FUNCTIONAL CHARACTERIZATION OF WILD-TYPE AND VARIANT (T202I AND M59I) HUMAN UDP-GLUCURONOSYLTRANSFERASE 1A10

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ABSTRACT:

UDP-glucuronosyltransferase (UGT) 1A10 is an isoform of UGT1A, which is expressed in extrahepatic, biliary and aerodigestive/gastrointestinal tissues. We have previously reported two nonsynonymous single nucleotide polymorphisms in exon 1 of human UGT1A10 gene; 177G>A and 605C>T resulting in amino acid alterations, M59I and T202I, respectively. In the present study, wild-type (WT) and these variant UGT1A10 cDNAs were transiently expressed in COS-1 cells for functional characterization. Glucuronidation activities in these COS-1 membrane fractions were assayed using 7-hydroxy-4-trifluoromethylcoumarin (HTFMC) and 17β-estradiol (E2) as substrates. WT and variant UGT1A10s catalyzed HTFMC glucuronidation with similar apparent Km values of approximately 5 μM, whereas the Vmax value of T202I normalized by the expressed UGT1A10 protein levels was nearly half of those of WT and M59I. High-performance liquid chromatography analysis of E2 glucuronide revealed that UGT1A10 catalyzed E2 3-O-glucuronidation but not 17-O-glucuronidation. Similarly, the three UGT1A10s catalyzed E2 3-O-glucuronidation with comparable apparent Km values (approximately 2 μM), whereas the normalized Vmax value of T202I was almost half that of WT and M59I. These results suggest that the lowered glucuronidation activity of T202I affects the gastrointestinal glucuronidation of orally administered chemicals and the enterohepatic circulation of biliary excreted metabolites.

UDP-glucuronosyltransferases (UGTs) are a group of phase II drug-metabolizing enzymes that are resident in endoplasmic reticulum. UGTs catalyze the conversion of hydrophobic endogenous compounds and xenobiotics to hydrophilic glucuronides, which subsequently undergo renal and biliary excretion (Burchell and Coughtrie, 1989). On the basis of their amino acid sequence homology and gene structures, UGTs have been classified into two families, UGT1 and UGT2 (Mackenzie et al., 1997; Burchell et al., 1998; de Wildt et al., 1989). On the basis of their amino acid sequence homology and gene structures, UGTs have been classified into two families, UGT1 and UGT2 (Mackenzie et al., 1997; Burchell et al., 1998; de Wildt et al., 1999).

The UGT1 family, to which UGT1A10 belongs, is known to include nine functional isoforms (UGT1A1, UGT1A3-UGT1A10). This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences (MPJ-1 and MPJ-6) of the Organization for Pharmaceutical Safety and Research of Japan.

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UDT1A2P, UGT1A11P, UGT1A12P, and UGT1A13P are thought to represent pseudogenes based on the existence of frame-shift mutations. The UGT1 family members have common carboxyl terminal sequences (245 amino acids in length) derived from four exons (exons 2–5) located at the 3’ end of the human UGT1 gene complex locus on chromosome 2q37 (Gong et al., 2001). A consecutively numbered array of first exon cassettes are positioned at the 5’ end of the common four exons and encode the amino terminal sequence of each UGT1 family member. Expression of each UGT1A isoform is under the control of its own unique promoter located upstream of exon 1, resulting in polymorphic expression of UGT1A isoforms. It has been reported that UGT1A10 is expressed in a tissue-specific manner. Expression of UGT1A10 has been detected in the biliary tissue, aerodigestive tract, stomach, small intestines (duodenum, jejunum, and ileum), and colon but not in the liver and lung (Strassburg et al., 1997, 1998, 2000; Mojarrabi and Mackenzie 1998; Cheng et al., 1999, Zheng et al., 2002). The limited expression profile of UGT1A10 implies that this enzyme plays an important role in the removal of orally administered drugs or ingested environmental toxicants. UGT1A10 has been shown to participate in the glucuronidation of mycophenolic acid, an active metabolite of mycophenolate mofetil (Mojarrabi and Mackenzie, 1997), a selective estrogen receptor modulator raloxifene, (Kemp et al., 2002) and 7-hydroxy-benz[a]pyrene (Zheng et al., 2002).
Recently, we found two novel non-synonymous single nucleotide polymorphisms in exon 1 of human UGT1A10 gene; 605C>T (T202I) and 177G>A (M59I) (Saeki et al., 2002). In the present study, wild-type and these variant human UGT1A10s were transiently expressed in COS-1 cells for functional characterization, and the kinetic analysis of their glucuronidation activities was carried out using 7-hydroxy-4-trifluoromethoxy coumarin (HTFMC) and 17ß-estradiol (E2) as substrates.

Materials and Methods

Materials. Human stomach polyA+ RNA was obtained from OriGene Technologies (Rockville, MD). HTFMC and HTFMC glucuronide were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Sigma-Aldrich (St. Louis, MO), respectively. E2 was purchased from Wako Pure Chemicals (Osaka, Japan), and 3-O- and 17-O-glucuronide were from Sigma-Aldrich.

Plasmid Construction. Oligo(dT) primed cDNA was synthesized from human stomach polyA+ RNA using a SuperScript First-strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The cDNA encoding wild-type UGT1A10 was amplified into the pDONR201 vector (Invitrogen) by the Gateway cloning technology (Walhout et al., 2000). Briefly, the UGT1A10 cDNA was first amplified by 10-cycle PCR using the gene specific primers (5'-AAAAAGCAGGCTG-CAGTTTCTCTATGGTCCG-3' and 5'-AAAGAAGCTTGTCATCAGTGGGTTCGTTG-3'). The 12-base pairs partial attB sequences at the 5'-end are underlined. Then an aliquot of the reaction mixture was subjected to a second round of PCR (25 cycles) using the following attB adaptor primers; 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3' and 5'-GGGACA-CACCTTTGTGACAGAAGCTGGTGG-3'. The resulting attB-flanked product was cloned into pDONR201 vector using the Gateway BP reaction, a recombination between attB site and attP site. Mutations were introduced into the wild-type UGT1A10 cDNA clone in pDONR201, using a QuikChange multi site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotide primers to construct T202I (605C>T) and M59I (177G>A) variants by PCR were 5'-phospho-GGGGACAATTTGTTTACAAAAAGCAGGCT-3' and 5'-GGGAC-CACCTTTGTGACAGAAGCTGGTGG-3', respectively. The single base changes are underlined. To ensure no mistakes had been introduced during the amplification process, all the plasmid constructs were verified by DNA sequencing of both strands. Subcloning of each UGT1A10 fragment from pDONR201 into pcDNA-DEST40 was performed by the Gateway LR reaction, a recombination between attL site and attR site.

Expression of Wild-Type and Variant UGT1A10s in COS-1 Cells. COS-1 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The day before transfection, COS-1 cells were plated in 100-mm culture dishes at a density of 5.5 × 104 cells/cm2. On the following day, the culture medium was replaced with 8 ml of Opti-MEM (Invitrogen), and the cells were transfected using a LipofectAMINE 2000 reagent (Invitrogen). Briefly, the plasmid DNA (14 µg in 810 µl of Opti-MEM) and LipofectAMINE 2000 reagent (48 µl in 810 µl of Opti-MEM) were combined, and incubated for 20 min at room temperature. The resulting DNA-Lipo-fectAMINE 2000 complexes were added directly to each dish.

Forty-eight hours after transfection, the COS-1 cells were washed twice with ice-cold phosphate-buffered saline and harvested in 0.25 M sucrose-5 mM HEPES, pH 7.4 (buffered-sucrose). The cell suspensions were disrupted by sonication (three 10-s bursts) using an ultrasonic processor (USP-300; Shizu- hadzu, Kyoto, Japan), followed by centrifugation at 105,000g for 60 min. The resulting pellets were resuspended in the buffered-sucrose and stored at −80°C.

Western Blotting. Twenty micrograms of the membrane fraction proteins from COS-1 cells were resolved by SDS-polyacrylamide gel electrophoresis (10% gel) and electrophoretically transferred onto polyvinylidene difluoride membranes. Immunoochemical detection of each UGT1A10 protein was performed by chemiluminescence using rabbit anti-human UGT1A (diluted at 1:5000; BD Gentest, Woburn, MA) and donkey anti-rabbit Ig coupled to horseradish peroxidase (diluted at 1:2000). Chemiluminescence (enhanced chemiluminescence-plus; Amersham Biosciences, Piscataway, NJ) was detected and quantified using the Typhoon 9400 Variable Mode Imager and ImageQuant analysis Amersham Biosciences Inc. To confirm that the samples were evenly loaded, the blot was subsequently stripped in a stripping buffer (2% SDS, 100 mM 2-mercaptoethanol and 62.5 mM Tris-HCl, pH 6.7) at 50°C for 30 min, and re-probed with a polyclonal anti-calnexin antibody (diluted at 1:10,000; StressGen Biotechnologies, San Diego, CA).

Enzyme Assays. A 0.2-ml reaction mixture containing 0.1 mg/ml of the membrane fractions, 1 mM uridine diphosphoglucuronic acid (UDPGA), 10 mM magnesium chloride, 0.025 mg/ml amethicin, and HMFTEC (1.3–50 µM) in 50 mM Tris, pH 7.5, was incubated at 37°C for 5 or 10 min. After incubation, the reaction was stopped by the addition of glacial acetic acid/ acetonitrile (6:9, 100 µl) and centrifuged at 10,000g for 3 min at 4°C. The HPLC system consisted of Shimadzu LC-10AD pumps, an SIL-10A auto injector, a CTO-10A column oven, an SPD-10AV UV-VIS detector and RF-10A spectrofluorometric detector. An aliquot (50 µl) of the supernatant was injected into a 4.6 × 150 mm C18 HPLC column (5 µm) (Inertsil ODS-80A; GL Sciences, Tokyo, Japan) and separated at 30°C using a linear gradient. Initial HPLC conditions were 80% of 10% methanol solution (mobile phase A), 10% of methanol (mobile phase B), and 10% of aqueous solution containing 30% acetonitrile and 1 mM perchloric acid (mobile phase C). Ratio of the mobile phase B was increased to 90% over 15 min, whereas the ratio of mobile phase C remained constant during the course of the HPLC run. The HPLC flow rate was 1 ml per minute. The glucuronide was detected by its absorbance at 325 nm and quantified using HTFMC glucuronide as a standard. The limit of quantification for HTFMC glucuronide was 0.15 nmol/ml, which corresponded to the enzyme activity of 0.2 nmol/min/mg of protein. The HTFMC glucuronidation production was linear for at least 60 min using an enzyme concentration up to 0.5 mg/ml.

E2 glucuronidation activities were assayed as described by Alkharfy and Frye (2001) except that product formation was monitored using an external standard method. The incubation mixture (0.25 ml) consisted of 30 µg of the membrane fraction, 15 µg/ml amethicin, 1 mM magnesium chloride, 5 mM UDPGA, E2 (0.63–50 µM), and 0.1 M potassium phosphate, pH 7.1. The reaction was initiated by adding UDPGA, and the mixture was incubated at 37°C for 5 or 10 min. The reaction was terminated by the addition of 6% perchloric acid (25 µl) and then clarified by centrifugation at 10,000g for 5 min at 4°C. An aliquot (50 µl) of the resulting supernatant was injected into a 4.6 × 150 mm phenyl HPLC column (5 µm) (Inertsil Ph; GL Sciences). Isotopic elution with acetonitrile and 50 mM ammonium phosphate buffer, pH 3.0 (32:68, v/v), was used at the flow rate of 1 ml/min. The E2 glucuronides were detected by fluorescence (210 nm excitation, 300 nm emission) and quantified using E2 3-O-glucuronide and E2 17-O-glucuronide as standards. The limit of quantification for each E2 glucuronide was 400 ng/ml, which corresponded to an enzyme activity of 0.1 nmol/min/mg of protein. The E2 3-O-glucuronide production was linear for at least 60 min using an enzyme concentration up to 0.5 mg/ml.

Data Analysis. Results were analyzed using one-way ANOVA followed by Dunnett’s test as a post hoc test. Kinetic parameters were calculated with Prism 3.0 (GraphPad Software, Inc., San Diego, CA) using nonlinear regression of Michaelis-Menten equation, and the results are shown as mean ± S.D. from four-independent preparations of UGT1A10 protein.

Results

Expression of Wild-Type and Variant UGT1A10s in COS-1 Cells. The wild-type human UGT1A10 cDNA was successfully cloned from stomach cDNA, according to the information on the gastrointestinal expression of UGT1A10 (Strassburg et al., 1997). The DNA sequence of our wild-type UGT1A10 cDNA was identical to that reported by Mojarra and Mackenzie (1997) and the current version of GenBank U39550.2 submitted by I. S. Owens, J. W. Cho, N. Gholami, and C. Potter (unpublished data). The sequencing results of the UGT1A10 gene from Japanese subjects (Saeki et al., 2002) were also consistent with the sequence of the wild-type UGT1A10 cDNA cloned here. However, our UGT1A10 cDNA was different from the published UGT1A10 cDNA sequence (GenBank accession number U89508) in several positions resulting in the following amino acid substitutions; the putative signal peptide “RAGWTSVPVLVCILL-
TCGFA” in our deduced amino acid sequence (residues 3–23) was replaced by “PRRDQPRSFMCVSTADLWLC” in U89508. U89508 contained additional three amino acid substitutions, T40A, H175R, F224L. At present, we have no information as to how these alterations influence the UGT1A10 activities. Thus the enzyme characteristics shown below might be different from those reported by Strassburg et al. (1998).

T202I and M59I substitutions were introduced into the wild-type UGT1A10 cDNA by PCR-based site-directed mutagenesis. The wild-type and variant UGT1A10s were transiently expressed in COS-1 cells. Figure 1A shows the representative Western blot of pooled samples from four-independent transfections. For each sample preparation, relative UGT1A10 levels were determined using one of the wild-type samples as a standard. As shown in Fig. 1B, the relative expression level of T202I was slightly lower (0.73 ± 0.10) than that of the wild-type, whereas that of M59I was marginally elevated (1.15 ± 0.21). These alterations in the expression levels, however, were not statistically significant by one-way ANOVA and Dunnett’s test (p > 0.05). These relative expression levels were used for the normalization of glucuronidation activities of UGT1A10s described below.

Glucuronidation Activities of Wild-Type and Variant UGT1A10s. Functional characterization of the expressed UGT1A10s was first carried out using HTFMC as a substrate. Table 1 summarizes the apparent kinetic parameters of the wild-type and variant UGT1A10s for HTFMC glucuronidation. Wild-type UGT1A10 catalyzed HTFMC glucuronidation with a $V_{\text{max}}$ value of 4.03 nmol/min/mg membrane protein, whereas those of T202I and M59I were 1.52 (38% of WT) and 4.28 (106%) nmol/min/mg of membrane protein, respectively. Their $K_m$ values were 4.9, 5.4, and 4.2 μM for wild-type, T202I, and M59I, respectively. With respect to the normalized efficiencies ($V_{\text{max}}/K_m$), where the $V_{\text{max}}$ values were normalized by the expressed UGT1A10 protein levels, the efficiency of T202I was just half those of wild-type and M59I. These results indicate that the T202I variant resulted in a lower HTFMC glucuronidation activity, whereas the M59I alteration had no significant influence on the activity.

The wild-type and variant UGT1A10s were further characterized using an endogenous substrate, E2. E2 is known to be glucuronidated at two different positions to form 3-O- and 17-O-glucuronides (as reviewed by Ritter, 2000). Figure 2 shows the representative HPLC chromatograms of E2 glucuronide standards (A) and a 10-min incubation sample with E2 (50 μM) and wild-type UGT1A10 (0.5 mg/ml). As depicted in Fig. 2B, wild-type UGT1A10 exclusively catalyzed E2 3-O-glucuronidation. The same specificity was also found for the T202I and M59I variants (data not shown). Table 2 summarizes the apparent kinetic parameters of wild-type and variant UGT1A10s toward E2 3-O-glucuronidation. The $V_{\text{max}}$ and normalized $V_{\text{max}}/K_m$ values of T202I were 36 and 50% of that for the wild-type enzyme, respectively, and no statistically significant difference was observed in the $K_m$ values (2.3, 2.3, and 1.9 μM for wild-type, T202I and M59I, respectively).

Therefor the kinetic analysis using both HTFMC and E2 revealed that the M59I variant was functionally identical to wild-type UGT1A10, whereas the catalytic activity of the T202I variant was almost half that of the wild-type enzyme without influencing the apparent $K_m$ values.

Discussion

The digestive tract represents the first metabolically active tissue for orally ingested compounds. Recently, there has been a growing concern about the intestinal first pass metabolism, which influences the overall bioavailability of orally administrated drugs (Lin et al., 1999). The limited expression of UGT1A10 in biliary and gastrointestinal tissues suggests that this isoform participates in the removal of such drugs and their metabolite. For instance, Kemp et al. (2002) suggested that intestinal glucuronidation by UGT1A8 and UGT1A10 was a significant contributor to the presystemic clearance of a selective estrogen receptor modulator raloxifene. Thus alterations in function resulting from genetic polymorphisms may influence systemic drug levels and therapeutic outcome.

To date, a number of single nucleotide polymorphisms have been identified in the human UGT1A locus including UGT1A1, UGT1A6, UGT1A7, and UGT1A8 (Burchell et al., 2000; Guillemette et al., 2000; Tukey and Strassburg, 2000; Huang et al., 2002). Recently, we found two novel nonsynonymous single nucleotide polymorphisms in UGT1A10 exon 1 which resulted in amino acid alterations, T202I and M59I (Saeki et al., 2002). So far, no other polymorphisms in
functionality characterization of UGT1A10 variants

TABLE 1
Kinetic parameters of wild-type and variant (T202I and M59I) UGT1A10s for the glucuronidation of 7-hydroxy-4-trifluoromethylcoumarin and the 3-O-glucuronidation of 17β-estradiol

<table>
<thead>
<tr>
<th>UGT1A10</th>
<th>HTFMC Glucuronidation Activity</th>
<th>E2 3-O-glucuronidation Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apparent $K_m$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td></td>
<td>µM</td>
<td>mmol/min/mg</td>
</tr>
<tr>
<td>WT</td>
<td>4.91 ± 0.40</td>
<td>4.03 ± 0.20</td>
</tr>
<tr>
<td>T202I</td>
<td>5.37 ± 0.74</td>
<td>1.52 ± 0.21‡</td>
</tr>
<tr>
<td>M59I</td>
<td>4.18 ± 0.41</td>
<td>4.28 ± 0.34</td>
</tr>
</tbody>
</table>

The results were expressed as the mean ± S.D. from four-independent transfection experiments. † and ‡ Statistically different from that of WT at the levels of $P < 0.05$ and $P < 0.01$ by one-way ANOVA and Dunnett's test, respectively.

* $V_{max}$ values were normalized by the relative protein expression level, where the ratios for T202I and M59I were 0.73 ± 0.10 and 1.15 ± 0.21, respectively.

The arrow indicates the position corresponding to the residue 202 of UGT1A10. Shaded amino acids show those shared among all the UGT1A isoforms. The GenBank accession number is shown in the parenthesis.

Amino acid sequences shown in Fig. 3 indicates that the residue T202 is located within a highly conserved region of UGT1A isoforms. The amino acid sequence "MTF" at the residues 200–202 in UGT1A10 is shared among all the UGT1A isoforms. Interestingly, a substitution at the same residue (T202A) was also found in one of the controversial cDNA sequences of UGT1A8, which led to the generation of a protein with no enzymatic activity (Strassburg et al., 1998). The reduced activity of the UGT1A10 T202I variant toward HTFMC and E2 suggests that the conserved T202 residue is functionally important for catalytic activity.

Another intriguing observation of this study is that UGT1A10 catalyzes the 3-O-glucuronidation of E2, but not the 17-O-glucuronidation. Previous studies have shown that the hydroxyl groups at positions 3 and 17 undergo glucuronidation mediated by at least three different UGT isoforms. The glucuronidation at 17β-OH is mediated by human UGT2B7 (Gall et al., 1999), while UGT1A1 and UGT1A3 glucuronidate E2 at the phenolic 3-OH (Senafi et al., 1994; Gall et al., 1999). Although UGT1A4 (Green and Tephly, 1996), UGT1A9 (Albert et al., 1999), and UGT1A10 (Strassburg et al., 1998) have also been shown to possess the E2 glucuronidation activities, the region-specificity has not been reported for these UGT1A isoforms. Human intestinal microsomes have been shown to glucuronidate E2 exclusively at 3-position with 2 to 15 times higher activities than those seen in human liver microsomes (Czerwik et al., 2000). The apparent $K_m$ values were approximately 40 µM with the $V_{max}$ values of 6.4–7.3 nmol/min/mg. Fisher et al. (2000) have also reported a comparable apparent $K_m$ value of 31 µM for the 3-O-glucuronidation of E2 by human small intestinal microsomes. In the human small intestine, several UGT1A isofrom transcripts have been detected, including UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A10 (Strassburg et al., 2000). As suggested by Fisher et al. (2000) and Czerwik et al. (2000), UGT1A1 and another UGT1A isoform would catalyze 3-O-glucuronidation in a cooperative manner. Our data suggest that...
UGT1A10 could be an important candidate for the UGT isoforms involved in the intestinal 3-O-glucuronidation of E2.

In conclusion, of the two UGT1A10 variants characterized in this study, the T202I variant had lower glucuronidation activities toward HTFMC and E2, compared with the wild-type enzyme, whereas the M59I variant had no effect on these activities. These results suggest that the T202I substitution may influence the intestinal absorption of orally administrated drugs and ingested environmental pollutants. In addition, enterohepatic circulation of biliary excreted chemicals may also be influenced.

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References


