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

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ABSTRACT:

Benoxaprofen (BNX), a nonsteroidal anti-inflammatory drug (NSAID) that was withdrawn because of hepatotoxicity, is more toxic than its structural analog flunoxaprofen (FLX) in humans and rats.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 1) BNX-gluconuride (G) is more reactive than FLX-G, and 2) IBP-G is the least reactive metabolite, which support previous in vivo studies in rats. The proportional increases of protein adduct formation for BNX, FLX, and IBP as acyl glucuronidation increased also support 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 showed that these in vitro methods can be applied using human tissues for the study of acyl glucuronide reactivity. More BNX-protein adduct was formed in sandwich-cultured human hepatocytes than FLX-protein adduct, which not only agreed with its relative toxicity in humans but also was consistent with the in vitro findings using 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.

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).

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 makes 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 UDP glucuronosyltransferase (UGT) activities that 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 humans 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 therefore is 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 canaliculter networks formed with rat hepatocytes. In addition, the normal polarized distribution of several different classes of functionally active canalicular transport systems, such as multidrug-resistant protein 2, is also re-established in this configuration (LeCluyse et al., 1994). It has been reported that the hepatobiliary transport of reactive diclofenac glucuronide via multidrug-resistant protein 2 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 because 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 (Fig. 1) with different degrees of hepatotoxicity in humans and rats, were selected as model compounds. BNX was marketed as the racemate mixture, whereas FLX was marketed as the S-isomer only. Several reports of fatal cholestatic jaundice, often associated with nephrotoxicity, led to the withdrawal of BNX from the world market only 5 months after its launch (Dahl and Ward, 1982). However, no fatal adverse events related to FLX have been reported. We have previously shown that BNX is more reactive than FLX with human serum albumin and plasma protein in vitro, as well as in vivo in a rat model (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 adduct formation of model compounds in the sandwiched rat hepatocyte culture. An in vitro and in vivo correlation in rats in terms of relative reactivity of glucuronide metabolites was then established. In addition, 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 radiolabeled 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 show 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.

FIG. 1. Structures of BNX and FLX. BNX was marketed as the racemate; FLX was marketed as the S-isomer.

Materials and Methods

Materials. Rac-BNX was extracted and purified from tablets of Oraflex previously marketed by Eli Lilly & Co. (Indianapolis, IL). (Analysis calculated for BNX (C16H15ClO4): C, 63.69; H, 0.41; N, 4.64; Cl, 11.75; O, 15.91. Found: C, 63.05; H, 0.31; N, 4.71; Cl, 12.00; O, 16.25.) The purity of BNX (>99%) was confirmed based on elemental analysis and analytical high-performance liquid chromatography (HPLC) using UV detection at wavelength of 210 nm. S-FLX, the marketed form, was contributed by Dr. A. Forgione (Ravizza Laboratories, Milan, Italy) and was determined to be pure (>98%) based on HPLC with UV detection. IBP, Triton X-100, EDTA, glyceral, glycine, phosphate-buffered saline (PBS), bovine, SKF-525A, lactate dehydrogenase (LDH) kit, Percoll, Hanks’ balanced salt solution, and dexamethasone were obtained from Sigma-Aldrich (St. Louis, MO). [3H]IBP (1 Ci/mmol) was purchased from ICN (currently Valeant Pharmaceuticals, Costa Mesa, CA). 1,7-Phentanthroline was purchased from Aldrich Chemical Co. (Milwaukee, WI). 1-Aminobenzotriazole (ABT) was a gift from Dr. J. Mathews at Triangle Research Institute (Research Triangle Park, NC). Protogel, ammonium sulfate, TEMED, dithiothreitol, bromphenol blue, and Tris were purchased from Bio-Rad Laboratories (Hercules, CA). Collagenase was obtained from Worthington Biochemicals (Freehold, NJ). Dulbecco’s modified Eagle’s medium (DMEM), modified Chee’s medium (MCM), fetal calf serum, and insulin were purchased from Invitrogen (Carlsbad, CA). Rat tail collagenogen (type I) was obtained from Collaborative Research (Bedford, MA). Matrigel was purchased from Thermo Fisher Scientific (Waltham, MA). Methanol and acetonitrile were HPLC grade from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). All of the other chemicals used were of reagent grade. Male Sprague-Dawley rats (250–350 g) were supplied by Harlan (Indianapolis, IN). Experiments were approved by the university’s animal care committee. Human liver microsomal preparations were obtained from BD Gentest (Woburn, MA).

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 before hepatocyte harvest. To obtain a gelled collagen substrate, neutralized collagen type I was prepared on ice (as described by the manufacturer’s instruction) and spread onto Petri dishes with a sterile Teflon (DuPont, Wilmington, DE) 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 of fresh medium to the dishes and storage in a humidified incubator until used. Just before cell isolation, the medium was aspirated from the precoated culture dishes.

Hepatocytes were isolated from male Sprague-Dawley rats (250–350 g b.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.) before 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 to 15 min. After 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% fetal calf serum, 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 were 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 one more time. The cell suspension was divided equally between two 50-ml sterile centrifuge tubes and centrifuged at 40g 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% isocitonic acid/Percoll (Percoll/10% Ficoll) balanced salt solution (9:1, v/v) was added to the suspension, which was mixed well by inverting the tube and centrifuged at 80g 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 60g 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 x 10⁶ cells/ml, and 3-ml aliquots were seeded into 60-mm collagen-coated culture dishes. After a 2-h attachment period at 37°C in a 5% CO₂/95% 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 h 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 pipettes. Matrigel-treated cultures were allowed to incubate overnight. Medium was then changed daily until studies were performed. All the experiments were done in 5-day postoverlay 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 dependence studies, 500 μM BNX, FLX, or IBP was dissolved in fresh MCM and directly added to the rat hepatocyte cultures. The dose of 500 μM IBP also contained trace amounts of radiolabeled IBP (2.3 μCi of [1H]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 nonspecific esterases. The cells were harvested in 2 ml of ice-cold PBS containing 0.2 mM PMSF, 1% Triton X-100, and 10 μM of 4% phosphoric acid and lysed by sonication at 80°C. Cytotoxicity of BNX and FLX at concentrations 100, 250, and 500 μM was selected in 0.5 ml of mobile phase before injection onto the HPLC. Activities of BNX and FLX protein adducts were determined by adding 10 ml of Ultima Gold scintillation fluid (PerkinElmer Life and Analytical Sciences, Waltham, MA) was added, and the radioactivity was determined by liquid scintillation counting.

Irreversible binding of BNX, FLX, and IBP metabolites to hepatocellular proteins was determined as described previously (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 h. BNX- and FLX-protein adducts were extracted from sodium hydroxide with dichloromethane. The subsequent 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 (PerkinElmer Life and Analytical Sciences) 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 was measured similarly to that described for rat liver microsomal studies by Dong et al. (1999). In brief, 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); 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 of 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 of 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.
dissolved in MCM at 500

(University of North Carolina at Chapel Hill, Chapel Hill, NC). BNX and FLX post-Matrigel overlay, five Petri dishes) were gifts from Dr. E. LeCluyse.

concentration on double-reciprocal plots and Eadie-Hofstee plots, then deter-

were first estimated by plotting the initial rate of activity and substrate

concentration of BNX 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; Pharsight, Mountain View, CA).

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

Results

Time Dependence and Concentration Dependence of Gluc-uronidation 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 as a result of 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. 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 was not different from that in the control cells for up to 12 h (data not shown). LDH release was increased after 24 h to ~24 and ~18% of total LDH activity in BNX- and FLX-treated hepatocytes, respectively, compared with ~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 was determined by exposing cultured hepatocytes to each at 500 μM for up to 24 h. BNX-G and FLX-G formed in the hepatocytes were quantified 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 was 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 glucuronidation 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 versus 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 dependence of glucuronidation and protein adduct formation of BNX and FLX ranging from 10 to 1000 μM was investigated at the 6-h incubation time. Nonlinear glucuronidation and protein adduct formation were observed for both BNX and FLX at concentrations higher than 100 μM, and the maximal glucuronidation and binding were reached at an initial concentration of 750 μM (Figs.
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 the 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-glucuronic acid (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 was only inhibited approximately 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 was significantly increased in hepatocyte cultures pretreated with PH (55 and 224% for BNX and FLX, respectively) compared with 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 is 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 cytochrome P450 (P450) activity on the glucuronidation of BNX, FLX, and IBP were first evaluated to ascertain that the P450 inhibitors did not block the glucuronidation pathway. SKF-525A and ABT, two general P450 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 the covalent protein binding of BNX, FLX, and IBP is not significantly increased in hepatocyte cultures pretreated with PH (55 and 224% for BNX and FLX, respectively) compared with untreated controls (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 is only partially mediated via the formation of reactive glucuronides.

**Human UGT Activities in Liver Microsomes and Enzyme Kinetic Studies.** The glucuronidation of BNX and FLX in human liver microsomes from three different donors was determined over a range of substrate concentrations. The dependence 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 the substrate concentration of 1 mM. The extent of glucuronidation varied dramatically between donors. The UGT activity of donor 3 was very low; only a 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. Donors 1 and 2 appeared to have similar formation rate versus substrate concentration profiles, with the formation of BNX-G consistently faster than that of FLX-G. Michaelis-Menten parameters (V_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 as a result of the high variability.
ular-multispecific organic anion transporters, were maintained in rat hepatocytes. Such as the canalicular bile acid transporter and the canaliculus, have been shown that the expression and function of primary active transporters such as the bile canalicular network that is usually fully developed have been identified for BNX and FLX in either humans or animals. However, our results of P450 inhibition studies suggested that BNX- and FLX-G formation was normalized to the glucuronide level, BNX-protein adduct formation was higher for BNX glucuronidation in all 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 h after they were exposed to their parent drugs at 500 μM, 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 adduct formation was normalized to the glucuronide 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 acyl glucuronide adducts formed in rat hepatocyte cultures and can bind covalently to hepatic proteins in a time- and concentration-dependent manner (Figs. 3, 4, 6, and 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 nonsteroidal anti-inflammatory drugs (NSAIDs) to hepatic proteins is 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 the 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 P450 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 acetylsalicylic acid 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), although 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 in either humans or animals. However, our results of P450 inhibition studies suggested that BNX-, FLX-, or IBP-protein adduct formation in vitro was 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 the carboxylic acids are thought to covalently modify proteins by this mechanism (Hertz and Barr, 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-glucuronide amino acid conjugate formed in vitro in freshly
isolated rat hepatocytes exposed to tolmetin. Their studies showed the chemical reactivity of xenobiotic acyl-CoAs and their potential to undergo nonenzymatic protein acylation. Sallustio et al. (2000) were the first to show 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 was examined. The preliminary studies showed that for part of the covalent binding of BNX and FLX to hepatic proteins that are not accounted for by their reactive acyl glucuronides (Figs. 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 showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues. Greater BNX-protein adduct formation was examined. The preliminary studies showed that the in vitro methods can be applied using human tissues.

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 h; 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.

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<th>Donor</th>
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TABLE 1

Michaelis-Menten parameters (V<sub>max</sub> and K<sub>M</sub>) of BNX-G and FLX-G formation in human liver microsomes

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References


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