Identification of UDP-Glucuronosyltransferases Responsible for the Glucuronidation of Darexaban, an Oral Factor Xa Inhibitor, in Human Liver and Intestine

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ABSTRACT:

Darexaban maleate is a novel oral direct factor Xa inhibitor, which is under development for the prevention of venous thromboembolism. Darexaban glucuronide was the major component in plasma after oral administration of darexaban to humans and is the pharmacologically active metabolite. In this study, we identified UDP-glucuronosyltransferases (UGTs) responsible for darexaban glucuronidation in human liver microsomes (HLM) and human intestinal microsomes (HIM). In HLM, the \( K_m \) value for darexaban glucuronidation was >250 \( \mu \)M. In HIM, the reaction followed substrate inhibition kinetics, with a \( K_m \) value of 27.3 \( \mu \)M. Among recombinant human UGTS, UGT1A9 showed the highest intrinsic clearance for darexaban glucuronidation, followed by UGT1A8, -1A10, and -1A7. All other UGT isoforms were inactive toward darexaban. The \( K_m \) value of recombinant UGT1A10 for darexaban glucuronidation (34.2 \( \mu \)M) was comparable to that of HIM. Inhibition studies using typical UGT substrates suggested that darexaban glucuronidation in both HLM and HIM was mainly catalyzed by UGT1A8, -1A9, and -1A10. Fatty acid-free bovine serum albumin (2%) decreased the unbound \( K_m \) for darexaban glucuronidation from 216 to 17.6 \( \mu \)M in HLM and from 35.5 to 18.3 \( \mu \)M in recombinant UGT1A9. Recent studies indicated that the mRNA expression level of UGT1A9 is extremely high among UGT1A7, -1A8, -1A9, and -1A10 in human liver, whereas that of UGT1A10 is highest in the intestine. Thus, the present results strongly suggest that darexaban glucuronidation is mainly catalyzed by UGT1A9 and UGT1A10 in human liver and intestine, respectively. In addition, UGT1A7, -1A8, and -1A9 play a minor role in human intestine.

Introduction

Anticoagulant prophylaxis substantially reduces the risk of venous thromboembolism after major orthopedic surgery. Factor Xa is a pivotal blood coagulation factor that works at the point where the extrinsic and intrinsic coagulation cascades cross. Darexaban maleate (YM150 monomaleate; Fig. 1) is an oral, specific, direct inhibitor of the pivotal blood coagulation factor that works at the point where the extrinsic and intrinsic coagulation cascades cross. Darexaban maleate (YM150 monomaleate; Fig. 1) is an oral, specific, direct inhibitor of factor Xa that has shown antithrombotic potency in preclinical model studies, with minimal effects on bleeding compared with vitamin K antagonists (Kaku et al., 2007; Saiith et al., 2007; Iwatsuki et al., 2011). Darexaban is being developed for the prevention of venous thromboembolism in patients undergoing hip or knee replacement surgery, prevention of stroke in patients with atrial fibrillation, and prevention of ischemic events after recent acute coronary syndrome (Eriksson et al., 2010). Clinical studies have shown that the major metabolite of darexaban in human plasma is its phenolic \( O \)-glucuronide (darexaban glucuronide; Fig. 1) and that the plasma concentration of parent darexaban is very low and does not exceed 1% of that of darexaban glucuronide (Eriksson et al., 2010), indicating that darexaban is extensively eliminated by first-pass metabolism via glucuronidation. After oral administration of \( [14C] \)darexaban to humans at a dose of 60 mg, darexaban glucuronide accounted for approximately 90% of total radioactivity in plasma (T. Hashimoto, K. Suzuki, T. Iwatsubo, A. Miyashita, and T. Usui, unpublished data). Of interest, the inhibitory activity of darexaban glucuronide against factor Xa is similar to that of darexaban in vitro (Iwatsuki et al., 2011). Therefore, darexaban glucuronide is the active metabolite and the primary determinant of the pharmacological effect of darexaban after oral administration in humans (Eriksson et al., 2010). Peak plasma concentrations of darexaban glucuronide were reached at 0.75 to 2.2 h after single or multiple doses of 3 to 720 mg and both maximal plasma concentration (\( C_{\text{max}} \)) and area under the plasma concentration-time curve generally increased proportionally with dose (Groenendaal-van de Meent et al., 2010). The elimination half-life of darexaban glucuronide ranged from 14.3 to 20.5 h after single or multiple doses (Groenendaal-van de Meent et al., 2010).

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; HLM, human liver microsomes; HIM, human intestinal microsomes; UDPGA, UDP-glucuronic acid; BSA, bovine serum albumin; LC, liquid chromatography; MS/MS, tandem mass spectrometry; HEK, human embryonic kidney.
Glucuronidation represents both a clearance and detoxification mechanism for a large number of compounds that include drugs, nondrug xenobiotics, and endogenous compounds (Miners and Mackenzie, 1991; Radominska-Pandya et al., 1999; Tukey and Strassburg, 2000; Miners et al., 2004). Glucuronidation reactions are catalyzed by enzymes of the UDP-glucuronosyltransferase (UGT) superfamily. In humans, 19 known functional UGTs are categorized into three subfamilies, 1A, 2A, and 2B, based on gene structure and sequence homology (Mackenzie et al., 2005). The UGTs are variably expressed in different tissues, such as liver, gastrointestinal tract, kidneys, and others (Turgeon et al., 2001; Gregory et al., 2004; Mackenzie et al., 2005; Nakamura et al., 2008; Ohno and Nakajin, 2009). The individual UGT enzymes exhibit distinct, but overlapping, substrate and inhibitor selectivities and differ in terms of gene regulation, including tissue distribution (Tukey and Strassburg, 2000; Kiang et al., 2005; Miners et al., 2010). The liver plays a central role in drug metabolism, including glucuronidation and oxidation. In addition, the intestine also plays a crucial role in the first-pass metabolism of drugs that are mainly catalyzed by CYP3A and UGTs. The importance of intestinal glucuronidation has been demonstrated for some drugs (Fisher et al., 2001; Dalvie et al., 2008; Cubitt et al., 2009).

The UGT isoforms involved in the metabolism of darexaban have not been clearly determined. The aim of this study was to identify the UGT isoforms responsible for the glucuronidation of darexaban in human liver and intestine using human liver microsomes (HLM), human intestinal microsomes (HIM), and recombinant UGTs.

Materials and Methods

Chemicals and Reagents. Darexaban maleate (YM150 monomaleate; purity 99.8%), darexaban glucuronide (YM-222714; purity 99.0%) (Fig. 1), and human liver and intestine using human liver microsomes (HLM), human UGT isoforms responsible for the glucuronidation of darexaban in not been clearly determined. The aim of this study was to identify the UGT enzymes of the UDP-glucuronosyltransferase (UGT) superfamily. In humans, 19 known functional UGTs were conducted under linear conditions for both protein concentrations and incubation times: HLM, 0.1 mg of protein/ml and 10 min; HIM, 0.1 mg of protein/ml and 5 min, and recombinant human UGTs, 0.1 mg of protein/ml and 5 or 10 min. A typical incubation mixture contained 30 μM darexaban, 5 mM MgCl2, alamethicin (50 μg/mg protein), 100 mM potassium phosphate buffer (pH 7.4), enzyme preparation (HLM, HIM, or recombinant human UGTs), and 5 mM UDPGA in a total volume of 500 μl. Darexaban maleate and alamethicin were dissolved in 50% acetonitrile and 50% methanol, respectively, and a 5-μl aliquot of each solution was added to the incubation mixture. HLM, HIM, and recombinant human UGTs were fully activated by the addition of the pore-forming polypeptide alamethicin (Fishier et al., 2000). After the incubation mixture without darexaban or UDPGA was allowed to stand for 15 min on ice, the mixture was preincubated at 37°C for 5 min, and then the reaction was started by adding darexaban and UDPGA. After incubation for the designated time, the reaction was terminated by precipitating proteins by the addition of 500 μl of acetonitrile. An internal standard solution ([2H6]darexaban glucuronide, 2 μg/ml, 50 μl) was added, mixed well, and then centrifuged at 17,800 g for 10 min at 4°C. The resulting supernatant was diluted 2-fold with purified water, and then a 15-μl aliquot was injected into an LC-MS/MS system. In the kinetic studies using HLM, HIM, and recombinant human UGTs, darexaban concentrations varied from 1 to 250 μM.

Effects of Typical UGT Substrates on Darexaban Glucuronidation by Pooled HLM and HIM. Final concentration and target UGT isoforms of each typical UGT substrate were as follows: bilirubin, 100 μM, UGT1A1; β-estradiol, 500 μM, UGT1A1, -1A9, and -1A10 and some other UGTs; imipramine, 100 μM, UGT1A3 and UGT1A4; emodin, 500 μM, UGT1A1, -1A3, -1A8, and -1A9; propofol, 500 μM, UGT1A8 and UGT1A9; and troglitazone, 20 μM, UGT1A1, -1A8, and -1A10. These UGT inhibitors were set at effective concentrations according to previous reports (Watanabe et al., 2002; Yamanaka et al., 2007; Itahata et al., 2008; Miners et al., 2010). The assays were performed as described above, with 50 μM substrate. The percentage glucuronidation rate relative to the vehicle control was calculated.

Correlation Analyses of Darexaban Glucuronidation by Individual HLM. The glucuronidation of darexaban was measured in HLM of 16 individual human livers. The assays were performed as described above, with 30 μM substrate. The activity of each UGT isoform in each HLM sample, determined by isoform-specific reaction markers (UGT1A1, 17β-estradiol 3-glucuronidation; UGT1A4, trifluoperazine glucuronidation; UGT1A6, 1-naphthol glucuronidation; UGT1A9, propofol glucuronidation UGT2B7, morphine 3-glucuronidation; and UGT2B7, morphine 6-glucuronidation) was provided by the supplier. Correlation between darexaban glucuronidation and other glucuronosyltransferase marker enzyme activities was determined by linear regression analysis. p < 0.05 was considered statistically significant.

LC-MS/MS Analysis of Darexaban Glucuronide. Darexaban glucuronide was determined using an LC-MS/MS system composed of an LC-10A series high-performance liquid chromatograph (Shimadzu, Kyoto, Japan), coupled with a triple quadrupole mass spectrometer using multiple reaction monitoring in positive electrospray ionization mode (API 4000; AB Sciex, Foster City, CA). A Capcell Pak C8 DD 3 × 250 mm column (3-μm particle size; Shiseido Co., Ltd., Tokyo, Japan) connected to a Capcell Pak C8 DD 2 × 10 mm guard column (5-μm particle size) was used. The mobile phase consisted of purified water-acetonitrile-50 mM ammonium acetate containing 1% formic acid (65:25:10, v/v/v) and was maintained at a constant flow rate of 0.425 ml/min. The temperature of the column was
kept at 40°C. Mass transitions were set for darexaban glucuronide and internal standard ([H3]darexaban glucuronide) at m/z 651.4 → 217.2 and m/z 657.4 → 220.2, respectively. The retention time of darexaban glucuronide and internal standard was 5.2 min. Quantitation of the analytes was performed using Analyst 1.2 software (AB Scica).

**Effects of BSA on Darexaban Glucuronidation by Pooled HLM and Recombinant Human UGT1A9.** The glucuronidation of darexaban was measured in pooled HLM and recombinant human UGT1A9 as described above, with 30 μM substrate. In this study, fatty acid-free BSA was used at final concentrations of 0, 0.1, 0.5, and 2%.

The binding of darexaban to HLM and recombinant human UGT1A9 in the presence and absence of BSA was measured by ultrafiltration using a Centri-free centrifugal filtration device (YM-30; Nihon Millipore, Tokyo, Japan). The mixture contained darexaban (1, 10, and 250 μM), 5 mM MgCl2, alamethicin (50 μg/mg protein), 100 mM potassium phosphate buffer (pH 7.4), 0.1 mg of protein/ml HLM or recombinant human UGT1A9, and fatty acid-free BSA. After incubation at 37°C for 10 min, 1-ml aliquots of the mixture were applied to an ultrafiltration device (n = 3) and centrifuged at 37°C for 5 min at 440g. Under these conditions, the resulting filtrate was less than 26% of the applied volume. Concentrations of darexaban in the mixture before ultrafiltration and the resulting filtrate were determined using LC-MS/MS as described below. An aliquot (100 μl) of the mixture before centrifugation or the filtrate was mixed with 1000 μl of acetonitrile and 20 μl of 150 μM darexaban glucuronide (internal standard) on ice. The sample was subsequently centrifuged at 1740g for 10 min at 4°C, and an aliquot of the supernatant (800 μl) was collected. The supernatant was evaporated to dryness under a stream of nitrogen at 38°C. The residue was reconstituted in 400 μl of the mobile phase, which was used for analysis of darexaban glucuronide, and the resultant solution was diluted 1000-fold with the same mobile phase. A 20-μl aliquot of the diluted sample was injected into the LC-MS/MS system described above. Mass transitions were set for darexaban and internal standard (darexaban glucuronide) at m/z 475.4 → 217.5 and m/z 651.3 → 217.6, respectively. The retention times of darexaban and internal standard were 7.6 and 5.0 min, respectively.

**Kinetic Analyses.** The mean values derived from duplicate determinations with either pooled HLM, HIM, or recombinant human UGTs were used for kinetic analyses. All data were analyzed by nonlinear regression analysis using WinNonlin (version 5.2.1; Pharsight, Mountain View, CA) with models for either Michaelis-Menten kinetics (eq. 1) or substrate inhibition kinetics (eq. 2) (Houston and Kenworthy, 2000). The appropriate equation for each dataset was selected on the basis of visual inspection of Michaelis-Menten and Eadie-Hofstee plots:

\[ V = V_{\text{max}} \times S/(K_m + S) \]  
\[ V = V_{\text{max}} \times S/(K_m + S + S^2/K_s) \]

where \( V \) is the velocity of the reaction, \( S \) is the substrate concentration, \( K_m \) is the Michaelis-Menten constant, \( V_{\text{max}} \) is the maximum velocity, and \( K_s \) is the substrate inhibition constant. Intrinsic clearance (\( CL_{\text{int}} \)) was determined from the ratio of \( V_{\text{max}}/K_m \) for both cases displaying Michaelis-Menten kinetics and substrate inhibition kinetics. In the study of the effects of BSA on darexaban glucuronidation, fitting was based on unbound substrate concentrations in the incubation mixture, and kinetic parameters of unbound \( K_m \) (\( K_{m,u} \)) and \( V_{\text{max}} \) were obtained. Unbound \( CL_{\text{int}} \) (\( CL_{\text{int,u}} \)) was determined from the ratio of \( V_{\text{max}}/K_{m,u} \).

**Results**

**Darexaban Glucuronidation in Pooled HLM and HIM.** We first investigated the effects of substrate concentrations on glucuronidation of darexaban glucuronidation by potted HLM and HIM. In HLM, Michaelis-Menten plots showed that darexaban glucuronidation rates were almost linear up to the highest darexaban concentration of 250 μM, indicating that the apparent \( K_m \) value was >250 μM (Fig. 2). Therefore, \( K_m \) and \( V_{\text{max}} \) values of darexaban glucuronidation by HLM could not be determined. The \( CL_{\text{int,u}} \) value calculated from the slope of the Michaelis-Menten plots was 8.71 μl · min⁻¹ · mg protein⁻¹. On the other hand, darexaban glucuronidation by HIM was best described by the substrate inhibition kinetics (Fig. 2). Substrate inhibition was obvious at substrate concentrations greater than 150 μM. \( K_{int} \), \( V_{\text{max}} \), and \( K_s \) values of darexaban glucuronidation in HIM were 27.3 μM, 4.53 nmol · min⁻¹ · mg protein⁻¹, 166 μl · min⁻¹ · mg protein⁻¹, and 463 μM, respectively. On the basis of these results, the final concentration of darexaban in the incubation mixture was set at 30 μM for the subsequent studies, except for kinetic studies.

**Darexaban Glucuronidation by Recombinant Human UGTs.** Twelve recombinant human UGT isoforms were used to determine their darexaban glucuronosyltransferase activities. UGT1A9 exhibited the highest darexaban glucuronidation activity of 2.43 nmol · min⁻¹ · mg protein⁻¹, followed by UGT1A10, 1A8, and 1A7 with the activities of 0.516, 0.393, and 0.251 nmol · min⁻¹ · mg protein⁻¹, respectively. All other UGT isoforms, UGT1A1, -1A3, -1A4, -1A6, -2B4, -2B7, -2B15, and -2B17, showed no glucuronidation activities for darexaban (<0.01 nmol · min⁻¹ · mg protein⁻¹). These results clearly indicate that UGT1A7, -1A8, -1A9, and -1A10 are capable of catalyzing darexaban glucuronidation.

**Kinetik Analysis of Darexaban Glucuronidation by Recombinant Human UGTs.** Kinetic analyses were performed on four recombinant human UGT isoforms showing darexaban glucuronidation activities. The kinetics of darexaban glucuronidation by recombinant human UGT1A7 and UGT1A9 fitted Michaelis-Menten kinetics (Fig. 3). In contrast, glucuronidation by recombinant human UGT1A8 and UGT1A10 fitted substrate inhibition kinetics (Fig. 3). Kinetic parameters obtained for darexaban glucuronidation by recombinant human UGT isoforms are shown in Table 1. The \( K_m \) values of UGT1A7, -1A8, -1A9, and -1A10 were 59.1, 11.8, 58.2, and 34.2 μM, respectively. All \( K_m \) values of UGT1A7, -1A8, -1A9, and -1A10 are 59.1, 11.8, 58.2, and 34.2μM, respectively. The \( K_{int} \) values of UGT1A7 and UGT1A10 were 354 and 235 μM, respectively. On the basis of these results, the final concentration of darexaban in the incubation mixture was set at 30 μM for the subsequent studies, except for kinetic studies.

**Other**
strongly inhibited by β-estradiol, emodin, propofol, and troglitazone to 4.2, 7.6, 9.0, and 8.4% of control values, respectively. Darexaban glucuronidation in HIM was strongly inhibited by emodin to 3.5% of control value and also inhibited by β-estradiol, propofol, and troglitazone to 22.6, 21.3, and 19.9% of control values, respectively. Bilirubin and imipramine weakly inhibited darexaban glucuronidation to 59.9 to 92.0% of control values in both HLM and HIM. These results suggest that darexaban glucuronidation might be mainly catalyzed by UGT1A8, 1A9, and 1A10 in both HLM and HIM.

Effects of Fatty Acid-Free BSA on Darexaban Glucuronidation by Pooled HLM and Reombinant Human UGT1A9. As previously demonstrated, the \( K_m \) value for darexaban glucuronidation in HIM (27.3 μM) was similar to those in recombinant UGT1A7 (59.1 μM), UGT1A8 (11.8 μM), UGT1A9 (58.2 μM), and UGT1A10 (34.2 μM). However, the \( K_m \) value for HLM (>250 μM) was at least 4.2-fold higher than those in recombinant UGTs. A recent study indicated that BSA and fatty acid-free human serum albumin reduced \( K_m \) values for UGT1A9 substrates by sequestering inhibitory long-chain fatty acids released by incubations of HLM and HEK293 cells expressing this enzyme, with little or no effect on \( V_{\text{max}} \) values (Rowland et al., 2008). Therefore, the effects of fatty acid-free BSA on the kinetics of darexaban glucuronidation by pooled HLM and recombinant human UGT1A9 were evaluated.

First, darexaban unbound fractions in the incubation mixtures containing HLM and recombinant human UGT1A9 were determined at darexaban concentrations of 0.1, 1, 10, and 250 μM. The recovery of darexaban from the ultrafiltration device at concentrations of darexaban in ultrafiltrate of 0.1 to 250 μM was 104 to 113%, indicating that adsorption to the ultrafiltration device was negligible. Darexaban unbound fractions in the incubation mixture did not vary with darexaban concentrations from 1 to 250 μM but varied with BSA concentrations from 0 to 2%. Mean darexaban unbound fractions in the incubation mixture containing 0% BSA were 0.873, 0.720, 0.517, and 0.251 in the presence of 0, 0.1, 0.5, and 2% BSA, respectively. Likewise, mean values in the incubation mixture containing recombinant human UGT1A9 were 0.879, 0.755, 0.553, and 0.237 in the presence of 0, 0.1, 0.5, and 2% BSA, respectively. Darexaban unbound fractions were decreased in a BSA concentration-dependent

**TABLE 1**

_Kinetic parameters for darexaban glucuronidation by recombinant human UGT isoforms_

\[ V_{\text{max}}(\mu M), \; K_m (\mu M), \; \text{and} \; \text{Cl}_{\text{int}} (\mu M) \text{ were expressed as best-fit values ± S.E. of the estimate.} \]

<table>
<thead>
<tr>
<th>Isoform</th>
<th>( K_m ) (μM)</th>
<th>( V_{\text{max}} ) (μM)</th>
<th>( K_m ) (μM)</th>
<th>( V_{\text{max}} ) (μM)</th>
<th>( \text{Cl}_{\text{int}} ) (μM)</th>
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</thead>
<tbody>
<tr>
<td>UGT1A7</td>
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<td>UGT1A8</td>
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<tr>
<td>UGT1A9</td>
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<td>7.70 ± 0.08</td>
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<tr>
<td>UGT1A10</td>
<td>34.2 ± 1.7</td>
<td>1.36 ± 0.04</td>
<td>235 ± 16</td>
<td>39.8</td>
<td></td>
</tr>
</tbody>
</table>

N.A., not applicable.

a Kinetic parameters were calculated by the Michaelis-Menten equation (eq. 1).

b Kinetic parameters were calculated by the substrate inhibition equation (eq. 2).
The unbound concentrations of darexaban in the incubation mixtures were corrected by using these values for calculation of kinetic parameters.

The effects of fatty acid-free BSA on darexaban glucuronidation by pooled HLM and recombinant human UGT1A9 are shown in Fig. 6 and kinetic parameters are shown in Table 2. Darexaban glucuronidation by HLM followed Michaelis-Menten kinetics in the presence and absence of BSA. In HLM, 0.1 to 2% BSA decreased $K_{m,u}$ values for darexaban glucuronidation from 216 to 17.6 to 17.7 $\mu$M and increased $CL_{int,u}$ values approximately 10-fold, with little effect on $V_{max}$ values. No clear BSA concentration dependence was observed in the range from 0.1 to 2% for darexaban glucuronidation in HLM. In recombinant human UGT1A9, 0.1 to 2% BSA decreased $K_{m,u}$ values for darexaban glucuronidation from 35.5 to 18.3 to 22.7 $\mu$M, and increased the $CL_{int,u}$ values approximately 2-fold, with little effect on $V_{max}$ values. These results indicate that $K_{m,u}$ values (17.6–17.7 $\mu$M) for darexaban glucuronidation by HLM in the presence of 0.1 to 2% BSA were similar to that of recombinant human UGT1A9 (18.3 $\mu$M) in the presence of 2% BSA.

**Discussion**

In general, glucuronides are less biologically active than the parent aglycone (Mulder, 1992). However, glucuronide conjugates of some drugs have been reported to possess significant pharmacological activities (Milne et al., 1996; van Heek et al., 2000). Darexaban is mainly eliminated in humans by first-pass metabolism via glucuronidation. In this study, we identified UGTs responsible for darexaban glucuronidation in human liver and intestine. The kinetic profiles of darexaban glucuronidation by pooled HLM and HIM were quite different from each other as shown in Fig. 2. $CL_{int}$ values for darexaban glucuronidation in HLM and HIM were 8.71 and 166 $\mu$M·min$^{-1}$·mg protein$^{-1}$, respectively, indicating that the value of HIM was 16-fold higher than that of HLM. Among 12 recombinant human UGT isoforms, UGT1A7, -1A8, -1A9, and -1A10 were capable of catalyzing darexaban glucuronidation. The kinetic profiles, $K_{m,u}$ and $K_{i,u}$ values of UGT1A8 and UGT1A10, were similar to those of HIM (Figs. 2 and 3). These results suggest that both UGT1A8 and UGT1A10 play a major role in darexaban glucuronidation in human intestine. On the other hand, the kinetic profiles of UGT1A7, -1A8, -1A9, and -1A10 were quite different from those of HIM (Figs. 2 and 3).

Significant correlations were observed between darexaban glucuronidation and UGT1A1 and UGT1A9 marker enzyme activities in HLM (Fig. 4). Because recombinant UGT1A1 was inactive toward...
parameters were calculated by the Michaelis-Menten equation (eq. 1). 

UGT1A3, apart from UGT1A1, to the 3-glucuronidation of 17β-estradiol (Miners et al., 2010). On the basis of relative activity data, UGT1A3 may account for up to one third of 17β-estradiol glucuronidation in HLM (Izukawa et al., 2000; Gregory et al., 2004). Recent reports indicated that UGT1A9 plays a major role in darexaban glucuronidation in human liver. Inhibition studies using typical UGT substrates suggested that darexaban glucuronidation was mainly catalyzed by UGT1A9, -A1, and -A10 in both HLM and HIM. Because inhibition by bilirubin, a selective substrate for UGT1A1, was weak, the contribution of UGT1A1 isoform to darexaban glucuronidation in both HLM and HIM is not considered significant.

Human UGTs are expressed in a tissue-specific manner (Tukey and Strassburg, 2000; Gregory et al., 2004). Recent reports indicated that among UGT1A7, -1A8, -1A9, and -1A10, the mRNA expression level of UGT1A9 is highest and those of the other isoforms are extremely low or are not detected in human liver (Izukawa et al., 2009; Ohno and Nakajin, 2009). In contrast, the expression level of UGT1A10 was highest among them, whereas those of UGT1A8, -A10, and -A17 were less than one tenth that of UGT1A10 in human small intestine (Ohno and Nakajin, 2009). Taken together with these mRNA expression levels of UGTs in human tissues, the results obtained in the present study strongly suggest that darexaban glucuronidation in human liver and intestine is mainly catalyzed by UGT1A9 and UGT1A10, respectively. In addition, in human intestine, UGT1A7, -A8, and -A9 play a minor role in darexaban glucuronidation.

Darexaban glucuronidation in human liver is strongly suggested to be mainly catalyzed by UGT1A9. However, K_m value for darexaban glucuronidation in HLM (>250 μM) was at least 4.3-fold higher than that in recombinant human UGT1A9 (58.2 μM). A recent study indicated that BSA and fatty acid-free human serum albumin reduced glucuronidation in HLM (250 μM) by 25% and 65%, respectively, as compared with that in recombinant human UGT1A9 (58.2 μM). A similar phenomenon was observed with regard to UGT2B7 (Rowland et al., 2007). In addition, arachidonic, linoleic, and oleic acids were suggested to be substrates for human UGT1A9. We therefore examined the effects of fatty acid-free BSA on darexaban glucuronidation by recombinant human UGT1A9.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>BSA Conc.</th>
<th>K_m, μM</th>
<th>V_max, nmol/min/mg protein</th>
<th>C_Lint, μM</th>
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<tr>
<td></td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLM</td>
<td>0</td>
<td>216 ± 32</td>
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<td></td>
<td>0.1</td>
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<td>3.25 ± 0.07</td>
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<tr>
<td></td>
<td>0.5</td>
<td>177 ± 0.9</td>
<td>3.25 ± 0.05</td>
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<td>2</td>
<td>176 ± 1.0</td>
<td>3.14 ± 0.06</td>
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<td>UGT1A9</td>
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<tr>
<td></td>
<td>0.1</td>
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<tr>
<td></td>
<td>2</td>
<td>18.3 ± 1.3</td>
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Fig. 6. Effects of fatty acid-free BSA on darexaban glucuronidation by pooled HLM (A and B) and recombinant human UGT1A9 (C and D). Michaelis-Menten plots (A and C) and Eadie-Hofstee plots (B and D) are shown. Darexaban concentrations in the incubation mixture were corrected with the unbound fraction of darexaban. Microsomes (0.1 mg of protein/ml) were incubated with 1 to 250 μM darexaban and 5 mM UDPGA at 37°C for 10 min in the presence and absence of fatty acid-free BSA. S, u is the unbound darexaban concentration. Data represent the mean of duplicate determinations, and curves are from model-fitting.
Recent reports indicated that in the presence of 2% BSA or fatty acid-free human serum albumin in incubations is likely to improve the accuracy of hepatic clearance by glucuronidation predicted from microsomal CL_{int} values for substrates of UGT1A9 and UGT2B7 (Rowland et al., 2007, 2008; Kilford et al., 2009). Thus, human in vivo intrinsic clearance ([CL_{int}, in vivo] milliliters per minute per kilogram body weight) in liver and intestine from in vitro CL_{int, u} was estimated using the following equation:

\[
\text{CL}_{\text{int, in vivo}} = \frac{\text{CL}_{\text{int, u}} \times \text{mg microsomal protein}}{\text{g tissue}} \times \frac{\text{g tissue}}{\text{kg b.wt.}}
\]

Because the CL_{int, u} of HLM was not determined in the present study, the value was estimated from CL_{int, u} divided by darexaban unbound fraction in the incubation mixture containing HLM (0.873), on the basis of the assumption that the unbound fraction does not differ between HLM and HIM. The estimated CL_{int, u} value of human intestine was 190 µl \cdot min^{-1} \cdot mg^{-1} protein^{-1} and that of human liver using for the estimation of CL_{int, in vivo} was 178 µl \cdot min^{-1} \cdot mg^{-1} protein^{-1}. According to a previous study (Soars et al., 2002), 45 and 3 mg/tissue were used as the contents of the microsomal protein in human liver and intestine, respectively. For the weights of human liver and intestine, 20 and 30 g/kg b.wt., respectively, were used. The CL_{int, in vivo} values in human liver and intestine were estimated to be 160 and 17 ml \cdot min^{-1} \cdot kg^{-1} b.wt., respectively, indicating that the value for the liver was 9.4-fold higher than that for the intestine. The CL_{int, in vivo} value of liver is 8-fold higher than liver blood flow (20 ml \cdot min^{-1} \cdot kg^{-1} b.wt.) (Soars et al., 2002) and that of intestine is 3.7-fold higher than mucosal blood flow of intestine (4.6 ml \cdot min^{-1} \cdot kg^{-1} b.wt.) (Soars et al., 2002). These estimates suggest that intestinal glucuronidation significantly contributes to the first-pass metabolism of darexaban. However, the relative contribution of the liver and intestine could not be accurately calculated because darexaban is almost completely eliminated presystemically by first-pass metabolism via glucuronidation in humans.

In summary, recombinant human UGT1A7, -A8, -A9, and -A10 are capable of catalyzing darexaban glucuronidation. Considering the mRNA expression levels of UGT isoforms in human tissues (Izukawa et al., 2009; Ohno and Nakajin, 2009), the results of the present study strongly suggest that darexaban glucuronidation is mainly catalyzed by UGT1A9 and UGT1A10 in human liver and intestine, respectively. In addition, in human intestine, UGT1A7, -A8, and -A9 play a minor role in glucuronidation. Thus, darexaban glucuronidation seems to be catalyzed by multiple UGT1A isoforms in human intestine and liver after intake of darexaban.

Authorship Contributions

Participated in research design: Shiraga, Yajima, and Hashimoto.

Conducted experiments: Yajima and Ke. Suzuki.

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Wrote or contributed to the writing of the manuscript: Shiraga, Yajima, Ka. Suzuki, Hashimoto, Iwatsubo, Miyaehata, and Usui.

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