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Role of benoxaprofen and flunoxaprofen acyl glucuronides in covalent binding to rat plasma and liver proteins in vivo

Jennifer Q. Dong [a], Jianhua Liu [b], Philip C. Smith [c,*]

[a] Department of Pharmacokinetics and Drug Metabolism, Allergan, Inc., Irvine, CA 92623, USA
[b] Department of Pharmacokinetics, Pharmacodynamics and Drug Metabolism, Pfizer, Inc., Groton, CT 06340, USA
[c] Division of Drug Delivery and Disposition, CB#7360, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7360, USA

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Abstract

Benoxaprofen (BNX) has been implicated in rare but serious hepatotoxicity which led to its withdrawal from the world market. Flunoxaprofen (FLX), a structural analog, appears to be less toxic. It has been postulated that the nonsteroidal antiinflammatory drugs associated toxicity may be related to covalent modification of proteins by their reactive acyl glucuronides, and the extent of covalent protein binding depends on both reactivity of the acyl glucuronide and the exposure to the reactive metabolite. The disposition of BNX and FLX in rats were compared upon intravenous administration of 20 mg/kg of BNX, FLX or their metabolites. Covalent binding of BNX and FLX to plasma and liver proteins were also determined, and an immunochemical approach was used to detect their hepatic targets. Similar concentrations of plasma protein adducts for BNX and FLX were detected even though the AUC of BNX-glucuronide (BNX-G) was almost twice that of FLX-glucuronide (FLX-G). Similar concentrations of liver protein adducts for BNX and FLX were also detected at 8 h, however, the hepatobiliary exposure of BNX-G was only 1/3rd that of FLX-G indicating that BNX-G was more reactive than FLX-G, which was in agreement with in vitro data. Proteins of 110 and 70 kDa were the major liver protein targets modified by covalent attachment of BNX and FLX. In conclusion, measuring covalent binding to tissue proteins in animals in addition to plasma adducts should be considered when evaluating and comparing carboxylic acid analogs that form reactive acyl glucuronides.

Keywords: Reactive acyl glucuronide; Covalent protein binding; NSAIDs; Reversible metabolism; Immunoblot; Liver protein targets

1. Introduction

A major metabolic pathway for drugs and endobiotics bearing carboxylic acid groups is coupling with glucuronic acid to yield acyl glucuronide conjugates. Unlike other types of glucuronides, acyl glucuronides are chemically unstable in vitro and in vivo [1,2]. This reactivity stems from the inherent susceptibility of the acyl (ester) group to nucleophilic substitution, and manifests itself along three related pathways: (1) hydrolysis, (2) intramolecular rearrangement, and (3) covalent binding to proteins via nucleophilic displacement and/or imine mechanisms. It has been suggested that acyl glucuronides of a number of carboxylic acids drugs are implicated in adverse reactions due to the observation that they are able to bind irreversibly to cellular proteins [3]. Drug plasma protein-adducts have been detected in humans and animals after the administration of diclofenac [4], diflunisal [5–7], suprofen [8], zomepirac [9], valproic acid [10], tolmetin [11,12], clofibric acid [13], salicylate [14], and ibuprofen [15]; all of these drugs form acyl glucuronides by metabolism and hypersensitivity reactions have been reported related to their use in some patients [16,17].

Abbreviations: $A(m)_{e,bile,0\rightarrow 8\ h}$, cumulative biliary excretion of metabolite up to 8 h; AUC, area under the plasma concentration curve; BNX, benoxaprofen; BNX-G, benoxaprofen glucuronide; CL, systemic clearance; $Cl_{f,bile}$, biliary formation clearance; $Cl_{e,bile}$, biliary excretion clearance; DMF, *N*,*N*-dimethylformamide; ECL, enhanced chemiluminescence; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; ELISA, enzyme-linked immunoabsorbance assay; FCS, fetal calf serum; FLX, flunoxaprofen; FLX-G, flunoxaprofen glucuronide; HPLC, high performance liquid chromatography; HSA, human serum albumin; IBP, ibuprofen; KLH, keyhole limpet hemocyanine; NSAIDs, nonsteroidal antiinflammatory drugs; PBS, phosphate buffered saline; RSA, rat serum albumin; SDS-PAGE, sodiumdodecyl sulfate polyacrylamide gel electrophoresis; $t_{1/2}$, half life

* Corresponding author. Tel.: +1 919 962 0095; fax: +1 919 966 0095.
E-mail address: pcs@email.unc.edu (P.C. Smith).

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Benoxaprofen (BNX) and flunoxaprofen (FLX) are a pair of structural analogs but with apparent divergent hepatotoxicity in humans. BNX was withdrawn from U.S. and British markets due to several fatal incidences of cholestatic jaundice in elderly patients [17]. FLX, which appeared to be less toxic, though data is limited, was only marketed in Italy and no fatal adverse events related to FLX have been reported in available sources. Formation of acyl glucuronide represents a major metabolic pathway for both BNX and FLX in humans [18,19]. Similar concentrations of BNX- and FLX-plasma protein adducts have been detected in the systemic circulation in human [20]. The major site of conjugation for BNX and FLX in humans is believed to be the liver. Experimental studies in mice and rats to examine organ tolerance to NSAIDs suggested that BNX was more toxic than FLX [21]. BNX was also shown to be more toxic than ibuprofen (IBP), a well-tolerated NSAID, in primary cultures of rat hepatocytes in vitro [22]. Disposition and covalent protein binding of BNX and FLX, however, have not been well characterized in rat. It has been suggested that the extent of irreversible tissue binding at a particular time depends on both the stability/reactivity of the reactive acyl glucuronide and the exposure of the organ to the reactive metabolite [6,8]. Differences in the disposition of these two NSAIDs may in part influence their protein adducts formations in vivo. In the present study, we first compared the reversible metabolism of BNX and FLX in rats where dosing of both parent compound and its respective acyl glucuronide are possible. In addition, covalent adduct formation of BNX and FLX in systemic circulation and in liver tissue was characterized to determine if protein adduct formation in plasma and liver tissue correlates with systemic or hepatobiliary exposure to labile and reactive acyl glucuronides. Furthermore, studies directed at identification of hepatic protein targets of BNX and FLX in rats, which could be causative factors or correlated with hepatotoxic responses, have been lacking. It has been demonstrated previously by Bailey and Dickinson [23] that the pattern of protein modification in liver varied from drug to drug. An immunochemical approach was therefore taken to compare the protein targets in livers from rats treated with BNX and FLX, and compared to livers from rats treated with IBP, the relatively nontoxic NSAID. Finally, the stability and reactivity of BNX acyl glucuronide (BNX-G) and FLX acyl glucuronide (FLX-G) were also evaluated in vitro.

2. Materials and methods

2.1. Materials

Rac-BNX was extracted and purified from Tablets of Oraflex® previously marketed by Eli Lilly (Indianapolis, IL). Anal. Calcd for BNX ($C_{16}H_{12}ClNO_3$): C, 63.69; H, 4.01; N, 4.64; Cl, 11.75; O, 15.91. Found: C, 63.05; H, 4.31; N, 4.71; Cl, 11.68; O, 16.25. The purity of BNX was confirmed based on elemental analysis and analytical HPLC using UV detection at wavelength of 210 nm. *S*-FLX, the marketed form, was obtained by a generous contribution from Dr. A. Forgione (Ravizza Laboratories, Milan, Italy), and was determined to be pure based on HPLC with UV detection. BNX-G and FLX-G were extracted and purified from urine using preparative HPLC, as described previously [24]. β-Glucuronidase (type B-10 from bovine liver), human serum albumin (HSA, fraction V), IBP, rat serum albumin (RSA), Triton X-100, EDTA, and thimerosal were obtained from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose membrane (0.45 μm), and Bradford reagent used for protein analysis were purchased from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence (ECL) reagents, goat anti-rabbit IgG (peroxidase conjugate), and hyperfilm ECL were obtained from Amersham (Arlington Heights, IL). Methanol and acetonitrile were HPLC grade from J.T. Baker (Pillipsburg, NJ). All other chemicals used were of reagent grade. Human plasma was provided by UNC Hospital Blood Center (Chapel Hill, NC). Male Sprague-Dawley rats (200–300 g) were supplied by Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Experiments were approved by the University's Institutional Animal Care and Use Committee.

2.2. Pharmacokinetic studies

Adult, male Sprague-Dawley rats (200–300 g) were maintained under a 12 h light/dark cycle in a temperature-controlled environment, with free access to food and water. All animals were anesthetized with intramuscular injection of ketamine:acepromazine (75:2 mg/kg) into the thigh. About 20 mg/kg of each parent drug was administered intravenously through cannulated jugular vein to six control, bile duct-intact animals and six bile duct-cannulated animals. Serial blood samples of 0.2 ml were collected through cannulated carotid artery over 8 h for analysis of plasma concentrations of both parent drugs and their glucuronide metabolites. At 1, 4, and 8 h after the administration of BNX and FLX, additional blood samples were drawn for measuring drug–protein adducts in plasma. For bile duct-cannulated animals, bile samples were also collected at hour intervals. The pH of both plasma and bile samples were adjusted with 43% phosphoric acid to between 2 and 4 to prevent degradation of acyl glucuronides. For the determination of the drugs covalently bound to liver tissues, the animals were euthanized 8 h after the drug administration, then livers were harvested. All biological samples were stored at −20 °C until analyzed. In addition, six bile duct-cannulated animals were each administered a bolus dose of 20 mg/kg (parent equivalents) of BNX-G or FLX-G as solutions in 0.15 M phosphate buffer at pH 5. Blood samples were collected as frequently as possible during the first 30 min to

assess the rapid loss of acyl glucuronides in vivo. After 30 min, sampling frequency was reduced and blood samples were drawn up to 8 h.

Plasma concentrations of BNX, FLX, and their acyl glucuronides were determined by analysis with a direct HPLC method as previously described [24]. During sample preparation and HPLC analysis, the pH was kept between 2 and 4 and samples were cooled on ice to minimize acyl migration and hydrolysis of the acyl glucuronides. Bile samples were also analyzed for concentrations of BNX, FLX, and their acyl glucuronides by direct injection of properly diluted samples onto HPLC.

Pharmacokinetic analysis was performed using model-independent analysis (WinNONLIN 2.0, Pharsight Corp., Palo Alto, CA). Plasma concentration–time curves were plotted for each parent drug and its acyl glucuronide. The elimination half-lives ($t_{1/2}$) of the log-linear terminal phase of the curves were estimated by linear least-square regression of the last three or four data points. The areas under the curves from time zero to the last measured concentration at 8 h ($AUC_{0\rightarrow 8\ h}$) were determined by the linear trapezoidal method. The areas under the concentration–time curves from time zero to infinity ($AUC_{0\rightarrow\infty}$) were estimated by summing $AUC_{0\rightarrow 8\ h}$ and the extrapolated area which was determined by dividing the final plasma concentration by the terminal rate constant. The plasma clearance was determined by the equation: $CL = Dose/AUC_{0\rightarrow\infty}$. Eight-hour cumulative amount of parents and glucuronides excreted in the bile ($A_{e,bile,0\rightarrow 8\ h}$) were also calculated.

2.3. Covalent protein binding studies

Covalent binding of BNX and FLX to plasma proteins in vivo were determined using the method developed by Pohl and Branchflower [25] with some modifications made by Smith et al. [9]. Plasma samples (0.5 ml) were treated with equal volume of ice-cold isopropanol containing 1% acetic acid and acetonitrile to precipitate proteins. After centrifugation the protein pellets were washed exhaustively with methanol–ether (3:1, v/v) to remove reversibly bound drug, dried briefly at 80 °C, then treated with 1 ml of 0.2 M sodium hydroxide at 80 °C for 60 min to hydrolyze and release covalently bound BNX or FLX. Both BNX and FLX were previously determined to be stable to this treatment. After hydrolysis, the liberated BNX or FLX were extracted with 5 ml dichloromethane and then measured by analytical HPLC. A standard curve was established by using blank protein that was treated similarly and spiked with BNX or FLX at a series of concentration (0–1000 ng/ml) after the washing procedure. Protein concentrations were measured according to the Bradford method [26] using HSA as the standard. The extent of covalent binding was normalized to protein content for each sample, and was expressed as mmol of drug that became covalently bound per mol of protein where protein for plasma was assumed to be HSA (MW = 66 kDa).

A similar procedure was employed for the analysis of the drugs covalently bound to tissues. The tissue samples (0.5 g) were thawed, chopped and homogenized (Polytron®, Brinkmann Instruments, Westbury, NY) on ice in 3 ml of homogenization buffer (100 mM Tris–acetate, 250 mM sucrose, 1 mM EDTA, pH 7.4). Proteins were precipitated, and the reversibly bound and unbound drug and metabolites were removed. Samples were incubated with sodium hydroxide at 80 °C for 60 min, and the released parent drugs were extracted with dichloromethane then measured by analytical HPLC.

2.4. Immunoblotting studies

For dose-dependency studies, rats were administered BNX or FLX at doses of 20, 50, 100 and 200 mg/kg by intraperitoneal injection. Control rats received only distilled water. The animals were euthanized at 8 h after dosing, then livers were removed and homogenized with a Tenbroeck tissue grinder in three volumes of ice-cold 100 mM Tris–acetate (pH 7.4) containing 250 mM sucrose and 1 mM EDTA (homogenization buffer). For time-dependency studies, rats given 100 mg/kg BNX or FLX by intraperitoneal injection were sacrificed at 0, 4, 8, 16 and 24 h after the dose, and liver homogenates were prepared. Similar dose- and time-dependency studies were also conducted by treating rats with IBP, generally considered a safe NSAID with a less reactive acyl glucuronide. Protein concentrations of liver homogenates were determined according to Bradford [26]. All the homogenates were frozen immediately in liquid nitrogen and stored at −80 °C in small aliquots until they were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with antiserum.

Both anti-BNX serum and anti-IBP serum were prepared by Strategic Biosolutions (Ramona, CA). In brief, BNX was dissolved in methanol and reacted with a five-fold excess of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). After the incubation was complete (approximately 4 h at room temperature), the solvent was evaporated. The residue was rehydrated with 0.5 ml of *N*,*N*-dimethylformamide (DMF) and mixed with the carrier protein Keyhole limpet hemocyanin (KLH) in the ratio of nine parts protein solution to one part activated antigen. The solution was stirred at room temperature for 2 h. After 2 h, the solution was desalted on a PD10 cartridge (Sigma, St. Louis, MO, USA) previously equilibrated with PBS. IBP was covalently coupled to KLH by a two-step conjugation method [4]. EDC in 20 mM sodium phosphate (pH 5.0) was added to a methanolic solution of IBP. After 2 min, the reaction mixture was added to KLH and incubated overnight at room temperature. The conjugate was dialyzed against PBS, with three changes. Coupling of drugs to carrier protein was assessed by the chemical cleavage method, described in detail by Castillo [27],

before immunization began. The epitope density was estimated to be approximately 11 BNX or IBP molecules per molecule of KLH.

Two female New Zealand white rabbits each were immunized with KLH–BNX conjugate or KLH–IBP conjugate, suspended in Freund's complete adjuvant, by a set of multi-site subcutaneous injections. Each animal received booster injections of KLH–BNX conjugate or KLH–IBP conjugate suspended in Freund's incomplete adjuvant after 3 weeks, and at 2 weeks intervals thereafter. Peripheral blood was collected from ear veins 1 week after boosting and at weekly intervals thereafter. Serum was prepared and stored at −80 °C.

ELISA was undertaken essentially as described by Pumford et al. [4] to monitor antibody titers. BNX, FLX and IBP were covalently coupled to rat serum albumin (RSA) by a two-step conjugation method [4] and used as test antigens. Test antigens (RSA–BNX, RSA–FLX, RSA–IBP conjugates or unconjugated RSA) at 15 μg/ml in 60 mM sodium carbonate (pH 9.6, 0.1 ml/well) were then incubated overnight at 4 °C in 96-well microtiter plates. Plates were washed four times in washing buffer (0.05% Nonidet P40 in PBS). Excess binding sites were blocked with 5% fetal calf serum (FCS) in PBS for 1 h, followed by addition of 100 μl of either anti-BNX serum or anti-IBP serum diluted in 2% FCS-PBS. After incubation for 2 h, the wells were washed as before and incubated for 1 h with 0.1 ml/well alkaline phosphate-conjugated goat anti-rabbit IgG (1:3000 dilution in 2% FCS-PBS). After washing, 100 μl of substrate solution (*p*-nitrophenyl phosphate, prepared according to manufacture's insert, Sigma, St. Louis, MO, USA) was added and the reaction was allowed to develop. Product absorbance was measured at 405 nm with Bio-Rad protein assay multi-plate reader.

Liver homogenates were subject to SDS-PAGE and transferred to nitrocellulose membrane as described by Ware et al. [28]. Prior to antibody development, the nitrocellulose was blocked for 1 h at room temperature by incubation in blocking buffer (65 mM Tris, 10 mM EDTA, 1% Triton X-100, 0.02% (w/v) thimerosal, 2% (w/v) nonfat dry milk, pH 7.5). The blocked nitrocellulose was first incubated for 90 min with primary antibodies, then for 1 h with horseradish peroxidase-conjugated secondary antibody. Labeled proteins were visualized by ECL, using ECL Western blotting reagent from Amersham (Arlington Heights, IL, USA) according to the manufacturer's instructions. BNX antiserum, IBP antiserum and goat anti-rabbit IgG (peroxidase-conjugated secondary antibody) were diluted 1:2500, 1:1000 and 1:10,000, respectively in washing buffer (1:6 dilution of blocking buffer with distilled water).

2.5. Stability and reactivity of acyl glucuronide in vitro

The degradation of β-1 acyl glucuronides were studied at pH 7.4 and 37 °C in three different media: buffer solution, human serum albumin (Fraction V, HSA), and plasma. BNX-G or FLX-G was added to a final concentration of 50 μg/ml in 150 mM sodium phosphate buffer (pH 7.4), 30 mg/ml (0.5 mM) of HSA solution, or pooled blank human plasma (pH 7.4). All samples were prepared in triplicate and incubated at 37 °C for up to 48 h. Aliquots (300 μl) of the incubation mixture were taken at various times and split into three treatment groups (100 μl each). The incubation reactions of all the samples in the first group were stopped by the addition of 200 μl ice-cold acetonitrile which contained 10 μl acetic acid and 200 ng the structural analog as the internal standard (FLX served as internal standard for BNX, BNX-G assay and vice versa). For the second treatment group, the pH of the incubation mixtures were adjusted to 5.0 to stabilize the acyl glucuronides, then 2000 units of β-glucuronidase was added to each sample followed by further incubation for 30 min at 37 °C to cleave β-1 glucuronide conjugates present in the incubation. A positive control with *p*-nitrophenyl glucuronide was examined to verify the reactivity of β-glucuronidase. The enzyme reactions for all solutions were then stopped with two volumes of ice-cold acetonitrile containing acetic acid and internal standard. The pH of each sample in the third treatment group was adjusted with 2 M sodium hydroxide to more than 10, then all the solutions were further incubated for 2 h at 37 °C to cleave both β-1 conjugates and all the other putative isomers. The chemical hydrolysis was then stopped with 2 M hydrochloride to neutralize the pH to 7, and two volumes of acetonitrile containing acetic acid and internal standard was added. The mixtures from all three treatment-groups were centrifuged, the supernatants were transferred to a clean tube and evaporated under nitrogen flow to dryness. Finally, the residues were dissolved in 0.5 ml HPLC mobile phase and injected onto HPLC to determine the concentrations of BNX or FLX formed and β-1 as well as its isomeric conjugates. The apparent first-order degradation half-life of each glucuronide in buffer, HSA and plasma was calculated from the slope of the log concentration–time graph of the β-1 glucuronide conjugate remaining in solution during the incubation using linear regression analysis. Student *t*-test was performed to verify statistical difference in the degradation half-lives between BNX-G and FLX-G in buffer, HSA and plasma at physiological conditions.

Covalent binding of BNX-G and FLX-G to model proteins were determined as described above. BNX-G or FLX-G was added to a final concentration of 50 μg/ml (BNX or FLX equivalents) in 150 mM sodium phosphate buffer (pH 7.4) containing 30 mg/ml of HSA or pooled blank human plasma (pH 7.4). All samples were prepared in triplicate and incubated at 37 °C for 48 h. Aliquots (1 ml) of the incubation mixtures were taken at various times to measure the total BNX or FLX equivalents bound irreversibly to HSA or plasma proteins. As a control, incubation of the parent drugs (50 μg/ml) with 30 mg/

ml HSA at pH 7.4 was conducted in triplicate at 37 °C for 24 h. Samples were taken over the time to measure the drug bound to proteins.

3. Results

3.1. Disposition of BNX and FLX in rats

Mean plasma concentration–time profiles of BNX, FLX, and their acyl glucuronides in bile duct-cannulated rats after the administration of 20 mg/kg of the parent drug are presented in Figs. 1 and 2. The plasma levels of BNX-G and FLX-G were nearly parallel to levels of BNX and FLX, respectively, which is common with reversible metabolism. Plasma levels of acyl glucuronides were much lower than their respective parent compounds at any given time in both control and bile duct-cannulated rats. Within each animal treatment group (i.e. normal rats and bile duct-cannulated rats), $t_{1/2}$ was significantly different between BNX and FLX after the administration of the parent drug ($P < 0.05$). BNX was eliminated from systemic circulation much slower than FLX with an estimated elimination $t_{1/2}$ of BNX (44 h) more than three-fold longer than that of FLX (13 h) in normal rats. Because of the slow clearance of BNX, the estimation of terminal $t_{1/2}$ may not be accurate. Pharmacokinetic parameters of each NSAID between normal and bile duct-cannulated rats were also compared. Though not statistically significant, estimated mean CL for both BNX and FLX were faster in bile duct-cannulated rats than those in normal rats (4.48 versus 3.54 ml/(h kg) for BNX, 15.5 versus 9.72 ml/(h kg) for FLX), suggesting that both BNX and FLX underwent enterohepatic circulation in rats.

Reversible metabolism of BNX and FLX was confirmed by the rapid appearance of parent compound in plasma after dosing of corresponding acyl glucuronide in bile duct-cannulated rats (Figs. 1 and 2). Pharmacokinetic parameters of the acyl glucuronides are presented in

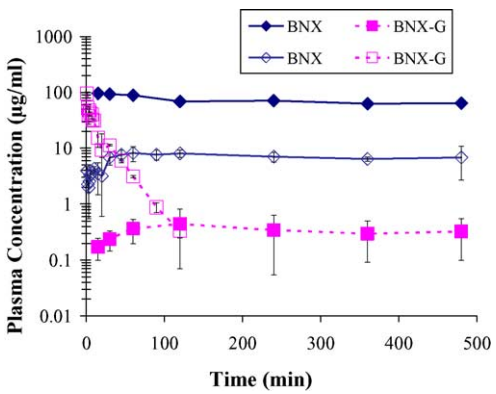

Fig. 1. Mean plasma concentration–time curve from six bile duct-cannulated rats after intravenous administration of 20 mg/kg of BNX (solid symbols) and BNX-G (open symbols) (in BNX equivalent).

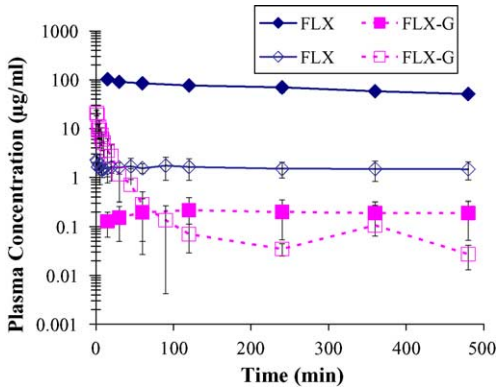

Fig. 2. Mean plasma concentration–time curve from six bile duct-cannulated rats after intravenous administration of 20 mg/kg of FLX (solid symbols) and FLX-G (open symbols) (in FLX equivalent).

Table 1. The average plasma clearance of BNX-G and FLX-G (4830 and 5560 ml/(h kg), respectively) when administered intravenously to rats were found to be much higher than that of their parent compounds (estimates of 4.48 and 15.5 ml/(h kg) for BNX and FLX, respectively), and exceeded estimates of hepatic blood flow in rats (~3300 ml/(h kg)) [29,30], indicating that extrahepatic clearance via hydrolysis was likely involved for these labile ester glucuronides. AUCs of BNX-G and its reformed parent compound were significantly higher than those of FLX-G and reformed FLX. However, apparent systemic clearance of BNX-G did not differ significantly from that of FLX-G, probably due to high variability and small sample size ($n = 2$ for dosing BNX-G due to limited availability of the metabolite).

Systemic and hepatobiliary exposures to BNX, FLX and their acyl glucuronides after given a dose of the parent drug or the acyl glucuronide are summarized in Table 2. Systemic exposure ($AUC_{0\rightarrow 8\ h}$) of BNX-G in rats over 8 h was two times higher than that of FLX-G when parent compound was administered, whereas hepatobiliary exposure ($A_{e,bile,0\rightarrow 8\ h}$) of BNX-G up to 8 h was only 1/3rd of that of FLX-G. Hepatobiliary exposure of BNX-G differed when rats were given equivalent molar dose of BNX versus BNX-G, whereas comparable exposure of FLX-G was observed when given equivalent molar dose of FLX or FLX-G.

Table 1
Pharmacokinetic parameters of BNX-G and FLX-G in bile duct-cannulated rats after intravenous administration of 20 mg/kg (parent equivalents) of the acyl glucuronide

Parameter	BNX-G ($n = 2$)	FLX-G ($n = 6$)
CL[a] (ml/(h kg))	4830 ± 396[b]	5560 ± 2240
$t_{1/2}$ (h)	0.313 ± 0.045	0.280 ± 0.104
$AUC_{0\rightarrow\infty,glu}$ (μg h/ml)	18.1 ± 1.5[c]	4.23 ± 1.85[c]

[a] Apparent plasma clearance of acyl glucuronide after dosing of acyl glucuronide, $Dose^m/AUC^m_m$.
[b] Parameters determined are given as the mean ± S.D.
[c] Significantly different at $P < 0.05$.

Table 2
Systemic and hepatobiliary exposures of BNX, FLX and their acyl glucuronides (in parent equivalents) in bile duct-cannulated rats after administration of 20 mg/kg either parent compound or acyl glucuronide (in parent equivalent)

Compound administered	$AUC_{0\rightarrow 8\ h}$ (μg h/ml)		$A_{e,bile,0\rightarrow 8\ h}$ (% of dose)	
	Parent	Glucuronide	Parent	Glucuronide
BNX	573 ± 22[a]	1.98 ± 0.59	0.67 ± 0.11	10.8 ± 1.4
FLX	560 ± 89	1.09 ± 0.24	3.28 ± 1.09	28.5 ± 6.1
BNX-G	54.1 ± 2.6	18.0 ± 1.5	1.11 ± 0.01	49.5 ± 0.3
FLX-G	12.3 ± 4.8	3.98 ± 1.84	1.63 ± 0.61	28.8 ± 5.0

[a] Parameters determined are given as the mean ± S.D.

3.2. Covalent protein binding in vivo

Drug–protein adduct measured by release of drug with strong base was detectable in plasma following the intravenous administration of 20 mg/kg BNX or FLX. Attempts to use acid for hydrolysis resulted in degradation of BNX and FLX. Concentrations of plasma protein adducts of BNX and FLX at 1, 4, or 8 h following the dose did not substantially differ. Protein adduct in liver tissues after the intravenous dose of 20 mg/kg BNX or FLX was below the detection limit of the fluorescent assay (∼1 pmol/mg protein). Higher doses of BNX and FLX were then given to rats intraperitoneally. Formation of liver protein adducts in rats, as measured by release of parent compound by base hydrolysis, were found to be dose-proportional for both BNX and FLX (Fig. 3). At 8 h following drug administration, similar concentrations of liver protein adducts were detected in rats treated with the same dose of BNX and FLX (50, 100 or 200 mg/kg).

Polyclonal anti-BNX serum was shown by ELISA to contain antibody which recognized conjugates prepared by coupling BNX to the carrier protein RSA (Fig. 4a). The antiserum also exhibited significant degree of cross-reactivity with RSA–FLX test antigen, but did not recognize RSA–IBP or unconjugated RSA. Anti-IBP serum was also prepared and tested against RSA–IBP, RSA–BNX, RSA–FLX conjugates, and unconjugated RSA by ELISA. Only

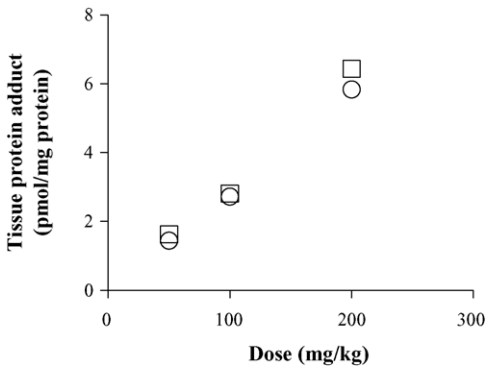

Fig. 3. Covalent binding of BNX (□) and FLX (○) to hepatic tissue proteins. Rats ($n = 2$) were given 50, 100, or 200 mg/kg BNX or FLX intraperitoneally and livers harvested after 8 h, then analyzed for the amount of drug irreversibly bound to protein by cleavage with base.

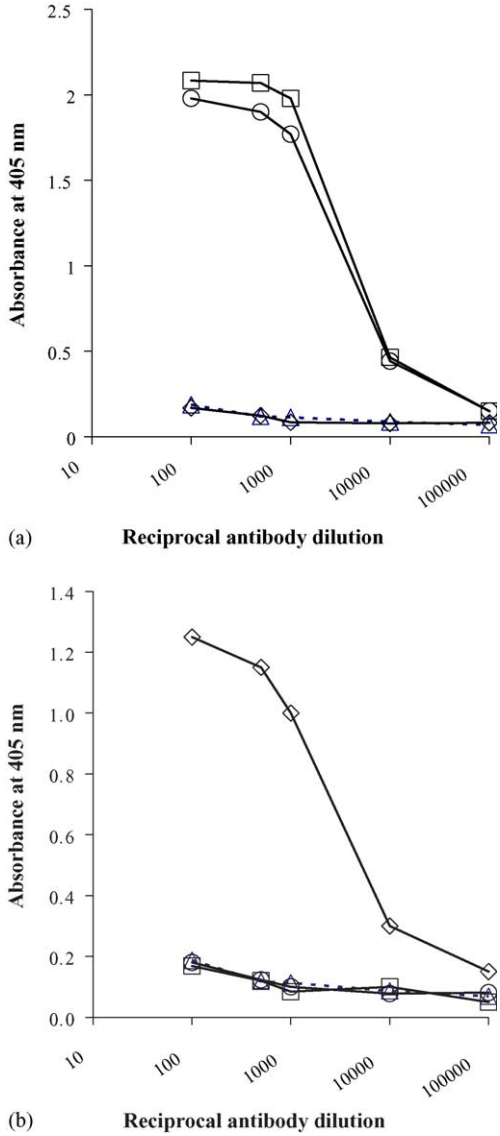

Fig. 4. (a) Recognition of NSAID-modified RSA conjugates by antisera raised against KLH–BNX conjugate. (b) Recognition of NSAID-modified RSA conjugates by antisera raised against KLH–IBP conjugate. Antigen tested were RSA–BNX (□), RSA–FLX (○), RSA–IBP (◇) and unconjugated RSA (△).

RSA–IBP conjugate was recognized by anti-IBP serum (Fig. 4b).

Liver homogenates were prepared from rats 8 h after intraperitoneal administration of BNX, FLX, or IBP. Representative immunoblots shown in Fig. 5 with anti-BNX serum had numerous proteins with putative adducts but revealed two major protein adducts with molecular masses of approximately 70 and 110 kDa in livers of rats treated with BNX and FLX. The intensity of the signal for the 70 and 110 kDa BNX- and FLX-labeled proteins increased in an apparent dose-dependent manner, with the signals more prominent for the BNX-treated livers. These two adducts were not detected in liver homogenate of control animals. In addition, the labeling of the 70 and

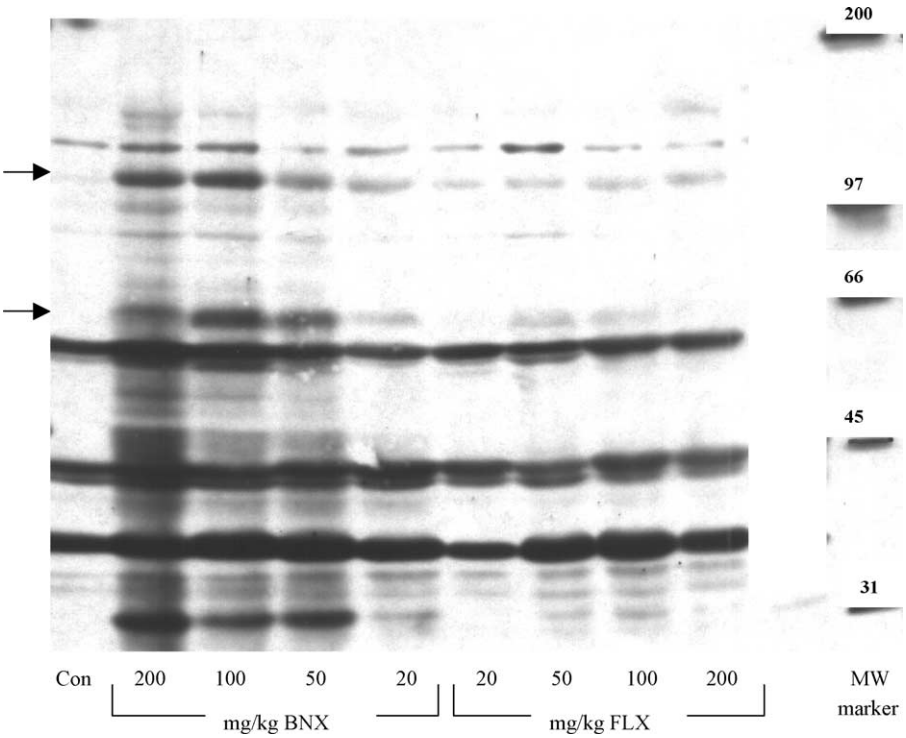

Fig. 5. Representative dose-dependent covalent modification of hepatic proteins in BNX- and FLX-treated rats. Rats were given vehicle control, 20, 50, 100, or 200 mg/kg BNX or FLX intraperitoneally and killed after 8 h. Proteins from liver homogenates were separated by SDS-PAGE (100 μg/lane), transferred to nitrocellulose, and immunoblotted with anti-BNX sera. Arrows indicate the two major protein targets of BNX, FLX at MW of approximately 110 and 70 kDa. MW of proteins was estimated based on their migration relative to standard MW marker.

110 kDa proteins in livers treated with BNX and FLX appeared to be time-dependent with the signals more prominent in BNX-treated group (Fig. 6). In contrast, antisera from rabbits immunized with KLH–IBP recognized only a single drug-induced 66 kDa protein with a trend toward a dose- and time-dependent manner in livers from IBP-treated rats (Fig. 7).

3.3. Stability and reactivity of acyl glucuronides in vitro

At physiological pH, the hydrolysis and acyl migration (loss of β-1 conjugate) of BNX-G and FLX-G were significantly faster in plasma than in buffer and HSA, probably because of the presence of plasma enzymes that may influence the degradation of these labile metabolites (Table 3). Loss of β-1 glucuronide were apparent first-order process. BNX-G is less stable than FLX-G in either HSA or pooled human plasma. An achial HPLC method was used for quantitation of β-1 glucuronides, thus potential chiral inversion was not assessed in this study.

Mean profiles of the in vitro covalent binding of BNX-G and FLX-G to HSA and pooled human plasma proteins (Fig. 8a and b) show a time-dependent covalent binding for the two glucuronide conjugates. Detectable covalent binding was observed after 1 h of incubation of β-1 acyl glucuronides with protein solutions and BNX-G had faster initial rates of covalent binding in vitro than FLX-G in for both HSA and plasma. The maximum formation of drug–protein adduct in HSA at 37 °C, pH 7.4 reached an apparent plateau after 24 h for both BNX-G and FLX-G with significantly greater adduct via BNX-G than FLX-G. The profiles of covalent binding of BNX-G and FLX-G bound to plasma (Fig. 8b) differed from those obtained with HSA (Fig. 8a). Under the same experimental conditions, the covalent binding to plasma proteins continue to increase even up to the last sampling time of the incubation due to the continued presence of isomeric conjugates in the medium. In summary, BNX-G is less stable and more reactive than FLX-G in vitro. No detectable binding was observed when the parent drugs were incubated with HSA solution and human plasma, indicating that the covalent binding in vitro was via the acyl glucuronides and their isomeric conjugates.

4. Discussion

The present study describes the disposition of BNX and FLX in both bile duct-intact rats and bile duct-cannulated rats under anesthesia. Limited by the bile drainage procedure, all the in vivo studies were only carried up to 8 h. As a result, $AUC_{0\rightarrow 8\ h}$ was accurately determined (Table 2). The elimination $t_{1/2}$ of BNX was estimated to be greater than three times that of FLX. The estimated $t_{1/2}$ values for BNX reported in the present study agree with that previously

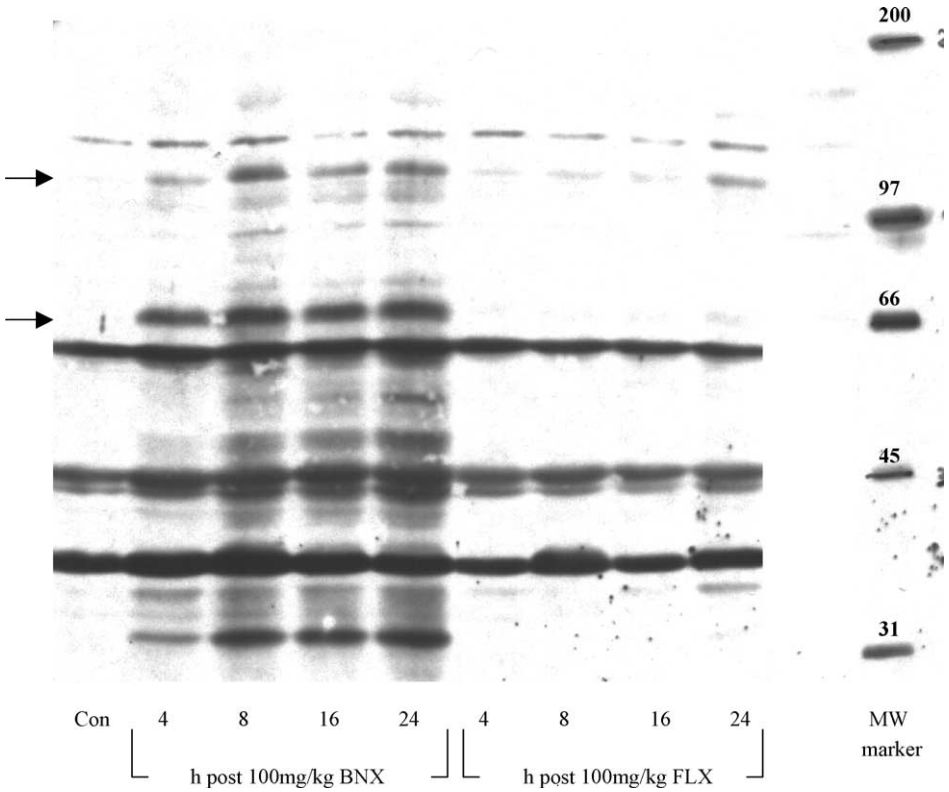

Fig. 6. Representative time-dependent covalent modification of hepatic proteins in BNX- and FLX-treated rats. Rats were given 100 mg/kg BNX or FLX intraperitoneally and killed after 4, 8, 16, or 24 h. Proteins from liver homogenates were separated by SDS-PAGE (100 μg/lane), transferred to nitrocellulose, and immunoblotted with anti-BNX sera. Arrows indicate the two major protein targets of BNX, FLX at MW of approximately 110 and 70 kDa. MW of proteins was estimated based on their migration relative to standard MW marker.

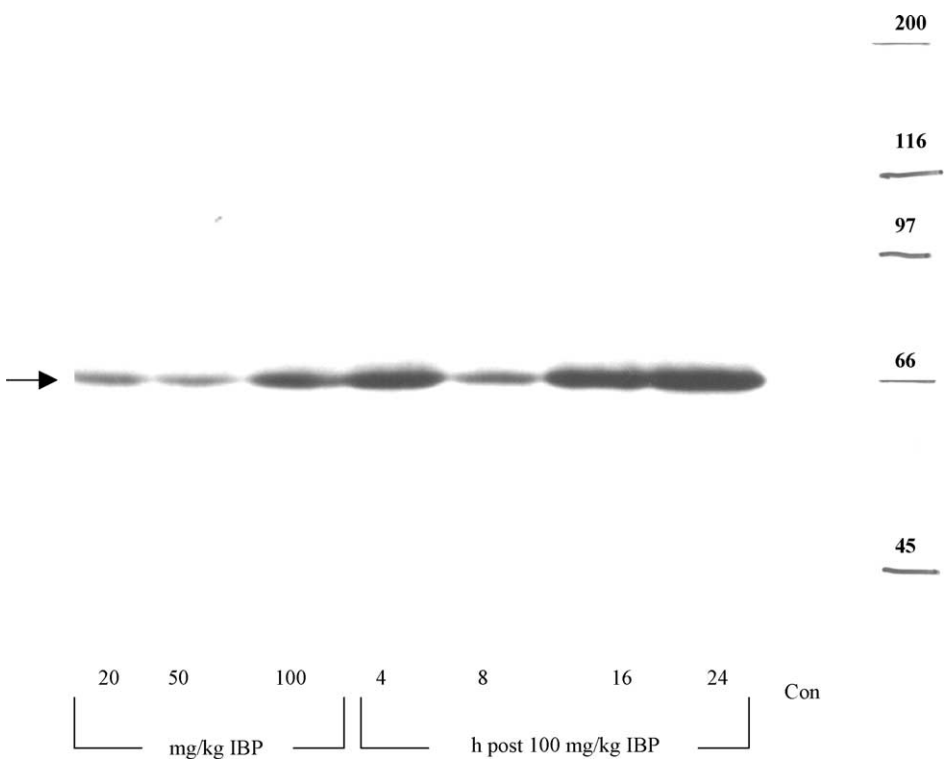

Fig. 7. Representative dose- and time-dependent covalent modification of hepatic proteins in IBP-treated rats. Rats were given vehicle control, 20, 50, or 100 mg/kg IBP intraperitoneally and killed after 8 h. Alternatively, rats were given 100 mg/kg IBP intraperitoneally and killed after 4, 8, 16, or 24 h. Proteins from liver homogenates were separated by SDS-PAGE (100 μg/lane), transferred to nitrocellulose, and immunoblotted with anti-IPB sera. Arrow indicates one major protein targets of IBP at MW of approximately 66 kDa. MW of protein was estimated based on their migration relative to standard MW marker.

Table 3
Apparent first-order degradation[a] half-lives[b] of BNX and FLX β-1 acyl glucuronides in different media at pH 7.4 and 37 °C

	β-1-BNX-G[c] (h)	β-1-FLX-G[c] (h)
Phosphate buffer	1.43 (0.067)	1.96 (0.10)
HSA[d]	1.55 (0.021)	2.01 (0.011)
Human plasma	0.89** (0.013)	1.25** (0.091)

[a] Degradation refers to loss of β-1 conjugate by either acyl migration or hydrolysis.
[b] The values represent mean (standard deviation, $n = 3$).
[c] Significantly different in all media, $P < 0.01$.
[d] HSA was dissolved in phosphate buffer (0.15 M) at pH 7.4.
** Significant effect of human plasma on degradation of acyl glucuronides, $P < 0.01$.

reported for male rats after the intravenous administration of 10 mg/kg of BNX by Mohri et al. [31]. The elimination $t_{1/2}$ of FLX has been reported previously to be much longer than the current finding. Segre et al. [32] reported a $t_{1/2}$ of ~70 h for FLX in rats after the administration of 40 mg/kg FLX intravenously. However, precautions to prevent hydrolysis of acyl glucuronide back to its parent drug during sample storage and extraction were not taken in this early study. Moreover, the $t_{1/2}$ was derived from the last exponential term of a multiexponential function that was fitted to the concentration–time profile, a methodology different from the current study. Since the current study was carried only up to 8 h compared to the 72 h study by Segre et al. [32], errors might be introduced in the current estimation of the terminal $t_{1/2}$ of FLX.

Rapid in vivo hydrolysis of the conjugates was observed for both BNX-G and FLX-G after the administration of glucuronides to rats (Figs. 1 and 2). From Figs. 1 and 2, it is apparent that only a small fraction of the acyl glucuronide dose was converted to parent drug in spite of their very short initial half-lives. This indicates that systemic hydrolysis clearance (i.e. reversible metabolism) was not the major clearance pathway for these labile glucuronides when administered intravenously. There were significant differences for AUCs of the acyl glucuronide between dosing BNX-G and dosing FLX-G, however, there was no significant difference when CL and $t_{1/2}$ were compared probably due to the high inter-animal variability and small sample size (Table 1).

The hepatobiliary excretion of acyl glucuronides differed when given equivalent molar intravenous doses of BNX and BNX-G, whereas similar biliary recovery of FLX-G was observed in rats given equivalent molar doses of FLX and FLX-G as shown in Table 2. Since dosing of the metabolites allowed AUCs to be manipulated, biliary formation clearance ($Cl_{f,bile}$) and biliary excretion clearance ($Cl_{e,bile}$) for both BNX and FLX acyl glucuronides were therefore determined by solving Eqs. (1) and (2)

$$A(m)^{p}_{e,bile} = Cl_{f,bile} \times AUC^{p}_{p} + Cl_{e,bile} \times AUC^{p}_{m} \quad (1)$$

$$A(m)^{m}_{e,bile} = Cl_{f,bile} \times AUC^{m}_{p} + Cl_{e,bile} \times AUC^{m}_{m} \quad (2)$$

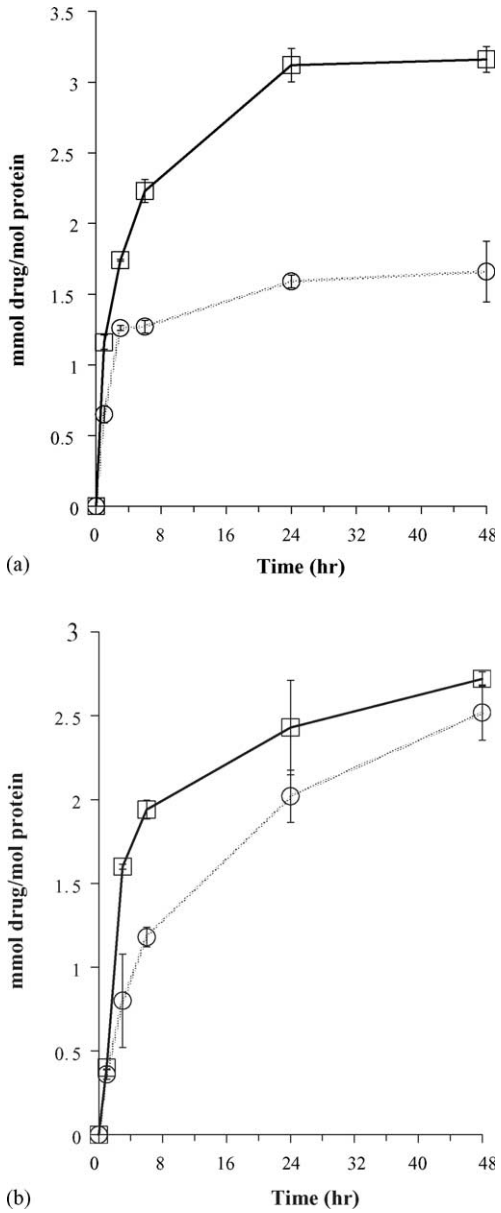

Fig. 8. (a) Mean profiles for formation of covalent drug–protein adducts following incubation of 50 μg/ml (0.17 mM) with HSA (30 mg/ml, 0.5 mM). (b) Mean profiles for formation of covalent drug–protein adducts following incubation of 50 μg/ml (0.17 mM) with pooled human plasma; BNX-G (□) and FLX-G (○) at 37 °C, pH 7.4. Data are the average of triplicate measurements (data for formed covalent adducts were corrected for the initial concentrations of β-1 conjugates).

In the equations the superscript indicates the dose administered (p, parent drug; m, metabolite) and the subscript refers to the compound measured in plasma. These apparent clearance terms for BNX-G reflect a mixture of enantiomers due to in vivo inversion (R → S) [33] that was not assessed with the achiral assay employed here. The AUC and $A(m)_{e,bile}$ determined up to 8 h were used in the calculations. The $Cl_{f,bile}$ for FLX-G (0.12 ml/(min kg)) was about three-fold higher than that for BNX-G (0.032 ml/(min kg)). The $Cl_{e,bile}$ of FLX-G was also much higher than that of BNX-G (24 and 9.1 ml/(min kg) for

FLX-G and BNX-G, respectively). The apparent $Cl_{bile,gluc}$ calculated simply as $A_{e,bile}/AUC_{gluc}$ from Table 2 were consistently higher when the parent drug was administered (18 versus 9.1 ml/(min kg) for BNX versus BNX-G, and 87 versus 24 ml/(min kg) for FLX versus FLX-G) which is apparently due to much of the glucuronide formed after administration of the parent being excreted into bile without exposure to the systemic circulation due to the efficiency of biliary excretion via transporters such as MRP2 (ABCC2).

Protein adducts of both BNX and FLX were found in the plasma of rats administered a single 20 mg/kg intravenous dose of the parent drug. Adduct formation in vivo has previously been shown to depend on the exposure of the organ to acyl glucuronide as reported for salicylate in rats [34] and zomepirac in guinea pig [35] and in humans [9]. However, though the systemic exposure ($AUC_{0\rightarrow 8\ h}$) of BNX-G in rats over 8 h was two times higher than that of FLX-G when administered the parent compound (Table 2), no significant differences in plasma protein adduct formation at 8 h were observed between BNX- and FLX-treated groups. Similar amounts of *R*- and *S*-BNX and *S*-FLX protein adducts have also been detected in systemic circulation of human [20], though in vitro studies reported here indicated that BNX-G was more reactive than FLX-G. This suggests that measuring covalent binding to plasma proteins might not be a relevant surrogate marker for predicting potential in vivo reactivity of acyl glucuronide metabolites with other tissues.

Organs where acyl glucuronides are formed or concentrated for excretion (e.g. liver and kidneys) are more likely to be exposed to higher concentrations of reactive metabolites, and therefore have the potential for greater protein adduct formation and higher epitope densities that may favor development of an immune response. Significant concentration gradients of acyl glucuronide between the sinusoidal circulation, hepatocyte and bile were evident in an isolated perfused rat liver study [36], which were most likely the effect of transporter systems on the basolateral and cannalicular membranes of hepatocytes. In the present study, mean values for percent excretion of the dose in bile over 8 h for BNX-G and unchanged BNX were 10.8 ± 1.4 and $0.67 \pm 0.11\%$, respectively. This is comparable to a previously reported 12 h biliary excretion of 13.2 ± 2.3 and $0.33 \pm 0.09\%$ for BNX-G and BNX, respectively [31]. Hepatobiliary exposure ($A_{e,bile,0\rightarrow 8\ h}$) of BNX-G was only 1/3rd of that of FLX-G determined from the 20 mg/kg dose (Table 2), yet similar concentrations of liver protein adducts were detected in rats 8 h after similar doses of BNX and FLX (Fig. 3). Though the present study did not characterize the relative covalent binding of the respective enantiomers, the data suggests that BNX-G was more reactive than FLX-G to liver tissue in vivo, which was supported by in vitro incubation studies with albumin where BNX-G was found to be less stable and more reactive with albumin in vitro than FLX-G (Fig. 8).

Discrepancies in terms of the relative covalent binding of BNX and FLX to hepatic proteins were observed when detected by the nonspecific alkaline cleavage method (BNX $\approx$ FLX, Fig. 3) versus the immunochemical method (BNX $>$ FLX, Fig. 5). This is probably due to (1) differences in the nature of the assays: the former is based on the quantification of the parent drug that would be released from the protein adduct by base hydrolysis, whereas the later detects the proteins modified covalently by NSAIDs; (2) different routes of administration (intravenous dosing and intraperitoneal dosing for base hydrolysis study and immunoblotting study, respectively) may have an impact on the liver exposure of acyl glucuronides, and consequently the protein adduct formation. However, both BNX and FLX are low extraction ratio compounds such that a first-pass effect should not enhance the total liver exposure of acyl glucuronides so much. The dose-proportional adduct formation in the liver (Fig. 3) also suggest that changes of covalent binding to proteins due to altered concentration profiles over time (i.e. first-pass) is less likely; (3) different antibody sensitivity for ELISA and immunoblots: only anti-BNX serum was prepared and used for detection of both BNX- and FLX-protein adducts. However, the cross-reactivity of anti-BNX serum for FLX-RSA demonstrated in ELISA may not be translated in the immunoblotting study as Western blots were run under denatured conditions. This underscores the need for alternative measures to quantitate SDS-PAGE gel fractions. Finally, this matter is further complicated by the possible involvement of other potentially reactive metabolites such as acyl-CoA thioesters which can also acylate proteins [37,38]. Indeed, formation of adducts with BNX and FLX to hepatocellular proteins were only partially inhibited (46 and 50% for BNX and FLX, respectively) in the rat hepatocyte cultures when treated with borneol, which depleted glucuronidation cofactor UDP-GA, relative to control (unpublished results). Thus, formation of reactive NSAID-CoA intermediate may be responsible for part of covalent binding of BNX and FLX to hepatic proteins that are not accounted by their reactive acyl glucuronides, and SDS-PAGE Western immunoblot analysis alone would not be expected to distinguish between adducts formed by acyl glucuronides and acyl-CoAs.

The 110 kDa protein target of BNX and FLX has been previously reported for diclofenac treated rat liver tissues by anti-diclofenac serum and has been identified as dipeptidyl peptidase IV (CD26) by subsequent purification and amino acid analyses of the target protein [39]. In addition, they reported an inhibitory effect of diclofenac adduct formation on the enzyme activity (decrease of 20%) of dipeptidyl peptidase IV. These results suggested that the 110 kDa adduct might have a role in hepatotoxicity induced by diclofenac and possibly BNX. Hargus et al. [40] also examined subcellular fraction of liver homogenate from diclofenac-treated rats for diclofenac labeled protein targets. Bile cannalicular domain of the plasma

membranes was shown to be a major site of diclofenac adduct formation. The 110 kDa was found to be a canalicular membrane protein which is important in bile transport and cell adhesion [41–43], and similar proteins may be present in human bile cannaliculi. The 70 kDa target protein has also been previously detected in zomepirac- and clofibric acid-treated rat livers [37]. The identity of this protein has yet to be revealed.

Besides the 110 and 70 kDa protein targets, other numerous less intense protein bands were also observed in livers of rats treated with either BNX or FLX (Figs. 5 and 6). The multitude of proteins identified as being adducted by BNX and FLX acyl glucuronides underscored the apparent lack of specificity in the reaction of these metabolites, which would be considered long-lived reactive metabolites by Gillette [3] and thus less likely to discriminate between proteins. In fact, tolmetin acyl glucuronide binds to numerous sites on albumin in vitro [12], also indicating that acyl glucuronides are nonspecific, long-lived reactive metabolites.

In contrast to BNX and FLX, a single protein adduct was detected in IBP-treated liver homogenates in a dose- and time-dependent manner. This protein target has a molecular weight the same as HSA (66 kDa). As suggested by a recent study of interaction of human serum albumin with furosemide glucuronide [44], HSA may work as a scavenger to eliminate reactive acyl glucuronide by both reversible and irreversible binding. This is also supported by the observation that acyl glucuronides are often reversibly bound to plasma proteins, as shown by 88.6 ± 2.7 and $79.5 \pm 4.9\%$ binding of zomepirac glucuronide and suprofen glucuronide to albumin [45]. Thus, preferential binding of IBP to HSA may in part explain the low incidence of adverse reactions associated with IBP. The relative reversible protein binding of BNX-G and FLX-G was not studied here. However, given their significant difference in lipophilicity, the chloride versus fluoride group may lead to differences in reversible binding and thus covalent binding for these acyl glucuronides. Related to this, Smith and Wang [45] also found that glycation via glucuronic acid was facilitated by conjugation to NSAIDs which was postulated to be due to differences in reversible binding to protein.

In conclusion, hepatobiliary exposure rather than systemic exposure of acyl glucuronide may be a more relevant measure when assessing potential for toxicity of these long-lived reactive metabolites, especially given that idiosyncratic toxicities of NSAIDs are often hepatic in nature. Moreover, adducts to hepatic tissue would include exposure to other more reactive metabolites such as the putative acyl-CoA intermediate, which is not likely to leave the hepatocyte. Similar concentrations of liver protein adducts for BNX and FLX were detected at 8 h even though the hepatobiliary exposure of BNX-G was only 1/3 of that of FLX-G, supporting the idea that BNX-G is more reactive than FLX-G, in agreement with in vitro findings. The 110 and 70 kDa proteins were the major liver protein targets modified by covalent attachment of BNX and FLX. Other less intense and less studied protein bands were also observed in hepatic tissue. The critical protein(s) that might cause immune reaction or direct toxicity associated with NSAIDs is still unknown, although HSA has been suggested to serve as a protective protein. However, using covalent adducts of acidic drugs to HSA or plasma proteins as a surrogate measure for potential in vivo reactivity and toxicity may not provide similar correlations to that seen in tissues such as the liver.

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