GLUCURONIDATION OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS: IDENTIFYING THE ENZYMES RESPONSIBLE IN HUMAN LIVER MICROSONES

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Received October 12, 2004; accepted April 19, 2005

ABSTRACT:

Nonsteroidal anti-inflammatory drugs (NSAIDs), used for the treatment of pain and inflammation, are eliminated primarily through conjugation with polar sugar moieties to form glucuronides. Glucuronidation is catalyzed by the UDP-glucuronosyltransferases (UGT) superfamily. An inverse relationship may exist between glucuronidation activity and NSAID efficacy; however, specific UGTs catalyzing conjugation of the structurally diverse NSAIDs have yet to be identified systematically. Therefore, NSAID glucuronidation activity by 12 individually expressed UGTs was investigated by liquid chromatography-tandem mass spectrometry. The relative rates of NSAID glucuronidation varied among UGT enzymes examined, demonstrating specificity of the individual UGTs toward selected NSAIDs. Kinetic parameters were determined for expressed UGT Supersomes and compared with parameters determined in pooled human liver microsomes (HLMs). Comparison of $K_m$ values suggested roles for UGTs 1A3 and 2B7 in indene glucuronidation and UGTs 1A9, 2B4, and 2B7 in profen glucuronidation. Inhibitory studies in pooled HLMs support the role of UGTs 1A1, 1A3, 1A9, 2B4, and 2B7 in the glucuronidation of ibuprofen, flurbiprofen, and ketoprofen. Bilirubin did not inhibit indomethacin or diclofenac glucuronidation, suggesting that UGT1A1 was not involved in catalysis. Imipramine did not inhibit glucuronidation of sulindac, sulindac sulfone, indomethacin, or naproxen in pooled HLMs, suggesting that UGT1A3 was not a principal hepatic catalyst. Nevertheless, multiple UGT enzymes, most notably UGTs 1A1, 1A9, 2B4, and 2B7, seem to be involved in the hepatic catalysis of NSAID glucuronidation.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are an important class of therapeutic agents used in the treatment of pain and inflammatory disease. Recent evidence suggests that NSAIDs may be useful in the chemoprevention of colorectal cancer (for review, see Baron and Sandler, 2000). There are interindividual differences in drug efficacy, which may be a result of variation in the ability to excrete these compounds as nonreactive metabolites. It is conceivable that an increase in the ability to metabolize these drugs results in a decrease in the duration and extent of therapeutic response. A thorough understanding of the enzymes involved in metabolizing these NSAIDs will facilitate our ability to maximize drug efficacy for individual patients.

One of the primary pathways of NSAID metabolism is glucuronidation, in which a polar sugar is conjugated to the NSAID, increasing its rate of elimination from the body. Glucuronidation is catalyzed by a superfamily of enzymes called UDP-glucuronosyltransferases (UGTs) (for review, see Tukey and Strassburg, 2000). Many different genes and pseudogenes have been identified in the UGT superfamily and are subdivided into the UGT1A and UGT2B families based on sequence identity (Mackenzie et al., 1997). Protein levels are a result of tissue-specific and inducible expression of each UGT. UGTs 1A1, 1A3, 1A4, 1A6, 1A9, 1A10, 2B4, 2B7, and 2B15 have been identified in different populations (Ciotti et al., 1997; Levesque et al., 1997, 1999; Lampe et al., 1999, 2000; Balram et al., 2002; Gagné et al., 2002; Huang et al., 2002; Villeneuve et al., 2003; Ehmer et al., 2004; Iwai et al., 2004; Verlaan et al., 2004; Yamanaka et al., 2004). Sometimes, these polymorphisms result in altered enzyme expression or activities toward a variety of endogenous and xenobiotic aglycones (Ciotti et al., 1997; Levesque et al., 1999; Huang et al., 2002; Ehmer et al., 2004; Iwai et al., 2004; Yamanaka et al., 2004).

A variety of chemically distinct compounds have been classified as NSAIDs, based on their ability to inhibit prostaglandin G/H synthase, also called cyclooxygenase (COX)-1 and -2. Prostaglandins formed by COX elicit an inflammatory response. NSAIDs are classified based on their ability to inhibit COX as follows: COX-2 specific inhibitors, COX-1 and COX-2 inhibitors, and COX-independent anti-inflammatory drugs (Frölich, 1997). For this study, we focused our investigation on the most commonly used NSAIDs that undergo glucuronidation in the absence of a principal oxidation step. These drugs were separated into three classes based on chemical composition: indene derivatives, including sulindac, sulindac sulfone, and indomethacin; propionic acid-containing drugs (profens), including ibuprofen, flurbiprofen, and ketoprofen; and others that are structurally distinct, including diclofenac and naproxen (Fig. 1). These compounds are all...
primarily nonspecific COX inhibitors (Frölich, 1997), except for sulindac sulfone, which acts in a COX-independent manner (Piazza et al., 1997). These drugs readily undergo glucuronidation at the carboxyl group to form acyl glucuronides.

Few studies have been carried out to examine the glucuronidation of a variety of NSAIDs by heterologously expressed UGTs. UGT2B7 has been suggested as the principal catalyst of the glucuronidation of a variety of NSAIDs such as naproxen, ketoprofen, ibuprofen, fenoprofen, tiaprofenic acid, benoxaprofen, zomepirac, diflunisal, and indomethacin (Jin et al., 1993). Expressed UGT1A3 has been reported to catalyze the glucuronidation of profens, including ibuprofen and ketoprofen (Green et al., 1998). Sabolovic et al. (2000) proposed that along with UGTs 1A3 and 2B7, 1A6 expressed in Caco-2 cells may also catalyze the glucuronidation of profen NSAIDs. More recently, Owens and coworkers have demonstrated that UGTs 1A3, 1A7, 1A8, and 1A10 catalyze the glucuronidation of several NSAIDs, including flurbiprofen, diclofenac, and indomethacin (Basu et al., 2004). Although these studies represent a significant contribution to our understanding of NSAIDs glucuronidation, a systematic comparison of the ability of individual members of the UGT superfamily to catalyze the glucuronidation of a variety of NSAIDs has not been accomplished. To this end, we developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to measure the glucuronidation of the NSAIDs mentioned previously, determined and compared the apparent kinetic parameters for glucuronidation catalyzed by selected UGTs, and measured the inhibition of NSAID glucuronidation in pooled HLMs in the presence of UGT-selective probes to confirm the role of individual enzymes in the hepatic catalysis of specific compounds.

Materials and Methods

Materials. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. A crude solution of β-glucuronidase from Helix pomatia was used (134,400 units/ml). UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 Supersomes and pooled HLMs were obtained from BD Gentest (Woburn, MA). Morphine was purchased from

![Fig. 1. Structures of commonly used NSAIDs (glucuronidation occurs at the carboxyl group).](image_url)
Cerilliant Corporation (Round Rock, TX). All reagents were of the highest grade commercially available.

**LC-MS/MS Analysis of NSAID Glucuronides.** Metabolites were separated on a Luna C5 column (5 μm, 100 × 2 mm) from Phenomenex (Torrance, CA) isocratic at 30% trifluoroacetic acid (TFA) (0.05%) and 70% acetonitrile (0.15 ml/min) with postcolumn infusion of ammonium hydroxide (7%) at a flow rate of 1 μl/min. The high-performance liquid chromatography (HPLC) system consisted of two Shimadzu LC-10AD pumps with a gradient controller and a Shimadzu SIL-10ADvp autoinjector or a Waters Alliance 2695 separation module. Glucuronides were quantified using a Micromass Quattro II mass spectrometer or a Micromass Quattro micro mass spectrometer (Waters, Milford, MA) with electrospray ionization (ESI) in negative ion mode. ESI source conditions on the Quattro II mass spectrometer were set as follows: capillary voltage, 3.5 kV; cone voltage, 30 V; source block temperature, 100°C; capillary temperature, 250°C; and collisional gas, 3 × 10⁻³ mbar. ESI source conditions on the Quattro micro mass spectrometer were set as follows: capillary voltage, 3.5 kV; cone voltage, 20 V; source temperature, 120°C; desolvation temperature, 500°C. Elution times and parent and daughter ions used in the quantification of NSAID glucuronides are given in Table 1, along with their limits of detection. Fragmentation patterns were consistent with previously reported literature (Bozle et al., 2002). Rates of glucuronidation were quantified by comparison with known concentrations of glucuronide standards. The response of the MS/MS detector was linear over the concentration ranges for NSAID glucuronides. Sample aliquots were analyzed before and after addition of NSAID glucuronides at concentrations consistent with those produced by each enzyme to confirm the accuracy of quantification.

**Generation of Ibuprofen Glucuronide.** The ibuprofen glucuronide was isolated from overnight urine samples excreted by a single individual dosed with 200 mg of ibuprofen (Advil). The Institutional Review Board of the Fred Hutchinson Cancer Research Center approved the study, and informed consent was obtained from this individual. Urine pH was adjusted to between 3 and 4 using concentrated phosphoric acid. Urinary metabolites were concentrated on a C18 Sep-Pak SPE cartridge (Waters) and eluted with methanol. Eluates were dried under nitrogen and resuspended in nanopure water. Fractions were analyzed with and without β-glucuronidase treatment. Metabolites were analyzed and purified by HPLC using a Hewlett-Packard series 1050 separation module with an ultraviolet detector and autoinjector (Agilent Technologies, Palo Alto, CA) eluted from a Waters C18 Nova-Pak column (5 μm, 3.9 × 300 mm) using a gradient of 0.1% TFA (A) and methanol (B) as follows: 100% A to 50% B over 40 min. Ibuprofen and glucuronide conjugate were detected by UV absorbance at 235 nm. Under these conditions, the ibuprofen glucuronide eluted at 33 min. The ibuprofen glucuronide eluate was dried under nitrogen. The glucuronide was resuspended in nanopure water, and aliquots were treated with β-glucuronidase in triplicate. No residual ibuprofen glucuronide peak was detected by HPLC-UV after treatment with β-glucuronidase. The concentration was determined by comparison of the aglycone released with known concentrations of ibuprofen standard. Mass spectrometry and MS/MS analysis was also used to confirm the identity of the ibuprofen glucuronide standard.

**Biosynthesis of Sulindac, Sulindac Sulfone, Indomethacin, Diclofenac, Flurbiprofen, Ketoprofen, and Naproxen Glucuronide Standards.** HLMs have been used previously to produce authentic glucuronide standards (Soars et al., 2002). Glucuronides of each NSAID were biosynthesized in pooled HLMs under the following conditions: 2 mg/ml protein, 50 μg of alamethicin/mg protein, 500 mM aglycone (in 1% dimethyl sulfoxide), 2 mM UDP-glucuronic acid, 3 mM saccharolactone, and 10 mM MgCl₂ in 50 mM potassium phosphate, pH 6. Reactions were carried out overnight at 37°C in a shaking water bath and terminated with half-volumes of methanol. Mixtures were vortexed and centrifuged at 13,000g to precipitate protein. Supernatants were transferred to fresh tubes and dried completely under nitrogen. Metabolites were then resuspended in nanopure water for purification of the glucuronides by HPLC. HPLC purification was carried out as described previously for ibuprofen glucuronide standard production, except that the following gradients were used: 100% TFA (0.1%) to 50% acetonitrile in 25 min (sulindac, sulindac sulfone, and indomethacin) or 100% TFA (0.1%) to 67% methanol in 30 min (diclofenac, flurbiprofen, ketoprofen, and naproxen). UV detection of sulindac, sulindac sulfone, and indomethacin was carried out at 300 nm (Fig. 2), whereas other NSAIDs were detected at 235 nm. Concentrations of each purified glucuronide were determined by β-glucuronidase treatment as described above. Residual glucuronide peaks were not observed by HPLC when NSAID glucuronides were treated with β-glucuronidase. Mass spectrometry and MS/MS analysis was carried out to confirm the identity of each NSAID glucuronide standard.

**Screening UGT Supersomes for NSAID Glucuronidation Activity.** Glucuronidation activity by UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 Supersomes was measured. The assay conditions were as follows: 0.05 to 2 mg/ml protein, 50 μg of alamethicin/mg protein, 2 mM UDP-glucuronic acid, and 500 μM concentrations of each NSAID, and 10

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<th>Daughter Ion (m/z)</th>
<th>Detection Limit (ng)</th>
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**TABLE 1**

**LC-MS/MS parameters for analysis of NSAID glucuronides**

**FIG. 2.** HPLC-UV separation of sulindac glucuronide (A), sulindac sulfone glucuronide (B), and indomethacin glucuronide (C) biosynthesized using pooled HLMs.
mM MgCl₂ in 50 mM potassium phosphate, pH 6. Optimal alamethicin/protein ratios have been determined previously for UGT Supersomes (Kuehl and Murphy, 2003) and were kept constant throughout the study. Reactions were allowed to progress for 2 h in a shaking water bath at 37°C before being terminated with equal volumes of methanol. Samples were vortexed and centrifuged at 13,000g for 5 min. The supernatants were then removed and dried completely under nitrogen. The dried supernatant was resuspended in nanopure water and glucuronides were quantified by LC-MS/MS analysis. Control Supersomes not transfected with any of the human UGT cDNAs did not catalyze the glucuronidation of any NSAID examined. The rates of NSAID glucuronidation were linear from 30 to 120 min over the protein concentration ranges examined.

**Kinetics of NSAID Glucuronidation by Selected UGTs.**

The mean plasma concentrations for several NSAIDs have been determined previously as follows: indomethacin, 1.4 μM; ibuprofen, 38.8 μM (low dose), 111.0 μM (high dose); flurbiprofen, 53.2 μM; ketoprofen, 9.4 μM; diclofenac, 6.1 μM; and naproxen, 1.3 μM (Cryer and Feldman, 1998). Rates of glucuronidation with 1 μM, 10 μM, 50 μM, 100 μM, 500 μM, and 1 mM concentrations of...
each NSAID by UGTs 1A1, 1A3, 1A9, and 2B7 for sulindac, sulindac sulfone, and indomethacin or UGTs 1A3, 1A9, 2B4, and 2B7 for the remaining NSAIDs were measured under the following conditions: 0.5 mg/ml protein, 50 μM of alamethicin/mg protein, 8.5 mM saccharolactone, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM MgCl₂, 50 mM potassium phosphate, pH 7, and 2 mM UDP-glucuronic acid. Reactions were allowed to progress for 30 min before being terminated with equal volumes of ice-cold methanol, vortexed, and centrifuged. Glucuronides were quantified by LC-MS/MS as described previously.

Kinetics of NSAID Glucuronidation in Pooled HLMs. Rates of glucuronidation in pooled HLMs were measured under the following conditions: 1 mg/ml protein, 50 μM of alamethicin/mg protein, 8.5 mM saccharolactone, 1 mM PMSF, 10 mM MgCl₂, 50 mM potassium phosphate, pH 7, and 2 mM UDP-glucuronic acid with 1 μM, 10 μM, 50 μM, 100 μM, 500 μM, and 1 mM concentrations of each NSAID. Reactions were allowed to progress for 20 min before being terminated with equal volumes of ice-cold methanol, vortexed, and centrifuged. Glucuronides were quantified by LC-MS/MS as described previously.

Glucuronidation of NSAIDs in HLMs in the Presence of UGT-Selective Probes. The glucuronidation of each NSAID catalyzed by pooled HLMs was measured in the absence and presence of UGT-selective probes. Bilirubin, estradiol, imipramine, propofol, hydroxyecholic acid (HDCA), and morphone were used to determine the contribution of UGTs 1A1, 1A2/2B7, 1A3, 1A9, 2B4/2B7, and 2B7, respectively, to the glucuronidation of each NSAID (Pillot et al., 1993; Bosma et al., 1994; Coffman et al., 1997; Ethell et al., 2002; Nakajima et al., 2002b; Mackenzie et al., 2003; Lepine et al., 2004). These aglycones have been used previously to determine whether individual UGTs contribute to aglycone glucuronidation in HLMs (Miners et al., 1988; Haniska et al., 2001; Nakajima et al., 2002a,b,c; Kuehl and Murphy, 2003). To determine that these UGT-selective probes acted as competitive inhibitors, rates of sulindac and flurbiprofen glucuronidation were measured in the absence and presence of 10, 20, and 50 μM concentrations of estradiol, imipramine, propofol, HDCA, and morphone. Rates of glucuronidation were quantified by LC-MS/MS analysis. Once we had determined that each inhibitor acted in a competitive manner, the percentage of inhibition observed upon addition of each UGT-selective probe to reactions was measured at 10 and 500 μM concentrations of the NSAIDs with 5 μM bilirubin or 50 μM estradiol, imipramine, propofol, HDCA, or morphone.

Data Handling and Analysis. Reactions were carried out in duplicate and are reported as the average of determinations varying by less than 10%. Rates of glucuronidation were fit to the Michaelis-Menten equation using GraphPad Prism 3.03 software (GraphPad Software Inc., San Diego, CA).

Results

Screening UGT Supersomes for NSAID Glucuronidation Activity. Individually expressed UGT enzymes were screened for their ability to catalyze the glucuronidation of a variety of NSAIDs. Rates of glucuronidation of different NSAIDs by an individual UGT enzyme were compared. These rates were not adjusted for levels of expressed protein or active enzyme in each of the Supersomes preparations.

Overall, rates of NSAID glucuronidation catalyzed by UGTs 1A1, 1A3, 1A9, 2B4, and 2B7 Supersomes were higher than the rates of NSAID glucuronidation catalyzed by all other UGT enzymes examined (Fig. 3, note different scales). UGT1A1 catalyzed the glucuronidation of sulindac sulfone, 184 pmol/min/mg protein, at the highest rate compared with other NSAIDs examined (Fig. 3). Rates of sulindac, flurbiprofen, and naproxen glucuronidation by UGT1A1 were less than 2-fold lower than the rate of sulindac sulfone glucuronidation. Unlike UGT1A1, UGT1A3 catalyzed flurbiprofen glucuronidation at the highest rate, 393 pmol/min/mg protein. Ibuprofen glucuronidation by UGT1A3 was catalyzed at the lowest rate. UGT1A9 catalyzed the glucuronidation of indomethacin at the highest rate, 361 pmol/min/mg protein. Rates of glucuronidation of the remaining NSAIDs were more than 4-fold lower than indomethacin glucuronidation. UGT2B4 was a better catalyst of propan glucuronidation than indene glucuronidation. Flurbiprofen and ketoprofen glucuronidation by UGT2B4 was higher than all other NSAIDs investigated. UGT2B7 catalyzed diclofenac glucuronidation at the highest rate, 748 pmol/min/mg protein. Like UGT2B4, UGT2B7 catalyzed the glucuronidation of profens, diclofenac, and naproxen at higher rates than indenes.

Kinetics of NSAID Glucuronidation by UGT Supersomes. Kinetics of indene glucuronidation catalyzed by UGTs 1A1, 1A3, 1A9, and 2B7 was determined and fit to the Michaelis-Menten equation (Table 2). UGT1A1 had the highest affinity for sulindac and sulindac sulfone glucuronidation, as demonstrated by a low binding constant (Kᵣ). UGT1A3 had the highest apparent affinity for indomethacin. Among the indenes investigated, sulindac sulfone glucuronidation was catalyzed with the highest maximal rates by UGTs 1A1, 1A3, and 2B7. UGT1A9 had a higher Vₘₐₓ for indomethacin glucuronidation than sulindac or sulindac sulfone glucuronidation. UGTs 1A1, 1A3, and 2B7 were more efficient catalysts (higher Vₘₐₓ/Kᵣ values) of sulindac sulfone glucuronidation than of sulindac or indomethacin glucuronidation. UGT1A9 was a more efficient catalyst of indomethacin glucuronidation than of sulindac or sulindac sulfone glucuronidation. Rates of sulindac and sulindac sulfone glucuronidation by UGT2B7 and sulindac sulfone by UGT1A1 decreased when NSAID concentrations were increased from 500 to 1000 μM. Rates of glucuronidation by UGTs 1A1, 1A3, 1A9, and 2B7 fit Michaelis-Menten kinetics.
kinetics only from 1 to 100 μM indomethacin, decreasing at higher NSAID concentrations.

Kinetics of profen glucuronidation catalyzed by UGTs 1A3, 1A9, 2B4, and 2B7 were determined and fit to the Michaelis-Menten equation (Table 2). UGT2B7 had the highest affinity for profen glucuronidation (flurbiprofen > ketoprofen > ibuprofen). The relative \( V_{\text{max}} \) values for profen glucuronidation were enzyme-specific; for example, UGTs 1A3, 2B4, and 2B7 had a higher \( V_{\text{max}} \) and were more efficient catalysts of ketoprofen glucuronidation than ibuprofen or flurbiprofen glucuronidation (ketoprofen > flurbiprofen > ibuprofen). The \( V_{\text{max}} \) values for UGT1A9-catalyzed flurbiprofen and ibuprofen glucuronidation were similar and higher than the \( V_{\text{max}} \) for ketoprofen glucuronidation. Decreased rates of glucuronidation by UGTs 1A3 and 1A9 were observed at 1000 μM flurbiprofen compared with glucuronidation at high flurbiprofen concentrations.

Kinetics of diclofenac and naproxen glucuronidation by UGTs 1A3, 1A9, 2B4, and 2B7 was also investigated and fit to the Michaelis-Menten equation (Table 2). UGT2B4 demonstrated the highest affinity for diclofenac (Table 2). Unlike diclofenac glucuronidation, naproxen glucuronidation was catalyzed with the highest affinity by UGT2B7. UGTs 1A3 and 2B7 catalyzed the glucuronidation of diclofenac and naproxen at similar maximal rates. However, the \( V_{\text{max}} \) of UGTs 1A9 and 2B4 for naproxen glucuronidation was 3-fold higher than the \( V_{\text{max}} \) for diclofenac glucuronidation. UGTs 1A3, 1A9, and 2B4 catalyzed naproxen glucuronidation less efficiently than diclofenac glucuronidation, whereas the reverse was true for UGT2B7. Rates of diclofenac glucuronidation fit Michaelis-Menten kinetics from 1 to 100 μM concentrations; however, glucuronidation rates decreased above 100 μM. Rates of naproxen glucuronidation catalyzed by UGT2B7 decreased above 500 μM, but no decrease in rates was observed for naproxen glucuronidation catalyzed by UGTs 1A3, 1A9, and 2B4.

Kinetics of NSAID Glucuronidation in Pooled HLMs. Rates of glucuronidation catalyzed in pooled HLMs were determined from 1 to 1000 μM of each NSAID. In pooled HLMs, sulindac sulfone and indomethacin glucuronidation was catalyzed with the highest affinities, \( K_m \) values of 34 and 30 μM, respectively (Table 3). Pooled HLMs had comparable affinities for sulindac, diclofenac, and naproxen glucuronidation and had the lowest affinity for ibuprofen glucuronidation with a \( K_m \) of 188 μM. The highest \( V_{\text{max}} \) and efficiency (\( V_{\text{max}}/K_m \)) was observed when diclofenac and naproxen glucuronidation was measured. Rates of glucuronidation fit the Michaelis-Menten equation from 1 to 500 μM of each NSAID. However, rates of sulindac, indomethacin, flurbiprofen, diclofenac, and naproxen glucuronidation in pooled HLMs decreased above 500 μM.

We compared the relative kinetic parameters determined for NSAID glucuronidation in HLMs with those determined by expressed UGT Supersomes. In HLMs, the affinities for sulindac sulfone and indomethacin glucuronidation were comparable, but higher than the affinity for sulindac. This is consistent with affinities for indene glucuronidation catalyzed by UGTs 1A3 and 2B7. Differences in the apparent \( V_{\text{max}} \) values for indene glucuronidation (sulindac ∼ indomethacin < sulindac sulfone) are consistent with UGT2B7 as a principal catalyst of glucuronidation. Among the profens, enzymes in pooled HLMs catalyzed flurbiprofen glucuronidation with the highest affinity followed by ketoprofen and ibuprofen. This is comparable with the affinities for profen glucuronidation by expressed UGTs 1A9, 2B4, and 2B7. However, the \( V_{\text{max}} \) of ketoprofen glucuronidation in pooled HLMs was higher than ibuprofen and flurbiprofen glucuronidation. The differences in the \( V_{\text{max}} \) of glucuronidation between profens were more pronounced in Supersomes than in pooled HLMs. The affinities of the UGTs for diclofenac and naproxen differ between the two assay systems used. In HLMs, diclofenac glucuronidation was catalyzed at a higher \( V_{\text{max}} \) than naproxen glucuronidation. This is different from the maximal rates of diclofenac and naproxen glucuronidation catalyzed by the expressed UGT Supersomes.

### TABLE 3

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<th>NSAID</th>
<th>Apparent ( K_m ) (μM)</th>
<th>Apparent ( V_{\text{max}} ) (μmol/mg protein)</th>
<th>( V_{\text{max}}/K_m )</th>
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of sulindac sulfate at high concentrations. However, inhibition was observed with both estradiol and HDCA, UGT-selective aglycones that likely also inhibit glucuronidation by UGT2B7, suggesting a role for UGTs 1A1, 1A9, 2B4, and 2B7 in hepatic indene glucuronidation.

Ibuprofen, flurbiprofen, and ketoprofen glucuronidation was inhibited by bilirubin, estradiol, imipramine, propofol, and HDCA (Table 4), whereas morphine inhibited only ibuprofen and flurbiprofen glucuronidation. However, both estradiol and HDCA resulted in decreases in ketoprofen glucuronidation, still implicating UGT2B7 in catalysis. UGT1A1 may not be a major player in flurbiprofen glucuronidation, because bilirubin did not inhibit glucuronidation at 500 μM flurbiprofen. Ketoprofen glucuronidation at 500 μM was not inhibited by imipramine or propofol, indicating that UGTs 1A3 and 1A9, respectively, may not play a role in catalysis.

Diclofenac and ketoprofen glucuronidation was inhibited in the presence of estradiol, propofol, and HDCA (Table 4). Bilirubin and morphine did not inhibit diclofenac glucuronidation, and imipramine did not inhibit naproxen glucuronidation. Again, although morphine failed to inhibit diclofenac glucuronidation, both estradiol and HDCA addition resulted in decreased glucuronidation rates, suggesting that UGT2B7 may still be involved in catalysis. The inhibition studies suggested a role for UGTs 1A3, 1A9, 2B4, and 2B7 in diclofenac glucuronidation and UGTs 1A1, 1A9, 2B4, and 2B7 in naproxen glucuronidation.

**Discussion**

This work is a comprehensive evaluation of the glucuronidation of structurally distinct groups of NSAIDs by heterologously expressed UGTs and pooled HLMs. UGTs 1A3, 1A6, 1A7, 1A8, 1A10, and 2B7 have been reported as catalysts of the acyl glucuronidation of carboxylic acid-containing drugs (Jin et al., 1993; Green et al., 1998; Sabolovic et al., 2000; Basu et al., 2004), but a thorough comparison of the activities of all members of the UGT family toward these aglycones has not previously been done. Therefore, we quantified the glucuronidation of three classes of commonly used NSAIDs: indene derivatives (sulindac, sulindac sulfate, and indomethacin), profens (ibuprofen, flurbiprofen, and ketoprofen), and others (diclofenac and naproxen) by heterologously expressed UGTs and in pooled HLMs. The glucuronidation of these NSAIDs by UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 was quantified by LC-MS/MS to identify the principal catalysts. These studies were complemented with the measurement of NSAID glucuronidation in pooled HLMs in the absence or presence of UGT-selective substrates.

Based on initial screening experiments with UGT Supersomes, UGTs 1A1, 1A3, 1A9, 2B4, and 2B7 were candidate catalysts involved in NSAID glucuronidation. However, a role for the other UGT enzymes could not be excluded based on the screening data. Currently, there exists no reliable method to quantify active UGT enzymes in cell preparations and rates of glucuronidation cannot be compared directly. In experiments with pooled HLMs and UGT-selective competitive inhibitors, we observed that estradiol and HDCA inhibited glucuronidation of all NSAIDs, supporting a role for UGTs 2B4 and 2B7 in hepatic glucuronidation of all NSAIDs examined. However, inhibition of UGTs 1A3, 1A6, and 1A9 has also been observed by Hanioka et al. (2001), albeit at higher concentrations, suggesting that HDCA may not be a selective inhibitor for UGT2B7. Significant inhibition of sulindac sulfate, indomethacin, flurbiprofen, ketoprofen, and diclofenac glucuronidation by morphine was not observed. This lack of inhibition may be due to the high KM of morphine glucuronidation (Stone et al., 2003), suggesting that morphine may not be a good competitive inhibitor of NSAID glucuronidation. Therefore, these results do not rule out a role for UGT2B7 in NSAID glucuronidation, especially since both estradiol and HDCA acted as inhibitors of NSAID glucuronidation. Indomethacin and diclofenac glucuronidation was catalyzed by expressed UGT1A1; however, bilirubin did not inhibit glucuronidation in pooled HLMs. Even though expressed UGT1A3 was a relatively good catalyst of indene glucuronidation, imipramine failed to inhibit glucuronidation.
of sulindac, sulindac sulfone, indomethacin, and naproxen in pooled HLMs. This result may be due to the relatively low UGT1A3 expression levels in human liver (Green et al., 1998). Imipramine did inhibit profen glucuronidation, suggesting that even at low expression levels, UGT1A3 still contributed to profen glucuronidation.

These discrepancies in glucuronidation between expressed UGTs and UGTs in HLMs show that simple screening of UGT activity in heterologous expression systems is not sufficient, by itself, to identify the principal catalysts of drug glucuronidation in vivo. Our results emphasize the need to compare glucuronidation activity of heterologously expressed UGT enzymes with inhibitory experiments in microsomes from human tissues to obtain a more accurate representation of the UGTs involved in drug glucuronidation. These studies also emphasize the need for competitive inhibitory antibodies to test the individual contribution of UGT enzymes to glucuronidation in microsomes from human tissues.

Rates of sulindac, sulindac sulfone, indomethacin, flurbiprofen, diclofenac, and naproxen glucuronidation catalyzed by multiple UGTs decreased at increasing aglycone concentrations. The most pronounced decreases were observed for indomethacin and diclofenac glucuronidation. Indomethacin and diclofenac glucuronidation by UGT2B7 decreased 3 and 8%, respectively, when NSAID concentrations were increased from 100 to 500 μM and further decreased 51% and 5%, respectively, when concentrations of NSAIDs were increased from 500 to 1000 μM. The inhibition of glucuronidation activity at increasing aglycone concentrations has been observed previously for entacapone glucuronidation by UGT1A9 (Lautala et al., 2000); (S)-oxazepam glucuronidation by UGTs 1A9, 2B15, and in HLMs (Court et al., 2002); and catechol estrogen glucuronidation catalyzed by UGTs 1A1 and 1A3 (Lepine et al., 2004). The cause of this decrease in glucuronidation rates at higher substrate concentrations is unknown. Acyl glucuronides have been reported to react with proteins (Bailey and Dickinson, 2003). It is possible that decreases in glucuronidation activity at higher substrate concentrations may be a result of acyl glucuronide reactivity with UGT enzymes. At higher NSAID concentrations, we also suspect that the membrane fluidity may be altered resulting in decreased enzymatic activity. Substantial decreases in the rates of NSAID glucuronidation were not observed in pooled HLMs and glucuronidation rates fit Michaelis-Menten kinetics from 1 to 1000 μM. The fact that such decreases in glucuronidation rates were not observed in HLMs suggests that this effect is unique to Supersomes.

Pooled HLMs demonstrated the highest affinity for sulindac sulphone and indomethacin glucuronidation. Results were consistent with the contribution of multiple UGT enzymes to the catalysis of NSAID glucuronidation in hepatic tissue. UGTs 1A3 and 2B7 were implicated in indene glucuronidation and UGTs 1A9, 2B4, and 2B7 in the glucuronidation of profen NSAIDs.

Here, we report the identification of UGTs 1A1, 1A3, 1A9, 2B4, and 2B7 as important catalysts of the glucuronidation of many structurally distinct NSAIDs. Consistent with our findings, Sakaguchi et al. (2004) reported that expressed UGTs 1A3, 1A9, and 2B7 catalyzed the glucuronidation of ibuprofen, flurbiprofen, ketoprofen, and diclofenac. However, UGT1A1 expressed in HK 293 cells did not catalyze the glucuronidation of NSAIDs (Sakaguchi et al., 2004). This difference probably is a result of higher UGT1A1 expression in the S9 cells than the HK 293 cells as well as the lower limits of detection of LC-MS/MS analysis used in our study compared with thin layer chromatography of radiolabeled compounds. Further support for the role of UGT1A1 in the glucuronidation of diclofenac, indomethacin, ketoprofen, and naproxen has been suggested recently (Mano et al., 2005).

UGTs 1A7, 1A8, and 1A10 were catalysts of NSAID glucuronidation when examined by individually expressed UGTs. Consistent with these findings, Basu et al. (2004) reported that UGTs 1A7, 1A8, and 1A10 catalyzed the glucuronidation of ibuprofen, flurbiprofen, and indomethacin. These enzymes are expressed in extrahepatic tissues only, most notably, the intestine and colon (Mojarrabi and Mackenzie, 1998; Cheng et al., 1999). Acyl glucuronides are highly unstable entities that readily undergo acyl migration and esterase cleavage in vivo (Spahn et al., 1989; Shipkova et al., 2003). It is possible that glucuronides formed in the liver are cleaved at other organ sites and the parent compounds then undergo enterohepatic circulation. It is therefore likely that extrahepatic UGT enzymes play a role in NSAIDs metabolism. A role for UGTs 1A7, 1A8, and 1A10 in the glucuronidation of NSAIDs in these tissues still warrants further investigation.

The relative efficacy of the drugs in target tissues is probably a combined effect of the effective concentrations of each NSAID, the level of UGT protein expression, and/or the existence of polymorphisms affecting glucuronidation activity. Polymorphisms in the hepatic UGTs 1A1, 1A9, 1A3, 1B4, and 2B7 have been reported in different populations; however, their role in the altered metabolism of NSAIDs remains to be investigated. The identification of the UGTs involved in glucuronidation now allows for further studies examining the variability in metabolism among populations to optimize the therapeutic benefits of NSAIDs use.

Acknowledgments. We thank members of the University of Washington Medicinal Chemistry Mass Spectrometry Center. William Howald was instrumental in the development of LC-MS/MS methods for analysis of NSAID glucuronidates.

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