Glucuronidation and Covalent Protein Binding of Benoxaprofen and Flunoxaprofen in Sandwich-Cultured Rat and Human Hepatocytes

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Abbreviations: ABT, 1-aminobenzotriazole; BNX, benoxaprofen; BNX-G, benoxaprofen glucuronide; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; FLX, flunoxaprofen; FLX-G, flunoxaprofen glucuronide; HBSS, Hank’s balanced salt solution; HPLC, high performance liquid chromatography; HSA, human serum albumin; LDH, lactate dehydrogenase; IBP, ibuprofen; IBP-G, ibuprofen glucuronide; MCM, modified Chee’s medium; NSAIDs, nonsteroidal antiinflammatory drugs; PBS, phosphate buffered saline; PH, 1,7-phenanthroline; PMSF, phenylmethylsulfonylfluoride; SA, saccharic acid 1,4-lactone; UGT, UDP-glucuronosyltransferase.
ABSTRACT

Benoxaprofen (BNX), a nonsteroidal anti-inflammatory drug (NSAID) which was withdrawn due to hepatotoxicity, is more toxic than its structural analog flunoxaprofen (FLX) in humans and in rats. Acyl glucuronides have been hypothesized to be reactive metabolites and may be associated with toxicity. Both time- and concentration-dependent glucuronidation and covalent binding of BNX, FLX and ibuprofen (IBP) were determined by exposing sandwich-cultured rat hepatocytes to each NSAID. The levels of glucuronide and covalent protein adduct measured in cells followed the order: BNX > FLX > IBP. These results indicate that BNX-G is more reactive than FLX-G, and IBP-G is the least reactive metabolite, which are in agreement with previous in vivo studies in rats. The proportional increases of protein adduct formation for BNX, FLX, and IBP as acyl glucuronidation increased supports the hypothesis that part of the covalent binding of all three NSAIDs to hepatic proteins is acyl glucuronide-dependent. Moreover, these studies confirmed the feasibility of using sandwich-cultured rat hepatocytes for studying glucuronidation and covalent binding to hepatocellular proteins. These studies also demonstrated that these in vitro methods can be applied using human tissues for the study of acyl glucuronide reactivity. Greater BNX-protein adduct was formed in sandwich-cultured human hepatocytes than FLX-protein adduct, which not only agreed with their relative toxicity in humans, but was also consistent with the findings in vitro utilizing rat hepatocyte cultures. These data support the use of sandwich-cultured human hepatocytes as an in vitro screening model of acyl glucuronide exposure and reactivity.
**Introduction**

Many types of acidic drugs form acyl glucuronides and other xenobiotics are metabolized to carboxylic acids (Phase I metabolites), which subsequently undergo Phase II conjugation to form acyl glucuronides. Often such a glucuronide conjugate constitutes the major metabolite. The major site of conjugation for most compounds in humans is believed to be the liver. Modification of critical hepatic proteins by covalent binding of acidic drugs through reactive acyl glucuronides may provide a basis for direct hepatocyte toxicity or immune-mediated adverse reactions (Gillette, 1974; Faed, 1984; Boelsterli 2002; Bailey and Dickinson, 2003).

At present some pharmaceutical companies have been conducting in vitro experiments by incubating acyl glucuronides with model proteins or peptides (Wang et al, 2004) to determine their reactivity, and hence possibly predict the relative extent of covalent protein binding in vivo in humans. This method requires chemical synthesis or biosynthesis of individual glucuronides for each drug candidate, a process that can be tedious and costly. In addition, hepatic tissue proteins are not present in the incubation, which make it less likely to correlate covalent protein binding with hepatotoxicity. *In vitro* models involving hepatic materials, including liver homogenates, microsomal subcellular preparation, and isolated hepatocyte suspensions and cultures, offer many advantages over *in vitro* incubation with model proteins. Not only are hepatic tissue proteins present, but UGT activities which catalyze the glucuronidation reactions are also maintained. However, membrane transport functions are lost or not well maintained in isolated hepatocytes or even primary hepatocyte cultures. Although the intact animal
model represents undisturbed hepatic physiology, high-throughput screenings using in vivo studies are not very practical. Moreover, extrapolation of animal data to human is often questionable, especially for drug metabolism.

We propose that hepatocyte culture with a “sandwich” configuration would be a good model for estimating covalent binding to hepatic tissue proteins in vivo. Hepatocyte monolayer is cultured between two layers of Matrigel, a matrix material resembling the mammalian cellular basement membrane, and this has been shown to prolong cell viability and has better preservation of liver-specific protein synthesis than primary hepatocytes maintained under conventional culture conditions (Dunn et al., 1989, 1991). This model system uses biologically relevant matrix components and is, therefore, more close to the native architecture of the hepatocyte environment. A unique feature of this sandwiched culture of hepatocytes is the formation of functional bile canalicular networks formed with rat hepatocytes. In addition, the normal polarized distribution of several different classes of functionally active canalicular transport systems such as Mrp2 is also reestablished in this configuration (LeCluyse et al., 1994). It has been reported that the hepatobiliary transport of reactive diclofenac glucuronide via Mrp2 is critical for diclofenac covalent binding to proteins in the biliary tree (Seitz et al., 1998). In addition to being a much more biologically relevant in vitro model in terms of morphology and biochemical properties, this sandwiched hepatocyte culture system also maintains a better functional enzyme/transport system than microsomes with which the hepatocytes can generate metabolites such as acyl glucuronides. Furthermore, the availability of sandwiched human hepatocyte cultures will allow in vitro evaluation of
covalent binding in hepatocytes using human tissues. This is essential for studies of compounds that are potentially toxic to humans, since direct detection of acyl glucuronide-derived drug-protein adducts in human liver is not feasible.

Benoxaprofen (BNX) and flunoxaprofen (FLX), a pair of structural analogs (Figure 1) with different degree of hepatotoxicity in human and in rat, were selected as model compounds. BNX was marketed as a racemic mixture, whereas FLX was marketed as the S-isomer only. Only 5 months after its launch, several reports of fatal cholestatic jaundice often associated with nephrotoxicity led to the withdrawal of BNX from world market (Dahl and Ward, 1982). On the other hand, no fatal adverse events related to FLX have been reported. Previously we have demonstrated that BNX is more reactive than FLX with human serum albumin and plasma protein in vitro and in rat model in vivo (Dong et al., 2005). Fluorescent adducts of BNX and FLX have also been detected with human plasma proteins from patients administered the drugs (Dahms and Spahn-Langguth, 1996). In this study, we first evaluated the glucuronidation and protein adducts formation of model compounds in the sandwiched rat hepatocyte culture. An in vitro and in vivo correlation in rat in terms of relative reactivity of glucuronide metabolites was then established. Additionally, the effects of modulating metabolism of BNX and FLX in vitro on the extent of protein adduct formation were investigated. Finally, we examined the applicability of sandwiched culture of human hepatocytes in estimating the relative covalent binding to hepatic proteins. Glucuronidation and protein adduct formation of radiolabelled ibuprofen (IBP), a well-tolerated drug with a low incidence of side effects, in the sandwiched rat hepatocyte culture were also determined.
and served as a reference. These studies demonstrate through a series of experiments with rat hepatocytes the applicability of sandwich culture hepatocytes for investigating reactive acyl glucuronides and confirm that similar studies can be conducted with human hepatocytes, thus allowing possible predictions to humans.
Materials and Methods

Materials. Rac-BNX was extracted and purified from Tablets of Oraflex® previously marketed by Eli Lilly (Indianapolis, IL). Anal. Calcd for BNX (C16H12ClNO3): 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 (>99%) 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 (>98%) based on HPLC with UV detection. IBP, Triton X-100, ethylenediaminetetraacetic acid (EDTA), glycerol, glycine, phosphate-buffered saline (PBS), borneol, SKF-525A, lactate dehydrogenase (LDH) kit, Percoll, Hank’s balanced salt solution (HBSS), and dexamethasone were obtained from Sigma Chemical Co. (St Louis, MO, USA). 3H-IBP (1 Ci/mmol) was purchased from ICN (currently Valeant Pharmaceuticals, Costa Mesa, CA, USA). 1,7-Phenanthroline was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). 1-Aminobenzotriazole (ABT) was a generous gift from Dr. J. Mathews at Triangle Research Institute (RTP, NC, USA). Protogel™, ammonium sulfate, TEMED, dithiothreitol (DTT), bromophenol blue, and Tris, were purchased from BioRad (Hercules, CA, USA). Collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ, USA). Dulbecco’s modified Eagle’s medium (DMEM), modified Chee’s medium (MCM), fetal calf serum and insulin were purchased from Gibco (Grand Island, NY, USA). Rat tail collagen (type I) was obtained from Collaborative Biochemical Research (Bedford, MA, USA). Matrigel was purchased from Fisher Scientific.
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(Pittsburg, PA, USA). Methanol and acetonitrile were HPLC grade from J. T. Baker (Phillipsburg NJ, USA). All other chemicals used were of reagent grade. Male Sprague Dawley rats (250-350 g) were supplied by Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). Experiments were approved by the University’s Animal Care Committee. Human liver microsomal preparations were obtained from Gentest Corp. (Woburn, MA, USA).

Isolation and Culture of Rat Hepatocytes. Plastic culture dishes (60 mm) were precoated with rat tail collagen, type I (0.1 ml/dish, 1.5 mg/ml), in a gelled state at least 1 day prior to hepatocyte harvest. To obtain a gelled collagen substratum, neutralized collagen type I was prepared on ice (as described by manufacture’s instruction) and spread onto Petri dishes with a sterile Teflon policeman. Freshly coated dishes were then placed at 37°C in a humidified incubator for at least 60 min to allow the matrix material to gel, followed by addition of 3 ml fresh medium to the dishes and storage in a humidified incubator until utilized. Just before cell isolation, the medium was aspirated from the precoated culture dishes.

Hepatocytes were isolated from male Sprague-Dawley rats (250-350 g body wt) by a modification of the two-step collagenase perfusion method as previously described by LeCluyse et al. (1994) and Liu et al. (1999). Animal studies were approved by the Institutional Animal Care and Use Committee at the University. Rats were anesthetized with ketamine/acepromazine (75:2 mg/kg, i.m.) prior to portal vein cannulation. The liver was perfused in situ with oxygenated calcium-free buffer containing 5.5 mM
glucose, 0.5 mM EDTA, 25.0 mM sodium bicarbonate, 1.2 mM potassium phosphate (pH 7.4), 4.7 mM potassium chloride, and 118.0 mM sodium chloride for ~10 min at 37°C, followed by perfusion with buffer containing 2 mM calcium and collagenase type II (0.3 mg/ml) for 10-15 min. Following collagenase perfusion, the digested liver was carefully removed and placed into a sterile, covered crystallization dish and transported to a sterile hood, where 30 ml of DMEM kept at room temperature and supplemented with 5% FCS, 1 μM dexamethasone and 4 mg/L insulin was immediately added. The hepatocytes were dispersed by tearing open Glisson’s capsule with the aid of two sterile forceps and gently shaking the liver until most of the cells are released into the medium. The released cells were then filtered through a sterile nylon 70-μm filter into a 250-ml beaker. Another 30 ml of medium was added to the crystallization dish, which was gently swirled to release any remaining cells from the liver remnant, and filtered as before. This rinsing step was then repeated for one more time. The cell suspension was divided equally between two 50-ml sterile centrifuge tubes and centrifuged at 40 × g for 2 min at room temperature. The supernatant fractions were discarded, and cell pellets were gently resuspended in fresh DMEM to attain a final volume of 25 ml. An equal volume of 90% isotonic Percoll (Percoll-10× HBSS, 9:1, v/v) was added to the suspension, which was mixed well by inverting the tube and centrifuged at 80 × g for 5 min at room temperature. The supernatant fraction was aspirated, and the cell pellets were resuspended in a combined volume of 40 ml of medium and transferred into one 50-ml centrifuge tube. The cells were washed again by centrifugation at 60 × g for 2 min. The final cell pellet was resuspended gently in 40 ml of medium. Hepatocyte viability was determined by trypan
blue exclusion and was typically > 90%. Cells were diluted with fully supplemented medium to a cell concentration of $1.0 \times 10^6$ cells/ml, and 3 ml aliquots were seeded into 60-mm collagen-coated culture dishes. After a 2-hr attachment period at 37°C in a 5% CO$_2$/95% air atmosphere, the medium was replaced with fresh DMEM supplemented insulin and dexamethasone. The culture dishes were then placed in the 37°C humidified incubator for 24 hr until the Matrigel overlay (Fig. 2A. shows the hepatocyte morphology just before the overlay).

To prepare cultures in a sandwich configuration, the Matrigel stock solution was slowly thawed at 4°C, diluted with ice-cold MCM to a final concentration of 250 μg/ml (Sidhu et al. 1993). The medium was aspirated from culture dishes, and 3 ml of medium/Matrigel mixture was overlaid onto dishes with precooled sterile pipets. Matrigel-treated cultures were allowed to incubate over night. Medium was then changed on a daily basis until studies were performed. All experiments were done in 5-day post-overlay hepatocyte cultures (Fig. 2B.) and in serum-free MCM.

**Studies with Rat Hepatocyte Culture.** The glucuronidation and covalent protein adduct formation in rat hepatocytes were first evaluated. For time-dependency studies, 500 μM BNX, FLX, or IBP were dissolved in fresh MCM and directly added to the rat hepatocyte cultures. The dose of 500 μM IBP also contained trace amounts of radiolabelled IBP (2.3 μCi of $^3$H-IBP/ml of culture medium). After incubation for designated times, the cultures were extensively washed with ice-cold PBS containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) to reduce possible hydrolysis of acyl
glucuronides by non-specific esterases. The cells were harvested in 2 ml of ice-cold PBS containing 0.2 mM PMSF, 1% Triton X-100, and 10 μl of 43% phosphoric acid, and lysed by Polytron® (Brinkmann Instruments, Westbury, NY, USA). The cell homogenates were then deproteinated by adding 4 ml of ice-cold acetonitrile (including 5 μg internal standard) to precipitate proteins and extract unbound (not covalently bound) drug and metabolites, followed by centrifugation at 4000 rpm for 10 min. The supernatant fractions and the protein pellets were stored at -80°C until analyzed for the concentrations of acyl glucuronide and protein adduct formed, respectively.

For concentration-dependency studies, cultured rat hepatocytes were incubated with various concentrations of BNX and FLX dissolved in MCM. BNX reaches peak plasma concentrations above 100 μM after oral doses of 600 mg to humans (Ridolfo et al., 1979), with higher concentrations expected in liver during absorption, therefore a range of up to 1000 μM was selected for these in vitro studies. After 6-hr exposure, the cultures were extensively washed with ice-cold PBS containing 0.2 mM PMSF, and the cells were harvested as described above. Four ml of ice-cold acetonitrile (20 ng, 50 ng, 2 μg, and 5 μg of internal standard was added for concentrations of 10 μM, 100 μM, 250 μM, and ≥ 500 μM, respectively) was added to precipitate the proteins. After the centrifugation of cell homogenates, the supernatant fractions and the cell pellets were stored separately at -80°C. Cytotoxicity of BNX and FLX at the highest concentration was estimated by determining lactate dehydrogenase (LDH) release into the extracellular medium. LDH activities were determined spectrophotometrically with a Sigma test kit.
Enzyme activity in the medium was expressed as percentage of total LDH present in the cells at the beginning of the incubation.

The effects of modulating metabolic pathways on the glucuronidation and covalent protein binding were determined. The cells were exposed to 500 μM BNX and FLX in the presence or absence of 1 mM borneol, 10 μM SKF-525A or 1 mM ABT following preincubation with each for 30 min. Rat hepatocytes were also pretreated with 200 μM 1,7-phenathroline (PH) for 3 days (fresh dose replaced daily) followed by the exposure of 500 μM BNX and FLX. Cells were also exposed to 500 μM IBP (containing 2.3 μCi of ^3^H-IBP/ml of culture medium) in the presence and absence of 1 mM borneol, or 1 mM ABT following preincubation with each for 30 min. All the incubations were carried out to 6 hr, at which time point cells were extensively washed and harvested as described above. Supernatant fractions and protein pellets separated by centrifugation were stored at -80°C until further analysis.

HPLC analyses using the native fluorescence of BNX and FLX (details described previously by Dong et al., 2005) were carried out to determine the formation of BNX glucuronide (BNX-G) and FLX glucuronide (FLX-G) in the rat hepatocytes. The supernatant fractions of harvested cells were evaporated to dryness. The residues were reconstituted with 0.5 ml of mobile phase and injected onto HPLC. For cells incubated with 10 μM and 100μM of either BNX or FLX, 100 μl and 10 μl of 0.5 ml reconstituted samples were injected, respectively. For cells incubated with concentrations ≥ 250 μM,
additional dilution of reconstituted samples were made before 20 μl of the diluted samples were injected onto HPLC.

For detection of IBP glucuronide (IBP-G) formed in the rat hepatocytes, a HPLC assay (Castillo et al., 1995) was first carried out to isolate and collect the IBP-G. The HPLC equipment used were essentially the same as for BNX and FLX assays (Dong et al., 2005), but with a Hewlett-Packard variable wavelength detector (set at 250 nm) replacing the Shimadzu fluorescence detector. The mobile phase consisted of methanol-10 μM TFA in water (60:40, v/v) and isocratic elution was performed at a flow rate of 1.2 ml/min. The IBP-G peak was then collected with a Gibson fraction collector, and the collections were evaporated to dryness under nitrogen. Aliquots of 2.5 ml of 1 M sodium hydroxide were added to dissolve the residues which were then transferred into scintillation vials. Finally, 10 ml Ultima Gold scintillation fluid (Packard) were added and the radioactivity was determined by liquid scintillation counting.

Irreversible binding of BNX, FLX, and IBP metabolites to hepatocellular proteins was determined as previously described (Dong et al., 2005). In brief, the protein pellets were extensively washed with methanol-ether (3:1, v/v). After centrifugation, the protein pellets were dried and incubated with 2.5 ml of 1 M sodium hydroxide at 80°C for 1 hr. BNX- and FLX-protein adducts were extracted from sodium hydroxide with dichloromethane. The subsequently dried residues were reconstituted in 250 μl of proper mobile phase and 200 μl was injected onto HPLC. IBP-protein adducts were determined by adding 10 ml of Ultima Gold scintillation fluid to the sodium hydroxide mixture. The total cell-associated radioactivity was determined by liquid scintillation counting.
Total protein content per dish was determined according to Bradford (1976) using bovine serum albumin as standard protein.

**Studies with Human Liver Microsomes and Human Hepatocyte Cultures.**

Human liver microsomes from three subjects were used. The glucuronidation of BNX and FLX in these preparations were measured similar to that described for rat liver microsomal studies by Dong et al. (1999). Briefly, microsomal protein was first incubated with Triton X-100 for 15 min at room temperature. The incubation mixture (0.5 ml), contained 2 mg/ml microsomal protein, 0.05% Triton X-100, 10 mM magnesium chloride, 100 mM Tris buffer, pH 7.4, and 0.1 to 2 mM BNX or FLX with the addition of inhibitors of hydrolytic enzymes (0.4 mM PMSF and 16 mM saccharic acid 1,4-lactone (SA)); it was then warmed to 37°C for 5 min. The reaction (over a 20-min period) was started by adding UDP-glucuronic acid (final concentration, 10 mM). Aliquots of 0.10 ml were taken from the assay mixture at various times and were pipetted into 0.20 ml ice-cold acetonitrile (including internal standard). The pH of the samples was adjusted to 2 to 4 to further stop the reaction. After centrifugation, the supernatants were dried under nitrogen gas and reconstituted in 0.5 ml mobile phase before injection onto the HPLC. Activities of UGT are expressed as nanomoles of BNX-G or FLX-G formed per minute per milligram of microsomal protein.

Kinetic analyses of UGT in the human liver were carried out by determining initial rates of activity at five different fixed BNX and FLX concentrations (0.1, 0.2, 0.5, 1, and 2 mM). The initial glucuronide formation rate was determined simply by using
linear regression analysis on the BNX−G and FLX-G formation over time profile. Kinetic constants ($V_{\text{max}}$ and $K_M$) for UGT were first estimated by plotting the initial rate of activity and substrate concentration on double-reciprocal plots and Eadie-Hofstee plots, then determined by fitting the data to the Michaelis-Menten equation (WinNONLIN 1.1, Parsight, Palo Alto, CA, USA).

Sandwiched human hepatocyte cultures (35-year-old male Caucasian, day 4 post Matrigel-overlay, 5 Petri dishes) were gifts from Dr. E. LeCluyse (UNC-Chapel Hill, Chapel Hill, NC, USA). BNX and FLX dissolved in MCM at 500 μM were added to the cell cultures (BNX, n = 3; and FLX, n = 2). After 6-hr incubation, cell cultures were washed extensively, and cells were harvested and lysed as previously described. The amounts of BNX-G and FLX-G formed in the hepatocytes and the extent covalent binding of each to hepatic cellular proteins were determined as described above.
Results

Time- and Concentration-Dependency of Glucuronidation and Covalent Protein Binding in Rat Hepatocyte Cultures. BNX- and FLX-induced hepatocyte injury was monitored throughout the present study to ensure that glucuronidation and covalent protein binding of BNX and FLX were not compromised due to acute cell injury. Exclusion of the vital dye trypan blue by the hepatocyte cultures indicated that more than 85% of the cells were still viable after treatment with various concentrations of BNX or FLX for various periods of time. LDH release into the extracellular medium was also measured as a marker for acute cell injury in the sandwich-cultured rat hepatocytes. In the presence of 1000 μM (the highest concentration used in the present study) of BNX and FLX, LDH leakage in the medium were not different from that in the control cells for up to 12 hr (data not shown). LDH release was increased after 24 hr to ~24% and ~18% of total LDH activity in BNX- and FLX-treated hepatocytes, respectively, compared to a ~5% of total LDH leakage in control cells. However, when the hepatocyte cultures were exposed to 500 μM BNX and FLX, no differences in LDH release were observed between the treated and untreated cells (data not shown), indicating that BNX and FLX treatment at the concentration of 500 μM did not cause apparent cell injury.

Time-dependent glucuronidation of BNX, FLX and IBP were determined by exposing cultured hepatocytes to each at 500 μM for up to 24 hr. BNX-G and FLX-G formed in the hepatocytes were quantitated by HPLC-fluorescence analysis, whereas IBP-G was first isolated by HPLC and then analyzed by liquid scintillation counting. Formations of all three glucuronides were found to increase with time, with the
concentrations of BNX-G higher than FLX-G at any given time and IBP-G was detected at the lowest level (Fig. 3). Exposure of cultured hepatocytes to BNX, FLX, and IBP were associated with the appearance of drug irreversibly bound to hepatic proteins. Covalent protein binding also accumulated over the incubation period, with the highest protein adduct concentrations detected in BNX-treated hepatocytes and the lowest in IBP-treated hepatocytes (Fig. 4). In addition, covalent binding profiles appeared to mimic their corresponding glucuronidation profiles. In fact, covalent binding was found to increase as a linear function of the acyl glucuronide concentrations (Fig. 5), indicating that the covalent adduct formation may be mediated via reactive acyl glucuronide. When normalized to glucuronide concentration, the extent of adduct formation in the rat hepatocyte cultures was highest after BNX treatment and the lowest after IBP, as indicated by trends of the slopes of linear regressions of adduct vs. glucuronide concentration profiles in Fig. 5. This suggests that BNX-G is the most reactive one among the three acyl glucuronides studied in this study, and IBP-G is the least reactive acyl glucuronide of the three examined here.

Concentration-dependency of glucuronidation and protein adduct formation of BNX and FLX ranging from 10 to 1000 µM was investigated at the 6 hr incubation time. Nonlinear glucuronidation and protein adducts formation were observed for both BNX and FLX at concentrations higher than 100 µM, and the maximal glucuronidation and binding was reached at initial concentration of 750 µM (Figs. 6 and 7). Higher mean levels of BNX-G and BNX-protein adduct were detected in the hepatocytes than FLX-G and FLX-protein adduct, respectively, at all concentrations examined.
Effects of Modulating Metabolism on the Covalent Protein Binding. To assess whether covalent binding of BNX, FLX, and IBP could be mediated via another metabolic pathway other than the formation of reactive acyl glucuronides, the effects of modulating glucuronidation (with borneol and PH) and oxidative metabolism (with ABT and SKF-525A) on the covalent adduct formation were examined. Borneol, which depletes glucuronidation cofactor UDP-GA (Watkins and Klaassen, 1983), almost completely inhibited the formation of BNX-G, FLX-G and IBP-G (Fig. 8). In contrast, formation of adduct with BNX, FLX and IBP to hepatocellular proteins were only inhibited about half (46% and 50% for BNX and FLX, respectively) in the hepatocyte cultures when treated with borneol relative to control (Fig. 9). PH is a selective phase II enzyme inducer, and has been shown to significantly increase BNX glucuronidation in vivo in rats (Dong et al., 1999). In the present study, we found that the glucuronidation of BNX and FLX were significantly increased in hepatocyte cultures pretreated with PH (55% and 224% for BNX and FLX, respectively) compared to untreated cultures (Fig. 8). However, no effect of PH on covalent protein binding of either BNX or FLX was detected (Fig. 9). These data suggest that the covalent protein binding of BNX, FLX and IBP are only partially mediated via the formation of reactive glucuronides.

An alternative metabolic pathway that may form reactive intermediate capable of binding covalently to proteins and releasing parent drug when treated with base was then investigated. The effects of inhibitors of CYP activity on the glucuronidation of BNX, FLX, and IBP were first evaluated to ascertain that the CYP inhibitors did not block the glucuronidation pathway. SKF-525A and ABT, two general CYP inhibitors, did not
appear to alter the glucuronidation of BNX, FLX, and IBP (Fig. 10), nor did they reduce the covalent bindings of drugs to cellular proteins (Fig. 11). These data suggest that BNX, FLX, and IBP covalent protein adducts, as measured by base hydrolysis, are not likely to be mediated via the formation of reactive oxidative metabolites.

**Human UGT Activities in Liver Microsomes and Enzyme Kinetic Studies.**
The glucuronidation of BNX and FLX in human liver microsomes from three different donors were determined over a range of substrate concentrations. The dependency of the initial formation rates of BNX-G and FLX-G as a function of the substrate concentration is illustrated in Fig. 12, with the maximum formation rates reached at substrate concentration of 1 mM. The extent of glucuronidation varied dramatically between donors. The UGT activity of donor #3 was very low, only small amount of BNX-G was detected in its liver microsomal preparation, and levels of FLX-G were below the detection limit of the assay. Donor #1 and #2 appeared to have similar formation rate vs. substrate concentration profiles, with the formation of BNX-G consistently faster than that of FLX-G. Michaelis-Menten parameters ($V_{\text{max}}$ and $K_M$) were determined and summarized in Table 1. The values of $K_M$ did not appear to differ between BNX and FLX glucuronidation due to the high variability. $V_{\text{max}}$, on the other hand, were higher for BNX glucuronidation in all three microsomal preparations.

**Glucuronidation and Covalent Protein Binding of BNX and FLX in Human Hepatocyte Cultures.** A pilot study was conducted in five dishes of sandwiched human
hepatocyte cultures from a single donor. Both BNX-G and FLX-G were detected in the cells 6 hr after they were exposed to 500 μM of their parent drugs, with the levels of BNX-G significantly higher than FLX-G (Fig. 13). Greater amounts of covalent binding to hepatocellular proteins were also detected in the hepatocyte cultures exposed to BNX (Fig. 13). When adducts formation were normalized to the glucuronides level, BNX-protein adduct was still higher than FLX-protein adduct. This suggests that BNX is more reactive than FLX in human hepatocyte cultures.
Discussion

The major site of glucuronidation for most compounds in humans is believed to be the liver. Hepatotoxicity in elderly patients reported for BNX was the cause of its withdrawal from the world market (Dahl and Ward, 1982). The liver was also found to be a tissue targeted by both BNX and FLX in vivo in rats (Dong et al., 2005). An in vitro model involving hepatic materials, such as intact cells or slices, may therefore be a better method for studying the acyl glucuronide-derived drug-protein adducts than in vitro incubation with model proteins. Hepatocyte cultures with a “sandwich” configuration were selected in this study. Overlaying cultured hepatocytes with a top layer of gelled collagen or Matrigel has been demonstrated to prolong cell viability and preserve liver-specific protein synthesis (Dunn et al., 1989, 1991; Hewitt et al., 2007), which are more stable than microsomal incubations and allows for studies of time-dependent covalent protein binding for up to 24 hr. Furthermore, rat hepatocytes maintained in the sandwich configuration also reestablish a structural and functional bile canalicular network which is usually fully developed between 3 and 7 days after the overlay (LeCluyse et al., 1994). It has been demonstrated that the expression and function of primary active transporters, such as the canalicular bile acid transporter and the canalicular–multispecific organic anion transporters, were maintained in rat sandwich-cultured hepatocytes for 96 to 120 hr (Liu et al., 1999). Cultures of rat hepatocytes at 5 days after Matrigel overlay was, therefore, used throughout the present study to ensure the functional hepatobiliary transport of formed acyl glucuronides across canalicular membrane. Drug uptake and enzyme-induction potential are also better maintained in the sandwiched hepatocyte
cultures (LeCluyse et al., 1994, Hewitt et al., 2007), which makes manipulation of metabolic pathways needed in the present study possible.

Three possible metabolic pathways that might lead to the covalent binding to liver proteins have been proposed for carboxylic acids (Skonberg et al., 2008), as exemplified by diclofenac, a popular model compound for studies of liver protein adducts (Hargus et al., 1994). The first pathway that involves the formation of reactive acyl glucuronide has been shown to be responsible for covalent binding of BNX, FLX, and IBP to serum albumin in vitro (Castillo and Smith, 1995; Dong et al., 2005) and to plasma and liver tissue proteins in vivo in rat (Dong et al., 2005). Acyl glucuronides may bind covalently to proteins by a transacylation mechanism and bind to the proteins via Schiff base formation after acyl migration (Smith et al., 1990; Qiu et al., 1998). The results of the present study have shown that acyl glucuronide metabolites of BNX, FLX, and IBP are formed in rat hepatocyte cultures and can bind covalently to hepatic proteins in a time- and concentration-dependent manner (Figs. 3–4 and 6–7). These results also indicate that BNX-G is more reactive than FLX-G, and IBP-G is the least reactive metabolite, which are in agreement with both the in vitro studies with model proteins and the in vivo studies in rats (Dong et al., 2005). The proportional increases of adduct formation for BNX, FLX, and IBP as the glucuronidation increases supported the hypothesis that some part of the covalent binding of all three NSAIDs to hepatic proteins are largely acyl glucuronide-dependent (Fig. 5). Moreover, it confirmed the feasibility of using sandwich-cultured rat hepatocytes for studying glucuronidation and covalent binding to hepatocellular proteins. However, studies with inhibitor and inducer of glucuronide conjugation reaction indicate
that the formation of reactive acyl glucuronides may be only partially (~50%) responsible for the observed covalent binding of BNX, FLX, and IBP to hepatocellular proteins in hepatocyte cultures (Figs. 8 and 9).

An alternative pathway for metabolic activation involving CYP450 was also investigated for its role in the covalent protein adduct formation of BNX, FLX, and IBP in the rat hepatocytes. The reactive metabolites of diclofenac and acetaminophen formed by CYP450 have been reported to cause covalent modification of proteins (Hargus et al., 1994). Studies on the metabolism and disposition of IBP have shown that its major metabolites in plasma are carboxy- and hydroxy-IBP (Mills et al., 1973), though 13% of the dose is excreted in urine as IBP-G (Geisslinger et al., 1989). No oxidative metabolites have yet been identified for BNX and FLX either in human or in animals. Our results of CYP inhibition studies, however, suggested that BNX-, FLX-, or IBP-protein adducts formation in vitro were not likely to be mediated by oxidative metabolism (Figs. 10 and 11). Moreover, the assay of adduct by cleavage with base to liberate parent drug is unlikely to measure adducts formed by oxidative metabolism of BNX or FLX.

A third pathway that is possible for the bioactivation of NSAIDs is the formation of reactive acyl-CoA intermediate (Boelsterli 2002, Skonberg et al., 2008). Practically all carboxylic acids, are thought to covalently modify proteins by this mechanism (Hertz and Bar-Tana, 1988). This pathway may also lead to the inclusion of xenobiotic compounds in pathways of lipid biosynthesis (Tracy et al., 1993; Dodds, 1995). Grillo and Benet (1996) first reported in abstract form a tolmetin-glycine amino acid conjugate...
formed in vitro in freshly isolated rat hepatocytes exposed to tolmetin. Their studies demonstrated the chemical reactivity of xenobiotic acyl-CoAs and their potential to undergo nonenzymatic protein acylation. Sallustio et al. (2000) were the first to demonstrate a direct relationship between xenobiotic-CoA formation, in this case nafenopin-CoA, and acylation of human liver proteins. No acyl-CoA synthase-dependent BNX, FLX, or IBP adduct formation were characterized here in the present study, due to a lack of specific and potent inducers or inhibitors of this metabolic pathway. However, formation of reactive NSAID-CoA intermediate may still be responsible for part of covalent binding of BNX and FLX to hepatic proteins that are not accounted for by their reactive acyl glucuronides (Fig. 8 and 9).

Once an in vitro and in vivo correlation of relative covalent protein binding of BNX and FLX had been established using the rat as our animal model, the applicability of using sandwiched human hepatocyte cultures to investigate the glucuronidation and drug-protein adduct formation was examined. The preliminary studies demonstrated that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct was formed in human hepatocytes than FLX-protein adduct (Fig. 13), which not only agreed with their relative toxicity in humans, but was also consistent with the findings in vitro utilizing rat hepatocyte cultures. The enzyme kinetic studies in the small number of human liver microsomes, however, suggested higher intersubject variability of UGT activity in humans (Fig. 12 and Table 1) than in inbred rats. With the exception of one donor who has very low UGT activity, $V_{\text{max}}$ of BNX glucuronidation in humans (0.865 and 1.24 nmol/mg/min for donor 1 and 2, respectively) are higher than that in rats (0.30...
± 0.18 nmol/mg/min), whereas Km are similar in humans (0.322 and 0.396 mM for donor 1 and 2, respectively) and in rats (0.52 ± 0.43 mM). Further characterization is still needed before sandwiched human hepatocyte cultures can be used as an in vitro screening model of acyl glucuronide exposure and reactivity, though the preliminary studies look promising and suggest the method is transferable to human tissues.

In summary, the studies conducted and results obtained here demonstrate the applicability of sandwiched hepatocyte culture for estimating relative covalent binding of reactive acyl glucuronides in vivo and validated the method as a useful in vitro/in vivo model. The formation of labile and reactive acyl glucuronides was found to be at least partly responsible for about half of the drug-protein adducts formation in the liver tissues in vitro. Whether covalent modification of proteins in vivo leads to the observed hepatotoxicity of compounds, such as BNX, is still uncertain due to a lack of animal hepatotoxicity model for this and other drugs that form acyl glucuronides. However, preliminary studies indicate that this in vitro method is applicable to human tissue, which would provide a useful preclinical screening method to rank order relative reactivity of acyl glucuronide metabolites of compounds containing carboxylic acid groups that are candidates in drug discovery.
DMD #028944

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References


DMD #028944


DMD #028944


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**Footnote**

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Legends for figures

Fig. 1. Structures of benoxaprofen and flunoxaprofen. Benoxaprofen was marketed as the racemate, flunoxaprofen was marketed as the S-isomer.

Fig. 2. Hepatocyte cultures just before Matrigel overlay (A) or 5 days after overlay in a sandwich configuration (B).

Fig. 3. Time-dependent glucuronidation profiles of BNX, FLX and IBP in cultured rat hepatocytes. Hepatocytes were exposed to 500 μM BNX (■), FLX (●), and IBP (▲). At various times indicated, the cells were harvested, and the amounts of glucuronides formed were determined. Values are mean ± S.D. of triplicate incubations.

Fig. 4. Time-dependent covalent binding profiles of BNX, FLX and IBP in cultured rat hepatocytes. Hepatocytes were exposed to 500 μM BNX (■), FLX (●), and IBP (▲). At various times indicated, the cells were harvested, and the concentrations of protein adducts formed were determined. Values are mean ± S.D. of triplicate incubations.

Fig. 5. Covalent protein binding of BNX (■), FLX (●), and IBP (▲) correlate well with their corresponding glucuronide formations in the rat hepatocyte cultures at drug concentration of 500 μM. Slopes of the linear regressions are 13.1, 10.1, and 9.76 for BNX, FLX, and IBP, respectively.

Fig. 6. Concentration-dependent glucuronidation of BNX and FLX in cultured rat hepatocytes. BNX (■) and FLX (●) were added at the concentrations indicated. After 6 hr, the amounts of glucuronide formed in the cells were determined. Values are the means ± S.D. of triplicate incubations.

Fig. 7. Concentration-dependent covalent binding of BNX and FLX in cultured rat hepatocytes. BNX (■) and FLX (●) were added at the concentrations indicated. After 6 hr, the covalent protein adducts in the cells were determined. Values are the means ± S.D. of triplicate incubations.

Fig. 8. Effects of UGT inhibitor and inducer on the glucuronidation of BNX, FLX, or IBP in hepatocyte cultures. Hepatocyte cultures were incubated with 500 μM BNX, FLX, and IBP in the absence (control) or presence of 1 mM borneol or 200 μM PH for 6 hr. For induction study, cells were also preincubated with PH for 3 days. The zero time glucuronide values were subtracted from all data. Values are the mean ± S.D. of triplicate incubations. Asterisks indicate significant difference from the appropriate control (p < 0.05). Note: 24-hr incubation was conducted for IBP, and the effect of PH on IBP glucuronidation was not studied.
Fig. 9. Effects of UGT inhibitor and inducer on the covalent binding of BNX, FLX, and IBP to hepatocellular proteins. Hepatocyte cultures were incubated with 500 μM BNX, FLX, or IBP in the absence (control) or presence of 1 mM bornel or 200 μM PH for 6 hr. For induction study, cells were also preincubated with PH for 3 days. The zero time binding values were subtracted from all data, but were generally less than 5% of the values reported. Values are the mean ± S.D. of triplicate incubations. Asterisks indicate significant difference from the appropriate control (p < 0.05). Note: 24-hr incubation was conducted for IBP, and the effect of PH on IBP covalent protein binding was not studied.

Fig. 10. Effects of CYP inhibitors on the glucuronidation of BNX, FLX, and IBP in hepatocyte cultures. Hepatocyte cultures were incubated with 500 μM BNX, FLX, or IBP in the absence (control) or presence of 1 mM ABT or 10 μM SKF-525A for 6 hr. The zero time glucuronide values were subtracted from all data. Values are the mean ± S.D. of triplicate incubations. Note: 24-hr incubation was conducted for IBP, and the effect of SKF-525A on IBP glucuronidation was not studied.

Fig. 11. Effects of CYP inhibitors on the covalent binding of BNX, FLX, and IBP to hepatocellular proteins. Hepatocyte cultures were incubated with 500 μM BNX, FLX, or IBP in the absence (control) or presence of 1 mM ABT or 10 μM SKF-525A for 6 hr. The zero time binding values were subtracted from all data. Values are the mean ± S.D. of triplicate incubations. Note: 24-hr incubation was conducted for IBP, and the effect of SKF-525A on IBP covalent protein binding was not studied.

Fig. 12. The dependence of the initial rates of BNX-G (closed symbols) and FLX-G (open symbols) formation with human liver microsomes as a function of substrate concentrations. The liver microsomes were from three donors: donor #1 (▲), donor #2 (♦), and donor #3 (■).

Fig. 13. Glucuronidation and covalent protein binding of BNX (closed bars) and FLX (open bars) in human hepatocyte cultures. Human hepatocyte cultures were incubated with 500 μM BNX and FLX for 6 hr, cells were washed and harvested, and BNX-G, FLX-G and the covalent protein adducts were determined. Significantly different values obtained are the mean ± S.D. of triplicate (for BNX) or duplicate (for FLX) incubations.
TABLE 1
Michaelis-Menten parameters ($V_{\text{max}}$, and $K_M$) of BNX-G and FLX-G formation in human liver microsomes.

<table>
<thead>
<tr>
<th>Donor</th>
<th>BNX</th>
<th></th>
<th>FLX</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_M$</td>
<td>$V_{\text{max}}$</td>
<td>$K_M$</td>
</tr>
<tr>
<td></td>
<td>(nmol/mg/min)</td>
<td>(mM)</td>
<td>(µl/min/mg)</td>
<td>(mM)</td>
</tr>
<tr>
<td>#1</td>
<td>0.865</td>
<td>0.322</td>
<td>2.69</td>
<td></td>
</tr>
<tr>
<td>#2</td>
<td>1.24</td>
<td>0.396</td>
<td>3.13</td>
<td></td>
</tr>
<tr>
<td>#3</td>
<td>0.049</td>
<td>0.286</td>
<td>0.171</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1

Benoxaprofen (BNX)  \( R_1 = \text{Cl} \)
Flunoxaprofen (FLX)  \( R_1 = \text{F} \)
Fig. 3

![Graph showing Glucuronide in hepatocytes over time.](image-url)
Fig. 4

Protein adduct in hepatocytes (pmol/mg protein) vs. Time (hr)

0 5 10 15 20 25
0 30 60 90 120 150 180 210
BNX: $y = 13.1x - 4.14$
FLX: $y = 10.1x + 1.15$
IBP: $y = 9.76x - 0.172$

Concentration of glucuronides in hepatocytes (nmol/mg protein)
Covalent protein adducts in hepatocytes (pmol/mg protein)
Fig. 6

The graph shows the concentration of parent compound (μM) on the x-axis and glucuronide in hepatocytes (μmol/mg protein) on the y-axis. There are two lines representing different conditions or treatments. The y-axis ranges from 0 to 6 and the x-axis ranges from 0 to 1000 μM.

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Fig. 7

Protein adduct in hepatocytes (pmol/mg protein)

Concentration of parent compound (μM)
Glucuronide in hepatocytes (nmol/mg protein)

- Control
- + 1 mM Borneol
- + 200 µM PH

**Fig. 8**

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Fig. 9

Protein adduct in hepatocytes (pmol/mg protein)

- Control
- + 1 mM Borneol
- + 200 µM PH

Compound administered
- BNX
- FLX
- IBP

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Fig. 10

The graph shows the glucuronide formation in hepatocytes for different compounds administered. The y-axis represents glucuronide formation in nanomoles per milligram of protein, ranging from 0 to 5. The x-axis lists the compounds: BNX, FLX, and IBP.

- **Control**: Solid black bars.
- **+ 10 µM SKF-525A**: Hatched bars.
- **+ 1 mM ABT**: White bars.

For each compound:
- BNX shows a significant increase in glucuronide formation with SKF-525A and ABT.
- FLX shows a moderate increase with SKF-525A.
- IBP shows a minimal increase with SKF-525A.

The error bars indicate the variability in the data.
Fig. 11

Protein adduct in hepatocytes (pmol/mg protein)

- Control
- + 10 µM SKF-525A
- + 1 mM ABT

Compound administered

- BNX
- FLX
- IBP
Fig. 13

![Graph showing glucuronide and protein adduct levels in nmol/mg protein and pmol/mg protein, respectively. The graph displays a comparison between the two categories, with bars indicating variability.](image-url)