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

Yuu Ishii, Hiroki Koba, Kousuke Kinoshita, Toshiya Oizaki, Yuki Iwamoto, Shuso Takeda,1 Yuu Miyachi, Yoshio Nishimura, Natsuki Egoshi, Futoshi Taura,2 Satoshi Morimoto, Shin’ichi Ikushiro, Kiyoshi Nagata, Yasushi Yamazoe, Peter I. Mackenzie, and Hideyuki Yamada

Laboratory of Molecular Life Sciences (Y.Is., H.K., K.K., T.O., Y.Iw., S.T., Y.M., Y.N., N.E., H.Y.) and Laboratory of Medicinal Resource Regulation (F.T., S.M.), Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan; Biotechnology Research Center, Faculty of Engineering, Toyama Prefectural University, Imizu, Toyama, Japan (S.I.); Tohoku Pharmaceutical University, Sendai, Japan (K.N.); Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan (Y.Y.); and Department of Clinical Pharmacology, Flinders Medical Centre and Flinders University, Adelaide, Australia (P.I.M.).

Received September 24, 2013; accepted November 19, 2013

ABSTRACT

Functional protein-protein interactions between UDP-glucuronosyltransferase (UGT)1A isofoms and cytochrome P450 (CYP)3A4 were studied. To this end, UGT1A-catalyzed glucuronidation was assayed in Sf-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_0P and K_m both which are 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 UGT1A1 subfamily in a 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 (Campto; Pfizer, New York, NY) (Guillemette, 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 (Campto; Pfizer, New York, NY) (Guillemette, 2000).

This work was supported in part by the Japan Society for Promotion of Science [Grants-in-Aid for Scientific Research (B) Research No. 25293039 and Grants-in-Aid for Scientific Research (C) Research No. 21590164 to Y. I.].

Presented in part at the 25th Annual Meeting of the Japanese Society for the Study of Xenobiotics, Ohmiya, Japan October, 2010 (Koba et al.); the 27th Kyushu Regional Meeting for the Pharmaceutical Society of Japan, Nagasaki, Japan, December 2010 (Koba et al.; the 19th Microsomes and Drug Oxidations (MDO) and 12th European International Society for the Study of Xenobiotics (ISSX) Meeting, Noordwijk aan Zee, The Netherlands, June 2012 (Ishii et al.; the 28th Annual Meeting of the Japanese Society for the Study of Xenobiotics, Tokyo, Japan, October 2013 (Kinoshita et al.).

Current affiliation: Faculty of Pharmaceutical Sciences, Hiroshima International University, Kure, Hiroshima, Japan.

This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: bp, base pair; ER, endoplasmic reticulum; GST, glutathione S-transferase; HPLC, high-performance liquid chromatography; 5-HT, serotonin; K_m, Michaelis constant; 4-MU, 4-methylumbelliferone; 4-OHBP, 4-hydroxybiphenyl; P450 or CYP, cytochrome P450; PCR, polymerase chain reaction; S_0P, substrate concentration giving 0.5 V_max; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UDP-GlcUA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase; V_max, maximum velocity.
reduced SN-38 glucuronidation (Gagné et al., 2002; Jinno et al., 2003). However, interindividually different in UGT1A1 activity seem unable to be explained fully by these polymorphisms. UGT1A6 catalyzes the glucuronidation of phenolic compounds such as serotonin (5-HT) (Busu et al., 2004; Hanioka et al., 2006), and UGT1A7 is involved in the conjugation of compounds such as mycophenolic acid and SN-38 (Busu et al., 2004; Emi et al., 2007; Rosner et al., 2008; Joy et al., 2010). Although UGT1A1 and 1A6 are mainly expressed in the liver, UGT1A7 is expressed in extrahepatic tissues especially in the gastrointestinal tract (Tukey and Strassburg, 2000). There are several polymorphisms of UGT1A7 (Vogel et al., 2001) and, of the allelic variants, the frequency of UGT1A7*3 is approximately 35% and 26% in Caucasian and Japanese populations, respectively (Guillemette et al., 2003; Huang et al., 2005). A previous study demonstrated that UGT1A7*3 lacks activity toward phenolic compounds that are typical UGT substrates, such as 4-methylumbelliferone (4-MU) (Strassburg et al., 2002). If this is true, UGT1A7*3 may be a risk factor for cancer because of the reduced ability to detoxify carcinogenic phenols (Vogel et al., 2001; Strassburg et al., 2002; Ockenga et al., 2003; Chen et al., 2006). Indeed, epidemiologic studies have provided evidence of a significant association between an allelic variant UGT1A7*3 and carcinogenesis (Ockenga et al., 2003; Chen et al., 2006). However, controversial results showing no relationship between UGT1A7*3 and cancer risk have also been reported (Verlaan et al., 2005; te Morsche et al., 2008).

Some research groups claim that UGT1A7*3 exhibits substantial activity toward SN-38, although it is significantly lower than that of the wild type (Gagné et al., 2002; Villeneuve et al., 2003). In addition, Uchaipichat et al. (2004) investigated the activity of UGT1A7*3-catalyzed 4-MU glucuronidation. We obtained the same cDNA from that laboratory and confirmed its sequence to be UGT1A7*3. Therefore, there remains a discrepancy concerning the catalytic activity of UGT1A7*3 among researchers. Thus, the risk for cancer and irinotecan toxicity associated with poorly active UGT1A7 variants still is an important hypothesis that needs detailed investigation (Gagné et al., 2002; Vogel et al., 2011, references therein).

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., 1994). After our first report of interactions between P450 and UGT (Takeda et al., 2000), we demonstrated that the regioselectivity of UGT2B7-catalyzed morphine glucuronidation is altered by CYP3A4, and the J-helix of this P450 may contribute to the interaction with the 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, 1A6, 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 UGT1A1 subfamily. Therefore, this study examined the effect of CYP3A4 on the activity of UGT1A1, 1A6, and 1A7. To this end, we established baculovirus-Sf-9 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.

**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 and its glucuronide were generous gifts from Yakult Honsha Co. Ltd (Tokyo, Japan). 4-Hydroxybiphenyl (4-OHBp) was purchased from Wako Pure Chemical Co. Ltd (Tokyo, Japan). 4-OHBp glucuronide was purified in this laboratory from urine obtained from rabbits given 4-OHBp, according to Hodgson et al. (1948). UGT1A7 supersome 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 plRES-UGT1A7 (Uchaipichat et al., 2004) as a template and the following primers: [BamH-I-UGT1A7 (1-271): 5' - CGG GAT CTT GGA CCA TTA GTA GAA TAC-3' and UGT1A7R (633-613): 5' - GAT GTG CTA AAC TAA CCC 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. Pu turbo DNA-polymeerase (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 mega-primer method using pFastBac1-UGT1A7*3 as a template. To obtain the mega-primer, UGT1A7-Meg1 (249 bp) covering a range between the 385th and 633rd base was used, and PCR was performed using the following primers: UGT1A7F (385-408): 5' - AAT GAC CGA AAA TTA GTA GAA TAC-3' and UGT1A7R (633-613): 5' - GAT GTG CTT GCA TAC TCT CTC-3'. Another mega-primer pFastBac1-UGT1A7 Meg2 (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' - TAA AAA AAC CTA ATA AAT TCC GCG-3' and mega-primer UGT1A7 Meg1. Then, to prepare the full-length cDNA, pFastBac1-UGT1A7*2. PCR was carried out using the following primers: pFastBacR (4167-4139) 5' - TTA GGA TCC TCT TGT ACT CTA GAG GC-3' and pFastBac1-UGT1A7 Meg2. The full-length cDNA obtained was restricted with BamHI and inserted 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. The XhoI/Sall fragment of UGT1A1*1 cDNA from pTarget-UGT1A1 (Nishimura et al., 2007) was inserted into the XhoI/Sall site of pFastBac1 vector. The UGT1A6 cDNA introduced with BamHI site was amplified with pu DNA polymerase from plRES-UGT1A6 (Uchaipichat et al., 2004), and subcloned into the BamHI site of the pFastBac1-vector. pFastBac1-UGT1A1*6 (G211A) was engineered by a site-directed mutagenesis procedure (Agilent Technology, Santa Clara, CA) using the following primers: [UGT1A1F (192,230): 5' - TTC ATT TTA CAC CTT GAA-3' and UGT1A1R (230,192): 5' - TTA AAG GTG TAA AAT GCT CTG TCT CTG ATG TAC AAC GAG G-3'.] The cDNA of CYP3A4 inserted with Sall and KpnI restriction sites was amplified with pu DNA polymerase from pCMV4-CYP3A4 (Takeda et al., 2005). This was digested with Sall and KpnI and then inserted into the SalI/KpnI sites of the pFastBac1-vector. All sequences of the expression plasmids were verified by DNA sequencing using an ABI 3130x Genetic analyzer (Applied Biosystems, Life Technologies (NASQAD: LIFE), Carlsbad, CA).

**Expression of CYP3A4 and UGT1A1 in Sf-9 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, Sf-9 cells (2 x 10^6) 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 Sf-9 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 Sf-9 cells transfected with baculovirus in a volume of 200 ml (2 x 10^6 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.
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 amelamin (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 D-7000 HPLC System Manager software (version 3.1; Hitachi High-Technologies Co.). Separation was achieved using a μBondapak 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 95% 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-OHB 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-OHB 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-OHB 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-OHB glucuronide was quantified by fluorescence detection (excitation 269 nm and emission 323 nm). The retention time of 4-OHB 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 17β-estradiol by UGT1A1 baculosomes was assayed by a method described elsewhere (Nishimura et al., 2007).

Immunoblotting. Proteins separated by 7.5% or 9% SDS-PAGE were electroblotted on to polyvinylidene difluoride membranes (Millipore, Bedford, MA). UGT1A1, UGT1A6, and UGT1A7 were detected using rabbit anti-rat UGT1A com (Kushiburi et al., 1995). Polyclonal rabbit anti-CYP3A4 (epitope Leu331 to Lys342), which was a custom-made sample obtained from Scrum Inc. (Tokyo, Japan), or monoclonal mouse anti-CYP3A antibody (BD Gentest) was used as a primary antibody. Immunochemical staining was performed with alkaline phosphatase-labeled secondary antibody. The band intensity of UGT was quantified using Image-J software (version 1.61). After several transfection trials, SF-9 cell microsomes expressing UGT1A7 algaeymes with a similar glycosylation pattern were selected and subjected to further investigation of their enzymatic properties. The amount of UGT1A7 protein was determined and UGT1A7*2 and *3 proteins, the levels of which were equivalent to that of *1, were used for kinetic experiments. Similarly, UGT1A1*6 was used for kinetic assay at a protein level equivalent to that of *1.

Overlay Assay with GST-CYP3A4 Fusion Protein. The microsomes of insect cells transformed with baculovirus carrying UGT1A7 cDNA (Super- somes, BD Gentest) were used as the source of UGT1A7. The procedures were described previously (Takeda et al., 2005).

Other Methods. Protein concentrations were determined according to Lowry et al. (1951). The kinetic parameters with standard errors were calculated using GraphPad Prism 5J (GraphPad software, La Jolla, CA), and the statistical significances of the kinetic parameters were also determined. In this procedure, the data were fitted to a Michaelis-Menten eq. 1 or a Hill eq. 2. For UGT1A6, the data were also fitted to the substrate inhibition model (eq. 3).

The Michaelis-Menten model

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

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).

\[ V = V_{\text{max}} \times S^n / (S^n_{0.5} + S^n) \]  

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

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

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 $V_{\text{max}}$ 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 $V_{\text{max}}$ of UGT1A1*6 was significantly lower than the wild-type *1 and almost half that of *1. Conversely, the $S_{50}$ 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 $V_{\text{max}}$ values of UGT1A1*1 and *6, whereas their $S_{50}$ 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 $V_{\text{max}}$ was significantly lower in UGT1A1*6 than in wild-type *1, and the $S_{50}$ of UGT1A1*6 was higher compared with the wild-type *1. Like 4-MU glucuronidation, the $V_{\text{max}}$ 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 $V_{\text{max}}$, 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 achieved by the addition of baculosome protein from Sf-9 cells infected with the baculovirus without passenger DNA, which served as controls. The proteins in the gel were electrically transferred to polyvinilidene difluoride membranes, and blotted with anti-UGT1A common and monoclonal mouse anti-CYP3A, antibodies, respectively.

**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 μM), the formation of 4-MU glucuronide by UGT1A7*1 was dependent on the amount of microsomal protein over the range 0.5 to 10 μg. Linear 4-MU glucuronide formation was confirmed up to 4 hours (data not shown). The amount of UGT1A7*1 microsomes was set at 5 μg protein, and the incubation was carried out for 1 hour. When the 4-MU glucuronidation activity was compared among UGT1A7*1, *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 nM. 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. 1B, the amount of UGT1A7*1 microsomal protein ranging from 10 to 100 μg (data not shown). The formation of 4-OHBP glucuronide was linear up to 3 hour (data not shown). The amount of microsomal UGT1A7*1 was set at 20 μg protein, and the incubation was performed for 1 hour. 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_max of UGT1A7*2 was smaller than that of the wild-type *1, the intrinsic clearance (V_max/K_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.

![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-UGT1A1 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 μg protein) were used as a positive control. Lane 1, microsomes expressing wild-type UGT1A1 (2.5 μg protein); lane 2, microsomes expressing wild-type UGT1A1 and CYP3A4 (4 μg protein); lane 3, microsomes expressing UGT1A1*6 (2 μg protein); lane 4, microsomes expressing UGT1A1*6 and CYP3A4 (4 μg protein). Mock represents microsomes (20 μg protein) obtained from Sf-9 cells transfected with control baculovirus. B. Western blotting was performed with nonspecific anti-UGT1A1 antibody as a primary antibody. Lanes 1-4 represent the same samples as those of (A).](image-url)

![Fig. 4. Michaelis-Menten plots of 4-MU glucuronidation catalyzed by UGT1A1 in the absence and presence of CYP3A4 coexpression. 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 μM (UGT1A1*1) and 20–800 μM (UGT1A1*6). The total amount of proteins added to the assay mixture was standardized at 40 μg. 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.](image-url)
whereas that of *3 was reduced. In particular, the effect of CYP3A4 on V_max was marked, with the V_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 studies were carried out for SN-38 glucuronidation catalyzed by UGT1A7 allozymes, and 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_max was significant, with the V_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. Like 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_max was significant, with the V_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 (1.4-fold) whereas that of *3 was reduced one-half by CYP3A4.

---

**TABLE 2**

Kinetic parameters for 4-methylumbelliflone, SN-38, and 17β-estradiol (3-hydroxy group) glucuronidation catalyzed by UGT1A1*1 and its allelic variant *6: effect of CYP3A4 cotransfection

Data were fitted to a Michaelis-Menten equation. Results are the estimated value ± S.E.

<table>
<thead>
<tr>
<th>UGT1A1 and a variant</th>
<th>S&lt;sub&gt;0&lt;/sub&gt;</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; pmol/mg protein</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-MU&lt;sup&gt;a&lt;/sup&gt;</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>211 ± 12**</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>SN-38&lt;sup&gt;b&lt;/sup&gt;</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 ± 0.4**</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>
<tr>
<td>17β-estradiol&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1</td>
<td>60.0 ± 1.4</td>
<td>150 ± 2</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>*1+CYP3A4</td>
<td>65.9 ± 1.9</td>
<td>231 ± 4**</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>*6</td>
<td>102 ± 2***</td>
<td>35.1 ± 0.6***</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>*6+CYP3A4</td>
<td>106 ± 1</td>
<td>66.3 ± 0.7**</td>
<td>5.4 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>UGT activity was determined in the presence of 2 mM UDP-GlcUA with 10 substrate concentrations ranging from 10 to 500 µM (UGT1A*1) and from 20 to 800 µM (UGT1A*6).

<sup>b</sup>Ranging from 2 to 100 µM.

<sup>c</sup>Ranging from 8 to 500 µM (UGT1A*1) and from 10 to 500 µM (UGT1A*6).

**Fig. 5.** Detection of interaction between CYP3A4 and UGT1A7 by overlay assay. Proteins in solubilized UGT1A7 supersome (5 µg) were separated by SDS-PAGE (10% gel) and transferred to a polyvinilidene 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," "UGT2B7," 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 microsomes 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 microscope samples of the single and double transfected 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 polyvinilidene difluoride membranes, and blotted with anti-UGT1A common (top) and polyclonal anti-CYP3A4 (bottom), antibodies, respectively.
Compared with the wild-type UGT1A7*1, 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 UGT1A7, significantly increased in allozymes *2 and *3 (Table 4). Therefore, it was examined for UGT1A7 allozymes *1, *2, and *3. Keeping the 4-MU glucuronidation determined using 4-MU as a substrate. The cofactor kinetics were measured for each UGT1A7 allozyme was investigated in two additional experiments. Then, the statistical significance of the estimated kinetic parameters was analyzed. Consequently, the intrinsic clearance of UGT1A7*2 was increased whereas that of *3 remained unchanged (Supplemental Fig. 3). The intrinsic clearance of the wild-type *1 remained unchanged, because both Km (UDP-GlcUA) and Vmax were increased by CYP3A4. It appears that N129K, R131K, and W208R of UGT1A7*3 affect not only its recognition of UDP-GlcUA but also the Vmax of *2 was not significantly different from *1, whereas the Vmax of *3 was significantly increased whereas that of *2 was significantly increased whereas that of *3 remained unchanged. The cofactor kinetics for each UGT1A7 allozyme was investigated in two additional experiments. Then, the statistical significance of the estimated kinetic parameters was analyzed. Consequently, the intrinsic clearance of UGT1A7*2 was increased whereas that of *3 was reduced (Supplemental Fig. 3). The intrinsic clearance of the wild-type *1 remained unchanged, because both Km (UDP-GlcUA) and Vmax were increased by CYP3A4. It appears that N129K, R131K, and W208R of UGT1A7*3 affect not only its recognition of UDP-GlcUA but also the modulation by CYP3A4.

Comparison of UGT1A and CYP3A4 Levels between Human Intestinal Microsomes and UGT1A7-CYP3A4 in the Dual Expression System. Although CYP3A4 alters UGT1A7-catalyzed glucuronidation in vitro, it is not known whether the dual expression system used mimics the situation of human intestinal microsomes. Figure 8 compares the level of CYP3A4 and UGT1As in human intestinal microsomes with a UGT1A7-CYP3A4 dual expression system (Fig. 8). Comparison with baculosomes for the CYP3A4-UGT1A7 dual expression system used mimics the situation of human intestinal microsomes. Although 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 a major isofrom involved in SN-38 glucuronidation. To date, 113 allelic variants of UGT1A1 have been identified, with most of the common variants occurring at nucleotide 309, resulting in the substitution of a threonine residue for glutamine. The CYP3A4-CYP3A5 cotransfection in baculosomes was used to determine the effect of CYP3A4 on UGT1A7 glucuronidation in vitro. Data were fitted to a Michaelis-Menten equation. Results are the estimated value ± S.E.

**TABLE 3**

<table>
<thead>
<tr>
<th>UGT1A7 allozymes</th>
<th>Km (μM)</th>
<th>Vmax (pmol/min/mg protein)</th>
<th>Vmax/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1</td>
<td>43.2 ± 3.0</td>
<td>46.5 ± 1.6</td>
<td>1.08</td>
</tr>
<tr>
<td>*1+CYP3A4</td>
<td>50.3 ± 3.2</td>
<td>72.6 ± 2.45</td>
<td>1.44</td>
</tr>
<tr>
<td>*2</td>
<td>34.1 ± 3.5</td>
<td>31.8 ± 1.5</td>
<td>0.93</td>
</tr>
<tr>
<td>*2+CYP3A4</td>
<td>26.8 ± 2.5</td>
<td>74.2 ± 3.0</td>
<td>2.76</td>
</tr>
<tr>
<td>*3</td>
<td>45.5 ± 6.4</td>
<td>56.8 ± 4.0</td>
<td>1.25</td>
</tr>
<tr>
<td>*3+CYP3A4</td>
<td>47.9 ± 2.2</td>
<td>32.5 ± 0.88</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Fig. 7. Michaelis-Menten plots of 4-MU glucuronidation in the UGT1A7 single expression system and CYP3A4/UGT1A7 coexpression system. The plots for allozymes UGT1A7*1 (A), *2 (B), and *3 (C) are shown. The 4-MU concentration ranged from 2 to 100 μM. The amount of protein used is described in the legend to Fig. 5. Kinetic parameters were calculated by curve fitting of a Michaelis-Menten equation and are listed in Table 3.
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. Alternatively, 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 stablization, 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*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 Gln399 in UGT1B7 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 sit(e)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/min/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 ± 22**</td>
<td>56.8 ± 0.9**</td>
<td>0.125</td>
</tr>
<tr>
<td>*2</td>
<td>461 ± 32**</td>
<td>33.6 ± 0.7</td>
<td>0.073</td>
</tr>
<tr>
<td>*2+CYP3A4</td>
<td>512 ± 45</td>
<td>85.2 ± 2.4**</td>
<td>0.166</td>
</tr>
<tr>
<td>*3</td>
<td>480 ± 36††</td>
<td>42.5 ± 1.0**</td>
<td>0.088</td>
</tr>
<tr>
<td>*3+CYP3A4</td>
<td>725 ± 60**</td>
<td>34.8 ± 1.0**</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 UGT1A2s 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.

---

online
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 W208R 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 UGT1A7*3 in addition to *2. However, it is possible that UGT1A7*3, but not *2, is a risk factor for carcinogenesis due to the reduced glucuronidation activity toward phenolic compounds.

**Acknowledgment**

We are grateful for technical support from the Research Support Center, Graduate School of Medical Sciences, Kyushu University.

**Authorship Contributions**

**Participated in research design:** Ishii, Koba, Kinoshita, Oizaki, Iwamoto, Takeda, Nishimura, Egoshi, Nagata, Yamazoe, Mackenzie, Yamada.

**Conducted experiments:** Ishii, Koba, Kinoshita, Oizaki, Iwamoto, Takeda, Egoshi.

**Contributed new reagents and analytical tools:** Koba, Kinoshita, Nishimura, Taura, Morimoto, Nagata, Yamazoe, Ikushiro, Mackenzie.

**Performed data analysis:** Ishii, Koba, Kinoshita, Oizaki, Iwamoto, Takeda, Miyachi, Yamada.

**Wrote or contributed to the writing of the manuscript:** Ishii, Koba, Kinoshita, Oizaki, Mackenzie, Yamada.

**References**


Ishii, Koba, Kinoshita, Oizaki, Mackenzie, Yamada.

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, Shuso 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
A

4-MU–G formed (pmol/min/mg protein)

PMF/min/mg protein

*1

*2

*3

B

SN-38–G formed (pmol/min/mg protein)

PMF/min/mg protein

*1

*2

*3

C

4-OHBP–G formed (pmol/min/mg protein)

PMF/min/mg protein

*1

*2

*3
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) vs pH for UGT1A7*1, UGT1A7*2, and 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 ($V_{\text{max}}/K_{\text{m}}$) 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 ($^{\ddagger\ddagger}p<0.01$) are indicated.