ACYL GLUCURONIDATION AND GLUCOSIDATION OF A NEW AND SELECTIVE ENDOTHELIN ETA RECEPTOR ANTAGONIST IN HUMAN LIVER MICROSOMES

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ABSTRACT:

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] is a new and selective endothelin ETa receptor antagonist. It underwent significant acyl glucuronidation and acyl glucosidation in human liver microsomes supplemented with UDP-glucuronic acid (UDP-GA) and UDP-glucose (UDP-G). These two conjugations were observed in a panel of human liver microsomal samples (n = 16) that gave rise to varying activities but with no significant correlation with each other in the native and activator-treated microsomal preparations (R² ≤ 0.4, p > 0.05). The lack of correlation may be explained by the involvement of multiple UDP-glucuronosyltransferases (UGTs; UGT1A1, 1A3, 1A9, 2B7 and 2B15) in the glucuronidation but essentially solely UGT2B7 in the glucosidation. Both reactions conformed to monophasic Michaelis-Menten kinetics in human liver microsomes. The glucuronidation reaction exhibited apparent Kₘ values (mean ± S.E.) for compound A and UDPGA of 8.4 ± 0.6 and 605 ± 35 μM, respectively, whereas the values for the glucosidation reaction were 10.2 ± 1.5 and 670 ± 120 μM, respectively. In both pooled human liver microsomes and expressed UGT2B7, UDPG and UDPGA competitively inhibited their counterpart conjugations with Kᵢ values close to their Kₘ values, indicating a comparable affinity of the enzyme toward these two nucleotide sugars. We herein report a drug acyl glucoside formed in human liver microsomes at a considerable turnover rate and provide the evidence for a UGT isoform (UGT2B7) capable of transferring both glucuronic acid and glucose from UDPGA and UDPG to an aglycone.

The UDP-glucuronosyltransferases (UGTs) catalyze the transfer of glucosyl group from a nucleotide sugar to an aglycone. The transfer of glucuronic acid from UDP-glucuronic acid (UDP-GA) leads to glucuronidation, a major conjugation reaction, which, in general, results in inactivation and excretion of endogenous compounds such as bilirubin and steroids as well as a wide variety of xenobiotics including drugs, carcinogens, and other environmental pollutants (Dutton, 1980). The past decades have witnessed significant progress in substrate specificity identification in several species, especially in rats and humans, with the availability of purified and/or cloned UGTs, and sequence analysis (Burchell, 1999). For instance, morphine glucuronidation was found to be catalyzed by rat UGT2B7 (King et al., 1996; Coffman et al., 1997), and diclofenac glucuronidation is believed to be primarily catalyzed by rat UGT2B1 and human UGT2B7 (King et al., 2001).

Interestingly, in addition to substrate specificity, there exists the specificity of UGTs toward UDP-sugars (cosubstrates). As early as in 1977, Fevery et al. demonstrated that although UDPGA is the primary UDP-sugar cosubstrate used for the bilirubin esterification in mammals, two other UDP-sugars, UDP-glucose (UDP-G), and UDP-xyllose, are also potential substrates (Fever, et al., 1977). Studies by Motoyama (1979) with 50 human liver samples showed a significant correlation (r² = 0.974) between bilirubin UGT and bilirubin xylosyltransferase activity, suggesting that a single enzyme was responsible for these activities. Later on, UDP-sugar specificity of bilirubin UGT has been studied in purified rat liver preparations, and it was proposed that a single bilirubin UGT could accept different sugar nucleotides (Burchell and Blanckaert, 1984). Similarly, Senafi et al. (1994) demonstrated that a cloned expressed human bilirubin UGT (UGT1A1) could use UDPGA, UDPG, and UDP-xyllose as a substrate with the catalytic potential (Vₘₐₓ/Kₘ(bilirubin)) for UDPGA 2- and 10-fold greater than that for UDP-xyllose and UDPG, respectively. Apparently, it was believed that a single enzyme, at least a common subunit, was responsible for the transfer of sugars from respective sugar nucleotides to bilirubin. However, formation of hyodeoxycholic acid (a 6-hydroxylated bile acid) glucoside was thought to be catalyzed by a novel UDPG-specific glucosyltransferase because the patterns of photo-affinity labeling by 5-[β-32P]azido-UDP-GA and 5-[β-32P]azido-UDP-GA in the 50- to 56-kDa range were significantly different and cloned hyodeoxycholic acid-specific UGT (UGT2B4) failed to generate the glucoside conjugate (Radominska et al., 1993). Therefore, the authors suggested the presence of a novel UDPG-specific glucosyltransferase catalyzing the biosynthesis of 6-O-glucoside of bile acid in human liver microsomes. The different scenarios may

1 Abbreviations used are: UGT, UDP-glucuronosyltransferases; UDPGA, UDP-glucuronic acid; UDPG, UDP-glucose; 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; compound B, (5S,6R,7R)-5-(1,3-benzodioxol-5-yl)-7-[2-(3-hydroxy-2-methylpropyl)-4-[3-(4-methoxyphenyl)]-2-[1-(methylamino)-6,7-dihydro-5H-cyclopenta[b]pyridine-6-carboxylic acid; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography mass spectrometry; ESI, electrospray ionization.

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stem from the combination of substrate dependence and the involvement of different UGT isoforms. For instance, an aglycone may undergo conjugation with more than one sugar nucleotide but catalyzed by different UGT isoforms, in which no correlation should be observed among different conjugations. A correlation could be observed in which a UGT isoform is capable of transferring different sugars from different sugar nucleotides to the same aglycone. Although a considerable number of drugs and other xenobiotics, as well as some endogenous compounds, have been reported to be conjugated with UDPGA, UDPG, and/or UDP-xylose in different tissue microsomes from different species (Senafi et al., 1994; Chmela et al., 2001; Shipkova et al., 2001), the specificity of UGTs toward sugar nucleotides has received scant attention.

In the present study, we found that 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, Fig. 1], a new selective and potent endothelin ET\textsubscript{A} receptor antagonist (Okada et al., 2000), underwent significant acyl glucuronidation and acyl glucosidation in human liver microsomes. To determine the mechanism of both conjugations, we evaluated the kinetics of the glucuronide and glucoside formation and mutual inhibition of UDPGA and UDPG on conjugation reactions. Additionally, by using recombinant UGTs, we also evaluated UGT isoforms responsible for both conjugations and demonstrate that multiple UGTs are involved in the glucuronidation but essentially only UGT2B7 is responsible for the glucosidation.

**Experimental Procedures**

**Chemicals and Reagents.** Compound A and its analog compound B [(5S,6R,7R)-5-(1,3-benzodioxol-5-yl)-7-[2-(3-hydroxy-2-methylpropyl)-4-(methoxyphenyl)]-2-[(1-methylethyl)amino]-6,7-dihydro-5H-cyclopenta[b]pyridine-6-carboxylic acid] used as an internal standard (Fig. 1) were synthesized by Banyu Pharmaceutical Co. (Ibaraki, Japan). UDPGA, UDPG, alamethicin, and Brij 58 were obtained from Sigma-Aldrich (St. Louis, MO). Solvents used for liquid chromatography were of analytical or HPLC grade. A pool of human liver microsomes from 10 individuals (HHM-0259) were obtained from IIAM (Scanton, PA). A bank of human liver microsomes (n = 16 different organ donors) was purchased from Xenotech LLC (Kansas City, KS). Recombinant human UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15 (Supersomes) were obtained from the BD Gentest Corporation (Woburn, MA) and baculosomes of UGT1A7 and 1A10 from PanVera (Madison, WI).

**Incubation Conditions.** For the quantitation of conjugate formation, incubations were carried out at 37°C in a Fisher shaking water bath, employing 1.1 ml of 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), MgCl\textsubscript{2} (10 mM), EDTA (1 mM), UDPGA or UDPG (fixed concentration of 5 mM or varied concentrations), human liver microsomes or expressed UGTs (0.1 mg of protein/ml), and compound A with desired concentrations (100-fold concentrated stock solution in 50% acetonitrile aqueous solution). The concentration of alamethicin and Brij 58, when applied, was 50 and 100 μg/mg of protein, respectively. The reaction was started by the addition of the UDPGA or UDPG and terminated with acetonitrile (200 μl) following a 15-min incubation. The internal standard (compound B) solution (water/acetonitrile, 50:50) was added to the samples (50 μl, 2.5 μM). Following brief vortexing and centrifugation (10 min at 2000 rpm), the supernatant was transferred to 96-well microtiter plates for LC-MS assay.
The incubations for structure identification by LC-MS were carried out for 60 min and terminated by adding 1.0 ml of acetonitrile. The mixture contained the same components as described above except different concentrations of microsomal protein (1.0 mg/ml) and compound A (10 μM) in 13 × 100 mm borosilicate glass disposable culture tubes in a total volume of 1.0 ml. The samples were mixed with acetonitrile by vortexing prior to centrifugation for 5 min at 2000 rpm, and the supernatant was transferred to a clean tube and evaporated to dryness under a nitrogen stream at 37°C. The residues were reconstituted in 100 μl of 30% acetonitrile aqueous solution.

The same incubation mixture scaled to a larger volume (10 ml) was employed to obtain the glucuronides and glucosides of compound A for structure determination by NMR. Ten sets of incubations were carried out for 120 min in 20-ml borosilicate glass scintillation vials, and 10 ml of acetonitrile was added to terminate the reaction. Following centrifugation (5 min at 2000 rpm), the supernatant was combined and concentrated to ~2 ml under a nitrogen stream. Purification of the glucuronides and glucosides was accomplished by the HPLC-UV method described below.

**LC-MS Analysis.** For structure identification, the chromatographic separation of compound A and its metabolites was performed on a reverse phase C18 column (DBS Hypersil, 2.0 × 150 mm, 5 μm; Thermo Hypersil, Keystone Scientific Operations, Bellefonte, PA) with a HP 1050 LC system (Hewlett-Packard, Palo Alto, CA). Solvent A consisted of 0.02% aqueous acetic acid with pH adjusted to 4.5 with NH4OH and acetonitrile (90:10) and solvent B of acetonitrile and water (90:10) and were delivered at a constant total flow rate of 0.2 ml/min. The initial mobile phase consisted of 10% of solvent B, which was linearly increased to 55% over 20 min, then to 80% in another 2 min and held for additional 3 min. The column was then equilibrated under initial conditions for 5 min.

Mass spectrometric analysis was performed on a LCQ ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Finnigan MAT, San Jose, CA). ESI was operated in a positive mode with other conditions set as follows: capillary temperature, 230°C; sheath gas flow, 70; auxiliary gas flow, 10; ESI spray voltage, 4.5 kV. The automatic gain control target values for full scan and product scan were set to 5 × 104 and 2 × 103, respectively. The mass isolation window for collision-induced dissociation was set at 3 m/z units and collision energy at 25%. Mass spectral data were analyzed using the manufacturer’s software package (Explore 1.1).

For the purpose of quantitation, the separation of compound A, its conjugates and internal standard (compound B), was accomplished on a Betasil C18 column (2.1 × 50 mm, 5 μm; Thermo Hypersil, Keystone Scientific Operations). The same 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 additional 1.5 min, giving a total chromatographic run time of 3.5 min. The flow rate of split such that 2.5 of the flow was introduced into mass spectrometer. Under these conditions, the glucuronide and glucoside eluted at 1.3 and 1.7 min, respectively.

Tandem mass experiments were performed on a MDS Sciex (Concord, Ontario, Canada) model API 3000 triple quadrupole mass spectrometer interfaced to the column eluent via a Sciex turbospray probe operated at 350°C. Operating conditions for all the analytes were optimized by infusion of a mixture of all analytes at a flow rate of 5 μl/min, along with the LC flow (200 μl/min, solvent A/B = 50:50), and were determined as follows: nebulizing gas flow, 8; curtain gas pressure, 15; ion spray voltage, 5000 V; declustering potential, 46 V; focusing potential, 200 V; collision gas (nitrogen) flow, 4 (the manufacturer’s setting number). Selected reaction monitoring experiments in the positive 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 519/339 (compound B). The lower limit of quantitation in this study was 4 nM. The assay was linear over the range of 4 to 1600 nM for each conjugate.

**HPLC-UV Conditions.** The chromatographic separation of compound A and the two conjugates generated in the large scale incubation was performed on a reverse phase C18 column (DBS Hypersil, 4.6 × 150 mm, 5 μm; Thermo Hypersil, Keystone Scientific Operations) with a Shimadzu LC-10AD HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD). The same mobile phase for LC-MS analysis was used and delivered at a constant flow rate of 1.0 ml/min. The initial mobile phase consisted of 30% of solvent B, which increased linearly to 75% over 5 min and then to 85% in 0.1 min. The value was then held for an additional 2 min, and the column was equilibrated for 5 min. The elution of the analytes was monitored by UV detection (286 nm). Under these conditions, the glucuronide, glucoside, and compound A eluted at 4.3, 7.8 and 9.6 min, respectively. The fractions of the glucuronide and glucoside were collected, dried in a lyophilizer, and stored at −20°C until further use. The purity of each conjugate isolated was >95% confirmed by HPLC-UV analysis.

**NMR Analysis.** The 1H and total correlation spectroscopy NMR spectra for compound A and the isolated conjugates were obtained in CD3OD (99.96% deuterium content; Isotec Inc., Miamisburg, OH) at 25°C on a Varian Inova 500 MHz spectrometer (Varian Medical Systems, Palo Alto, CA) equipped with a MIDG-3 probe (Nalorac Corp., Martinez, CA). The 1H chemical shifts (in parts per million) are relative to the solvent CD3OD signal, which is set at 3.33 ppm.

**Quantiﬁcation of Glucuronide and Glucoside of Compound A.** The puriﬁed conjugates were dissolved in a given volume of buffered 50% acetonitrile aqueous solution (pH 6.0). A small quantity of the solution was added to phosphate buffer at pH 10 and incubated at 37°C for 2 h. Analysis by LC-MS conﬁrmed the complete hydrolysis of the conjugates to compound A, which was then calibrated with its standard curve. The quantified stock solution of each conjugate was kept at −20°C for the generation of standard curves.

**Data Analysis.** The apparent enzyme kinetic parameters were determined by ﬁtting the reaction velocities versus substrate concentrations to eq. 1 or eq. 2 (Gravit; Erithacus Software Ltd., Staines, UK), which describe Michaelis-Menten kinetics alone or Michaelis-Menten kinetics coupled with uncompeti- tive substrate inhibition, respectively.

\[
\begin{align*}
\nu &= \frac{V_{\text{max}} \cdot S}{K_s + S} \\
\nu &= \frac{V_{\text{max}} \cdot S}{K_s + (1 + S/K_i)}
\end{align*}
\]

Where \(\nu, V_{\text{max}}, K_s, \text{and } K_i\) are the velocity, concentrations, compound A concentration, the maximal conjugation velocity, apparent Michaelis constant, and substrate inhibition constant, respectively.

In the evaluation of the inhibition of UDPGA and UDPG toward their counterpart conjugations, the \(K_s\) values were determined by nonlinear regression analysis of the experimental data using Mathematica 4.0 (Wolfram Research, Inc., Champain, IL).

**Results**

**Structure Characterization of Compound A Conjugates.** Compound A appeared as its protonated molecule (MH+) at m/z 533 under positive ion electrospray ionization. In a pilot study where compound A was incubated in fresh human hepatocytes, two major metabolites were detected with MH+ at m/z 709 and 695 (data not shown). They were also generated in human liver microsomes supplemented with either UDPGA (MH+ at m/z 709) or UDPG (MH+ at m/z 695). Upon tandem mass spectrometry fragmentation, the metabolite with MH+ at m/z 709 gave rise to a prominent fragment at m/z 533 via loss of 176 (Fig. 2A), whereas that with MH+ at m/z 695 generated the same fragment (m/z 533) via loss of 162 (Fig. 2B). The involvement of UDP-sugars and fragmentation pattern suggest the metabolites as a glucuronide and glucoside of compound A, respectively.

The site of glucuronidation and glucosidation was established by the 500-MHz NMR analysis of the isolated conjugates. Proton NMR data confirm that the isolated conjugates are 1β-O-glucoside or 1β-O-glucuronide acyl conjugates at the 6-carboxyl group of the cyclopenteno [1,2-b] pyridine ring. The 1H chemical shifts of protons in the cyclopenteno-pyridine ring and the sugar ring for the parent and the metabolites are listed in Table 1. Compared with the parent compound A spectrum, the glucose conjugate 1H NMR data reveals a distinctive new resonance at 5.53 ppm (H1’, doublet, 8.2 Hz) indicative of a β-anomer. Similarly, the glucuronic conjugate NMR data reveals a new resonance at 5.55 ppm (H1’, doublet, 8.3Hz), also
indicative of a β-anomer. The chemical shift of H1' in both conjugates is indicative of an acyl conjugation. The chemical shifts of cyclopenteno ring protons (H5, H6, H7) also undergo small but commensurate down field shifts (+0.11 to +0.20 ppm) in the metabolite spectra. All other proton chemical shifts are practically unchanged.

**Kinetic Properties of Compound A Acyl Glucuronidation and Glucosidation in Human Liver Microsomes.** Optimal assay conditions were established using pooled human liver microsomes with varying concentrations of compound A, UDPGA, UDPG, microsomal protein, and time of incubation. Both reactions were shown to be linear up to 30 min, and all subsequent incubations were carried out for 15 min and contained 0.1 mg/ml of microsomal protein and 5 mM of either UDPGA or UDPG.

The rate of glucuronidation and glucosidation as a function of compound A concentration was measured at a fixed concentration of UDPGA (5 mM) or UDPG (5 mM). Over the range of compound A concentration tested (0.25–50 μM), both reactions followed Michaelis-Menten kinetics (Fig. 3) with low apparent $K_m$ for compound A ($K_{m(Compound A)}$) and relatively high $V_{max}$ (Table 2). A linear Eadie-Hofstee plot suggested a one-site (one $K_m$) Michaelis-Menten model for both reactions (data not shown). Pretreatment with alamethicin and Brij 58 of microsomal preparation led to 1.4- to 1.6-fold increase in intrinsic clearance ($V_{max}/K_m$) of glucosidation, but only alamethicin apprecia-

![Fig. 2. Tandem mass spectrometry spectra of compound A glucuronide (A) and glucoside (B) generated in a pool of human liver microsomes (HHM-0259) in the presence of UDPGA or UDPG.](image)

<table>
<thead>
<tr>
<th>Chemical shifts of cyclopenteno-pyridine ring and the sugar protons of compound A, its glucose and glucuronide conjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L-753091 Glucoside Glucuronide</strong></td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>1'</td>
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<tr>
<td>2',3',4'</td>
</tr>
<tr>
<td>5'</td>
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<tr>
<td>6'</td>
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bly enhanced glucuronidation (intrinsic clearance was increased by 2.5-fold) with minimal impact of Brij 58 on this reaction (Table 2).

Kinetic analysis of glucuronidation and glucosidation as a function of UDPGA and UDPG at a fixed concentration of compound A afforded the comparable apparent $K_m$ values for UDPGA ($K_m(UDPGA)$, 605 ± 35 μM) and UDPG ($K_m(UDPG)$, 672 ± 121 μM). Both reactions appeared to obey Michaelis-Menten kinetics when assayed with compound A as a second substrate. Interestingly, pretreatment with alamethicin resulted in significant increase in apparent $V_{max}$ for both conjugations without effect on the $K_m$ of UDPGA, although the value for UDPG was decreased by ~40% (Table 3).

Both UDPGA and UDPG were evaluated as potential inhibitors of the glucosidation or glucuronidation, respectively, and the inhibition constant ($K_i$) and the kinetic mode of the inhibition for either reaction were determined. As shown by the double reciprocal plot in Fig. 4A, the presence of various amounts of UDPG in the incubation for compound A glucuronidation changed only the apparent $K_m$($UDPGA$) and did not affect the $V_{max}$, suggesting competitive inhibition of glucuronidation by UDPG. The $K_i$ value determined using nonlinear regression was 0.97 ± 0.12 mM, close to its apparent $K_m$.

Similarly, addition of various amounts of UDPGA to the incubation system for glucoside formation resulted in the change only in the apparent $K_m$($UDPG$) without alteration of $V_{max}$, also indicating competitive inhibition of glucosidation by UDPGA (Fig. 4B). The $K_i$ value estimated in the same way was 0.52 ± 0.07 mM, also close to its apparent $K_m$.

### TABLE 2

<table>
<thead>
<tr>
<th>Disturbing agent</th>
<th>Glucuronidation</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Glucosidation</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (compound A)</td>
<td>$V_{max}$</td>
<td>$V_{max}/K_m$</td>
<td></td>
<td>$K_m$ (compound A)</td>
<td>$V_{max}$</td>
<td>$V_{max}/K_m$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alamethicin</td>
<td>8.4 ± 0.6</td>
<td>352 ± 8</td>
<td>42</td>
<td></td>
<td>10.2 ± 1.5</td>
<td>187 ± 9</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brij 58</td>
<td>5.9 ± 0.6</td>
<td>629 ± 18</td>
<td>107</td>
<td></td>
<td>10.7 ± 1.3</td>
<td>282 ± 12</td>
<td>26</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>10.2 ± 1.67</td>
<td>482 ± 11</td>
<td>47</td>
<td></td>
<td>8.7 ± 0.9</td>
<td>253 ± 9</td>
<td>29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A pooled human liver microsomal preparation ($n = 10$) purchased from IIAM.

* $K_m$ for compound A, μM.

* $V_{max}$, pmol/ (min·mg protein).

* $K_i$, μM.

* Data are expressed as mean ± S.E. from triplicates.

### TABLE 3

<table>
<thead>
<tr>
<th>UDP-sugar</th>
<th>Alamethicin</th>
<th>$K_m$ (UDP-sugar)</th>
<th>$V_{max}$</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDPGA</td>
<td>–</td>
<td>605 ± 35 μM</td>
<td>402 ± 7</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>603 ± 43</td>
<td>860 ± 18</td>
<td>1.43</td>
</tr>
<tr>
<td>UDPG</td>
<td>–</td>
<td>672 ± 121</td>
<td>147 ± 8</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>400 ± 71</td>
<td>208 ± 9</td>
<td>0.52</td>
</tr>
</tbody>
</table>

* A pooled human liver microsomal preparation ($n = 10$) purchased from IIAM.

* Data are expressed as Mean ± S.E. from triplicates.

* Concentration of compound A is 50 μM.
4.9 in native, almethicin-treated and Brij 58-treated human liver microsomes, respectively. There was no correlation between these two conjugations (Fig. 5). The correlation coefficients and the reaction rates of glucuronidation and glucosidation are summarized in Table 4. It appeared that glucosidation was more resistant than glucuronidation to the effect of alamethicin and Brij 58 in all microsomal preparations.

Acyl Glucuronidation and Glucosidation by Stably Expressed UGTs. The screening for compound A glucuronidation and glucosidation activity in all commercially available microsomes expressing specific UGT isoforms revealed that compound A was glucuronidated by multiple UGTs (1A1, 1A3, 1A9, 2B7 and 2B15), whereas it was glucosidated essentially only by UGT2B7, which gave rise to a comparable activity of both glucuronidation and glucosidation (Fig. 6). It was also found that the activity of UGTs toward both conjugations was decreased in the presence of alamethicin and Brij 58. As a result, no latency-disrupting agents were used in studies using expressed UGTs. There was no activity of glucuronidation and very low level of glucosidation (\(< 2\) pmol/(min \cdot mg of protein)) in control microsomes from cells infected with wild-type vector.

Kinetic Properties of Compound A Acyl Glucuronidation and Glucosidation by Expressed UGTs. Glucuronidation by four UGT isoforms (UGT1A1, 1A3, 2B7 and 2B15) and glucosidation by UGT2B7 displayed typical Michaelis-Menten kinetics. However, glucuronidation by UGT1A9 showed a more complex kinetics with decreasing velocity at higher substrate concentration (Fig. 7), suggesting the effect of substrate inhibition. Fitting the data points to the eq. 1 or 2 yielded the kinetic parameters listed in Table 5. As for glucuronidation, relatively low apparent \(K_m\) values (\(\leq 3\) \(\mu\)M) for compound A were observed with all three isoforms from the 1A family, especially UGT1A9 (0.24 \(\mu\)M), whereas a greater value (\(> 2.5\)-fold) was obtained with UGT2B7 and 2B15. The kinetic parameters of glucosidation by UGT2B7 were close to those of glucuronidation by the same isoform (Table 5).

Similar to what was observed in human liver microsomes, UDPG and UDPGA competitively inhibited their counterpart conjugations catalyzed by UGT2B7 with a \(K_i\) value (0.68 \(\pm\) 0.15 mM for UDPGA and 0.75 \(\pm\) 0.15 mM for UDPG) close to those determined with human liver microsomes.

Discussion

Glucuronidation reactions are catalyzed by the microsomal UGT. Consistent with its broad substrate profile, UGT is known to exist as a superfamily of enzymes (Mackenzie et al., 1997). Parallel to the progress in expression and characterization of UGT isoforms in human and laboratory animals (Tukey and Strassburg, 2000), recognition of UGT isoforms responsible for glucuronidation of given compounds has received increasing attention recently (Coffman et al.,...
and glucoside conjugates by UGT2B7. In addition, compound A
comparable catalytic capacity in the formation of both glucuronide
1A7 for benzo(a)pyrene-3,6-quinol), the present study revealed a
active glucuronide conjugate (UGT1A1 for bilirubin and UGT1A6 and
formation of glucoside conjugate was much lower than their respec-
tive isoforms. In contrast to the cases of bilirubin (Senafi et al., 1994) and
arations. In contrast to the cases of bilirubin (Senafi et al., 1994) and
UGT2B7 can use both UDPGA and UDPG as cosubstrates for me-
tabolism of this compound to transfer the respective sugar moiety to
responsible for its glucosidation as well as glucuronidation. Clearly,
UGT2B7 can use both UDPGA and UDPG as cosubstrates for meta-
tabolism of this compound to transfer the respective sugar moiety to
this compound with a comparable catalytic capacity ($V_{\text{max}}$) for both
UGPAs and UDPG (Table 5), but other isoforms can only have
UGPGA as a cosubstrate. This finding may explain the lack of
correlation between these two conjugations among 16 subjects (Fig.
5). Possible involvement in the glucosidation of other isoforms that
are not commercially available cannot be ruled out at the present time.

Glucuronidation has been considered as the most common phase II
reaction for a wide variety of endogenous substrates and xenobiotics
in mammals. Usually, if glucuronidation is possible, the remaining
types of glycosidations would be of minor importance in animals,
although they are common in plants and invertebrates (Tang, 1990).
Interestingly, we found that compound A significantly underwent both
acyl glucuronidation and acyl glucosidation in human liver
microsomes with the glucoside formation accounting for ~30 to 80% of
glucuronide conjugation in 16 native human liver microsomal
preparations. In contrast to the cases of bilirubin (Senafi et al., 1994) and
benzo(a)pyrene-3,6-quinol (Gschaidmeier et al., 1995), where the
formation of glucoside conjugate was much lower than their respec-
tive glucuronide conjugate (UGT1A1 for bilirubin and UGT1A6 and
1A7 for benzo(a)pyrene-3,6-quinol), the present study revealed a
comparable catalytic capacity in the formation of both glucuronide
and glucoside conjugates by UGT2B7. In addition, compound A

1997; King et al., 2001; Vashishtha et al., 2001). However, after
Senafi et al. (1994) used a cloned human bilirubin UGT (UGT1A1) to
investigate UDP-sugar specificity, little effort has been reported on
the UDP-sugar specificity of UGT isoforms in phase II metabolism of
drugs. Using stably expressed UGTs in the present study, we demon-
strate that a single UGT isoform can catalyze both glucuronidation
and glucosidation of an endothelin ETA receptor antagonist in human
liver microsomes. Although multiple UGTs (1A1, 1A3, 1A9, 2B7,
and 2B15) were involved in its glucuronidation, only UGT2B7 was
responsible for its glucosidation as well as glucuronidation. Clearly,

TABLE 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Correlation Coefficient $r^2$</th>
<th>Glucuronidation Activity (pmol/min mg protein)</th>
<th>Glucosidation Activity (pmol/min mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No activator</td>
<td>0.14 ($p &gt; 0.05$)</td>
<td>254 ± 60</td>
<td>132 ± 45</td>
</tr>
<tr>
<td>+ Alamethicin</td>
<td>0.40 ($p &gt; 0.05$)</td>
<td>853 ± 232</td>
<td>205 ± 79</td>
</tr>
<tr>
<td>+ Brij 58</td>
<td>0.12 ($p &gt; 0.05$)</td>
<td>504 ± 121</td>
<td>200 ± 63</td>
</tr>
</tbody>
</table>

Glucosidation may serve as an alternative de-
conjugation pathway when levels of UDPGA are depleted due to
toxico logical or pathological consequences or in cases where agly-
cones preferentially undergo glucosidation.

The relative significance of glucuronidation and glucosidation of
compound A may depend on a couple of factors. The concentration of
the nucleotide sugars is an important determinant. These two conju-
gations may compete for the UDP-sugars based on the fact that
UDPAGA and UDPG competitively inhibited their counterpart conju-
gations with comparable $K_i$ values (Fig. 4), and similar $K_m$ values

![Fig. 6. Rate of compound A glucuronidation and glucosidation catalyzed by stably expressed UGT isoforms.](image)

Data are expressed as mean ± S.E. of triplicates.

![Fig. 7. Concentration-dependent rate of glucuronide formation of compound A by UGT1A9.](image)

Data are expressed as mean ± S.E. of triplicates.
were observed for both UDPGA and UDPG (Table 3). Therefore, the actual concentrations of individual UDP-sugars are critical to the ratio of these two conjugations. It has been found that in a number of vertebrates, hepatic UDPGA concentration is not significantly different from UDPG (except for guinea pig liver where UDPGA level exceeds about two times that of UDPG), but UDPG is synthesized at a higher rate than UDPGA in rat, guinea pig, and chicken livers (Zhitkov and Toshova, 1986). It is worth noting that considerable fluctuations in UDP-sugar concentration have been observed after drug and hormone treatment (Toshova and Zhitkov, 1976, 1977). Therefore, the change in relative significance of compound A glucuronidation and glucosidation is likely where UDPGA and UDPG are altered due to toxicological and pathological impacts.

The relative activity of UGT2B7 in different subjects may also be a source of variability. The variation of the rate of compound A glucosidation in liver microsomal preparations from different donors may be derived mainly from the different activity of UGT2B7, which was characterized in the present study as the only UGT isofrom catalyzing acyl glucosidation of compound A in human liver microsomes. Of human isoforms comprising the UGT2B subfamily, UGT2B7 is of particular significance in drug metabolism. Notably, it glucuronidates numerous carboxylic acid-containing drugs (nonsteroidal anti-inflammatory drugs, clofibric acid and valproic acid) and opioids (Jin et al., 1993; Coffman et al., 1998). Compound A, also a carboxylic acid, was found to be comparably conjugated with UDPG and UDPGA by UGT2B7. Since its glucuronidation was mediated by multiple UGT isoforms (Fig. 6), any change in UGT2B7 activity would exert more significant impact on its glucuronidation than its glucosidation. Beside liver, UGT2B7 also has been found to a varying degree in extrahepatic tissues (gastrointestinal tract, brain, and kidney) in human (King et al., 2000). As a result, it is reasonable to expect different glucosidation activity for compound A in different tissues. Interestingly, several studies have shown higher glucosidation activity in kidney microsomes than in liver microsomes (Chmela et al., 2001; Shipkova et al., 2001). Whether it could be the same case with compound A needs to be explored in future studies.

While for P450 catalyzed reactions, where more than one isoforms with different Michaelis-Menten kinetics participate, the primary determinant is concentration of a substrate at the active site of the isoform (Chesne et al., 1998; Ko et al., 1998). Such is not a case with compound A conjugations by either sugar due to the comparable apparent $K_m$ values determined in human liver microsomes (Table 2). The relatively low $K_m$ values (~10 μM) for compound A in both conjugations suggest that compound A is a good substrate for UGTs, whereas most compounds reported for glucuronidation and glucosidation tend to show much higher $K_m$ values (Senafi et al., 1994; Innocenti et al., 2001; Shipkova et al., 2001). Interestingly, much lower $K_m$ values for compound A were obtained in the expressed UGT1A isoforms, especially in UGT1A9 (only 0.23 μM), but the values obtained in the expressed UGT2B isoforms remained comparable to those measured in human liver microsomes.

There have been several reports of the formation of either glucosides in the literature. To the best of our knowledge, this is the first description of an acyl glucoside conjugate of a drug generated by human liver microsomes. Another drug acyl glucoside formed in human previously reported is mycophenolic acid glucoside, but it was only generated in human kidney microsomes at a low level (Shipkova et al., 2001). Chemically, acyl glucuronides and acyl glucosides belong to the same class of electrophilic metabolites. Acyl glucuronides are known for their nonenzymatic reactions including acylation and/or glycation of endogenous macromolecules, hydrolysis to reform the parent aglycone, and intramolecular rearrangement (Salustio et al., 2000). However, the reactivity of acyl glucosides has attracted little attention probably due to their rare occurrence, especially in man. At present, our laboratory is active in evaluating the relative reactivity of acyl glucuronide and acyl glucoside conjugates of compound A, and this will be the subject of a separate manuscript.

In summary, the present study demonstrates that UGTs in human liver microsomes catalyze the transfer of glucuronic acid and glucose from UDPGA and UDPG, respectively, to compound A, a new, selective and potent endothelin ETα antagonist, resulting in extensive acyl glucuronidation and acyl glucosidation. Using commercially available microsomes expressing UGT isoforms, it is found that the glucosidation is mediated by a single isoform (UGT2B7), whereas the glucuronidation is catalyzed by multiple isoforms (UG1A1, 1A3, 1A9, 2B7 and 2B15). UGT2B7 possesses a comparable catalytic capacity for both conjugations.

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