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Glycosidation of an Endothelin ET_A Receptor Antagonist and Diclofenac in Human Liver Microsomes: Aglycone-dependent UDP-sugar Selectivity

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Abbreviations: UGTs: UDP-glucuronosyltransferases; UDP-GlcA: UDP-glucuronic acid; UDP-Glc: UDP-glucose; UDP-gal: UDP-galactose; UDP-GlcNAc: UDP-*N*-acetyl-glucosamine; K_m, apparent Michaelis constant; Ki: inhibition constant; V_{max}, maximal velocity.

ABSTRACT

Following the finding that UGT2B7 catalyzes the transfer of the glycosyl group from both UDP-glucuronic acid (UDP-GlcA) and UDP-glucose (UDP-Glc) to an endothelin ET_A receptor antagonist Compound A to form an acyl glucuronide and a glucoside (Tang et al., 2003), two additional nucleotide sugars (UDP-galactose, UDP-gal and UDP-*N*-acetyl glucosamine, UDP-GlcNAc) were examined as cosubstrates in human liver microsomes. It was found that UDP-gal, but not UDP-GlcNAc, also served as a sugar donor primarily through catalysis by UGT2B7, although at a significantly reduced catalytic rate. These three UDP-sugars showed pH-dependent kinetics and appeared to compete with each other, with IC_{50} values parallel to their respective apparent K_m values. In contrast, only UDP-GlcA served as the sugar donor for the conjugation of diclofenac, a known UGT2B7 substrate, with an apparent K_m for UDP-GlcA of 96 ± 17 μM. UDP-Glc and UDP-gal, two futile sugar donors for diclofenac, were found to competitively inhibit the glucuronidation of this aglycone. Different from the case with Compound A, UDP-Glc and UDP-gal displayed Ki values of 2054 ± 108 μM and 1277 ± 149 μM, > 10-folds greater than the K_m for UDP-GlcA, indicating that these two nucleotide sugars were also capable of binding to the enzyme but with a lower affinity. The findings of this study indicate that the selectivity of UGT2B7 towards UDP-sugars is aglycone-dependent. With Compound A as the acceptor substrate, human UGT2B7 becomes more accommodative in the transfer of the glycosyl group from UDP-sugars beyond UDP-GlcA. The mechanism may involve enzyme conformational changes associated with Compound A binding to the enzyme.

The UDP-glycosyltransferases are members of a superfamily of enzymes that catalyze the covalent conjugation of a variety of aglycone substrates (acceptors) with the glycosyl moiety of UDP-sugars (donors) to form a glycosidic bond. The UDP-sugar donor may be UDP-glucuronic acid (UDP-GlcA), UDP-galactose (UDP-gal), UDP-glucose (UDP-Glc), or UDP-xylose. It has been established that vertebrate UDP-glycosyltransferases preferentially use UDP-GlcA (Bock, 2003), while insect and plant UDP-glycosyltransferases typically use UDP-Glc (Ahmad and Forgash, 1976; Hansen et al., 2003; Rausell et al., 1997). This high selectivity for the common donor substrate could be related to the highly conserved donor-binding domain of the enzymes, and has been found to be determined by critical amino acid residues in the catalytic domain in some UDP-glycosyltransferases. For example, Ouzzine et al. (2002) identified His^{308} and Arg^{277} residues as essential determinants for the donor sugar selectivity of human β1,3-glucuronosyltransferase I. Substitution of His^{308} by arginine induced major changes in the UDP-sugar donor selectivity of this enzyme. Similarly, a single point mutation altered the sugar donor selectivity of plant glycosyltransferases (Kubo et al., 2004).

Human UDP-glycosyltransferases involved in drug metabolism are membrane-bound enzymes located in the endoplasmic reticulum with their catalytic sites facing the lumen. They highly prefer UDP-GlcA as the sugar donor as opposed to other UDP-sugars, and therefore, are named as UDP-glucuronsyltransferases (UGTs). Interestingly, the preference for UDP-sugar donors of wild-type UGTs has been found not absolute. There are a considerable number of cases where the glycosyl group in the conjugate could also be derived from other UDP-sugars, especially UDP-Glc (Tang et al., 1979; Arima, 1990; Senafi et al., 1994; Shipkova et al., 2001; Chmela et al., 2001; Tang et al., 2003; Mackenzie et al., 2003). Even more intriguing is the reversed sugar donor specificity found by Toide et al. (2004) showing that human UGT2B isoforms utilize only UDP-Glc, rather than UDP-GlcA, as a

cofactor to conjugate an aldose reductase inhibitor. Although it is possible that the tridimensional structure of the sugar donor binding site is not fully conserved among UGT isoforms, it appears that sugar donor selectivity may be aglycone-dependent. For instance, only with bilirubin can UGT1A1 use UDP-GlcA, UDP-xylose and UDP-Glc as sugar donors to form respective glycosides (Senafi et al., 1994). It is very fascinating that some aglycones are able to influence the sugar donor selectivity of certain UGTs.

We have observed that human UGT2B7 is capable of utilizing both UDP-GlcA and UDP-Glc to conjugate an endothelin ET_A receptor antagonist Compound A (Figure 1) with a comparable catalytic potential ($V_{max}/K_{m\ (Compound\ A)}$) for both glucuronidation and glucosidation (Tang et al., 2003). In addition, these two sugar donors competitively inhibit their counterpart conjugations with the inhibition constant (Ki) close to their K_m values. In expansion of these findings, we examined two additional UDP-sugars, UDP-gal and UDP-*N*-acetyl-glucosamine (UDP-GlcNAc), as co-substrates in human liver microsomes. The study also was extended to diclofenac, a known UGT2B7 substrate (King et al., 2001), to examine the impact of aglycone substrate on the selectivity of the enzyme towards UDP-sugars. We demonstrated that in addition to UDP-GlcA and UDP-Glc, UDP-gal also served as a sugar donor for the formation of a galactoside of Compound A in human liver microsomes, and the conjugation was again primarily mediated via UGT2B7. In contrast, diclofenac underwent only glucuronidation in human liver microsomes.

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Methods

Chemicals and Reagents. Compound A ((+)-(5S, 6R, 7R)-2-isopropylamino-7-[4-methoxy-2-((2R)-3-methoxy-2-methylpropyl)-5-(3,4-methylenedioxyphenyl) cyclopenteno [1,2-*b*] pyridine 6-carboxylic acid) and its analog Compound B ((5***S***,6***R***,7***R***)-5-(1,3-benzodioxol-5-yl)-7-[2-(3-hydroxy-2-methylpropyl)-4-(methyloxy)phenyl]-2-[(1-methylethyl)amino]-6,7-dihydro-5***H***-cyclopenta[***b***]pyridine-6-carboxylic acid) used as an internal standard (Figure. 1) were synthesized by Banyu Pharmaceutical Co. (Ibaraki, Japan). UDP-GlcA, UDP-Glc, UDP-gal, UDP-GlcNAc, ^{3}H-UDP-gal (galactose-6-^{3}H), alamethicin, diclofenac and phenytoin were obtained from Sigma-Aldrich (St. Louis, MO). Solvents used for liquid chromatography were of analytical or HPLC grade. Pooled male human (n = 10) liver microsomal preparations were purchased from Xenotech LLC (Kansas City, KS). Recombinant human UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15 (Supersomes^{TM}) were obtained from the Gentest Corporation (Woburn, MA) and baculosomes of UGT1A7 and 1A10 from Panvera (Madison, WI).

Incubation Conditions. The incubations for identification of Compound A and diclofenac glycosides by LC-MS were carried out in the presence of respective acceptor substrates (25 μM), four respective tested UDP-sugars (2 mM), human liver microsomes (1 mg/mL), $MgCl_2$ (10 mM) and alamethcicin in the phosphate buffer (0.1 M, pH 6.0). After the desired length of incubation (30 and 60 min for compound A with UDP-GlcA and UDP-Glc, and 120 min for the remaining incubations), an equal volume of acetonitrile was added to terminate the reactions. Following brief vortexing and centrifugation (10 min at 3800 rpm), the supernatant was subjected to LC-MS assay.

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For the quantitation of Compound A conjugate formation, incubations were carried out at 37°C in a Fisher shaking water bath, employing 1.1 mL polypropylene disposable deep well tubes purchased from Matrix Technologies Corp. (Hudson, NH). The incubation mixture (final volume of 250 μL) consisted of the following: 0.1 M potassium phosphate buffer (pH 7.4 or 6.0), $MgCl_2$ (10 mM), UDP-sugars (5 mM or varied concentrations), liver microsomes (0.05 mg protein/mL) or expressed UGTs (0.1 mg protein/mL), and Compound A (25 μM or varied concentrations from 100-fold concentrated stock solution in water/acetonitrile: 50/50). The concentration of alamethicin, when applied, was 100 μg/mg protein. The reaction was started by the addition of the respective UDP-sugars, and terminated with acetonitrile (200 μL) following a 15-min (for glucuronidation and glucosidation) or 30-min (for galactosidation and *N*-acetyl-glucosamidation) incubation. The internal standard (Compound B) solution (50 μL at 2.5 μM in water/acetonitrile: 50/50) was added to the samples. Following brief vortexing and centrifugation (10 min at 3800 rpm), the supernatant was transferred to 96-well microtiter plates for LC-MS assay.

As for diclofenac glucuronidation, the incubation mixture (final volume of 250 μL in 100 mM phosphate buffer at pH 6.0) contained diclofenac at 60 μM (~ 3-fold of its reported K_m (King et al., 2001)) or varied concentrations, $MgCl_2$ (10 mM), human liver microsomes (0.1 mg protein/mL), UDP-sugars (5 mM or varied concentrations) and alamethicin (100 μg/mg protein). The incubation was carried out for 15 min and terminated with acetonitrile (200 μL). The internal standard (phenytoin) solution (100 μL at 5.0 μM in water/acetonitrile: 50/50) was added to the samples.

Determination of UDP-sugar IC_{50} or Ki Values. The values of IC_{50}, rather than Ki, were determined for glycosidation of Compound A due to the involvement of multiple UDP-sugars. All incubations contained Compound A (25 μM), liver microsomes (0.05 mg/mL) and alamethicin (100

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μg/mg microsomal protein) in a total volume of 250 μL. Concentrations of UDP-sugars as co-substrates were close to their K_m values in human liver microsomes determined at pH 6.0 and 7.4. As inhibitors, their concentrations spanned from 0.01 to 5000 μM. The experimental design is detailed in Table 1.

The Ki value was determined for UDP-Glc and UDP-gal as inhibitors towards diclofenac glucuronidation in human liver microsomes. The incubation mixture consisted of diclofenac (60 μM), liver microsomes (0.1 mg/mL) and alamethicin (100 μg/mg microsomal protein) in a total volume of 250 uL). Concentrations of UDP-GlcA and UDP-Glc/UDP-gal ranged from 0.1 to 5 mM.

Identification of Glycosides of Compound A and Diclofenac. The chromatographic separation of Compound A and its respective glycosides was performed on a reverse phase C18 column (Synergi MAX-RP, 2.0 x 150 mm, 4 μm; Phenomenex, Torrance, CA) using a Rheos 4000 binary pump (LEAP Technologies, Carrboro, NC) with a flow rate of 200 μL/min. The same mobile phases and gradient described previously (Tang et al., 2003) were applied. The separation of diclofenac and its resultant glycosides was achieved with the same system except for a different gradient (The initial mobile phase consisted of 5% of solvent B, which was linearly increased to 45% over 20 min, then to 80% in another 2 min and held for an additional 3 min). Mass spectrometric analysis was performed on an LCQ ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (Finnigan MAT, San Jose, CA), as described previously (Tang et al., 2003), but ESI was operated in positive and negative modes for Compound A and diclofenac, respectively. Mass spectral data were analyzed using the manufacturer's software package (Xcaliber 1.2).

Quantitation of Glycosidic Conjugates of Compound A and Diclofenac. The purification and quantitation of glucuronide and glucoside conjugates of Compound A have been described

previously (Tang et al., 2003), but radiochromatographic separation and scintillation counting were utilized to quantify the galactoside due to its low yield. Compound A (50 μM) was incubated with human liver microsomes (0.5 mg/mL) supplemented with UDP-gal (2 mM) at pH 6.0 overnight. UDP-gal was mixed with its tritium-labeled analog to obtain a specific radioactivity of 0.0132 μCi/nmol. The biosynthesis was terminated by the addition of acetonitrile. Following centrifugation (5 min at 3800 rpm), the supernatant was subjected to brief evaporation under a nitrogen stream to remove the organic solvent. The resultant samples were allowed to pass through a pretreated OASIS® HLB extraction cartridge (1 cc, Waters Corporation, Milford, MA). The cartridge was washed with water and then with 20% aqueous methanol to remove the remaining UDP-gal. The eluant from the final wash with methanol was collected, evaporated to dryness, and reconstituted with a given volume of 50% acetonitrile. The radioactivity in a 0.05 mL aliquot of the reconstituted solution was measured by liquid scintillation counting, and the purity of ^{3}H-galactoside of Compound A in the solution assessed by radiochromatography. Values obtained from both methods were used to determine the concentration of the galactoside in the solution, which was then used for the generation of calibration curves.

The separation of Compound A, its conjugates, and internal standard was accomplished on a Betasil C18 column (2.1 x 50 mm, 5 μm, Keystone, Bellefonte, PA). Solvent A consisted of 0.02% aqueous acetic acid, pH adjusted to 4.5 with NH_4OH, and acetonitrile (90:10), and solvent B consisted of acetonitrile and water (90:10). The mobile phase was delivered at a flow rate of 0.5 mL/min with a linear increase of solvent B from 15% to 85% over 1 min and held for another 1 min. Equilibration was allowed for an additional 1.5 min, giving a total chromatographic run time of 3.5 minutes. Under these conditions, the glucuronide, glucoside, and galactoside conjugates eluted at 1.3, 1.7, and 1.7 min,

respectively. The separation of diclofenac, its glucuronide, and internal standard (phenytoin) was achieved with the same conditions except that solvent B was increased to 75% rather than 85%.

A tandem mass experiment was performed on a Sciex (Concord, Ontario, Canada) Model API 3000 triple quadrupole mass spectrometer interfaced to the column eluant *via* a Sciex turbospray probe operating at 350°C. Operating conditions for Compound A (and its glucuronide and glucoside conjugates), diclofenac and their respective internal standards were optimized by infusion along with the LC flow (200 μL/min, solvent A/B = 50/50). Selected reaction monitoring (SRM) experiments in the positive (for Compound A and its conjugates) or negative (for diclofenac and its conjugates) ionization mode were performed using a dwell time of 200 ms per transition to detect ion pairs at *m/z* 533/339 (Compound A), 709/533 (Compound A glucuronide), 695/533 (Compound A glucoside and galactoside), 519/339 (Compound B), 294/259 (diclofenac), 470/294 (diclofenac glucuronide), 456/294 (diclofenac glucoside) and 251/208 (phenytoin). The lower limit of quantitation for Compound A glucuronide, glucoside, and galactoside in this study was 4 nM. The assay was linear over the range of 4 to 1600 nM for each conjugate. However, only the peak area ratio of diclofenac glucuronide to its internal standard was used for the measurement of this conjugate due to the lack of the authentic standard.

Data Analysis. Estimates of apparent K_m and V_{max} were obtained by fitting the untransformed data to Michaelis-Menten kinetics (eq.1) using KaleidaGraph (Synergy Software, Reading, PA).

$$v = \frac{V_{\max} \cdot S}{K_m + S} \qquad (1)$$

Where v is the rate of reaction, V_{max} is the maximum velocity, K_m is the Michaelis constant, and S is the substrate concentration.

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The IC_{50} values were determined by fitting the data to the Dose Response Logistic model (eq.2) with the same software.

$$\%v_{remaining} = \frac{100}{1+(I / IC_{50})} \qquad (2)$$

Where $\%v_{remaining}$ is the remaining reaction rate in the presence of an inhibitor, and I is the inhibitor concentration.

The K_i value of UDP-Glc toward diclofenac glucuronidation was determined by the model of competitive inhibition described in equation 3.

$$v = \frac{V_{\max} \cdot S}{K_s(1+I / K_i)+S} \qquad (3)$$

Where K_s is apparent K_m for UDP-GlcA in the presence of UDP-Glc, and I is the concentration of UDP-Glc.

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Results

Glycosidation of Compound A and Diclofenac in Human Liver Microsomes. In addition to the previously characterized acyl glucuronide and glucoside of Compound A formed in human liver microsomes in the presence of UDP-GlcA and UDP-Glc (Tang et al., 2003), a metabolite with a protonated molecule (MH^+) at *m/z* 695 (identical to that of Compound A glucoside) was detected when supplemented with UDP-gal. Upon MS/MS fragmentation, this new conjugate gave rise to a dominant fragment at *m/z* 533, the ion of protonated Compound A. Based on the supplemented sugar donor (UDP-gal) and corresponding mass change, this conjugate can be assumed to be a galactoside of Compound A, while the site of the glycosyl group on the aglycone remains to be determined. However, no product was observed when UDP-GlcNAc was used as a sugar donor. The formation of glycosides of Compound A in the presence of their respective sugar donors is illustrated in Figure 2A. On the contrary, diclofenac only accepted the glucuronic acid from UDP-GlcA to form a glucuronide. No conjugation was detected when the incubation mixture was supplemented with UDP-Glc or UDP-gal. The extracted ion current chromatogram for diclofenac glucuronide ($[M-H]^-$ at m/z 470) revealed additional minor peaks which appeared to increase with longer incubation (data not shown). They may be derived from acyl migration of acyl glucuronide of diclofenac, a reaction reported by other investigators (Grillo et al., 2003). When incubation was performed at pH 6.0, the formation of these migrated conjugates was negligible in fresh samples (Figure 2B). Therefore, incubations for the quantitation of diclofenac glucuronide were carried out in a buffer at pH 6.0.

Kinetic Properties of Glycosidation of Compound A. The rate of glycosidation as a function of Compound A concentration (0.25 – 50 μM) was measured at a fixed concentration of each UDP-sugar (5 mM) in a buffer at pH 6.0 and 7.4. The resultant three glycosidation reactions appeared

to follow Michaelis-Menten kinetics with comparable $K_{m(Compound\ A)}$ but different V_{max} (glucuronidation > glucosidation > galactosidation, Table 2). The pH appeared to have minimal impact on V_{max} of glucuronidation and glucosidation while their $K_{m(Compound\ A)}$ was ~ 2-fold greater at pH 7.4 than at pH 6.0. Galactosidation displayed a V_{max} ~ 2-fold greater at pH 6.0 than at pH 7.4, but showed a similar $K_{m(Compound\ A)}$ at both pH values. In general, greater catalytic potential ($V_{max}/K_{m(Compound\ A)}$) of all three glycosidation reactions in human liver microsomes was obtained at pH 6.0, due to either a lower $K_{m(Compound\ A)}$ or a higher V_{max}.

Meanwhile, the rate of glycosidation as a function of UDP-sugar concentration (0.025 – 5 mM) was measured at a fixed concentration of Compound A (25 μM) in a buffer at pH 6.0 and 7.4. Kinetic analysis of glucuronidation, glucosidation and galactosidation indicated that they all obeyed Michaelis-Menten kinetics. Consistent with the results described previously (Tang et al., 2003), glucuronidation and glucosidation displayed comparable apparent K_m values for UDP-GlcA ($K_{m(UDP\text{-}GlcA),}$ 761 ± 42 μM) and UDP-Glc ($K_{m(UDP\text{-}Glc),}$ 540 ± 20 μM) when reactions were carried out at pH 7.4. In fact, the apparent K_m value for UDP-gal ($K_{m(UDP\text{-}Glc),}$ 1250 ± 190 μM) also was close to that for UDP-GlcA. However, the sugar donor associated kinetics of glucuronidation and glucosidation were sensitive to pH. As summarized in Table 2, the $K_{m(UDP\text{-}GlcA)}$ and $K_{m(UDP\text{-}Glc)}$ obtained at pH 6.0 was 5 ~ 25-fold lower than that obtained at pH 7.4 while the V_{max} remained comparable. In contrast, galactosidation responded to pH changes by altering its V_{max} (~ 2-fold lower at pH 7.4) instead of its $K_{m(UDP\text{-}gal)}$.

Kinetic Properties of Diclofenac Glucuronidation. The rate of glucuronidation of diclofenac as a function of diclofenac concentration (5 – 100 μM) was measured at a fixed concentration of UDP-GlcA (2 mM) in a buffer at pH 6.0. The reaction appeared to follow Michaelis-Menten kinetics with

an average K_m for diclofenac ($K_{m(diclofenac)}$ from three determinations of 20.6 ± 3.3 μM, comparable to the value reported by other investigators (King et al., 2001).

Similarly, the glucuronidation of diclofenac as a function of UDP-GlcA (0.25 to 5 mM) was measured at a fixed concentration of diclofenac (60 μM) in a buffer at pH 6.0. The result was indicative of typical Michaelis-Menten kinetics with apparent K_m value for UDP-GlcA ($K_{m(UDP-GlcA)}$) of 96 ± 17 μM.

UGT2B7 Catalyzed Both Glucosidation and Galactosidation of Compound A in Human Liver Microsomes. The screening for Compound A galactosidation activity in 10 commercially available microsomes expressing specific human UGT isoforms revealed that UGT2B7 showed the highest activity (2.60 pmol/(min·mg protein)) for this conjugation (Figure 3). The activity of other UGT isoforms was only at the basal level. This result was substantiated by another finding that the rate of galactosidation correlated well with that of glucosidation in human liver microsomal preparations from 16 subjects (Figure 4, $r^2 = 0.95$, $p < 0.01$). Since UGT2B7 is also primarily responsible for the glucosidation of Compound A in human liver microsomes (Tang et al., 2003), it is conceivable that both glucosidation and galactosidation of Compound A in human liver microsomes were catalyzed primarily by UGT2B7.

Effect of UDP-sugars on the Counterpart Glycosidation. The three nucleotide sugars were evaluated as potential inhibitors towards their counterpart glycosidation of Compound A, and the IC_{50} values were determined (Table 3). Although the glucoside and galactoside of Compound A were inseparable under the current experimental conditions, the IC_{50} of UDP-gal towards the glucosidation still could be estimated due to the large difference of these two conjugations. Since the catalytic capacity of the galactosidation was > 100-folds lower than that of the glucosidation (Table 2), the

amount of the galactoside formed in the presence of UDP-Glc should be negligible. It has been established that UDP-GlcA and UDP-Glc competitively inhibit glucosidation and glucuronidation of Compound A in human liver microsomes with their Ki values being close to their apparent K_m at pH 7.4 (Tang et al., 2003). Consistent with this finding, a comparable IC_{50} of UDP-GlcA (1195 μM) and UDP-Glc (1050 μM) was obtained at the same pH in the present study. However, the respective IC_{50} values obtained at pH 6.0 were ~ 10- and 2-fold lower, an observation in agreement with their lower K_m at pH 6.0 (Table 2). The high affinity of UDP-GlcA ($K_{m(UDP\text{-}GlcA)}$ of 28 μM) at pH 6.0 was also translated into its potent inhibition of galactosidation with IC_{50} of 30 μM, a potency > 10-fold greater than that obtained at pH 7.4 (IC_{50} of 525 μM). Such pH-dependent inhibitory effect was not appreciable with UDP-gal as an inhibitor towards glucuronidation and glucosidation. This nucleotide sugar demonstrated higher IC_{50} values (1200 – 2400 μM), in agreement with its higher $K_{m(UDP\text{-}gal)}$ (≥ 1250 μM), relative to the value of UDP-GlcA and UDP-Glc (Table 2). In general, the IC_{50} values of individual UDP-sugars obtained in this study approximated 2-folds of their respective K_m values, indicating a common competitive inhibition mechanism at both pH 7.4 and 6.0 (Segel 1993).

Although they were not accepted as glycosyl moiety donors to conjugate diclofenac, UDP-Glc and UDP-gal were inhibitory towards diclofenac glucuronidation in human liver microsomes. Their kinetic mode of inhibition was characterized, and their inhibition constant (Ki) values determined. As shown by the double reciprocal plot in Figure 5, the presence of various amounts of UDP-Glc and UDP-gal changed only the apparent $K_{m(UDP\text{-}GlcA)}$ and did not affect the V_{max}, suggesting a competitive inhibition of diclofenac glucuronidation by these two nucleotide sugars. The Ki values determined using nonlinear regression were 2054 ± 108 and 1277 ± 149 μM for UDP-Glc and UDP-gal, respectively. These values are > 10-fold greater than the $K_{m(UDP\text{-}GlcA)}$.

Discussion

In this study, we have demonstrated that, in addition to UDP-GlcA and UDP-Glc (Tang et al., 2003), UGT2B7 is capable of transferring the glycosyl group from UDP-gal as well to an endothelin ET_A antagonist, Compound A. This isoform has been reported to catalyze glucuronidation and glucosidation of hyodeoxycholic acid and to have some capacity to use UDP-xylose (Mackenzie et al., 2003). It even becomes highly selective towards UDP-Glc over UDP-GlcA to conjugate an aldose reductase inhibitor (Toide et al., 2004). On the contrary, only UDP-GlcA serves as a sugar donor to glucuronidate diclofenac, which is also a substrate of UGT2B7 (King et al., 2001). It appears that the sugar donor selectivity of UGT2B7 is aglycone-dependent.

It is believed that in the process of glucuronidation, the carboxyl group in UDP-GlcA is the functionality that interacts with a crucial residue, arginine, in the active site (Ouzzine et al., 2002; Senay et al., 1997; Zakim et al., 1983) to allow for an optimal orientation/alignment of an acceptor-donor-enzyme complex. The lack of a carboxyl group in UDP-Glc and UDP-gal may deprive such optimization in the case of diclofenac, although these two donors are able to bind to the same binding site for UDP-GlcA, as indicated by their competitive inhibition of diclofenac glucuronidation (Figure 5). In contrast, with Compound A as an acceptor, the lack of a carboxyl group in UDP-Glc and UDP-gal seems to be compensated for and UGT2B7 becomes more promiscuous. While more studies are needed to understand the mechanism, one possibility may involve distinct interaction of Compound A and diclofenac with the enzyme.

It is well documented that substrate binding can induce conformational changes in many enzymatic systems (Zhou et al., 2000; Segura-Pena et al., 2004; Davydov et al., 2004; Lopez-Corcuera

et al., 2001). Recent advances in this regard have been described for glycosyltransferases with the availability of X-ray crystal structures of the catalytic domain of a number of enzymes (Qasba et al., 2005). Such changes appear to be necessary in aligning two substrates for catalysis and in configuring the active site. An ordered binding has been proposed by some researchers for mammalian UGTs involved in drug metabolism (Koster and Noordhoek, 1983; Potrepka and Spratt, 1972; Sanchez and Tephly, 1975), by which the acceptor and UDP-sugar donor are the first and second binding substrate, respectively. With this mechanism, it is reasonable to expect acceptor substrate-induced conformational changes and to anticipate such changes to be dependent on the chemical and structural properties of a particular substrate. Supporting this hypothesis is the evidence of aglycone-dependent sugar donor kinetics in human and animal liver microsomes. The wide range of apparent K_m values of UDP-GlcA with different aglycones (Lin and Wong, 2002) has suggested that the binding affinity of this sugar donor is dependent on the chemical nature of the acceptor substrate, in other words, the interaction of acceptor substrate with the enzyme. It appears that such interaction not only affects the affinity of enzyme to its preferred UDP-sugar, but in some cases alters the selectivity of sugar donors. Depending on the aglycones, UGTs can be highly selective for UDP-GlcA (most common), accommodative for other sugar donors (Compound A), or highly selective for UDP-Glc rather than UDP-GlcA (an aldose reductase inhibitor (Toide et al., 2004)).

Compared with diclofenac, Compound A is bulkier and possesses more hydrogen bond donors and acceptors. Thus, once binding to the enzyme, presumably through additional Van der Waals forces or hydrogen bonds, Compound A may elicit conformational changes that assist in positioning Compound A and UDP-Glc in an orientation/alignment optimal for an in-line displacement reaction mechanism (S_N2), a situation identical to the glucuronidation catalyzed by mammalian UGTs (Radominska-Pandya et al., 1999; Qasba et al., 2005). However, the binding of diclofenac to

UGT2B7 may be unable to induce such conformational changes favorable for UDP-Glc. On the other hand, it appears that the lack of carboxyl group is more readily compensated in UDP-Glc than in UDP-gal, as exemplified in this study by the large difference in the activities of glucosyltransferase and galactosyltransferase with respect to Compound A. This finding indicates the importance of configuration of the C4 hydroxyl group on sugar moiety, because the only difference between UDP-Glc and UDP-gal is the equatorial versus axial hydroxyl group. In addition, UDP-GlcNAc lacks 2-hydroxyl on the sugar portion and it is not accommodated at all. Clearly, chemical and structural properties of sugar donors could constitute a contributing factor to this occurrence.

In summary, we herein report that UGT2B7 is capable of transferring the glycosyl group from additional sugar nucleotide, UDP-gal, to Compound A, and have demonstrated that the sugar donor selectivity of this enzyme is aglycone dependent. While conformational changes induced by aglycone binding to UGT2B7 are postulated to contribute to altered sugar donor selectivity, a full understanding of the mechanism is awaiting the availability of the crystal structure of UGT2B7. However computational methodologies have been developed to predict acceptor substrate selectivity and to provide a measure of substrate binding (Smith et al., 2003 and 2004; Sorich et al., 2002 and 2004). Therefore, with this approach, compounds known to have the ability to change sugar donor selectivity could be utilized for further investigation of the properties of the corresponding UGTs.

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Legends of Figures

Figure 1. *Structures of Compound A, Compound B, UDP-GlcA, UDP-Glc, UDP-gal and UDP-GlcNAc.*

Figure 2. *Base peak chromatograms of human liver microsomal incubates with Compound A (**A**) and diclofenac (**B**) supplemented with UDP-GlcA, UDP-Glc and UDP-gal, respectively.*

Incubations were carried out at pH 6.0.

Figure 3. *Formation of Compound A galactoside by stably expressed UGT isoforms.*

Incubations were carried out at pH 6.0. Data are expressed as mean ± SD of triplicates.

Figure 4. *Formation of Compound A glucoside versus galactoside in 16 human liver microsomal preparations.*

Incubations were carried out at pH 6.0.

Figure 5. *Double reciprocal plots for the inhibition of diclofenac glucuronidation by UDP-Glc (**A**) and UDP-gal (**B**).*

Incubations were carried out at pH 6.0. Data are expressed as mean ± SD of triplicates.

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Tables

Table 1. Experiment design for the determination of IC_{50} values of UDP-sugars towards the formation of respective Compound A glycosides in human liver microsomes.

pH	UDP-sugar as co-substrate (μM)			UDP-sugar as inhibitor (μM)		
	UDP-GlcA	UDP-Glc	UDP-gal	UDP-GlcA	UDP-Glc	UDP-gal
6.0	50	100	1000	10-1000	10-1000	25-5000
7.4	500	1000	1000	25-2000	25-2000	25-5000

Concentration of Compound A is 25 μM in all incubations.

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Table 2. Kinetic parameters of glycosidation for Compound A and UDP-sugars in human liver microsomes at pH 6.0 and 7.4

Co-substrate	pH	Parameter	Glucuronidation	Glucosidation	Galactosidation
Compound A	6.0	$K_{m(Compound\ A)}$	6.0 ± 0.5	8.12 ± 1.40	20.7 ± 2.0
		V_{max}	1348 ± 29	642 ± 34	10.8 ± 0.5
	7.4	$K_{m(Compound\ A)}$	11.3 ± 0.6	18.0 ± 1.64	24.6 ± 4.4
		V_{max}	1323 ± 25	649 ± 25	5.4 ± 0.5
UDP-sugar	6.0	$K_{m(UDP\text{-}sugar)}$	28 ± 3	104 ± 10	1250 ± 190
		V_{max}	1051 ± 14	652 ± 7	4.92 ± 0.37
	7.4	$K_{m\ (UDP\text{-}sugar)}$	761 ± 42	543 ± 20	1305 ± 90
		V_{max}	1040 ± 18	551 ± 9	2.74 ± 0.09

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Table 3. IC_{50} values of UDP-sugars for the inhibition of their counterpart glycosidations[a]

Glycosidation	IC_{50} (μM) (pH 6.0/pH 7.4)[b]		
	UDP-GlcA	UDP-Glc	UDP-gal
Glucuronidation		550/1050	2400/2400
Glucosidation	115/1196		1200/2400
Galactosidation	30/525	ND	

[a]The details of experimental designs are described in Table 1.

[b]The data are expressed as mean of duplicate determinations.

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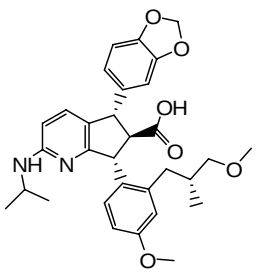
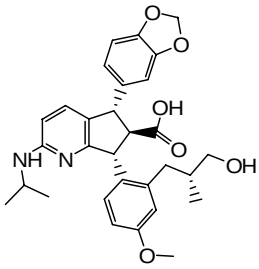
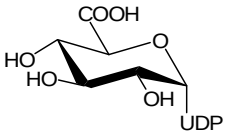


Compound A



Compound B (internal standard)



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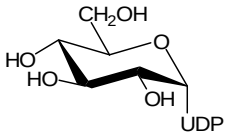

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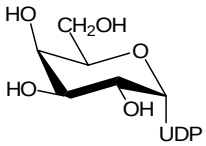
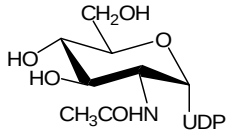


UDP-gal



UDP-GlcNAc

Figure 1

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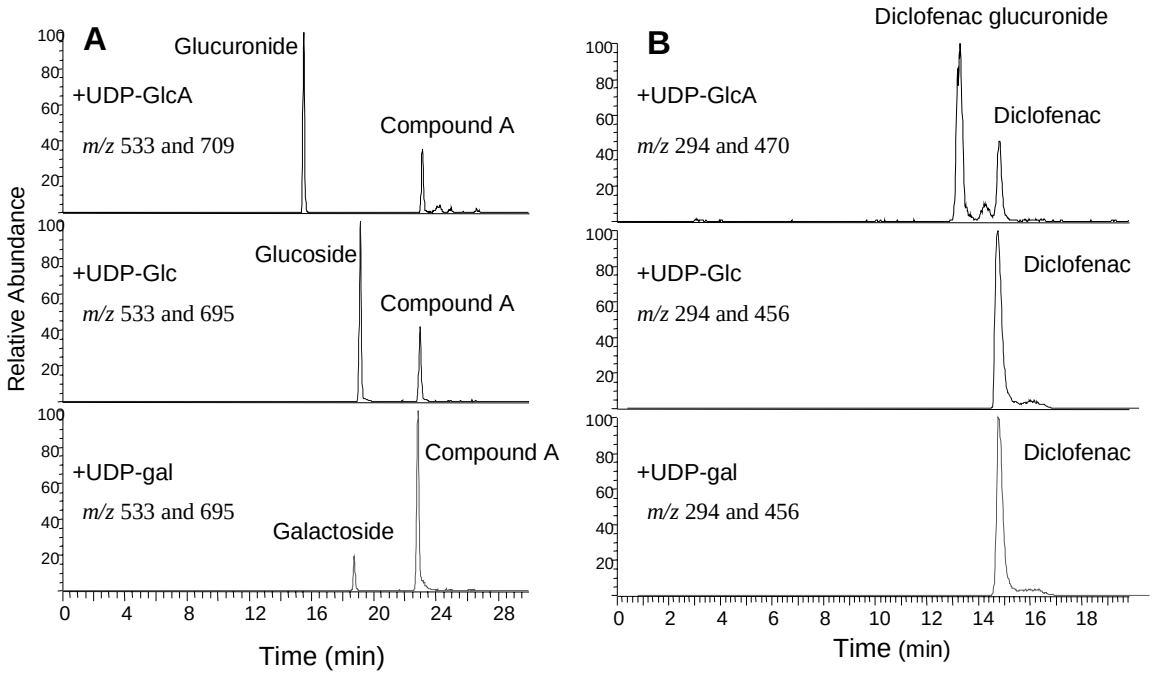

Figure 2

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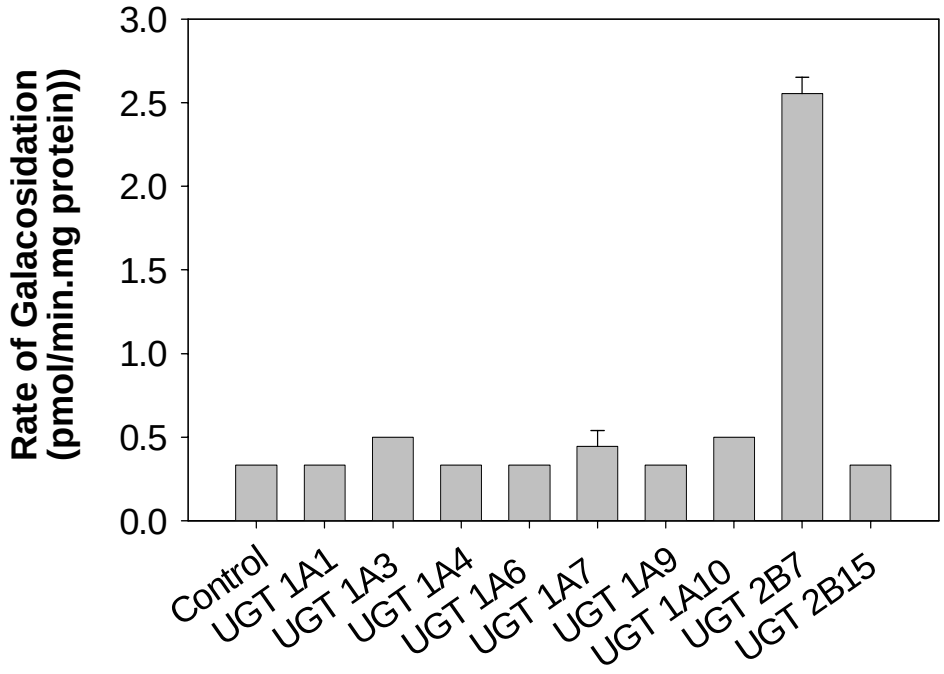

Figure 3

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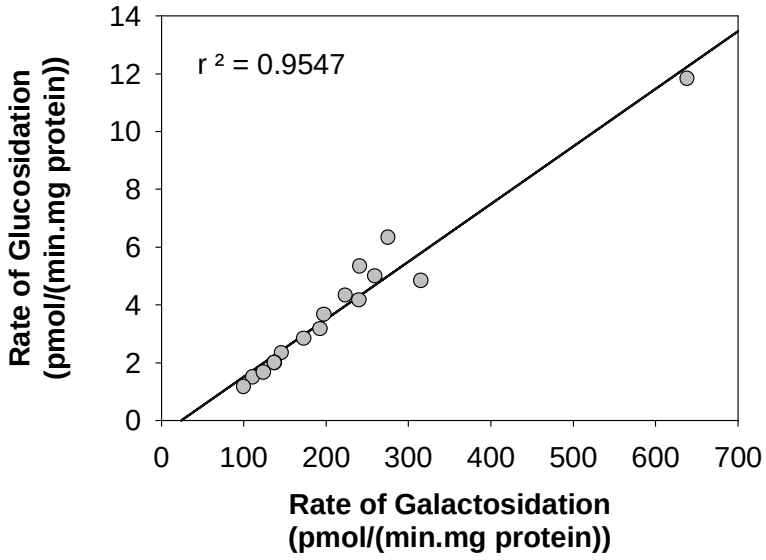

Figure 4

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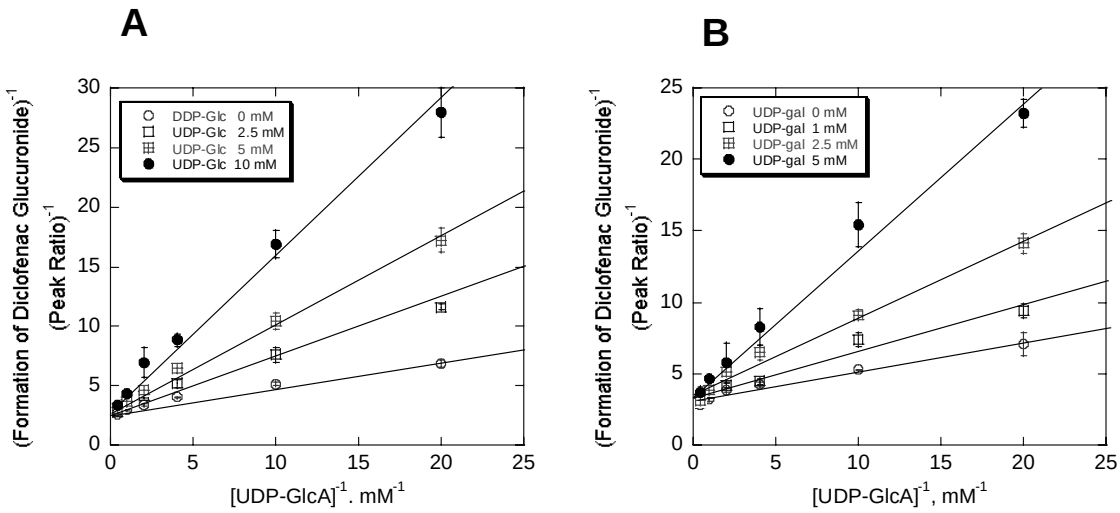

Figure 5