Alteration of the Function of the UDP-Glucuronosyltransferase 1A Subfamily by Cytochrome P450 3A4: Different Susceptibility for UGT Isoforms and UGT1A1/7 Variants

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ABSTRACT

Functional protein–protein interactions between UDP-glucuronosyltransferase (UGT)1A isoforms and cytochrome P450 (CYP)3A4 were studied. To this end, UGT1A-catalyzed glucuronidation was assayed in Si-9 cells that simultaneously expressed UGT and CYP3A4. In the kinetics of UGT1A6-catalyzed glucuronidation of serotonin, both Michaelis constant ($K_m$) and maximal velocity ($V_{max}$) were increased by CYP3A4. When CYP3A4 was coexpressed with either UGT1A1 or 1A7, the $V_{max}$ for the glucuronidation of the irinotecan metabolite (SN-38) was significantly increased. $S_{50}$ and $K_m$ both are which the substrate concentration giving 0.5 $V_{max}$ were little affected by simultaneous expression of CYP3A4. This study also examined the catalytic properties of the allelic variants of UGT1A1 and 1A7 and their effects on the interaction with CYP3A4. Although the UGT1A1-catalyzing activity of 4-methylumbelliferone glucuronidation was reduced in its variant, UGT1A1*6, the coexpression of CYP3A4 restored the impaired function to a level comparable with the wild type. Similarly, simultaneous expression of CYP3A4 increased the $V_{max}$ of UGT1A7*1 (wild type) and *2 (N129K and R131K), whereas the same was not observed in UGT1A7*3 (N129K, R131K, and W208R). In the kinetics involving different concentrations of UDP-glucuronic acid (UDP-GlcUA), the $K_m$ for UDP-GlcUA was significantly higher for UGT1A7*2 and *3 than *1. The $K_m$ of UGT1A7*1 and *3 was increased by CYP3A4, whereas *2 did not exhibit any such change. These results suggest that (1) CYP3A4 changes the catalytic function of the UGT1A subfamily in an UGT isoform-specific manner and (2) nonsynonymous mutations in UGT1A7*3 reduce not only the ability of UGT to use UDP-GlcUA but also CYP3A4-mediated enhancement of catalytic activity, whereas CYP3A4 is able to restore the UGT1A1*6 function.

Introduction

Glucuronidation is one of the major pathways contributing to the elimination of many exogenous and endogenous compounds. This step is catalyzed by UDP-glucuronosyltransferases (UGTs). The UGT isoforms are classified into two families (UGT1 and UGT2) based on sequence identity (Mackenzie et al., 1997, 2005). These UGTs exhibit an overlapping but distinct substrate specificity (Tukey and Strassburg, 2000). For example, human UGT1A1 is the predominant enzyme for bilirubin conjugation (Ritter et al., 1991), and this UGT also plays an important role in the hepatic glucuronidation of SN-38, an active metabolite of irinotecan (Camptosar; Pfizer, New York, NY) (Guillemette, 2003; Rosner et al., 2008). A partial reduction in UGT1A1 activity is believed to cause moderate hyperbilirubinemia, so called Gilbert syndrome (Iyanagi et al., 1998). Of the allelic variants reported thus far, UGT1A1*6 (G71R) and *28 (wild-type protein incapable of being covalently attached to the mitochondria due to Gilbert syndrome) were the only variants reported to exhibit only...
and its glucuronide were generous gifts from Yakult Honsha Co. Ltd (Tokyo, Japan). 4-Hydroxybiphenyl (4-OHB) was purchased from Wako Pure Chemical Co. Ltd (Tokyo, Japan). 4-OHB glucuronide was purified in this laboratory from urine obtained from rabbits given 4-OHB, according to Dodgson et al. (1948). UGT1A7 superosome and pooled human intestinal microsomes were purchased from BD Gentest (BD Bioscience, Franklin Lakes, NJ). All other reagents were of the highest quality commercially available.

Construction of Expression Plasmids. The open reading frame of UGT1A7 (1.6 kbp) was amplified by polymerase chain reaction (PCR) using pRES-UGT1A7 (Uchaipichat et al., 2004) as a template and the following primers: [BamHI-G-UGT1A7 (-2,17): 5'-CGG GAT CAG TTA GGG CTC GGT CGT GAG GGT G-3' and BamHI-UGT1A7 (1599,1580): 5'-CGG GAT CCC ACT TCT CAA TGG GTG CTG TTG G-3']. The numbers in parentheses represent the position of the first and last bases counted from the initiation codon (ATG), and underlines represent the BamHI restriction sites introduced. Pfu turbo DNA-polymerase (Stratagene, Agilent Technologies, Santa Clara, CA) was used for the PCR amplification. The PCR products were then digested with BamHI and cloned into the pFastBac1 vector. The sequence was confirmed to be UGT1A7*3, although it carries a synonymous base change at C33A. Then, UGT1A7*1 and *2 cDNAs (-2nd and 1599th) were prepared by the megaprimer method using pFastBac1-UGT1A7*3 as a template. To obtain the megaprimer, UGT1A7-Meg#1 (249 bp) covering a range between the 385th and 633rd base was used, and PCR was performed using the following primers: UGT1A7F (358-405): 5'-AAT GAC CGA AAA TTA GTA GAA TAC-3' and UGT1A7R (633-613): 5'-GAT GTG GTC CCA TAC TCT CTC-3'. Another megaprimer pFastBac1-UGT1A7 Meg#2 (689 bp) having a sequence coupling a vector region (3979-4032) and the *2 region (-2-633) was prepared by PCR amplification using the following primers: pFastBacF (3979-4002) 5'-AAA AAC CTA TAA ATA TTC CGG-3' and pFastBacR (4167-4139) 5'-AAT GAC CGA AAA TTA GTA GAA TAC-3' as a vector region (3979-4002) 5'-AAA AAC CTA TAA ATA TTC CGG-3' and mega-primer UGT1A7 Meg#1. Then, to prepare the full-length cDNA, pFastBac1-UGT1A7*2 PCR was carried out using the following primers: pFastBacF (4167-4139) 5'-TTA GGA TTC TCT TCT AGT CTA GAG CAC AGA CAT GAA GCA AAT TTA GAT CTA GAG GCT GTC-3' and pFastBacR (1948). UGT1A7 supersome and pooled human intestinal microsomes were digested with Sau3AI and KpnI restriction sites was amplified with pfu DNA polymerase using the following primers: pFastBacF (4167-4139) 5'-TTA GGA TTC TCT TCT AGT CTA GAG CAC AGA CAT GAA GCA AAT TTA GAT CTA GAG GCT GTC-3' and pFastBacR (1948). UGT1A7 supersome and pooled human intestinal microsomes were digested with Sau3AI and KpnI restriction sites were used as a template. The expressing vector for UGT1A7*1 was prepared as above, using pFastBac1-UGT1A7*2 as a template. The vectors for the expression of UGT1A1*1 and 1A6 were constructed by the methods described below. The XhoI/SalI fragment of UGT1A1*1 cDNA from pTarget-UGT1A1 (Nishimura et al., 2007) was inserted into the XhoI/SalI site of pFastBac1 vector. The UGT1A6 cDNA introduced with Sau3AI sites was amplified from pfu DNA polymerase using pRES-UGT1A6 (Uchaipichat et al., 2004), and subcloned into the BamHI site of the pFastBac1-vector. The expressing vector for UGT1A7*1 was prepared as above, using pFastBac1-UGT1A7*2 as a template. The vectors for the expression of UGT1A1*1 and 1A6 were constructed by the methods described below.

Expression of CYP3A4 and UGT1A1 in Sf9 Cells. The expression of UGT and CYP3A4 was carried out in a Bac-to-Bac system (Invitrogen). Briefly, a recombinant pFastBac1 clone carrying UGT/P450 cDNA(s) with the right orientation was transfected into the competent Escherichia coli of the Max Efficiency DH10Bac strain. Then, Sf9 cells (2 x 10^7) were transfected with Bacmid produced by the E. coli using Cellfection reagent (Invitrogen). Primary virus was collected 48 hours after transfection. To obtain a high titer of recombinant baculovirus for UGTs and CYP3A4, several rounds of amplification were repeated. For protein expression, baculovirus-infected Sf9 cells were collected 60 hours after infection and washed with phosphate-buffered saline. The phosphate-buffered saline was prepared according to the manual (Sambrook et al., 1989). We routinely cultured Sf9 cells transfected with baculovirus in a volume of 200 ml (2 x 10^7 cells/ml). Cellular microsomes were prepared according to the protocols described previously (Ishii et al., 2001) and suspended in phosphate-buffered saline containing 20% glycerol. The above scale of cultivation gave us a sufficient quantity of microsomal protein for all assays and experiments.

Materials and Methods

4-MU and 4-MU glucuronide were purchased from Sigma-Aldrich Co. Ltd (St. Louis, MO) and Nacalai Tesque Co. Ltd. (Kyoto, Japan), respectively. SN-38 was generously donated by Yakult Honsha Co. Ltd (Tokyo, Japan). 4-Hydroxybiphenyl (4-OHB) was purchased from Wako Pure Chemical Co. Ltd (Tokyo, Japan). 4-OHB glucuronide was purified in this laboratory from urine obtained from rabbits given 4-OHB, according to Dodgson et al. (1948). UGT1A7 superosome and pooled human intestinal microsomes were purchased from BD Gentest (BD Bioscience, Franklin Lakes, NJ). All other reagents were of the highest quality commercially available.

Cytochromes P450 (P450s, CYPs) are very important drug metabolizing enzymes because they are involved in the metabolism of most drugs (Evans and Relling, 1999). Of these P450s, CYP3A4 is the most abundant and involved in the metabolism of many drugs (Shimada et al., 2004). After our first report of interactions between P450 and UGT (Takeda et al., 2005, 2009), in these studies, the interaction between CYP3A4 and UGT2B7 was proven by overlay assay, coimmunoprecipitation, and crosslinking. Another group also showed that UGT1A1, UGT1A6, as well as UGT2B7 in solubilized human liver microsomes can be coimmunoprecipitated with CYP3A4 coupled to its antibody (Fremont et al., 2005). Furthermore, our preliminary evidence suggested that CYP3A4 interacts with UGT1A7. Thus, CYP3A4 appears to interact with UGT1A1, 1A6, and 1A7. However, to the best of our knowledge, it remains unknown whether CYP3A4 is able to modify the functions of the UGT1A subfamily. Therefore, this study examined the efficacy of CYP3A4 on the activity of UGT1A1, 1A6, and 1A7. To this end, we established baculovirus-Sf9 cells coexpressing UGT1A1, 1A6, and UGT1A7 as well as CYP3A4. Expression systems for UGT allelic variants, UGT1A1*6 and UGT1A7*2 and *3, were also established to examine the effect of the mutations on the P450-UGT interaction.
Glucuronidation Assay. Glucuronidation of 4-MU was assayed according to Nishimura et al. (2007). Unless otherwise stated, the incubation mixture (300 µl) consisted of 50 mM potassium phosphate buffer (pH 7.4), 5 mM MgCl₂, 2 mM UDP-GlcUA, recombinant UGT1A1 and 1A7 baculosomes, and 4-MU (see legends to figures for the amount of protein added and substrate concentration). Microsomes were pretreated with alamethicin (50 µg/mg protein; 4°C, 30 minutes) before assay. 4-MU was dissolved in methanol, and the final concentration of methanol in the incubation medium was 0.5%. Incubation was performed at 37°C for 1 hour. The linearity of the time-dependent increase in 4-MU glucuronide was confirmed up to an incubation period of 4 hours. In the kinetics for UGT1A7 catalysis by varying cofactor concentration, the UDP-GlcUA concentration ranged from 31.25 to 4000 µM. The 4-MU glucuronide produced was analyzed by high-performance liquid chromatography (HPLC) coupled to a fluorescence detector (Nishimura et al., 2007). Chromatographic analysis was carried out using a LaChrome Elite HPLC system equipped with an automatic sampler (model L-2200), pump (L-2130 HTA), and fluorescence detector (L-2485) (Hitachi High-Technologies Co., Tokyo, Japan). Data were stored and processed using a D-7000 HPLC System Manager software (version 3.1: Hitachi High-Technologies Co.). Separation was achieved using a µBondaspher Phenyl column (5 µm, 150 × 3.9 mm, Waters Co., Milford, MA) housed in an oven (HITACHI L-2300) that was set at 25°C. The column was stepwise eluted by changing the acetonitrile concentration in 100 mM sodium phosphate, pH 2.4 as follows: 5% for 9.9 minutes, raised to 15% over 0.1 minute and holding it for the next 10 minutes, then reducing it to 5% over 0.1 minute and maintaining the starting condition for 10 minutes for the next sample. The flow rate was set at 0.8 ml/min. 4-MU glucuronide was quantified by fluorescence detection (excitation wavelength 315 nm and emission wavelength 375 nm). The retention time of 4-MU glucuronide was 4.1 minutes under the above conditions. Glucuronidation of SN-38 was determined by HPLC using the procedure reported by Hanioka et al. (2001). Unless otherwise stated, the incubation conditions were the same as those for 4-MU described above, except that the substrate was SN-38 and incubation was performed at 37°C for 2 hours. A time-dependent increase in SN-38 glucuronide was confirmed for an incubation period up to 4 hours. The column was eluted with a linear gradient solution prepared by mixing eluent A (100 mM sodium phosphate, pH 2.4) and eluent B (acetonitrile); the concentration of acetonitrile was increased from 5 to 50% for 0 to 18 minutes and then increased from 50 to 90% over the period 18 to 23 minutes. Finally, it was reduced from 90 to 5% over the period 23 to 30 minutes. The flow rate was set at 0.8 ml/min. SN-38 glucuronide was quantified by fluorescence detection (excitation 370 nm and emission 425 nm), and the retention time of SN-38 glucuronide was 9.2 minutes. Determination of 4-OHBP glucuronide was carried out by HPLC. Unless otherwise stated, the assay conditions were the same as those for the assay of 4-MU glucuronidation, except that the substrate was 4-OHBP ranging from 50 to 1500 µM. Incubations were performed at 37°C for 1 hour. The reaction was stopped with 100 µl cold 1 M trichloroacetic acid. Samples were chilled on ice for 30 minutes and then transferred to 1.5-ml plastic tubes. The samples were then centrifuged at 12,000 rpm for 10 minutes, and the supernatants were subjected to HPLC analysis. The linearity of the time-dependent increase in 4-OHBP glucuronide was confirmed for incubation periods up to 2 hours. Chromatographic analysis was performed using the apparatus described above. The operating conditions were the same as those for the analysis of 4-MU glucuronide, and 4-OHBP glucuronide was quantified by fluorescence detection (excitation 269 nm and emission 323 nm). The retention time of 4-OHBP glucuronide was 9.3 minutes. Glucuronidation of 5-HT by UGT1A6 baculosomes was determined according to Hanioka et al. (2006). Glucuronidation at the 3-hydroxy group of 5-HT by UGT1A6 was confirmed in incubation periods up to 2 hours. A concentration-dependent increase in 4-MU glucuronide was confirmed up to an incubation period of 4 hours. The data were fitted to a Michaelis-Menten equation, substrate concentration needed to achieve a half-maximum enzyme velocity. The sigmoidal model

\[ V = \frac{V_{\text{max}} \cdot S}{K_m + S} \]

where \( V \) is the rate of reaction, \( S \) is the substrate concentration, \( V_{\text{max}} \) is the maximum enzyme velocity, and \( K_m \) is the Michaelis constant (substrate concentration needed to achieve a half-maximum enzyme velocity). The substrate inhibition model

\[ V = \frac{V_{\text{max}} \cdot S^n}{S^n + S^s} \]

where \( S_n \) is the substrate concentration giving 0.5 \( V_{\text{max}} \) and \( n \) is the Hill coefficient.

The substrate inhibition model

\[ V = \frac{V_{\text{max}} \cdot S}{K_m + S - (1 + S/K_i)} \]

where \( V_{\text{max}} \) is the maximum enzyme velocity if the substrate does not also inhibit enzyme activity and \( K_i \) is the substrate inhibition constant. The significance of differences between two groups was examined by Student’s t test using STATView software (ver. J 4.02, SAS Institute, Cary, NC). The differences among more than three groups were examined by one-way ANOVA followed by Scheffe’s F test. The statistical significance of the estimated kinetic parameter of repeated kinetics was determined by ANOVA followed by Dunnett’s test. A value of \( P < 0.05 \) was considered statistically significant. Results Effect of CYP3A4 on UGT1A6 Function. UGT1A6 was expressed in SF-9 cells, and the protein bands immunoreactive toward anti-UGT1A antibody and absent in control microsomes was judged to be UGT1A6 having different sugar chains (Fig. 1). CYP3A4 was coexpressed in SF-9 cells with UGT1A6. SF-9 cells, the microsomes of which express UGT1A6 with a similar glycosylation pattern to that in the single expression system, were selected and subjected to further investigation of their enzymatic properties (Fig. 1). In addition to the glycosylation pattern, the amount of UGT1A6 and microsomal protein used for the assay was also unified between the single and dual expression systems. For this, we rendered the protein level uniform with baculosomes obtained from SF-9 cells infected with control baculovirus. When CYP3A4 was expressed together with UGT1A6, the \( K_m \) of UGT1A6-catalyzed 5-HT glucuronidation was significantly increased (Fig. 2, Table 1). Although kinetic profiles for both the single and double expression could be fitted to a Michaelis-Menten equation, substrate inhibition or its trend was observed in UGT1A6 single expression at higher substrate concentrations. However, the same was not seen in a system coexpressing CYP3A4 and UGT1A6. This suggests that coexpressed CYP3A4 eliminates the substrate inhibition that occurred
in UGT1A6-catalyzed 5-HT glucuronidation. Furthermore, the \( V_{max} \) was significantly increased by CYP3A4.

**Interaction between CYP3A4 and UGT1A1*1/*6.** To examine whether CYP3A4 also modifies UGT1A1 function, UGT1A1*1 (wild type) and *6 (an allelic variant) were expressed together with CYP3A4 in SF-9 cells. Just like the experiments for a UGT1A6/CYP3A4 system, the SF-9 cell microsomes expressing UGT1A1 with a similar glycosylation pattern to that in the CYP3A4 coexpression system were selected (Fig. 3). Also, the amount of expressed UGT1A1 used for kinetic assay was the same between the single and dual expression systems (Fig. 3). Under the expression conditions used, the level of expressed CYP3A4 did not differ greatly from that in human liver microsomes.

Figure 4 shows the Michaelis-Menten plotting of 4-MU glucuronidation catalyzed by UGT1A1*1 and *6 and the effect of CYP3A4 coexpression on the profiles. The kinetic parameters together with those for other substrates (SN-38 and 17β-estradiol) are summarized in Table 2. As expected, the \( V_{max} \) of UGT1A1*6 was significantly lower than the wild-type *1 and almost half that of *1. Conversely, the \( S_0 \) of UGT1A1*6 was significantly higher than *1. The cotransfection of CYP3A4 with UGT1A1*1 and *6 affected their kinetic parameters. More specifically, CYP3A4 markedly increased the \( V_{max} \) values of UGT1A1*1 and *6, whereas their \( S_0 \) values were little affected (Fig. 4 and Table 2). Consequently, CYP3A4 coexpression rendered UGT1A1*6 function comparable with that of wild-type *1.

A similar picture was seen for SN-38 glucuronidation (Table 2). For example, the \( V_{max} \) was significantly lower in UGT1A1*6 than in wild-type *1, and the \( S_0 \) of UGT1A1*6 was higher compared with the wild-type *1. Like 4-MU glucuronidation, the \( V_{max} \) of SN-38 glucuronidation of both *1 and *6 was significantly enhanced by CYP3A4. Thus, simultaneous expression of CYP3A4 with UGT1A1*6 caused an ~2-fold increase in \( V_{max} \), and the value recovered to approximately 70% of that specific to *1. The same difference between UGT1A1*1 and *6 and the same effect of CYP3A4 coexpression on *1 function were also observed for 17β-estradiol glucuronidation (Table 2).

**Protein-Protein Interaction between CYP3A4 and UGT1A7.** To examine whether CYP3A4 interacts with UGT1A7, an overlay assay using GST-tagged CYP3A4 was performed. As a positive control, an overlay assay of UGT2B7 was carried out. As we reported previously (Takeda et al., 2005), UGT2B7 was recognized by GST-tagged CYP3A4 (Fig. 5). Like UGT2B7, UGT1A7 was also recognized by GST-tagged CYP3A4. This was supported by the observation that the same was not true for control baculosomes. No reacting band was observed when the overlay assay was performed with GST. These data suggest that CYP3A4 interacts with UGT1A7 as well as UGT2B7.

**Comparison of Catalytic Properties among UGT1A7*1, *2, and *3.** UGT1A7 allozymes *1, *2, and *3 were expressed in SF-9 cells by the baculovirus-based expression system. The protein immunoreactive toward anti-UGT1A antibody and not present in control microsomes was judged to be UGT1A7 protein (Fig. 6). Incubation mixtures contained equivalent amounts of UGT protein, as assessed by Western blotting, and equivalent amounts of total protein. The latter was achieved by the addition of baculosome protein from SF-9 cells infected with control baculovirus. Then, the catalytic properties of UGT1A7 allozymes *1, *2, and *3 were compared using 4-MU as a substrate. At a constant 4-MU concentration (100 \( \mu \)M), the formation of 4-MU glucuronide by UGT1A1*1 was dependent on the amount of microsomal protein over the range 0.5 to 10 \( \mu \)g. Linear 4-MU glucuronide formation was confirmed up to 4 hours (data not shown). The amount of UGT1A1*1 microsomes was set at 5 \( \mu \)g protein, and the incubation was carried out for 1 hour. When the 4-MU glucuronidation activity was compared among UGT1A1*7, *2, and *3, the activity was highest in *3, followed by *1 and, finally, *2 (Supplemental Fig. 1A). The pH-dependence of all the allozymes was similar with an optimum pH of 8.0 (Supplemental Fig. 2). Their thermostabilities were also similar (data not shown). Thus, although the differences between them were significant, they were subtle.

Next, we compared the glucuronidation of SN-38, an active metabolite of irinotecan, by UGT1A7 allozymes. Referring to a previous
substrate concentration was set at 400 M. It is known that rabbit UGT1A7 is capable of glucuronidating 4-OHBP (Bruck et al., 1997), so we next examined whether human UGT1A7 also catalyzes 4-OHBP glucuronidation. As shown in Supplemental Fig. 1C, UGT1A7 allozymes *1, *2, and *3 were capable of glucuronidating 4-OHBP. Thus, in this study, it was shown for the first time that 4-OHBP is a substrate of human UGT1A7. When the 4-OHBP glucuronidation activity in UGT1A7 allozymes *2 and *3 was significantly lower than in wild-type *1, being 65% and 70% of *1, respectively (Supplemental Fig. 1B).

Comparison of Kinetic Parameters among UGT1A7*1, *2, and *3: Effect of CYP3A4. When UGT1A7 allozymes *1, *2, and *3 were compared, the differences in 4-MU glucuronidation activity between them was significant but subtle. Kinetic studies were carried out to better understand the differences in the catalytic properties of UGT1A7 allozymes and the effect of CYP3A4. Keeping the UDP-GlcUA concentration constant, the concentration of each substrate was varied. Figure 7 shows the Michaelis-Menten plot of 4-MU glucuronidation catalyzed by UGT1A7 allozymes. The fitted kinetic parameters are summarized in Table 3. Although the \( V_{\text{max}} \) of UGT1A7*2 was smaller than that of the wild-type *1, the intrinsic clearance (\( V_{\text{max}}/K_{\text{m}} \)) of UGT1A7 allozymes *1, *2, and *3 was not markedly different. However, cotransfection of CYP3A4 with each UGT1A7 allozyme affected their kinetic parameters. As shown in Fig. 7, 4-MU glucuronidation in *1 and *2 was enhanced by CYP3A4.

### TABLE 1

Kinetic parameters for 5-HT glucuronidation catalyzed by UGT1A6: effect of CYP3A4 cotransfection

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>( K_{\text{m}} ) (mM)</th>
<th>( V_{\text{max}} ) (pmol/min/mg protein)</th>
<th>( V_{\text{max}}/K_{\text{m}} )</th>
<th>( K_i ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Michaelis-Menten</td>
<td>UGT1A6</td>
<td>2.84 ± 1.94</td>
<td>42.3 ± 8.4</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>UGT1A6 + CYP3A4</td>
<td>11.2 ± 4.4 ( ^{48} )</td>
<td>62.6 ± 9.9 ( ^{48} )</td>
<td>5.6</td>
</tr>
<tr>
<td>Substrate inhibition</td>
<td>UGT1A6</td>
<td>146 ± 717 (0.0 to 1637)</td>
<td>0.792 ± 3.716 (–6.939 to 8.522)</td>
<td>—</td>
</tr>
</tbody>
</table>

UGT activity was determined with 10 substrate concentrations ranging from 0.4 to 50 mM in the presence of 2 mM UDP-GlcUA.

\(^{48}\)Value in parentheses represents the 95% confidential interval.

\(^{48}P = 0.0100; \, ^{48}P = 0.0015\) from single expression.

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Fig. 3. Western blot analysis of UGT1A1 and CYP3A4 in Sf-9 microsomes expressing UGT1A1 alone and the UGT plus CYP3A4. A, Western blotting was performed with a monoclonal anti-CYP3A antibody, WB-MAB-3A. All microsomal samples applied to wells were adjusted in advance so that they contain comparable amounts of UGT1A1. Human liver microsomes (HLM) (5 \( \mu \)g protein) were used as a positive control. Lane 1, microsomes expressing wild-type UGT1A1 (2.5 \( \mu \)g protein); lane 2, microsomes expressing wild-type UGT1A1 and CYP3A4 (4 \( \mu \)g protein); lane 3, microsomes expressing UGT1A1*6 (2 \( \mu \)g protein); lane 4, microsomes expressing UGT1A1*6 and CYP3A4 (4 \( \mu \)g protein). Mock represents microsomes (20 \( \mu \)g protein) obtained from Sf-9 cells transfected with control baculovirus. B, Western blotting was performed with nonspecific anti-UGT1A antibody in the absence and presence of CYP3A4 expression. The plots for wild-type UGT1A1*1 (A) and its allelic variant *6 (B) are shown. The 4-MU concentration was varied over the range 10–500 \( \mu \)M (UGT1A1*1) and 20–800 \( \mu \)M (UGT1A1*6). The total amount of proteins added to the assay mixture was standardized at 40 \( \mu \)g. For this, when necessary, control baculosomes were added to the reaction mixture. Kinetic parameters were calculated by fitting the curve to a sigmoidal equation, and they are listed in Table 2.
whereas that of *3 was reduced. In particular, the effect of CYP3A4 on $V_{\text{max}}$ was marked, with the $V_{\text{max}}$ of UGT1A7*1 and *2 being significantly increased whereas that of *3 was significantly reduced. Consequently, when CYP3A4 was cotransfected, the intrinsic clearance was increased ~3-fold in *2 followed by *1 (1.5 times) whereas that of *3 was reduced one-half by CYP3A4.

Kinetic experiments were carried out for SN-38 glucuronidation catalyzed by UGT1A7 allozymes, and the fitted kinetic parameters are summarized in Table 3. Although the $V_{\text{max}}$ of UGT1A7*2 was smaller than that of the wild-type *1, the intrinsic clearance of UGT1A7 allozymes *1, *2, and *3 was not markedly different. However, like 4-MU, cotransfection of CYP3A4 with each UGT1A7 allozyme affected their kinetic parameters. SN-38 glucuronidation of *2 was significantly enhanced by CYP3A4 whereas that of *3 was reduced. In particular, the effect of CYP3A4 on the $V_{\text{max}}$ of *2 was marked, with the $V_{\text{max}}$ of UGT1A7 *1 and *2 being increased whereas that of *3 was reduced. Consequently, when CYP3A4 was cotransfected, the intrinsic clearance was increased ~3-fold in *2 whereas that of *3 was reduced to 80% by CYP3A4.

Kinetic studies for 4-OHBP glucuronidation catalyzed by UGT1A7 allozymes were also performed. The fitted kinetic parameters are summarized in Table 3. The intrinsic clearance of UGT1A7 allozymes *1, *2, and *3 was not markedly different. However, cotransfection of CYP3A4 with each UGT1A7 allozyme affected their kinetic parameters. Unlike 4-MU and SN-38, 4-OHBP glucuronidation involving *1 and *2 was enhanced by CYP3A4 whereas that of *3 was reduced. The effect of CYP3A4 on $V_{\text{max}}$ was significant, with the $V_{\text{max}}$ of UGT1A7*1 and *2 being increased whereas that of *3 was reduced. Consequently, when CYP3A4 was cotransfected, the intrinsic clearance was increased ~2- to 6-fold in *2 followed by *1 (4-fold) whereas that of *3 was reduced one-half by CYP3A4.

### Table 2

Table 2 presents the kinetic parameters for 4-MU and SN-38 glucuronidation catalyzed by UGT1A7*1 and its allelic variant *6: effect of CYP3A4 cotransfection.

<table>
<thead>
<tr>
<th>UGT1A1 and a variant</th>
<th>$S_{50}$</th>
<th>$V_{\text{max}}$</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4-MU</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1</td>
<td>58.8 ± 4.1</td>
<td>385 ± 13</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>*1+CYP3A4</td>
<td>53.6 ± 2.7</td>
<td>133 ± 13</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>*6</td>
<td>122 ± 13</td>
<td>306 ± 13</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>*6+CYP3A4</td>
<td>124 ± 11</td>
<td>413 ± 20</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td><strong>SN-38</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1</td>
<td>14.0 ± 0.3</td>
<td>28.6 ± 0.2</td>
<td>1.35 ± 0.03</td>
</tr>
<tr>
<td>*1+CYP3A4</td>
<td>16.6 ± 0.3</td>
<td>51.1 ± 0.4</td>
<td>1.25 ± 0.02</td>
</tr>
<tr>
<td>*6</td>
<td>18.1 ± 0.6</td>
<td>7.1 ± 0.1</td>
<td>1.48 ± 0.06</td>
</tr>
<tr>
<td>*6+CYP3A4</td>
<td>30.4 ± 1.1</td>
<td>18.9 ± 0.4</td>
<td>1.34 ± 0.03</td>
</tr>
</tbody>
</table>

### Table 3

Table 3 summarizes the kinetic parameters for SN-38 glucuronidation catalyzed by UGT1A7*1 single-expressing Sf-9 cells and CYP3A4 cotransfected Sf-9 cells. A summary of the single and double transfect cells. Baculosomes (5 μg protein) from Sf-9 cells infected with the baculovirus without passenger DNA and served as controls. The proteins in the gel were electrophoretically transferred to polyvinylidene difluoride membranes and blotted with anti-UGT1A7 common (top) and polyclonal anti-CYP3A4 (bottom), antibodies, respectively.

**Fig. 5.** Detection of interaction between CYP3A4 and UGT1A7 by overlay assay. Mouse monoclonal antibody (M) for CYP3A4 and CYP3B7 supernatant (10% gel) and transferred to a polyvinylidene difluoride membrane. The membrane was overlaid with either GST-CYP3A4 fusion protein (left) or GST (right) and then treated with anti-GST antibody. The lanes of "UGT1A7*1," "UGT2B7*1," and "control" mean baculosomes containing UGT1A7, UGT2B7, and those lacking the UGT, respectively. The arrowhead indicates the UGT1A7 position.

**Fig. 6.** Immunoblots of the expression of each UGT1A7 allozyme and CYP3A4 in Sf-9 cells. To obtain micromsome simultaneously expressing CYP3A4 and UGT1A7, Sf-9 cells were transfected with recombinant baculovirus for CYP3A4 and UGT1A7. The lanes labeled "+1," "+2," "+3," and "+CYP3A4" show micromsome samples of the single and double transfect cells. Baculosomes (5 μg protein) from UGT1A7*1 single-expressing Sf-9 cells were electrophoresed (SDS-PAGE). For other baculosomes, amounts of protein were used that were equivalent to the UGT1A7 content in the single-expressing UGT1A7*1. Mock represents the baculosomes (20 μg protein) from Sf-9 cells infected with the baculovirus without passenger DNA and served as controls. The proteins in the gel were electrically transferred to polyvinylidene difluoride membranes and blotted with anti-UGT1A7 common (top) and polyclonal anti-CYP3A4 (bottom), antibodies, respectively.
Compared with the wild-type UGT1A7*1, the however, the UGT1A7*2 and *3, have an effect on the recognition of UDP-GlcUA. It appears that N129K and R131K, which are common mutations in *2, was not significantly different from *1, whereas the V$_\text{max}$ of *3 was significantly higher than that of *1 (Table 4). Therefore, it appears that N129K and R131K, which are common mutations in UGT1A7*2 and *3, have an effect on the recognition of UDP-GlcUA.

Comparison of Kinetic Parameters among UGT1A7*1, *2, and *3: Effect of CYP3A4 at Different UDP-GlcUA Concentrations. Like 4-MU, SN-38, and 4-OHBP as substrates, CYP3A4 similarly alters the V$_\text{max}$ of UGT1A7 allozymes *1, *2, and *3 in kinetic studies. Thus, the affinity for UDP-GlcUA, a common cofactor of the reactions, was determined using 4-MU as a substrate. The cofactor kinetics were examined for UGT1A7 allozymes *1, *2, and *3. Keeping the 4-MU concentration at 100 μM, the UDP-GlUCUA concentration was varied. Compared with the wild-type UGT1A7*1, the K$_\text{m}$ for UDP-GlUCUA was significantly increased in allozymes *2 and *3 (Table 4). Therefore, it appears that N129K and R131K, which are common mutations in UGT1A7*2 and *3, have an effect on the recognition of UDP-GlUCUA.

However, the V$_\text{max}$ of *2 was not significantly different from *1, whereas the V$_\text{max}$ of *3 was significantly higher than that of *1 (Table 4). Therefore, it appears that N129K and R131K, which are common mutations in UGT1A7*2 and *3, have an effect on the recognition of UDP-GlUCUA.

TABLE 3

<table>
<thead>
<tr>
<th>UGT1A7 allozymes</th>
<th>K$_\text{m}$ (μM)</th>
<th>V$_\text{max}$ (pmol/mg protein)</th>
<th>V$<em>\text{max}$/K$</em>\text{m}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1</td>
<td>31.8 ± 2.3</td>
<td>642 ± 14.9</td>
<td>20.4</td>
</tr>
<tr>
<td>*2</td>
<td>2.08 ± 0.12</td>
<td>2.03 ± 0.03</td>
<td>0.100</td>
</tr>
<tr>
<td>*3</td>
<td>1.04 ± 0.07</td>
<td>1.00 ± 0.00</td>
<td>0.010</td>
</tr>
</tbody>
</table>

$^\text{a}$ Kinetic parameters for 4-methylumbellifere, SN-38, and 4-hydroxybiphenyl glucuronidation catalyzed by UGT1A7 allozymes *1, *2, and *3: effect of CYP3A4 cotransfection. Data were fitted to a Michaelis-Menten equation. Results are the estimated value ± S.E.

Discussion

CYP3A4 alters the function of UGT1A1, 1A6, and 1A7. Although CYP3A4 markedly activated UGT1A1*1- and UGT1A7*1-catalyzed glucuronidation, the effect of CYP3A4 on UGT1A6-catalyzed 5-HT glucuronidation was different. UGT1A1 is major isofrom involved in SN-38 glucuronidation. To date, 113 allelic variants of UGT1A1 have...
been reported (UGT home page: http://www.flinders.edu.au/medicine/sites/clinical-pharmacology/ugt-homepage.cfm). Of these variants, UGT1A1*6, resulting in reduced catalytic activity, seems to be one of the causes of Gilbert syndrome (Aono et al., 1993). It is well known that UGT1A1*28, which is another candidate for the genomic defect explaining Gilbert syndrome, has polymorphic (TA) repeats in the promoter that cause reduced expression of UGT1A1 wild-type protein (Rosner et al., 2008; Sugatani, 2013). Although both UGT1A1*6 and *28 are important, the former is more frequent (15.5%) in the Japanese population than the latter (11%) (Akaba et al., 1998; Huang et al., 2000; Kurose et al., 2012). UGT1A1*28 is considered to be an index that allows effective prediction in terms of the tolerability to irinotecan, an anticancer drug (Ando et al., 2007). However, it should be noted that there are patients showing a normal ability to glucuronidate SN-38 despite carrying the mutant genotypes (Sai et al., 2004). It is, therefore, controversial whether the sensitivity to irinotecan can be predicted only by analyzing the UGT1A1*28 genotype (Deeken et al., 2008). In addition, the allelic frequency (15%) of UGT1A1*6 is higher than that (5%) of Gilbert syndrome in Japanese subjects. In summary, more work is still needed to obtain a better understanding of interindividual differences in UGT1A1 activity. As reported here, CYP3A4 has the potential to enhance the activity of wild-type UGT1A1*1 and a variant UGT1A1*6 with low activity. This observation suggests that a change in CYP3A4 level is involved, at least in part, in interindividual differences in UGT1A1-catalyzed SN-38 glucuronidation. For instance, the observation that CYP3A4 restored the lowered activity of UGT1A1*6 to the level comparable with *1 (Fig. 4, Table 2) suggests a mechanism explaining the reason why some patients exhibit a normal activity although they carry the defect-type UGT1A1 gene.

The function of UGT1A7 allozymes *1, *2, and *3 was virtually identical when substrate and cosubstrate are present in excess concentrations. Although UGT1A7*3 has been reported to be catalytically inactive toward 4-MU (Strassburg et al., 2002), in our tests, *3 exhibited comparable activity with the wild-type *1. This is also supported by the work of Uchaipichat et al. (2004) who reported UGT1A7-catalyzed 4-MU glucuronidation that was determined using the same cDNA as the UGT1A7*3 of this study. The difference is unclear but the former study used transient expression, whereas the latter used stable expression in HEK293 cells. The assay systems were also different. In the former study, 4-MU glucuronide was detected by thin-layer chromatography, whereas in the latter it was assayed by the fluorescence produced from 4-MU glucuronide in alkaline solution. In the present study, we used a baculovirus-Sf-9 cell system for expressing UGT1A7*3 and detected the 4-MU glucuronide formed by HPLC. Probably other factors that we have not considered also contributed to the difference. Alternately, it has been reported that UGT1A7*3 exhibited SN-38 glucuronidation activity of ~60% of the wild-type *1 (substrate concentration 1.5 μM) (Villeneuve et al., 2003). UGT1A7*3 that exhibited SN-38 UGT activity of 70% of the wild type (substrate concentration 2 μM) (Supplemental Fig. 1B) corresponded well to that in a previous report (Villeneuve et al., 2003). Taking these factors into consideration, UGT1A7*3 is catalytically active toward 4-MU, SN-38, and 4-OHBP, at least under the assay conditions we used.

The activity of UGT1A7 allozymes *1, *2, and *3 was significantly reduced to ~80% by a 60-minute preincubation at 37°C (data not shown). The addition of UDP-GlcUA during the preincubation step did not prevent this inhibition. Thus, the interaction with UDP-GlcUA does not help to stabilize the UGT1A7 allozymes tested in this study. Without stabilization, the activity should be reduced to 40% by a 4-hour incubation at 37°C. However, the linearity of 4-MU glucuronidation of UGT1A7 was confirmed up to 4 hours in our preliminary experiments (data not shown). Therefore, there are mechanism(s) to stabilize UGT1A7 during incubation at 37°C. Interaction with substrate or other factors during catalysis is likely to be involved in maintaining UGT1A7 stability. In the kinetic study with different 4-MU, SN-38, and 4-OHBP concentrations, the intrinsic clearance of UGT1A7 allozymes *1, *2, and *3 was comparable. However, when the kinetics of 4-MU glucuronidation were investigated using different UDP-GlcUA concentrations, the Km for UDP-GlcUA was significantly higher in UGT1A7*2 and *3 than in the wild-type *1, whereas their Vmax values were comparable. This suggests that N129K and R131K of UGT1A7*2 and *3 are involved in the recognition of UDP-GlcUA. It is known that the UDP-GlcUA binding site is present in the carboxy-terminal domain of UGT (Mackenzie et al., 2005). The residues from Trp356 to Gin399 in UGT2B7 have been identified as the UDP-GlcUA-binding site on the basis of crystallographic data (Miley et al., 2007). This forms the signature sequence of UDP-glycosyltransferase (Mackenzie et al., 2005) that binds the UDP-moiety. Photo-affinity labeling experiments showed that the glucuronic acid moiety of UDP-GlcUA interacts with positively charged residue(s) in the N-terminal domain of UGT2B4 (Pillot et al., 1993). It is reasonable to suppose that these should be the site(s) interacting with the glucuronic acid moiety in the N-terminal domain of

### TABLE 4

<table>
<thead>
<tr>
<th>Variants</th>
<th>Km (μM)</th>
<th>Vmax (pmol/mg protein)</th>
<th>Vmax/Km (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1</td>
<td>317 ± 10</td>
<td>33.7 ± 0.3</td>
<td>0.106</td>
</tr>
<tr>
<td>*1+CYP3A4</td>
<td>455 ± 22s</td>
<td>56.8 ± 0.9s</td>
<td>0.125</td>
</tr>
<tr>
<td>*2</td>
<td>461 ± 32s</td>
<td>33.6 ± 0.7</td>
<td>0.073</td>
</tr>
<tr>
<td>*2+CYP3A4</td>
<td>512 ± 45</td>
<td>85.2 ± 2.4s</td>
<td>0.166</td>
</tr>
<tr>
<td>*3</td>
<td>480 ± 36s</td>
<td>42.5 ± 1.0s</td>
<td>0.088</td>
</tr>
<tr>
<td>*3+CYP3A4</td>
<td>725 ± 60s</td>
<td>34.8 ± 1.0s</td>
<td>0.048</td>
</tr>
</tbody>
</table>

††† P < 0.0001 from *1; **P < 0.0001 from single expression.

---

**Fig. 8.** Immunoblots of the expression of UGT1As and CYP3A4 in pooled human intestinal microsomes and comparison with UGT1A7 and CYP3A4 dual expressed microsomes. The lane “HIM” represents human intestinal microsomes pooled from five donors (20 μg protein). The lanes “*1”, “*2,” “*3,” and “UGT1A7+CYP3A4” show microsome samples of double transfected cells from UGT1A7*1, *2, and *3, respectively. Baculosomes (20 μg protein) coexpressing UGT1A7*1 and CYP3A4 were electrophoresed (SDS-PAGE). For other baculosomes, amounts of protein equivalent to the UGT1A7 content in the UGT1A7*1 + CYP3A4 were used. CYP3A4 represents the baculosomes (20 μg protein) from Sf-9 cells infected with the recombinant baculovirus for CYP3A4. Recombinant purified CYP3A4 (1.2 pmol) was on the lane at the extreme left. The proteins in the gel were electrically transferred to polyvinylidene difluoride membranes and blotted with anti-UGT1A common (top) and monoclonal anti-CYP3A (bottom) antibodies, respectively.
other UGT isoforms. In UGT1A7*2 and *3, the affinity for UDP-GlcUA is altered by N129K and R131K. These are located in the N-terminal domain and distinct from the above mentioned UDP-binding site. Taking these factors into consideration, it appears that the amino acid substitutions at residues 129 and 131 of UGT1A7 alter its recognition of the glucuronic acid moiety of UDP-GlcUA. However, it remains unclear whether either residue 129 or 131 or both are important. CYP3A4 interacts with UGT1A7 as well as UGT2B7 (Fig. 4). In the present study, the glucuronidation activity of UGT1A7 allozymes *1, *2, and *3 was altered by cotransfection of CYP3A4. However, the effect differed among the three allozymes. The effects of CYP3A4 on UGT activity toward the three different substrates were similar, except that the effect on UGT1A7*1 in SN-38 glucuronidation was not very marked. The $K_m$ for UDP-GlcUA was significantly increased in *3 and the wild-type *1 whereas that of *2 remained unchanged. The resulting affinity for UDP-GlcUA was further reduced in *3, whereas it was comparable between *2 and the wild-type *1. However the $V_{max}$ of UGT1A7*1 and *2 increased. Therefore, it appears that CYP3A4 interacts with all the UGT1A7 allozymes tested to alter their function. Consequently, the intrinsic clearance of *3 was reduced by CYP3A4 whereas that of *2 was increased. Furthermore, it is reasonable to consider that $V_{max}$ represents the activity with the substrate and cosubstrate at maximum concentrations. To the best of our knowledge, the concentration of UDP-GlcUA in the lumen of the endoplasmic reticulum (ER) has yet to be measured. Because UGT exhibits latency in intact microsomes, it is reasonable to consider that the luminal concentration of UDP-GlcUA is limited to a lowered concentration under in vivo conditions. Thus, the reduced affinity for UDP-GlcUA probably results in an insufficient glucuronidation ability. It is well known that there is large interindividual variability in the level of CYP3A4 (Shimada et al., 1994). It is assumed that the role of UGT1A7*3 in epidemiologic studies is evaluated in terms of the influence of CYP3A4. Taking these factors into consideration, it is likely that UGT1A7*3, but not *2, is a risk factor for carcinogenesis due to the reduced glucuronidation activity toward phenolic compounds.

The coexpression of UGT1A7 and CYP3A4 alters the $K_m$ for the UDP-GlcUA of UGT1A7*3 but not *2. The $V_{max}$ of UGT1A7*3 is the sole difference from *2. However, it is curious that the $K_m$ for the UDP-GlcUA of wild-type *1 is affected similar to *3. This reason remains to be clarified. As has been established, the main bodies of UGT and P450 are situated on opposite sides of the ER membrane (Ishii et al., 2005). Thus, the respective interaction domains of UGT1A7 and CYP3A4 may meet together in the ER membrane. It is clear that there is another ER retention signal in UGT2B1 other than the carboxy terminal double-lysine motif (Meech and Mackenzie, 1998). Interestingly, an ER retention signal of UGT1A6 has been suggested to be present between residues 140 and 240 (Ouizenne et al., 1999). On the other hand, CYP3A4 is thought to bind to the ER membrane via the N-terminal helix and other hydrophobic surface regions (Nath et al., 2007). Furthermore, it has been reported that the membrane environment has some effects on P450 conformation and activity (Ahn et al., 1998). Therefore, the residues 129, 131, and 208 of UGT1A7 or the surrounding region are candidates for involvement in the CYP3A4-UGT1A7 interaction. We suggested that the J-helix region of CYP3A4 may interact with UGT2B7 (Takeda et al., 2009). However, there is no information about the region of UGT involved in the interaction. We believe that our study provides important information about the region of UGT involved in the protein-protein interaction with CYP3A4. Further studies are necessary to clarify the detailed mechanism of the modulation of UGT1A7 activity by CYP3A4.
Sugatani J (2013) Function, genetic polymorphism, and transcriptional regulation of human UDP-

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Pharmacokinetics and Pharmacodynamics of UGT1A7 and UGT1A9 glucuronidating enzymes in Caucasian and African-American subjects and their impact on the metabolism of 7-ethyl-10-pulegone and 1-naphthol glucuronidation, effects of organic solvents, and inhibition by dichloromethane and probenecid. Drug Metab Dispos 32:413–423.
Alteration of the Function of the UDP-Glucuronosyltransferase 1A Subfamily by Cytochrome P450 3A4: Different Susceptibility for UGT Isoforms and UGT1A1/7 Variants

Yuji Ishii, Hiroki Koba, Kousuke Kinoshita, Toshiya Oizaki, Yuki Iwamoto, Shusaku Takeda, Yuu Miyauchi, Yoshio Nishimura, Natsuki Egoshi, Futoshi Taura, Satoshi Morimoto, Shin’ichi Ikushiro, Kiyoshi Nagata, Yasushi Yamazoe, Peter I. Mackenzie, and Hideyuki Yamada

Drug Metabolism and Disposition
Fig. S1. Comparison of glucuronidation activity for 4-MU, SN-38 and 4-OHBP among UGT1A7 allozymes*1, *2 and *3 Sf-9 cell microsomes expressing each UGT1A7 allozyme were used for the assay of UGT1A7 activity towards 4-MU (A), SN-38 (B) and 4-OHBP (C). Substrate concentrations were set at 100 µM (4-MU), 2 µM (SN-38) and 400 µM (4-OHBP). For 4-MU, SN-38 and 4-OHBP glucuronidations, baculosomes (5, 10 and 20 µg protein) from UGT1A7*1 single-expressing Sf-9 cells were used, respectively. For other baculosomes, amounts of protein equivalent to the UGT1A7 content of single-expressing UGT1A7*1 were used. Total protein was standardized by adding control baculosomes. Each bar represents the mean ± S.D. of triplicate assays. Significant differences from the wild-type *1 (†p<0.05, ††p<0.01, †††p<0.001) are indicated. Statistical significance was calculated by Scheffe’s F-test.
4-MU-G formed (relative activity)

pH

- UGT1A7*1
- UGT1A7*2
- UGT1A7*3
Fig. S2. pH-dependence in 4-MU glucuronidation among UGT1A7 allozymes *1, *2 and *3 The activity of each microsomal allozyme was measured at pH 6.5, 7.0, 7.4, 8.0, 8.5 and 9.0. The substrate concentration was set at 100 µM. The amount of protein used is described in the legend to Fig. 6. Relative activity to that at pH 7.4 (=1.0) is shown. Each bar represents the mean ± S.D. of triplicate assays.
Fig. S3. Comparison of intrinsic clearance ($V_{\text{max}}/K_m$) for 4-MU glucuronidation in the UGT1A7 single expression system and CYP3A4/UGT1A7 co-expression system. The 4-MU concentration was fixed at 100 µM and the UDP-GlcUA concentration ranged from 31.25 to 4000 µM. The amount of protein used is described in the legend to Fig. 6. Three independent kinetic experiments were carried out for each UGT1A7 allozyme. Kinetic parameters of each experiment were calculated by curve fitting of a Michaelis-Menten equation. Values represent the Mean ± S.E.M. Then, the resulting intrinsic clearance was compared using Dunnett's test. Significant differences from the single expression of each UGT1A7 allozyme (††$p<0.01$) are indicated.