediated degradation, whereas the activity of this enzyme is known to be negligible in rat liver tissue (Hinchman and Ballatori, 1990). This rapid degradation is similar to that shown in published reports with zomepirac-SG, diclofenac-SG, and ibuprofen-SG thioesters, for which the degradation rates were also rapid (measured $t_{1/2}$ values of 0.8, 1.0, and 4.0 min, respectively) (Grillo and Hua, 2003, 2008; Grillo et al., 2003). More importantly, *S*-acyl-glutathione thioesters are themselves chemically reactive species that can transacylate nucleophiles such as *N*-acetylcysteine and therefore might also contribute to the transacylation of protein nucleophiles (Grillo and Benet, 2002); however, we did not characterize the chemical reactivity of FLX-SG in the present studies.

Concentration-Dependent Formation of FLX-CoA and FLX-SG in Incubations with Rat Hepatocytes. When rat hepatocytes (2 million cells/ml) were incubated for 6 min with increasing concentrations of (*R*)-(-)-FLX, results showed a sharp concentration-dependent formation of FLX-CoA from 3.90 to 62.5 μ M (*R*)-(-)-FLX, reaching 39.5 nM FLX-CoA (Fig. 7A). No statistically significant increase in FLX-CoA formation was observed at concentrations of (*R*)-(-)-FLX greater than 62.5 μ M. From these same incubations, FLX-SG formation was also found to increase sharply with increasing concentrations of (*R*)-(-)-FLX (Fig. 7B). The concentration of FLX-SG reached a maximum of ~2.6 nM at 62.5 μ M (*R*)-(-)-FLX. At greater than 62.5 μ M (*R*)-(-)-FLX, the FLX-SG concentration did not change significantly. A similar trend was observed for incubations conducted with the (*S*)-(+)-FLX isomer, except that there was much less FLX-CoA and FLX-SG detected. Based on the *R/S* ratio of calculated AUC_{3.9→250 μ M} values, the average amounts of FLX-CoA and FLX-SG formed in incubations with (*S*)-(+)-FLX were 12.5 and 6.2%, respectively, as much as that formed in incubations with (*R*)-(-)-FLX. More importantly, a significant portion or all of the FLX-CoA and FLX-SG formed in incubations with the (*S*)-(+)-FLX isomer may have come from the 3.8% (*R*)-(-)-FLX isomer contamination. For instance, hepatocyte incubations containing 100 μ M

FIG. 6. Time course for the formation of FLX-CoA (A), FLX-SG (B), and FLX-1-*O*-G in rat hepatocytes (2 million cells/ml) incubated with 100 μ M (*R*)-(-)- or (*S*)-(+)-FLX (C). D, concentration of FLX free acid [(*R*)-(-)- and/or (*S*)-(+)-FLX] over the 60-min incubation. Values are expressed as the mean $\pm$ S.D. from three incubations.

FIG. 7. Concentration-dependent formation of FLX-CoA (A) and FLX-SG (B) in rat hepatocytes (2 million cells/ml) incubated with increasing concentrations of (R)-(-) or (S)-(+)-FLX for 6 min. Values are expressed as the mean $\pm$ S.D. from three incubations.

TABLE 1

Effect of inhibitors of FLX-1-O-G and FLX-CoA formation on the production of FLX-SG in incubations of (R)-(-)-FLX (10 μ M) with rat hepatocytes (2 million cells/ml, 6-min incubation time)

Values are expressed as the mean $\pm$ S.D. from three incubations.

Inhibitor	FLX-1-O-G		FLX-CoA		FLX-SG	
	Conc.	%Control	Conc.	% Control	Conc.	%Control
	nM		nM		nM	
Control	N.D.		24.0 $\pm$ 1.20		1.60 $\pm$ 0.50	
(-)-Borneol (100 μ M)	N.D.	2.50 $\pm$ 0.50	24.1 $\pm$ 4.60	100 $\pm$ 19.2	1.57 $\pm$ 0.26	98.4 $\pm$ 16.1
Lauric acid (1000 μ M)	N.D.	12.5 $\pm$ 0.70	0.00	0.00	0.04 $\pm$ 0.06	2.3 $\pm$ 4.1

N.D., not determined.

FIG. 8. Time-dependent formation of the respective S-acyl-glutathione adducts obtained from the coinubation of (R,S)-FLX-CoA (2 μ M) and (R,S)-I-CoA (2 μ M) with GSH (10 mM) in potassium phosphate buffer (0.1 M, pH 7.4, 37°C). Values are expressed as the mean $\pm$ S.D. from three incubations.

(S)-(+)-FLX would have been contaminated with 3.8 μ M (R)-(-)-FLX isomer. Results from these incubations showed that the concentrations of FLX-CoA and FLX-SG formed after 6 min of incubation were $\sim$ 50% of those formed in incubations with 3.9 μ M (R)-(-)-FLX (Fig. 7).

Inhibition Study. An inhibition experiment was performed with (R)-(-)-FLX (10 μ M) in incubations with rat hepatocytes (2 million cells/ml, 6 min) in the presence or absence of (-)-borneol (100 μ M), for the inhibition of FLX-1-O-G formation (Watkins and Klaassen, 1982), or lauric acid (1000 μ M), for the inhibition of FLX-CoA formation (Xiaotao and Hall, 1993). Results showed that (-)-borneol inhibited FLX-1-O-G formation by 98%; however, no significant inhibition of FLX-SG production was observed (Table 1). In contrast, coinubation of (R)-(-)-FLX with lauric acid led to a complete inhibition of FLX-CoA formation and also to 98 $\pm$ 4% inhibition of FLX-SG formation (Table 1).

Reaction of GSH with (R,S)-FLX-CoA and (R,S)-I-CoA in Buffer. Coinubation of (R,S)-FLX-CoA and (R,S)-I-CoA (2 μ M

each) with GSH (10 mM) in buffer (0.1 M potassium phosphate, pH 7.4, 37°C) resulted in a rapid transacylation of GSH forming $\sim$ 1.0 μ M FLX-SG and $\sim$ 0.7 μ M I-SG, respectively, after 20 min of incubation (Fig. 8). The reaction of FLX-CoA with GSH forming FLX-SG occurred at a rate of 0.21 nmol/min over the first 20 min of incubation, which was 1.5-fold more rapid than the reaction of (R,S)-I-CoA forming I-SG over the same incubation time period. Both (R,S)-FLX-CoA and (R,S)-I-CoA reacted quantitatively with GSH to form the respective GSH thioesters by the 180-min incubation time point.

Identification of Thioether-Linked FLX-GSH Adducts. LC-MS/MS analysis of extracts from (R)-(-)- and (S)-(+)-FLX-treated rat hepatocyte incubations for thioether-linked GSH adducts potentially formed through common P450-mediated bioactivation pathways showed the presence of two isobaric GSH adducts, both having MH^+ ions at m/z 589, which is consistent with a GSH adduct composition consisting of FLX + GSH (306 Da) + oxygen (16 Da) - F (19 Da). LC-MS/MS analysis by CID of the MH^+ m/z 589 ion provided

tandem mass spectra that were similar for both conjugates and consistent with product ions usually observed for GSH adducts (Supplemental Fig. 5) (Baillie and Davis, 1993). The major fragment ion for both conjugates was m/z 316 because of the loss of GSH (except for the sulfur atom) by fragmentation between the cysteinyl-carbon-sulfur bond. The extracts from the (R) -(-)- and (S) -(+)-FLX-treated rat hepatocyte incubations were then analyzed by LC-MS/MS MRM detection using the transition MH^+ m/z 589 to m/z 316 and provided qualitative evidence showing that the adducts were ~10-fold more abundant (peak height comparison) in the (R) -(-)- compared with the (S) -(+)-FLX hepatocyte incubation extracts (Fig. 9). Therefore, as was the case for FLX-SG, thioether-linked GSH adduct formation was also highly stereoselective for the (R) -FLX-(-)-isomer.

Discussion

One hypothesis used to explain toxicities mediated by some carboxylic acid-containing NSAIDs is that the covalent modification of tissue proteins by chemically reactive metabolites leads to the generation of immunogenic drug-protein adducts (Boelsterli, 2002). Reactive metabolites in common for profen drugs include acyl glucuronides and acyl-CoA thioesters (Skonberg et al., 2008). Studies have shown that both of these derivatives are able to react with GSH and protein, leading to thioester-, ester- and/or amide-linked covalent adducts (Benet et al., 1993; Sallustio et al., 2000; Grillo and Benet, 2002; Grillo et al., 2003; Sidenius et al., 2004).

It is interesting to note that FLX was withdrawn from clinical use because of its close structural similarity to BNX, even though it was never shown to cause toxicity in humans (Bakke et al., 1995). Major differences between these drugs are that BNX contains a 4-chlorine atom instead of the 4-fluorine atom found in FLX but also, more importantly, that BNX was dosed as the racemate, unlike FLX, which was given only as the pharmacologically active (S) -(+)-isomer. We propose that the differences in hepatotoxic potential between these two drugs may be related to differences in

their stereoselective metabolism to chemically reactive acyl-CoA thioester derivatives.

Another significant difference between these drugs was the dose used clinically. Although we were unable to locate the dose of (S) -(+)-FLX used before its withdrawal, the oral dose used in published human pharmacokinetic studies was 100 mg (Furlanut et al., 1985; Segre et al., 1987; Dahms and Spahn-Langguth, 1996). In one pharmacokinetic study, the C_{max} of (S) -(+)-FLX (Priaxim, 100-mg tablet) was ~7.3 $\mu\text{g/ml}$ (Dahms and Spahn-Langguth, 1996), which was consistent with the C_{max} of (S) -(+)-FLX measured in elderly subjects (Segre et al., 1987). In that same study, the C_{max} values of (R) -(-)- and (S) -(+)-BNX after a single racemic 600-mg dose (coated tablet) were ~25 and 30 $\mu\text{g/ml}$, respectively (Dahms and Spahn-Langguth, 1996). Therefore, the C_{max} of (S) -(+)-FLX was ~13% as much as the combined C_{max} values of (R) -(-)- and (S) -(+)-BNX. The hepatotoxicity caused by BNX was shown to occur in patients taking 600 mg daily for 1 to 12 months (Lewis, 2003). Therefore, the lower concentration of (S) -(+)-FLX relative to that of BNX under such dosing regimens may have contributed to the lack of reported (S) -(+)-FLX idiosyncratic toxicity compared with BNX (Utrecht, 2001).

The metabolism of FLX has been characterized in rat and in humans; the major route of metabolism is acyl glucuronidation, which exhibits a preference for the (S) -(+)-FLX isomer in both rat and human (Palatini et al., 1988; Iwakawa et al., 1991). In rats, FLX undergoes extensive stereoselective chiral inversion in favor of the (R) -(-)-FLX isomer being converted to the (S) -(+)-FLX isomer. After administration of (R) -(-)-FLX to rats, the exposure to the (S) -(+)-FLX isomer was ~1.5-fold greater than exposure to the (R) -(-)-FLX-isomer, whereas after administration of the (S) -(+)-FLX isomer to rats, the exposure to the (R) -(-)-FLX isomer was only ~2% compared with that of its antipode (Iwakawa et al., 1991). The proposed stereoselective step for the overall chiral inversion process occurs via the stereoselective formation of an (R) -FLX-CoA thioester intermediate (Fig. 1); however, the formation of FLX-CoA has not been directly identified before these studies.

In addition to the acyl glucuronidation pathway, bioactivation of FLX by acyl-CoA formation has also been proposed to contribute to the covalent binding of FLX to hepatocellular proteins, because it was shown that upon inhibition of FLX acyl glucuronidation by coinubation with (-)-borneol in rat hepatocyte cultures, covalent binding to protein was inhibited by only 50% (Dong et al., 2005). The authors of that study proposed that the FLX-CoA thioester might be responsible for the covalent binding of FLX to hepatic proteins that was not accounted for by the FLX acyl glucuronide metabolite. Because stereoselective differences in the bioactivation of (R) -(-)- and (S) -(+)-FLX isomers occur, we predicted that these differences might lead to stereoselective differences in transacylation of GSH in studies with rat hepatocyte preparations. Therefore, we proposed that if the formation of FLX-CoA represents an important bioactivation route leading to the formation of a chemically reactive acyl-linked intermediate, then results from in vitro GSH conjugate detection studies in hepatocytes would show preferential FLX-SG adduct formation in incubations containing the (R) -(-)-FLX isomer.

Results from the present studies showed that FLX (100 μM) was metabolized stereoselectively in incubations with rat hepatocytes in favor of the (R) -(-)-isomer to FLX-SG, which corresponded to the high stereoselectivity of FLX-CoA formation in favor of the (R) -(-)-isomer (Fig. 6). We also determined that (R,S) -FLX-CoA reacted readily with GSH in phosphate buffer (pH 7.4 and 37°C), leading to the formation of the FLX-SG thioester derivative (Fig. 8). We did not perform experiments to characterize the relative rates of reaction of

FIG. 9. Representative reverse-phase gradient LC-MS/MS chromatograms of FLX-GSH thioether-linked adducts from the analysis of untreated rat hepatocytes (A), extract from rat hepatocytes incubated for 60 min with 50 μM (S) -(+)-FLX (B), and extract from rat hepatocytes incubated for 60 min with 50 μM (R) -(-)-FLX (C). The transition used for the LC-MS/MS MRM analysis of the thioether linked FLX-GSH adducts (proposed structures shown) was MH^+ m/z 589 to m/z 316. The relative abundances for each trace on the y-axes are equal.

the separate (*R*)-FLX- and (*S*)-FLX-CoA isomers in the present work; however, such studies with 2-phenylpropionic acid showed no differences in the reactivity of the (*R*)- or (*S*)-2-phenylpropionic acid-*S*-acyl-CoA derivatives with GSH (Li et al., 2002a).

In the present work, the importance of FLX-CoA formation leading to the transacylation of GSH in incubations with hepatocytes was clearly demonstrated from time- and concentration-dependent experiments with the separate isomers in which a strong correlation between FLX-CoA and FLX-SG formation was observed (Figs. 6 and 7). The importance of the acyl-CoA pathway was confirmed further when results from inhibition studies with lauric acid showed a complete inhibition of both FLX-CoA and FLX-SG formation in incubations with rat hepatocytes (Table 1). We also determined that the FLX-1-*O*-G metabolite, the formation of which was almost completely inhibited by coinubation with (–)-borneol, did not contribute to the transacylation of GSH because there was no significant inhibition of FLX-SG formation. These results are consistent with analogous experiments performed with (*R*)-(–)-ibuprofen (Grillo and Hua, 2008).

In addition to FLX-SG thioester detection, analysis of rat hepatocyte extracts from incubations with (*R*)-(–)-FLX and (*S*)-(+)-FLX by LC/MS led to the detection of two thioether-linked FLX-GSH adducts (Fig. 9). These GSH adducts were isobaric, having MH^+ ions at m/z 589, which is consistent with the loss of the fluorine atom and the addition of an oxygen atom and the elements of GSH to FLX. Preliminary qualitative results indicated stereoselective bioactivation, whereas an apparent ~10-fold increased abundance of the thioether-linked adducts was detected in the (*R*)-(–)- compared with the (*S*)-(+)-FLX isomer incubation extracts (Fig. 9). We propose that these adducts may be formed via an unstable epoxide intermediate that reacts with GSH followed by elimination of HF (Tang et al., 2005; Zheng et al., 2007). We have not confirmed the identity of these GSH adducts; however, LC-MS/MS analysis (CID MH^+ m/z 589) provided product ions consistent with their structures (Supplemental Fig. 5). From these results, a proposal could be made that similar bioactivation might be occurring for BNX and that covalent binding to protein in the liver might occur by a combination of multiple bioactivation pathways including P450-mediated oxidation, acyl glucuronidation, and/or *S*-acyl-CoA-formation. However, in a recent report that included studies on the covalent binding of [^{14}C]BNX to protein in NADPH-fortified human liver microsomes, results showed no detectable covalent binding to protein (Obach et al., 2008). In that same report, two other carboxylic acid-containing drugs known to form cytochrome P450-mediated reactive intermediates and corresponding GSH adducts, namely diclofenac and tienilic acid, both showed NADPH-dependent covalent binding to protein (Valadon et al., 1996; Tang, 2003). Such results suggest that BNX may not be metabolized by cytochrome P-450 to reactive intermediates (e.g., epoxides) that react with hepatocyte protein. Further covalent binding studies performed in incubations of BNX with human hepatocytes showed measurable covalent binding of the drug to protein; however, neither metabolism nor consumption of BNX was detected (Bauman et al., 2009). We propose that the observed in vitro covalent binding to human hepatocyte protein may have been mediated by BNX-*S*-acyl-CoA; however, the *S*-acyl-CoA derivative was not searched for in by Bauman et al. (2009) in their report. BNX acyl glucuronide is known to be the major metabolite of BNX formed in humans; however, it was shown not to be an important reactive intermediate when metabolic activation experiments conducted in UDP-glucuronic acid-fortified human liver S-9 fraction showed a complete blockage of covalent binding to protein (Bauman et al., 2009). Such results are consistent with observations obtained from mechanistic hepatocyte covalent

binding studies with 2-phenylpropionic acid, in which inhibition of acyl glucuronidation had only a minor effect on covalent binding to protein (Li et al., 2002b). We are currently investigating the metabolism of BNX to BNX-*S*-acyl-CoA and the ability to form varied types of GSH adducts (no literature reports to date) in vitro in rat and human hepatocytes and in vivo in rat.

In summary, results from the present studies showed that the metabolic activation of FLX is mediated by acyl-CoA formation and not by acyl glucuronidation, leading to the transacylation of GSH to form FLX-SG. Finally, from the results presented here, we propose that metabolic activation of (*R*)-(–)-FLX by acyl-CoA formation may also lead to covalent binding of the isomer to protein nucleophiles. Unlike its structural congener, namely racemic BNX, we propose that the lack of reported FLX toxicity in humans may have been due to the clinical use of the (*S*)-(+)-isomer and the lack of significant (*S*)-FLX-CoA formation.

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