Glucuronidation of OTS167 in Humans Is Catalyzed by UDP-Glucuronosyltransferases UGT1A1, UGT1A3, UGT1A8, and UGT1A10

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ABSTRACT

OTS167 is a potent maternal embryonic leucine zipper kinase inhibitor undergoing clinical testing as an antineoplastic agent. We aimed to identify the UDP-glucuronosyltransferases (UGTs) involved in OTS167 metabolism, study the relationship between UGT genetic polymorphisms and hepatic OTS167 glucuronidation, and investigate the inhibitory potential of OTS167 on UGTs. Formation of a single OTS167-glucuronide (OTS167-G) was observed in pooled human liver (HLM) (K_m = 3.4 ± 0.2 μM), intestinal microsomes (HIM) (K_m = 1.7 ± 0.1 μM), and UGTs. UGT1A1 (64 μl/min/mg) and UGT1A8 (72 μl/min/mg) exhibited the highest intrinsic clearances (CL_i) for OTS167, followed by UGT1A3 (51 μl/min/mg) and UGT1A10 (47 μl/min/mg); UGT1A9 was a minor contributor. OTS167 glucuronidation in HLM was highly correlated with thyroxine glucuronidation (r = 0.91, P < 0.0001), SN-38 glucuronidation (r = 0.79, P < 0.0001), and UGT1A1 mRNA (r = 0.72, P < 0.0001). Nilotinib (UGT1A1 inhibitor) and emodin (UGT1A8 and UGT1A10 inhibitor) exhibited the highest inhibitory effects on OTS167-G formation (HLM 68%, HIM 47%). We hypothesize that OTS167-G is an N-glucuronide according to mass spectrometry. A significant association was found between rs6706232 and reduced OTS167-G formation (P = 0.03). No or weak UGT inhibition (range: 0–21%) was observed using clinically relevant OTS167 concentrations (0.4–2 μM). We conclude that UGT1A1 and UGT1A3 are the main UGTs responsible for hepatic formation of OTS167-G. Intestinal UGT1A1, UGT1A8, and UGT1A10 may contribute to first-pass OTS167 metabolism after oral administration.

Introduction

OTS167 is a novel synthetic molecule undergoing clinical development as an anticancer agent. It has been found to be a very potent inhibitor (IC_{50} of 0.41 nM) (Chung et al., 2012) of maternal embryonic leucine zipper kinase, a protein kinase aberrantly upregulated in many types of cancer and essential for survival and proliferation of undifferentiated cancer cells (Ganguly et al., 2014).

Glucuronidation is a common conjugation reaction catalyzed by UDP-glucuronosyltransferase (UGT) enzymes. The reaction involves the transfer of glucuronic acid from UDP-glucuronic acid (UDPGA) to an acceptor substrate containing hydroxyl, carboxyl, amino, or sulfhydryl groups to generate more polar and excretable compounds (Tukey and Strassburg, 2000). The human UGTs are classified into families UGT1, UGT2, and subdivided into UGT1A, UGT1B, UGT1C, UGT2A, UGT2B, UGT2C, and UGT2D subfamilies based on gene structure and sequence homology (Guillemette, 2003). The UGT1A enzymes are generated by alternative splicing of unique exon 1 to the common exons 2–5, whereas the UGT2Bs are individually transcribed. There are nine UGT1As (UGT1A1, UGT1A3–UGT1A10), all of which are expressed in the gastrointestinal tract. UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, and UGT1A9 are expressed in the liver, the major site of glucuronidation, whereas UGT1A7 and UGT1A8 and UGT1A10 are extrahepatic (Tukey and Strassburg, 2000, 2001; Finel et al., 2005). All seven UGT2B enzymes (UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28) are found in the liver (Tukey and Strassburg, 2000, 2001; Lévesque et al., 2001). Many polymorphisms exist in the UGT genes (http://www.pharmacogenomics.pha.ulaval.ca/cms/ugt_alleles/; accessed Nov. 26, 2014). UGT genetic variation could have a significant impact on metabolism of endogenous compounds such as bilirubin (Bosma, 2003), and on variability in response to drugs such as irinotecan among others (Ramírez et al., 2010).

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ABBREVIATIONS: CL_{int}, intrinsic clearance; DMSO, dimethylsulfoxide; HIM, human intestinal microsomes; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; imipramine-G, imipramine N-β-D glucuronide; K-S, Kolmogorov-Smirnov; MS, mass spectrometry; 4-MU, 4-methylumbellifereone; 4-MU-G, 4-methylumbelliferyl-β-D-glucuronic acid hydrate; OTS167-G, OTS167-glucuronide; single nucleotide polymorphisms (SNPs); UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.
in human livers; and (3) evaluate the potential inhibitory effects of OTS167 on glucuronidation reactions.

Materials and Methods

Chemicals and Reagents
OTS167 was provided by OncoTherapy Science (Kawasaki City, Kanagawa, Japan). Coumarin, 4-methylumbelliferone (4-MU), 4-methylumbelliferyl-β-D-glucuronic acid (4-MU-G), diclofenac sodium salt, sulfipyrazone, imipramine, hecogenin, niflumic acid, emodin, potassium phosphate monobasic, β-glucuronidase (type IX-A, from Escherichia coli), and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Imipramine N-β-D glucuronide (imipramine-G) and nilotinib were purchased from Toronto Research Chemicals (North York, Ontario, Canada). UGT Reaction Mix Solution A (25 mM UDPGA) and Solution B (250 mM Tris-HCl, 40 mM MgCl2, and 0.125 mg/ml glucuronide hydrate) were acquired from BD Biosciences (Bedford, MA). Acetonitrile, methanol, formic acid, glacial acetic acid, ammonium acetate, 7-hydroxycoumarin, and sodium phosphate monobasic and dibasic were purchased from Thermo Fisher Scientific (Hanover Park, IL).

Microsomal Preparations
Normal human livers were obtained with human subjects’ approval through the Liver Tissue Cell Distribution System (Pittsburgh, PA; National Institutes of Health Contract HHSN276201200017C) and the Cooperative Human Tissue Network. HLM were prepared using differential centrifugation methods (Ramirez et al., 2007). Pooled HLM and microsomes from cDNA-transfected baculovirus-insect cells (Supersomes) expressing UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B10, UGT2B15, UGT2B17, and HLM) were investigated in the presence of UGT inhibitors. Incubations were performed, as described above, using OTS167 at 305 nm. Retention times for OTS167, OTS167-G, and coumarin were 15.2, 12.7, and 17.7 minutes, respectively.

As OTS167-G was not available, its relative formation was estimated using a standard curve generated with OTS167. Standards were made by serial dilutions of OTS167 in methanol and further diluted 100× in deionized water. To be consistent with the microsomal incubations, standards (100 μl) were maintained at 37°C for 30 minutes (incubations with UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, UGT2B10, UGT2B15, UGT2B17, and HLM) or 35 minutes (incubations with HIM, UGT1A7, UGT1A8, and UGT1A10), and processed, as described above. Calibration curves were linear over the concentration range of 0.31–10 μM (r² > 0.99).

Enzymatic hydrolysis using β-glucuronidase was used to confirm OTS167-G formation by HLM and HIM. OTS167 incubations were dried down under nitrogen gas and reconstituted in 2000 μl 0.1 M sodium phosphate buffer. After 24-hour incubation at 37°C, 400 μl ice-cold methanol was added. Samples were dried under nitrogen gas and reconstituted with a 50/50 (v/v) mix of 2 mM ammonium acetate containing 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B) prior to injection into the HPLC. Control incubations were performed simultaneously with 0.1 M sodium phosphate buffer only (no β-glucuronidase added).

Measurement of OTS167 Glucuronidation
HPLC separation was achieved at room temperature using an Atlantis dC18 column (5 μM, 3.0 × 250 mm; Waters, Milford, MA) preceded by an Atlantis dC18 guard column (5 μM, 3.9 × 20 mm). The following mobile phase gradient was applied at a flow rate of 1 ml/min: 0–2 minutes, 90% A and 10% B; 2.1–17 minutes, switch from 90% A and 10% B to 65% A and 35% B; 17.1–20 minutes, hold at 65% A and 35% B; and 20.1–25 minutes, hold at 90% A and 10% B. The eluate was monitored for 25 minutes using UV detection at 205 nm. Retention times for OTS167, OTS167-G, and coumarin were 15.2, 12.7, and 17.7 minutes, respectively.

Inhibition of OTS167 Glucuronidation
OTS167 glucuronidation by HLM, HIM, and UGTs (UGT1A1, UGT1A3, UGT1A8, UGT1A9, and UGT1A10) was investigated in the presence of UGT inhibitors. Incubations were performed, as described above, using OTS167 at

Experimental design and data analysis
Experiments were conducted to study the linearity of OTS167 glucuronidation (OTS167-G) formation with respect to time and protein using pooled HLM and HIM. Initially, OTS167 (10 μM in methanol) was combined with UGT Reaction Mix Solution B (50 mM Tris–HCl, 8 mM MgCl2, and 0.025 mg/ml hecogenin) and 1 mg/ml pooled HLM or HIM on ice for 15 minutes. The concentration of methanol in the reaction was kept at 1% (v/v). After equilibration for 5 minutes in a 37°C water bath, reactions were initiated by addition of UGT Reaction Mix Solution A (2 mM UDPGA). The final reaction volume was 100 μl. Samples were incubated for various times (range: 10–120 minutes, 8 time points). Reactions were terminated with 100 μl cold acetonitrile, followed by addition of 10 μl internal standard (200 μM coumarin in acetonitrile). Samples were vortexed for 10 seconds and centrifuged at 20,817g for 15 minutes at 4°C. Aliquots (20–40 μl) of supernatant were injected into a high-performance liquid chromatography (HPLC) system (Hitachi High Technologies America, Schaumburg, IL). For determining optimal protein concentration, samples were incubated for 30 or 35 minutes (optimum time for HLM and HIM, respectively). Control incubations were simultaneously performed in the absence of OTS167, UDPGA, HLM, and HIM.

Correlation Studies in HLM
Incubations with 46 individual HLM (34 male, 12 female) were performed, as described above, and contained 3.4 μM OTS167. Measurements of glucuronidation activities toward thyroxine (UGT1A1 and UGT1A3 substrate) (Tong et al., 2007; Yamanaka et al., 2007; Yoder Gruber et al., 2007; Kato et al., 2008), SN-38 (UGT1A1 substrate) (Iyer et al., 1998; Hanioka et al., 2001; Gagné et al., 2002), mycophenolic acid (UGT1A9 substrate) (Bernard and Guillemette, 2004; Miles et al., 2005), and testosterone (UGT2B17 substrate) (Torgeon et al., 2001), and quantitation of mRNA levels (UGT1A1, UGT1A3, UGT1A9, and UGT2B17) in this set of HLM were previously described (Iyer et al., 1999; Ramírez et al., 2007; Yoder Gruber et al., 2007; Kang et al., 2010; Liu et al., 2014).
the respective $K_{m}$ values (HLM, 3.4 $\mu$M; HIM, 1.7 $\mu$M; UGT1A1, 5.7 $\mu$M; UGT1A3, 2.2 $\mu$M; UGT1A8, 3.7 $\mu$M; UGT1A10, 0.9 $\mu$M) except for UGT1A9. Incubations with UGT1A9 were performed at 3.4 $\mu$M ($K_{m}$ for HLM) as its $K_{m}$ could not be estimated. Incubations included the following inhibitors: 2.5 $\mu$M nitroimid, (dissolved in DMSO) for UGT1A1 (Ai et al., 2013), 500 $\mu$M imipramine (in deionized water) for UGT1A3 (Lu et al., 2009), 2.5 $\mu$M niflumic acid (in DMSO) for UGT1A9 (Miners et al., 2011), and 100 $\mu$M emodin (in DMSO) for UGT1A8 and UGT1A10 (Watanabe et al., 2002). Control incubations contained vehicle (no inhibitor added). Total concentration of organic solvents in incubations was 1% (v/v).

Genotyping

UGT polymorphisms significantly associated with UGT1A1 and UGT1A3 phenotypes (Yamamoto et al., 1998; Jinno et al., 2003; Liu et al., 2014) were genotyped, as previously described (Innocenti et al., 2004; Liu et al., 2014). These gene variants were the functional single nucleotide polymorphisms (SNPs) UGT1A1*28 (rs175347, -53[TA]) in the UGT1A1 promoter) and UGT1A1*6 (rs148323, 211G>A, G71R, in UGT1A1 exon 1), and the tag SNPs (Liu et al., 2014), rs706232 (UGT1A3 E27E), rs10203853 (in the UGT1A3 3'-flanking region), and rs33979061 (in UGT1A1 intron 1).

Inhibition of UGTs by OTS167

4-MU Glucuronidation. 4-MU was used as nonspecific substrate to evaluate the inhibitory potential of OTS167 on the majority of the UGTs (UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17). Incubations contained 4-MU (concentrations from Dong et al., 2012), UGTs (concentrations reported by Liu et al., 2010, except that UGT1A10 was used at 0.25 mg/ml), 2.5 mM UDPGA, 50 mM Tris-HCl (pH 7.5), 8 mM MgCl$_2$, 25 $\mu$g/ml alamethicin, 500 $\mu$M imipramine, 0.5 mg/ml protein, and OTS167 (0.4 $\mu$M, 2 $\mu$M, and 10 $\mu$M), or hecogenin (200 $\mu$M in methanol as positive control for inhibition). The negative (vehicle) control contained 1% methanol. Reactions were stopped after 60 minutes with ice-cold acetonitrile containing 100 $\mu$M 7-hydroxyxocoumarin as internal standard and centrifuged at 20,817 relative centrifugal force for 15 minutes (4°C). Supernatants (10 $\mu$l) were analyzed by HPLC. The mobile phase consisted of 28/72 (v/v) acetonitrile/10 mM potassium phosphate monobasic (pH 2.6) at a flow rate of 1 ml/min. Separation was achieved with a XTerra RP18 column (4.6 x 100 mm, 5 mm; Waters Corporation, Milford, MA) and a Nova-Pak C18 (4 $\mu$m) guard column (Waters Corporation). UV detection (254 nm) was used. Imipramine-G standards ranged from 1.9 $\mu$M to 30 $\mu$M.

Identification of OTS167-G

To determine the mass and structure for OTS167-G, microsomes (1 mg/ml HLM, 0.5 mg/ml UGT1A1, and 0.5 mg/ml UGT1A8) were incubated with 10 $\mu$M OTS167 for 90 minutes, as described above. The reaction was stopped with addition of 100 $\mu$L ice-cold acetonitrile, subjected to HPLC, and the metabolite peak (eluting around 13 minutes) was collected for mass spectrometry (MS). The latter was dried down under nitrogen gas, reconstituted in methanol, and subjected to MS using an API 2000 MS/MS triple-quadrupole system (Applied Biosystems, Foster City, CA). Additionally, OTS167 was analyzed. OTS167 and its metabolite (from 3 different incubation mixtures: HLM, UGT1A1, and UGT1A8) were infused at 40 $\mu$L/min with a syringe pump. The electrospray ionization voltage was set at $+5500$ V with a temperature of 50°C. Positive ion Q1 monitoring was performed along with product ion monitoring (+MS2) of OTS167-G. The curtain, collision, nebulizer, and heater gases were set at 35, 5, 50, and 70 psi, respectively. The declustering potential, focusing potential, entrance potential, collision energy, and collision cell entrance potential voltages were 90, 400, 7, 50, and 18 V, respectively.

Data Analysis and Statistics. Formation rates of OTS167-G, 4-MU-G, and imipramine-G were expressed as pmol/min/mg total protein. Results represent the mean of a single experiment performed in triplicate ( assay optimization, UGT screening, kinetic studies, and inhibition of OTS167) or duplicates (correlation and inhibition studies by OTS167). Apparent kinetic parameters for HLM, HIM, and UGTs were estimated by fitting OTS167 glucuronidation rates versus OTS167 concentrations by nonlinear regression. The Enzyme Kinetics Module from SigmaPlot 12.3 (Systat Software, San Jose, CA) was used to analyze and plot kinetics data. The Kolmogorov-Smirnov (K-S) test was used to test whether glucuronidation rates and mRNA expression levels followed a normal distribution. The glucuronidation rates of thyroxine, mycophenolic acid, and testosterone were not normally distributed and were log transformed prior to statistical analyses, after which they passed the normality test (K-S distance = 0.09–0.11, P > 0.10). Formation rates of OTS167 and SN-38
glucuronides were apparently normal (K-S distance = 0.10, \( P < 0.01 \) for both). The mRNA levels of UGT1A1, UGT1A3, UGT1A9, and UGT2B17 were also log transformed to pass the normality test (K-S distance = 0.08-0.12, \( P > 0.10 \)). Pearson correlation was used to test the association among glucuronidation activities measured with different substrates, and between activities and mRNA expression. Multivariate analysis to investigate the contribution of UGT mRNA levels to variability in OTS167 glucuronidation was done with Microsoft Excel 2010. In inhibition experiments, residual activity was calculated by dividing the amount of glucuronide formed in the presence of inhibitor by that formed in its absence. Correlations between UGT genotypes and OTS167 glucuronidation were tested using linear regression analysis. The \( P \) values of the linear regressions test the null hypothesis that the slope is equal to 0. Results were considered statistically significant when \( P < 0.05 \). Data were analyzed using GraphPad Prism 6.00 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com), unless specified otherwise.

### Results

#### Identification of OTS167-G in Microsomal Incubations

In incubations containing OTS167, UDPGA, and HLM showed formation of a single product with a shorter retention time (12.7 minutes) than OTS167 (15.2 minutes) (Fig. 2A), suggesting formation of a metabolite that is more polar than its parent compound. The conjugated

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**Fig. 3.** Michaelis-Menten and Eadie-Hofstee plots for formation of OTS167-G by HLM (A), HIM (B), UGT1A1 (C), and UGT1A8 (D). Incubations were performed over a substrate concentration range of 0.5–100 \( \mu M \). Data are shown as the mean ± S.E.M. of triple determinations in a single experiment.

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**TABLE 1**

<table>
<thead>
<tr>
<th>UGTs</th>
<th>( r^2 )</th>
<th>( Km (\mu M) )</th>
<th>( V_{max} (\text{pmol/min/mg}) )</th>
<th>( Ki (\mu M) )</th>
<th>( CL_{int} (\mu l/min/mg) )</th>
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</thead>
<tbody>
<tr>
<td>HLM</td>
<td>0.991</td>
<td>3.4 ± 0.2</td>
<td>164 ± 4</td>
<td>168 ± 15</td>
<td>48</td>
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<tr>
<td>HIM</td>
<td>0.985</td>
<td>1.7 ± 0.1</td>
<td>151 ± 3</td>
<td>276 ± 33</td>
<td>89</td>
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<tr>
<td>UGT1A1</td>
<td>0.991</td>
<td>5.7 ± 0.4</td>
<td>365 ± 13</td>
<td>179 ± 24</td>
<td>64</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>0.992</td>
<td>2.2 ± 0.1</td>
<td>112 ± 2</td>
<td>382 ± 41</td>
<td>51</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>0.997</td>
<td>3.7 ± 0.1</td>
<td>265 ± 4</td>
<td>382 ± 42</td>
<td>72</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>0.888</td>
<td>0.9 ± 0.1</td>
<td>42 ± 0.7</td>
<td>428 ± 57</td>
<td>47</td>
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</table>
The difference in intrinsic metabolic clearances (CLint) for OTS167 (Ki = 168 and 276 pmol/min/mg) was less than twofold. Glucuronidation affinity for the substrate (Km = 3.4 M) for HLM and HIM was less than twofold. Kinetic parameters are summarized in Table 1. Both microsomal systems showed high substrate inhibition kinetics (Fig. 3, A and B). Kinetic parameters are shown for OTS167 (72 pmol/min/mg) and OTG1A1 (64 pmol/min/mg) had the highest CLint for OTS167, followed by OTG1A3 (51 pmol/min/mg) and OTG1A10 (47 pmol/min/mg) (Table 1). These UGTs showed high affinity for the substrate (Km = 0.9–5.7 M). OTS167-G formation was inhibited with substrate concentrations higher than 25 μM (UGT1A1, UGT1A3, and UGT1A8) or 10 μM (UGT1A10). K values were high (range: 179–428 μM).

Correlation of OTS167 Glucuronidation with UGT Activities and mRNA Levels. OTS167-G formation was measured in 46 Caucasian HLM. The average rate of OTS167 glucuronidation (mean ± S.D.) was 100.7 ± 35.8 pmol/min/mg (range: 35.6–195.2 pmol/min/mg). The interindividual variation in OTS167-G formation was 36%. Rates of OTS167 glucuronidation correlated strongly with thyroxine (r = 0.91, P < 0.0001) (Fig. 5A) and SN-38 (r = 0.79, P < 0.0001) (Fig. 5B) glucuronidation, and with UGT1A1 mRNA levels (r = 0.72, P < 0.0001) (Fig. 5C). Moderate correlations were observed with mycophenolic acid glucuronidation (r = 0.50, P = 0.001) and mRNA levels of UGT1A3 (r = 0.36, P = 0.01) (Fig. 5D) and UGT1A9 (r = 0.42, P = 0.004). Multivariate analysis performed to investigate the contribution of UGT1A1, UGT1A3, and UGT1A9 mRNA levels to variability in OTS167 glucuronidation identified mRNA levels of UGT1A1 (P < 0.0001) as more important predictors than those of UGT1A3 (P = 0.30) and UGT1A9 (P = 0.11). Testosterone glucuronide formation and UGT2B17 mRNA levels were used as negative controls for correlations. OTS167 and testosterone glucuronidation rates were not correlated (r = –0.05, P = 0.77), and the correlation between OTS167-G and UGT2B17 mRNA levels was weak and insignificant (r = 0.25, P = 0.10).

Inhibition of OTS167 Glucuronidation. The effect of chemical inhibitors was studied to confirm the UGTs involved in OTS167 glucuronidation. Results are shown in Fig. 6. Nilotinib showed strong inhibitory effects on OTS167-G formation by UGT1A1 (83%) (Fig. 6A) and HLM (68%) (Fig. 6B), whereas inhibition of metabolism by HIM (25%) (Fig. 6B) was modest. Imipramine was found to inhibit OTS167-G production by 45%, 34%, and 19% in incubations with UGT1A3 (Fig. 6A), HLM, and HIM (Fig. 6B), respectively. In incubations with UGT1A1, UGT1A3, UGT1A8, and UGT1A10. Representative kinetic profiles are shown for UGT1A1 (Fig. 3C) and UGT1A8 (Fig. 3D). UGT1A8 (72 pmol/min/mg) and UGT1A1 (64 pmol/min/mg) had the highest CLint for OTS167, followed by UGT1A1A3 (51 pmol/min/mg) and UGT1A10 (47 pmol/min/mg) (Table 1). These UGTs showed high affinity for the substrate (Km = 0.9–5.7 M). OTS167-G formation was inhibited with substrate concentrations higher than 25 μM (UGT1A1, UGT1A3, and UGT1A8) or 10 μM (UGT1A10). K values were high (range: 179–428 μM).

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incubations with emodin, OTS167 glucuronidation was inhibited by 76%, 100%, 65%, and 47% in incubations with UGT1A8 (Fig. 6A), UGT1A10 (Fig. 6A), HLM (Fig. 6B), and HIM (Fig. 6B), respectively. Niflumic acid completely inhibited OTS167 glucuronidation in the presence of UGT1A9 (Fig. 6A). Very low inhibition was found when coincubated with HLM (10%) and HIM (5%) (Fig. 6B).

Association of OTS167 Glucuronidation with Genotypes. Reduced OTS167 glucuronidation rates were observed across UGT1A1*28, rs6706232, and rs33979061 genotypes; the opposite effect was observed with rs10203853 (Table 2). A modest and significant association was observed between rs6706232 genotypes (Supplemental Fig. 1, A–D, 3, 4, and 5). The UGT1A1*6 carrier did not have the UGT1A1*28 mutation. Other genotypes (rs6706232, rs33979061, and rs10203853) genotypes did not reach statistical significance (P > 0.05).

To assess the effect of the UGT1A1*6 polymorphism on OTS167 glucuronidation, incubations were performed using HLM from a donor heterozygous for UGT1A1*6. Additional samples with UGT1A1*6 were not available. The OTS167-G formation rate was low (57.8 pmol/min/mg) compared with the average velocity (100.7 pmol/min/mg) in 46 Caucasian HLM (all G/G). This UGT1A1*6 carrier did not have the UGT1A1*28 mutation. The other genotypes (rs6706232, rs33979061, and rs10203853) were not available.

Inhibition of UGTs by OTS167. To investigate whether OTS167 is a UGT inhibitor, we performed coincubations of OTS167 with 4-MU (nonspecific substrate for all UGTs except for UGT1A4 and UGT2B10) and imipramine (specific UGT1A4 substrate). OTS167 showed no marginal inhibition (<15%) of UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT2B4, UGT2B7, UGT2B15, and UGT2B17 at all concentrations tested (range: 0.4–10 μM) (Fig. 7). Similar results were observed for UGT1A1 (0–7% inhibition with 0.4 and 2 μM OTS167), UGT1A7 (0–11% with 0.4 and 2 μM OTS167), and UGT1A10 (7% with 0.4 μM OTS167) (Fig. 7A). At higher concentrations, relatively weak inhibition of UGT1A1 (36% with 10 μM OTS167), UGT1A7 (22% with 10 μM), and UGT1A10 (21% and 33% with 2 and 10 μM OTS167) was obtained (Fig. 7A). Residual activity (%) left after treatment with diclofenac, hecogenin, and sulfipyrazone (positive controls for inhibition) is shown in Table 3.

Identification of OTS167-G by MS/MS. Based on the structure of OTS167, the metabolite could be either an O-glucuronide or an N-glucuronide. We hypothesize that the metabolite is an N-glucuronide due to the fragmentation patterns of the molecule in Q1 (Supplemental Fig. 1). OTS167 (486.9 m/z) fragmented ions at 140.1, 244.3, and 347.8 (Supplemental Figs. 1, B and C, and 2). For all three experiments (HLM, UGT1A1, and UGT1A8), OTS167-G (663 m/z) similarly fragmented ions at 347.9, 365, and 487 (Supplemental Figs. 1, A–D, 3, 4, and 5). The masses at 347.8 m/z (Supplemental Fig. 1C), 486.9 m/z (Supplemental Fig. 1B), and 663 m/z (Supplemental Fig. 1A) were characteristic of having two chlorine atoms in the molecule due to peaks of M+1, M+2, and M+4 in a 9:6.1 ratio, respectively, whereas the fragment at 365 m/z (Supplemental Fig. 1D) lacked this characteristic and is consistent with a glucuronide attached to one of two aromatic N-heterocycles. A product ion scan of 663 m/z was not conclusive, as it produced fragments of 95.1, 139.8, 148, 347.6, and 486.7 m/z (consistent with the fragments found in Q1, but missing the identifying fragment of 365 m/z; data not shown).

**Discussion**

This is the first in vitro study to report glucuronidation as a metabolic pathway involved in OTS167 clearance. We demonstrated that UGT1A1 and UGT1A3 are the major hepatic UGTs involved in OTS167 metabolism. Among these, UGT1A1 appears to play the main role based on our experimental data and its high level of hepatic and intestinal expression (Nakamura et al., 2008; Izukawa et al., 2009; Ohno and Nakajin, 2009; Wu et al., 2011; Court et al., 2012; Harbourt et al., 2012; Rowland et al., 2013). Supporting data include high CLint

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>N</th>
<th>OTS167-G Formation (pmol/min/mg) (mean ± S.D.)</th>
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<th>P</th>
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<tr>
<td>UGT1A1*28</td>
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<td>18</td>
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<tr>
<td></td>
<td>*1/*28</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*2/*28</td>
<td>6</td>
<td>78.9 ± 28.2</td>
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<td>rs6706232</td>
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<td>11</td>
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<td>0.11</td>
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<tr>
<td></td>
<td>G/A</td>
<td>26</td>
<td>102.5 ± 32.2</td>
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<td>T/T</td>
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<td>rs33979061</td>
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<td>78.6 ± 31.5</td>
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for OTS167, significant correlations of OTS167 glucuronidation with UGT1A1 activity and mRNA levels in HLM, and high level of HLM inhibition with nilotinib, a highly selective UGT1A1 inhibitor at low concentrations (Ai et al., 2013). The decreased glucuronidation observed in HIM after treatment with nilotinib reflects inhibition of intestinal UGT1A8 and unaffected OTS167 glucuronidation activity by intestinal UGT1A8 and UGT1A10. We hypothesize that UGT1A3 is involved in OTS167-G formation to a lesser extent than UGT1A1, as the correlation between OTS167-G formation and UGT1A3 mRNA \( (r = 0.36) \) was not as strong as with UGT1A1 mRNA \( (r = 0.72) \), and UGT1A3 is expressed in the intestines at lower levels than UGT1A1 (Nakamura et al., 2008; Iizuka et al., 2009; Ohno and Nakajin, 2009; Wu et al., 2011; Court et al., 2012; Harbourt et al., 2012; Rowland et al., 2013), but it did not glucuronidate OTS167 at a significant extent, the correlations with UGT1A9-marker activity and mRNA levels were weak compared with the results with UGT1A1, and glucuronide production in HLM and HIM was weakly inhibited (5–10%) by a very strong UGT1A9 inhibitor.

Because UGT1A8 and UGT1A10 are expressed in the intestine but not in the liver (Ohno and Nakajin, 2009; Harbourt et al., 2012), we hypothesize that these enzymes could contribute to first-pass drug metabolism after oral administration of OTS167. UGT1A8 had higher CL\(_{int}\) for OTS167 than UGT1A10. Correlation studies could not be performed, as individual HIM were not available. The inhibition of OTS167-G formation observed in HIM very likely reflects complete inhibition of UGT1A10, high inhibition of UGT1A8, and inhibition of UGT1A1, as this enzyme is highly expressed in the intestine (Ohno and Nakajin, 2009; Wu et al., 2011; Court et al., 2012; Harbourt et al., 2012) and emodin inhibited glucuronidation in HLM. Emodin is a good UGT1A8 and UGT1A10 inhibitor, but is not highly selective (Yamanaka et al., 2007).

We hypothesize that OTS167 undergoes N-glucuronidation according to MS data. Various nitrogen-containing compounds are converted to N-glucuronides, a reaction catalyzed by multiple enzymes, including UGT1A1, UGT1A3, UGT1A4, UGT1A8, UGT2B4, UGT2B7, and UGT2B10 (Kaivosaari et al., 2011). This reflects the fact that aromatic heterocyclic substrates are structurally very diverse and UGTs involved in their glucuronidation are substrate-dependent (Kaivosaari et al., 2011). Our hypothesized structure for OTS167-G includes three possible sites where the glucuronide molecule might attach to the amine. Further NMR studies will be required to confirm our results.

We also identified potential genotypic predictors of OTS167 glucuronidation. The trends observed, although not always statistically significant, are in agreement with those previously observed with the UGT1A1 substrate SN-38: decreased glucuronidation activities with \( UGT1A1*28 \), rs6706232, and rs33970961, and increased glucuronide formation with rs10203853 (Liu et al., 2014). However, as the associations of rs6706232 and \( UGT1A1*28 \) with OTS167-G formation rates were modest, the effect of these SNPs on OTS167 clearance may not be significant. \( UGT1A1*6 \), an allele present in Asians with a 13–23% frequency (Guillemette, 2003), reduces the catalytic
efficiency of UGT1A1 (Premawardhena et al., 2003) and predicts irinotecan-related toxicity in that population (Ramírez et al., 2010). We observed reduced OSTS167 glucuronidation observed in a liver with UGT1A1*6. Additional studies are needed to evaluate its impact on OSTS167-G formation in individuals of Asian ancestry. It seems unlikely that OSTS167 will have clinically significant effects on the glucuronidation of other drugs. The degree of relevance of the in vitro inhibition of UGT1A1 (36%) and UGT1A10 (33%) by 10 μM OSTS167 is of no concern, and it is unlikely that significantly higher plasma concentrations will be maintained in patients.

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Authorship Contributions

Participated in research design: Ramírez, Mirkov, House, Ratain. Conducted experiments: Mirkov, House. Performed data analysis: Ramírez, Mirkov, House. Wrote or contributed to the writing of the manuscript: Ramírez, Mirkov, House, Ratain.

References


