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Benoxaprofen (BNX), a nonsteroidal anti-inflammatory drug (NSAID) that was withdrawn because of hepatotoxicity, is more toxic than its structural analog flunoxaprofen (FLX) in humans and rats.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-glucuronide (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 hepatic 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).

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ABBREVIATIONS: UGT, UDP glucuronosyltransferase; BNX, benoxaprofen; FLX, flunoxaprofen; IBP, ibuprofen; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; SKF-525A, proadifen hydrochloride; LDH, lactate dehydrogenase; ABT, 1-aminobenzotriazole; DMEM, Dulbecco’s modified Eagle’s medium; MCM, modified Chee’s medium; PMSF, phenylmethylsulfonyl fluoride; PH, 1,7-phenanthroline; BNX-G, benoxaprofen glucuronide; FLX-G, flunoxaprofen glucuronide; IBP-G, ibuprofen glucuronide; P450, cytochrome P450; NSAID, nonsteroidal anti-inflammatory drug.
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 canaliculur networks formed with rat hepatocytes. In addition, the normal polarized distribution of several different classes of functionally active canaliculur 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 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.
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 × 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 [3H]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, 1% Triton X-100, 10 mM magnesium chloride, 100 mM Tris 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 BNХ 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 BNХ 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 BNХ-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 BNХ 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_{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. LeChuyse (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 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 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 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 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 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 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.
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 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 likely to be 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 (\(V_{\text{max}}\) 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 as a result of the high variability.
ular-multispecific organic anion transporters, were maintained in rat
porters, such as the canalicular bile acid transporter and the canalic-
been shown that the expression and function of primary active trans-
3 and 7 days after the overlay (LeCluyse et al., 1994). It has
somal incubations and allow for studies of time-dependent covalent
1989, 1991; Hewitt et al., 2007), which are more stable than micro-
viability and preserve liver-specific protein synthesis (Dunn et al.,
selected in this study. Overlaying cultured hepatocytes with a top
proteins. Hepatocyte cultures with a sandwich configuration were
slices, may be a better method for studying the acyl glucuronide-
in vitro model involving hepatic materials, such as intact cells or
adduct was still higher than FLX-protein adduct. This suggests that
formation was normalized to the glucuronide level, BNX-protein
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 target of BNX and FLX in vivo in rats (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 hepatoproteins 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 hepatoproteins 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 hepatic 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 acetaminophen formed by P450 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 Bar-Tana, 1988). This pathway may also lead to the inclusion of xenobi-
compositions 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

\[ V_{\text{max}} \] on the other hand, was 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 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.

![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 (■).](image-url)

Graph showing 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 (■).
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 characterized in the present study because of 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 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 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 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 was formed in human hepatocytes than FLX-protein adduct (Fig. 13), which not only agreed with their relative toxicity in humans but also was consistent with the findings in vitro using rat hepatocyte cultures. However, the enzyme kinetic studies in the small number of human liver microsomes suggested higher inter-subject variability of UGT activity in humans (Fig. 12; Table 1) than in inbred rats. With the exception of one donor who has very low UGT activity, V_{max} of BNX glucuronidation in humans (0.865 and 1.24 nmol/mg/min for donors 1 and 2, respectively) is higher than that in rats (0.30 ± 0.18 nmol/mg/min), whereas K_{M} values are similar in humans (0.322 and 0.396 mM for donors 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, although the preliminary studies look promising and suggest the method is transferable to human tissues.

In summary, the studies conducted and results obtained here show 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 approximately half of the drug-protein adduct 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 because of 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.

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References


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