DMD Fast Forward. Published on October 2, 2008 as doi:10.1124/dmd.108.022913
This article has not been copyedited and formatted. The final version may differ from this version.

DMD#022913

Key amino acid residues responsible for the differences in substrate specificity of human UDP-glucuronosyltransferase (UGT) 1A9 and UGT1A8

Ryoichi Fujiwara, Miki Nakajima, Hiroyuki Yamanaka, and Tsuyoshi Yokoi *

Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920-1192, Japan

* *Corresponding author*

Running title: Arg42 and Asn152 are important for the substrate specificity

To whom all correspondence should be sent:

Tsuyoshi Yokoi, Ph.D.,

Drug Metabolism and Toxicology

Faculty of Pharmaceutical Sciences

Kanazawa University

Kakuma-machi, Kanazawa 920-1192, Japan

Tel / Fax +81-76-234-4407

E-mail: tyokoi@kenroku.kanazawa-u.ac.jp

Number of text pages: 13

Number of tables: 3

Number of figures: 5

Number of references: 24

Abstract: 247 words

Introduction: 353 words

Discussion: 845 words

Abbreviations: UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; 4-MU, 4-methylumbelliferone; *p*-NP, *p*-nitrophenol.

Abstract

Human UDP-glucuronosyltransferase (UGT) 1A9 is one of the major isoforms in liver and extrahepatic tissues, catalyzing the glucuronidation of a variety of drugs, dietary constituents, steroids, fatty acids, and bile acids. UGT1A9 shows high amino acid homology with UGT1A7, UGT1A8, and UGT1A10 with overlapping substrate specificity. However, the affinities for substrates are different among them. Amino acid alignment analysis revealed that 14 amino acids, Cys3, Arg42, Lys91, Ala92, Tyr106, Gly111, Tyr113, Asp115, Asn152, Leu173, Leu219, His221, Arg222, and Glu241, are unique to UGT1A9 compared with UGT1A7, UGT1A8, and UGT1A10. In this study, we constructed expression systems in HEK293 cells for seven mutants UGT1A9, Mut 1 (Arg42Gln), Mut 2 (Lys91Met, Ala92Asp), Mut 3 (Tyr106Phe, Gly111Ser, Asp115Gly), Mut 4 (Asn152Ala), Mut 5 (Leu173Ala), Mut 6 (Leu219Phe, His221Gln, Arg222Tyr), and Mut 7 (Glu241Ala), in which the amino acids were substituted to those of UGT1A8. Using these mutants, the effects of the amino acid changes on the activities of 4-methylumbelliferone (4-MU), *p*-nitrophenol (*p*-NP), and 3-hydroxydesloratadine glucuronidations were investigated. For 4-MU and *p*-NP *O*-glucuronidations, Mut 1 and Mut 4 exhibited higher K_m values and Mut 3 and Mut 4 exhibited higher V_{max} values compared to wild-type UGT1A9. Interestingly, only Mut 4 was active toward 3-hydroxydesloratadine *O*-glucuronidation that is specific for UGT1A8. The findings reveal that the residues Arg42 and Asn152 may have large contribution to the difference in the substrate specificity with that of UGT1A8, although all of the unique amino acids of UGT1A9 would be collectively involved in the catalytic property.

Introduction

Human UDP-glucuronosyltransferases (EC 2.4.1.17; UGTs) play a key role in the metabolism of endogenous and exogenous compounds (Tukey and Strassburg, 2000). Human UGT superfamily is classified into UGT1 and UGT2 families, and further classified into subfamilies and isoforms, based on similarities between their amino acid sequences and gene organization (Mackenzie et al., 2005). Characterization of genomic DNA clones encoding the *UGT1A* gene has shown that the *UGT1A* locus comprises multiple first exons that encode isoform-specific sequences and a single set of commonly used exons 2-5 that encodes the same sequence of all UGT1 isoforms (Ritter et al., 1992; Bosma et al., 1992). The C-terminal domain conserved in UGT1 and UGT2 families is responsible for the UDPGA binding, whereas the N-terminal halves contain the aglycone binding site (Mackenzie, 1990).

Human UGT1A9 is one of the major isoforms in liver and extrahepatic tissues (Tukey and Strassburg, 2000). UGT1A7, UGT1A8, UGT1A9, and UGT1A10 share very high amino acid similarity (93 – 95%) within the UGT1A family members and exhibit overlapping substrate specificity (Strassburg et al., 1998; Tukey and Strassburg, 2000). However, the binding affinities of substrates and catalytic efficiency are different between the four isoforms. Besides, some substrates are specific for certain UGT1A enzyme as follows; 5-(4'-hydroxyphenyl)-5-phenylhydantoin (UGT1A9) (Nakajima et al., 2007), phenylbutazone (UGT1A9) (Nishiyama et al., 2006), and 3-hydroxydesloratadine (UGT1A8) (Ghosal et al., 2004). By a sequence alignment analysis, 14 amino acid residues (Cys3, Arg42, Lys91, Ala92, Tyr106, Gly111, Tyr113, Asp115, Asn152, Leu173, Leu219, His221, Arg222, and Glu241) were found to be specific to UGT1A9 by comparison with UGT1A7, UGT1A8, and UGT1A10 (Fig. 1). In this study, we constructed expression systems in HEK293 cells for seven mutant UGT1A9s, Mut 1 (Arg42Gln), Mut 2 (Lys91Met, Ala92Asp), Mut 3 (Tyr106Phe, Gly111Ser, Asp115Gly), Mut 4 (Asn152Ala), Mut 5 (Leu173Ala), Mut 6 (Leu219Phe, His221Gln, Arg222Tyr), and Mut 7 (Glu241Ala), in which amino acids were substituted to those of UGT1A8. Since Cys3 is in the signal peptide sequence, the mutant including this residue was

not constructed. We performed kinetic analysis using seven mutants to understand the residues determining the differences in the substrate specificity and catalytic efficiency between UGT1A9 and UGT1A8.

Materials and Methods

Chemicals and reagents

UDPGA, alamethicin, 4-MU, 4-MU *O*-glucuronide, *p*-NP, and *p*-NP *O*-glucuronide were purchased from Sigma-Aldrich (St. Louis, MO). 3-Hydroxydesloratadine was obtained from Toronto Research Chemicals (Toronto, Canada). Recombinant UGT1A7, UGT1A8, UGT1A9, and UGT1A10 expressed in baculovirus-infected insect cells (Supersomes) and rabbit anti-human UGT1A antibody were from BD Gentest (Woburn, MA). Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). All other chemicals and solvents were of the highest or analytical grade commercially available.

Construction of mutants human UGT1A9 and expression in HEK293 cells

The expression vector for human UGT1A9 was previously constructed (Fujiwara et al., 2007b). Site-directed mutagenesis was carried out using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacturer's instruction. The forward mutagenic primers are shown in Table 1. The nucleotide sequences of the constructs were confirmed by DNA sequencing analyses. Transfection of the expression vectors into HEK293 cells and the preparation of the total cell homogenates were performed as described previously (Fujiwara et al., 2007a and 2007b).

Immunoblot analysis

The UGT protein levels were determined by immunoblot analysis performed as described previously (Fujiwara et al., 2007a and 2007b). The UGT expression levels were defined on the basis on a standard curve using the wild-type UGT1A9 expression system (1 unit per 1 mg of

cell homogenates).

Enzymatic assays

Glucuronidations of 4-MU and *p*-NP were measured as described previously (Fujiwara et al., 2007b) with slight modifications. Briefly, a typical incubation mixture (200 µL of total volume) contained 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 2.5 mM UDPGA, 25 µg/mL alamethicin, 0.1 mg/mL total cell homogenates or recombinant UGTs, and substrates. The reaction was initiated by the addition of substrate and the mixture was incubated at 37°C for 10 min (the enzymatic activities were confirmed to be linear at least 20 min). The reaction was terminated by the addition of 100 µL of ice-cold methanol. After removal of the protein by centrifugation at 13,000 g for 5 min, a 20-µL portion of the sample was subjected to HPLC. HPLC was performed using an L-7100 pump (Hitachi, Tokyo, Japan), an L-7200 autosampler (Hitachi), a D-2500 integrator (Hitachi), and a CAPCELL PAK column (4.6 × 150 mm; 5 µm, Shiseido, Tokyo, Japan). The flow rate was 0.5 mL/min and the column temperature was 35°C. The eluent was monitored at 320 nm using an L-7405 UV detector (Hitachi). The mobile phase was 30% methanol including 20 mM KH_2PO_4. The quantification of 4-MU *O*-glucuronide and *p*-NP *O*-glucuronide was performed by comparing the HPLC peak heights to those of the authentic standards.

3-Hydroxydesloratadine *O*-glucuronide formation was determined according to the method of Ghosal et al. (2004) with slight modifications. Briefly, a typical incubation mixture (200 µL of total volume) contained 50 mM Tris-HCl or potassium phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 2.5 mM UDPGA, 25 µg/mL alamethicin, 1.0 mg/mL total cell homogenates or recombinant UGTs, and 30 µM 3-hydroxydesloratadine. The reaction was initiated by the addition of UDPGA following a 3-min preincubation at 37°C and the mixture was incubated at 37°C for 60 min (the enzyme activity was confirmed to be linear at least 90 min). The reaction was terminated by the addition of 100 µL of ice-cold methanol. After removal of the protein by centrifugation at 13,000 g for 5 min, a 50-µL portion of the sample was subjected to HPLC. HPLC apparatus, column, column temperature, and mobile phase were the same above. The

flow rate was 1.0 mL/min and the eluent was monitored at 241 nm. The 3-hydroxydesloratadine *O*-glucuronide formation was determined by the decrease of the peak area of the 3-hydroxydesloratadine. The retention times of 3-hydroxydesloratadine and its *O*-glucuronide were 21.9 and 6.3 min, respectively. The detection limit of the 3-hydroxydesloratadine was 0.5 pmol per injection.

Kinetic parameters were estimated from the fitted curve using a computer program (KaleidaGraph, Synergy Software, Reading, PA) designed for non-linear regression analysis. The following equation was used: $V = V_{max} \cdot [S] / (K_m + [S])$

where V is the velocity of the reaction at substrate concentration *S*, K_m is Michaelis-Menten constant, V_{max} is the maximum velocity. All the activities were normalized with the expression level of UGT proteins.

Native-PAGE analysis of wild-type and mutants UGT1A9

Total cell homogenates of each expression system in HEK293 cells were lysed in the solubilizing buffer (1.0 mg/mL in 0.5% NP-40, 0.25% sodium deoxycholate, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 1 mM EDTA) on ice for 2 h and were centrifuged at 13,000 g for 30 min. To the 20-µL portion of supernatant, 1 µL of 60% glycerol containing 0.2% bromophenol blue was added. The samples were applied to Perfect NT Gel M (5-20% gradient, DRC, Tokyo, Japan). Immunoblotting was carried out using anti-human UGT1A antibody.

Statistical analyses

Data are expressed as mean ± SD of three independent determinations. Statistical significance of the kinetic parameters was determined by analysis of variance (ANOVA) followed by Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

Results

Expression of wild-type and mutants UGT1A9 in HEK293 cells

The expression levels of UGT protein in HEK293 cells were determined by immunoblot analysis (Fig. 2). The UGT levels of Mut 1, Mut 2, Mut 3, Mut 4, Mut 5, Mut 6, and Mut 7 were 1.43, 0.07, 0.37, 1.88, 0.39, 1.05, and 0.49 unit/mg protein, respectively. By contrast, the expression levels of recombinant UGT1A9, UGT1A7, UGT1A8, and UGT1A10 in insect cells (Supersomes) were 13.7, 12.5, 17.6, and 10.4 unit/mg protein (data not shown). The lower UGT1A levels in HEK293 cells compared to the Supersomes would be due to the usage of the total cell homogenates instead of membrane fractions. In the subsequent study, enzymatic activities were normalized with the relative expression level.

4-MU *O*-glucuronidation by wild-type and mutants UGT1A9

First, we performed kinetic analysis for 4-MU *O*-glucuronidation using UGT Supersomes to compare the kinetic parameters by UGT1A9 with those by UGT1A7, UGT1A8, and UGT1A10 (Table 2). The reactions followed the Michaelis-Menten kinetics. The K_m, V_{max}, and V_{max}/K_m values of the UGT1A9 Supersomes were 9.6 ± 1.3 μM, 1.2 ± 0.1 nmol/min/unit, and 123 ± 14 μL/min/unit, respectively. The K_m value of UGT1A7 was 2.5 fold higher than that of UGT1A9. UGT1A8 exhibited a 20-fold higher K_m value and a 6-fold lower V_{max} value than UGT1A9. UGT1A10 showed a 8-fold higher K_m value and 60-fold lower V_{max} value than UGT1A10. These results suggest that UGT1A9 has the lowest apparent affinity for 4-MU among four UGT1A enzymes, consistent with previous studies (Uchaipichat et al., 2004, Luukkanen et al., 2005, Xiong et al., 2006).

Next, we performed kinetic analysis using wild-type and mutants UGT1A9 expressed in HEK293 cells. The reactions followed Michaelis-Menten kinetics. The K_m, V_{max}, and V_{max}/K_m values of wild-type UGT1A9 were 4.4 ± 0.5 μM, 3.9 ± 0.3 nmol/min/unit, and 891 ± 45 μL/min/unit, respectively (Fig. 3, Table 2). Mut 1 significantly increased the K_m and V_{max} values (2-fold) compared to those of wild-type UGT1A9. Mut 4 also significantly increased the K_m and V_{max} values. Mut 3 showed an increased V_{max} value without a change in the K_m value. The other mutants did not alter the K_m and V_{max} values. These results suggest that Arg42 and

DMD Fast Forward. Published on October 2, 2008 as DOI: 10.1124/dmd.108.022913
This article has not been copyedited and formatted. The final version may differ from this version.

Asn152 might contribute to the substrate specificity of UGT1A9.

p-NP *O*-glucuronidation by wild-type and mutants UGT1A9

Kinetic parameters for *p*-NP O-glucuronidation by the UGT Supersomes were determined (Table 3). The reactions followed the Michaelis-Menten kinetics. The K_m, V_{max}, and V_{max}/K_m values of the UGT1A9 Supersomes were 13.9 ± 0.8 μM, 0.5 ± 0.05 nmol/min/unit, and 33 ± 2 μL/min/unit, respectively. UGT1A7 showed a 20-fold higher K_m value and a 2-fold lower V_{max} value than those of UGT1A9. UGT1A10 exhibited a 140-fold higher K_m value and a 15-fold lower V_{max} value than those of UGt1A9. Kinetic parameters by UGT1A8 could not be calculated because of the enzyme's low affinity for the substrate. These results suggest that UGT1A9 has the lowest apparent affinity for *p*-NP among four UGT1A enzymes, consistent with previous studies (Luukkanen et al., 2005, Xiong et al., 2006).

Next, the kinetic parameters by wild-type and mutants UGT1A9 expressed in HEK293 cells were determined (Fig. 3, Table 3). The reactions followed the Michaelis-Menten kinetics. The K_m, V_{max}, and V_{max}/K_m values of wild-type UGT1A9 were 16.9 ± 1.2 μM, 1.0 ± 0.1 nmol/min/unit, and 58 ± 4 μL/min/unit, respectively. Mut 1 and Mut 4 significantly increased K_m values (2- and 3-fold) compared to that of wild-type UGT1A9. Mut 3, Mut 4, and Mut 5 exhibited higher V_{max} values than that of wild-type UGT1A9. Mut 6 and Mut 7 exhibited lower V_{max} values than that of wild-type UGT1A9. These results suggest that Arg42 and Asn152 might contribute to the substrate specificity of UGT1A9.

3-Hydroxydesloratadine *O*-glucuronidation by wild-type and mutants UGT1A9

First, we determined the 3-hydroxydesloratadine *O*-glucuronidation by UGT1A9, UGT1A7, UGT1A8, and UGT1A10 Supersomes (Fig. 4). Among them, only UGT1A8 showed the catalytic activity (0.4 ± 0.02 pmol/min/unit), supporting a previous study (Nishiyama et al., 2006). Then, we determined the 3-hydroxydesloratadine *O*-glucuronidation by wild-type and mutants UGT1A9 expressed in HEK293 cells. In accordance with the results using Supersomes, UGT1A9 did not exhibit the catalytic activity (Fig. 4). The most interesting finding is that Mut

4 exhibited catalytic activity toward 3-hydroxydesloratadine with a higher turnover rate than that of UGT1A8 Supersomes (2.9 ± 0.2 pmol/min/unit). These results suggest that the residue at 152 would play an important role for the recognition of 3-hydroxydesloratadine as a substrate.

Native-PAGE analysis of wild-type and mutants UGT1A9

It has been demonstrated that UGTs form dimmers and the dimerization affects the catalytic property of UGTs (Finel and Kurkela, 2008). To investigate whether the changes of activities in the mutants might be linked to the changes in the ability to form dimmers, we performed native-PAGE analysis. As shown in Fig. 5, all mutants showed an obvious band representing the dimer with similar extent of wild type. Thus, the changes of the kinetics in mutants may not be due to the changes in the ability to form dimmers.

Discussion

It is generally accepted that the N-terminal domain in UGT is responsible for the substrate binding, whereas the C-terminal domain is responsible for the UDPGA binding (Mackenzie, 1990). Although amino acid substitutions in the C-terminal region also affected the substrate binding, recent studies suggested that it might be due to the altered interaction between the N-terminal domain and C-terminal domain (Kurkela et al., 2004; Finel and Kurkela, 2008). In the present study, we investigated the effects of substitution of amino acids in the N-terminal region of UGT1A9 to corresponding residues of UGT1A8 on the enzyme kinetics using seven mutants.

The recombinant UGT1A9 (wild-type) in HEK293 cells showed higher catalytic activities than the UGT1A9 Supersomes when the activities were normalized with the relative expression level (Tables 2 and 3). Such higher activities in HEK293 system than Supersomes were also observed for the glucuronidations of 5-(4'-hydroxyphenyl)-5-phenylhydantoin (Nakajima et al., 2007) and *N*-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (Malfatti and Felton, 2004: Dellinger et al., 2007). The differences in the membrane environment or lipid components and/or the differences in the post-translational modulation of UGT such as

glycosylation or phosphorylation between the host cells (Barbier et al., 2000; Basu et al., 2005) may be involved in the inconsistency. In the present study using Supersomes, the V_{max} values of UGT1A8 and UGT1A10 were considerably lower than those of UGT1A7 and UGT1A9 for 4-MU and *p*-NP *O*-glucuronidations. In contrast, previous studies using their own recombinant systems have demonstrated that the enzymatic activities for 4-MU and *p*-NP *O*-glucuronidation by the four enzymes were almost the same (Uchaipichat et al., 2004; Luukkanen et al., 2005; Xiong et al., 2006). Supporting our data, the manufacture's data sheet shows that 7-hydroxy-4-trifluoromethylcoumarin glucuronidation activities by UGT1A8 (610 pmol/min/mg) and UGT1A10 (150 pmol/min/mg) Supersomes were substantially lower than those by UGT1A7 (5100 pmol/min/mg) and UGT1A9 (8100 pmol/min/mg) Supersomes. Therefore, it is assumed that UGT1A8 and UGT1A10 Supersomes may include large amount of inactive protein.

Although 4-MU and p-NP *O*-glucuronidations were catalyzed by UGT1A7, UGT1A8, UGT1A9, and UGT1A10, UGT1A9 showed the lowest K_m values. Interestingly, we found that Mut 1 and Mut 4 exhibited higher K_m values for 4-MU and *p*-NP than wild-type UGT1A9. Therefore, it is suggested that the substitutions of Arg42Gln and Asn152Ala provided a critical impact on binding affinities for 4-MU and *p*-NP. When we dissect the reported three dimensional structure of bacterial GtfA (TDP-*epi*-vancosaminyltransferase, PDB ID: 1PN3), belonging to GT1 family similar to human UGTs, which adopts the GT-B fold, the residue corresponding to Arg42 of UGT1A9 was in the cleft between N- and C-terminal domains (data not shown). Therefore, it was proposed that Arg42 of UGT1A9 might be responsible for the substrate binding. The importance of this region was supported by a previous paper reporting that the residues at 36 and 40 of UGT1A3 and UGT1A4 had critical roles in the substrate selectivities (Kubota et al., 2007). In addition to the changes of K_m values, Mut 4 exhibited higher V_{max} values for 4-MU and *p*-NP than wild-type UGT1A9. The residue of GtfA corresponding to Asn152 of UGT1A9 was not in the cleft between the N- and C-terminal domains, and was far from key residues for catalysis. Therefore, the Asn152 seems to unlikely affect the role of these catalytic residues. The changes in the kinetic parameter by the

substitution of Asn152 to Ala might be due to the steric effect.

Mut 3 (Tyr106Phe, Gly111Ser, Asp115Gly) also showed significantly higher V_{max} values than the wild type for 4-MU and *p*-NP *O*-glucuronidations. The region of GtfA corresponding to the residues 106, 111, and 115 of UGT1A9 were not in the cleft between the N- and C-terminal domains. A previous paper (Caillier et al., 2007) has reported that mutations of Phe110, Met114, and Ala158 of UGT1A3 caused the changes of V_{max} value. Thus, this region may be a determinant for catalytic activity.

Of particular interest was that Mut 4 catalyzed the 3-hydroxydesloratadine *O*-glucuronidation, which is a specific activity for UGT1A8. Most concern was that how similar the K_m value by Mut 4 with that of UGT1A8. Unfortunately, the kinetic analysis could not be accomplished in our system because the activity of 3-hydroxydesloratadine *O*-glucuronidation was substantially low. However, it should be received attention that the mutants UGT1A9 we constructed were HEK293 expression system, whereas the UGT1A8 was from baculovirus-infected insect cell system. Accumulating evidences revealed that K_m values of certain glucuronidation vary among enzyme sources (Soars et al., 2003; Rowland et al., 2008; Fujiwara et al., 2007a and 2007b). Therefore, even if we could perform the kinetic analysis, we may not draw a definitive conclusion from the comparison of K_m values between Mut 4 and UGT1A8.

The present study revealed that some UGT1A9 mutants showed the high K_m values for 4-MU and *p*-NP *O*-glucuronidations, but these values were still far from the K_m values by UGT1A8. In addition, the activity for 3-hydroxydesloratadine *O*-glucuronidation by a mutant UGT1A9 was surprisingly over the activity by UGT1A8. Collectively, it was surmised that all of unique amino acid residues would collectively determine the substrate specificity and catalytic property, although the Arg42 and Asn152 may have large contribution.

Acknowledgements

We acknowledge Brent Bell for reviewing the manuscript.

References

Barbier O, Girard C, Breton R, Belanger A, and Hum DW (2000) *N*-Glycosylation and residue 96 are involved in the functional properties of UDP-glucuronosyltransferase enzymes. *Biochemistry* 39: 11540-11552.

Basu NK, Kovarova M, Garza A, Kubota S, Saha T, Mitra PS, Banerjee R, Rivera J, and Owens IS (2005) Phosphorylation of a UDP-glucuronosyltransferase regulates substrate specificity. *Proc Natl Acad Sci USA* 102: 6285-6290.

Bosma PJ, Chowdhury NR, Goldhoorn BG, Hofker MH, Oude Elferink RP, Jansen PL, and Chowdhury JR (1992) Sequence of exons and the flanking regions of human bilirubin-UDP-glucuronosyltransferase gene complex and identification of a genetic mutation in a patient with Crigler-Najjar syndrome, type I. *Hepatology* 15: 941-947.

Caillier B, Lépine J, Tojcic J, Ménard V, Perusse L, Bélanger A, Barbier O, and Guillemette C (2007) A pharmacogenomics study of the human estrogen glucuronosyltransferase UGT1A3. *Pharmacogenet Genomics* 17: 481-495.

Dellinger RW, Chen G, Blevins-Primeau AS, Krzeminski J, Amin S, and Lazarus P (2007) Glucuronidation of PhIP and *N*-OH-PhIP by UDP-glucuronosyltransferase 1A10. *Carcinogenesis* 28: 2412-2418.

Finel M and Kurkela M (2008) The UDP-glucuronosyltransferases as oligomeric enzymes. *Curr Drug Metab* 9: 70-76.

Fujiwara R, Nakajima M, Yamanaka H, Katoh M, and Yokoi T (2007a) Interactions between human UGT1A1, UGT1A4, and UGT1A6 affect their enzymatic activities. *Drug Metab Dispos* 35: 1781-1787.

Fujiwara R, Nakajima M, Yamanaka H, Nakamura A, Katoh M, Ikushiro S, Sakaki T, and Yokoi T (2007b) Effects of coexpression of UGT1A9 on enzymatic activities of human UGT1A isoforms. *Drug Metab Dispos* 35: 747-757.

Ghosal A, Yuan Y, Hapangama N, Su AD, Alvarez N, Chowdhury SK, Alton KB, Patrick JE, and Zbaida S (2004) Identification of human UDP-glucuronosyltransferase enzyme(s)

responsible for the glucuronidation of 3-hydroxydesloratadine. *Biopharm Drug Dispos* 25: 243-252.

Kubota T, Lewis BC, Elliot DJ, Mackenzie PI, and Miners JO (2007) Critical roles of residues 36 and 40 in the phenol and tertiary amine aglycone substrate selectivities of UDP-glucuronosyltransferases 1A3 and 1A4. *Mol Pharmacol* 72: 1054-1062.

Kurkela M, Hirvonen J, Kostiainen R, and Finel M (2004) The interactions between the N-terminal and C-terminal domains of the human UDP-glucuronosyltransferases are partly isoform-specific, and may involve both monomers. *Biochem Pharmacol* 68: 2443-2450.

Luukkanen L, Taskinen J, Kurkela M, Kostiainen R, Hirvonen J, and Finel M (2005) Kinetic characterization of the 1A subfamily of recombinant human UDP-glucuronosyltransferases. *Drug Metab Dispos* 33: 1017-1026.

Mackenzie PI (1990) Expression of chimeric cDNAs in cell culture defines a region of UDP glucuronosyltransferase involved in substrate selection. *J Biol Chem* 265: 3432-3435.

Mackenzie PI, Walter Bock K, Burchell B, Guillemette C, Ikushiro S, Iyanagi T, Miners JO, Owens IS, and Nebert DW (2005) Nomenclature update for the mammalian UDP glycosyltransferase (*UGT*) gene superfamily. *Pharmacogenet Genomics* 15: 677-685.

Malfatti MA and Felton JS (2004) Human UDP-glucuronosyltransferase 1A1 is the primary enzyme responsible for the *N*-glucuronidation of *N*-hydroxy-PhIP in vitro. *Chem Res Toxicol* 17: 1137-1144.

Nakajima M, Yamanaka H, Fujiwara R, Katoh, M, and Yokoi T (2007) Stereoselective glucuronidation of 5-(4'-hydroxyphenyl)-5-phenylhydantoin by human UDP-glucuronosyltransferase (UGT) 1A1, UGT1A9, and UGT2B15: effects of UGT-UGT interactions. *Drug Metab Dispos* 35: 1679-1686.

Nishiyama T, Kobori T, Arai K, Ogura K, Ohnuma T, Ishii K, Hayashi K, and Hiratsuka A (2006) Identification of human UDP-glucuronosyltransferase isoform(s) responsible for the C-glucuronidation of phenylbutazone. *Arch Biochem Biophys* 454: 72-79.

Ritter JK, Chen F, Sheen YY, Tran HM, Kimura S, Yeatman MT, and Owens IS (1992) A novel

complex locus UGT1 encodes human bilirubin, phenol, and other UDP-glucuronosyltransferase isozymes with identical carboxyl termini. *J Biol Chem* 267: 3257-3261.

Rowland A, Knights KM, Mackenzie PI, and Miners JO (2008) The "albumin effect" and drug glucuronidation: bovine serum albumin and fatty acid-free human serum albumin enhance the glucuronidation of UDP-glucuronosyltransferase (UGT) 1A9 substrates but not UGT1A1 and UGT1A6 activities. *Drug Metab Dispos* 36: 1056-1062.

Soars MG, Ring BJ, and Wrighton SA (2003) The effect of incubation conditions on the enzyme kinetics of UDP-glucuronosyltransferases. *Drug Metab Dispos* 31: 762–767.

Strassburg CP, Manns MP, and Tukey RH (1998) Expression of the UDP-glucuronosyltransferase 1A locus in human colon. Identification and characterization of the novel extrahepatic UGT1A8. *J Biol Chem* 273: 8719-8726.

Tukey RH and Strassburg CP (2000) Human UDP-glucuronosyltransferases: metabolism, expression and disease. *Annu Rev Pharmacol Toxicol* 40: 581-616.

Uchaipichat V, Mackenzie PI, Guo XH, Gardner-Stephen D, Galetin A, Houston JB, and Miners JO (2004) Human udp-glucuronosyltransferases: isoform selectivity and kinetics of 4-methylumbelliferone and 1-naphthol glucuronidation, effects of organic solvents, and inhibition by diclofenac and probenecid. *Drug Metab Dispos* 32: 413-423.

Xiong Y, Bernardi D, Bratton S, Ward MD, Battaglia E, Finel M, Drake RR, and Radominska-Pandya A (2006) Phenylalanine 90 and 93 are localized within the phenol binding site of human UDP-glucuronosyltransferase 1A10 as determined by photoaffinity labeling, mass spectrometry, and site-directed mutagenesis. *Biochemistry* 45: 2322-2332.

DMD Fast Forward. Published on October 2, 2008 as DOI: 10.1124/dmd.108.022913
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Fig. 1. Sequence alignment of human UGT1A9 compared with UGT1A7, UGT1A8, UGT1A10, and GtfA. Amino acid residues, which are not common between UGT1A9, UGT1A7, UGT1A8, and UGT1A10, are shown in gray boxes. Especially, those of UGT1A9 are marked with arrowheads. Asterisks show consistency between UGT1A9 and GtfA.

Fig. 2. Immunoblot analysis of recombinant wild-type and mutants UGT1A9 expressed in HEK293 cells. Five μg of total cell homogenates were separated by SDS-10% PAGE. The transferred membrane was probed with a rabbit anti-human UGT1A antibody. The UGT expression levels were defined on the basis of a standard curve using the wild-type UGT1A9 (1 unit per 1 mg of cell homogenates). Each column is the mean ± SD of three independent determinations.

Fig. 3. Kinetic analyses of 4-MU (A) and *p*-NP (B) *O*-glucuronidations by wild-type and mutants UGT1A9 expressed in HEK293 cells. (A) 4-MU *O*-glucuronidation was determined with 0.25 mg/ml cell homogenates, 0.5 - 100 μM 4-MU, and 2.5 mM UDPGA at 37 °C for 15 min. The curves were fitted to the Michaelis-Menten kinetics. (B) *p*-NP *O*-glucuronidation was determined with 0.1 mg/ml cell homogenates, 5 - 200 μM *p*-NP, and 2.5 mM UDPGA at 37 °C for 10 min. The curves were fitted to the Michaelis-Menten kinetics. Data are the mean of three independent determinations.

Fig. 4. 3-Hydroxydesloratadine *O*-glucuronidation by recombinant UGTs in insect cells and HEK293 cells. 3-Hydroxydesloratadine *O*-glucuronidation was determined with 1.0 mg/ml protein, 30 μM 3-hydroxydesloratadine, and 2.5 mM UDPGA at 37°C for 60 min. Each column is the mean ± SD of three independent determinations. ND, not detected.

Fig. 5. Native-PAGE analysis of wild-type and mutants UGT1A9. Twenty μg of total cell

homogenates of each expression system in HEK293 cells were lysed and subjected to the native-PAGE analysis. Immunoblotting was carried out using anti-human UGT1A antibody.

Table 1. Forward mutagenic primers used for site-directed mutagenesis.

Name	Mutation	Primer	Sequence
Mut 1	Arg42Gln	Mut 1	5'-G AGC CAC TGG TTC ACC ATG **CA**G TCG GTG GTG GAG AAA CTC ATT C-3'
Mut 2	Lys91Met, Ala92Asp	Mut 2	5'-GAT CTG GAC CGG GAG TTC A**T**G G**A**T TTT GCC CAT GCT CAA TG-3'
Mut 3	Tyr106Phe, Gly111Ser	Mut 3-1	5'-CA CAA GTA CGA AGT ATA T**T**T TCT CTA TTA ATG **A**GT TCA TAC AAT G-3'
	Asp 115Gly	Mut 3-2	5'-G GGT TCA TAC AAT G**GT** ATT TTT GAC TTA TTT TTT TCA AAT TGC AGG-3'
Mut 4	Asn152Ala	Mut 4	5'-CA GTG TTT CTC GAT CCT TTT GAT **GC**C TGT GGC TTA ATT GTT GCC-3'
Mut 5	Leu173Ala	Mut 5	5'-GCT TTC GCC AGG GGA ATA **GC**T TGC CAC TAT CTT GAA GAA GGT G-3'
Mut 6	Leu219Phe, His221 Gln, Arg222Tyr	Mut 6	5'-G CAC TTG GAG GAA CAT TTA TT**T** TGC CA**G** **TA**T TTT TTC AAA AAT GC-3'
Mut 7	Glu241Ala	Mut 7	5'-CTC CAA ACA CCT GTT ACG G**CA** TAT GAT CTC TAC AGC CAC AC-3'

Mutated sites are indicated by underlined bold letters.

DMD Fast Forward. Published on October 2, 2008 as DOI: 10.1124/dmd.108.022913
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. Kinetic parameters for 4-MU *O*-glucuronidation by UGT1A9, UGT1A7, UGT1A8, and UGT1A10 expressed in insect cells (Supersomes) and wild-type and mutants UGT1A9 expressed in HEK293 cells.

	K_m (μM)	V_{max} (nmol/min/unit)	V_{max} / K_m (μL/min/unit)
Supersomes			
UGT1A9	9.6 ± 1.3	1.2 ± 0.1	123 ± 14
UGT1A7	23.9 ± 3.9	1.4 ± 0.3	57 ± 3
UGT1A8	191.7 ± 8.8	0.2 ± 0.01	1.1 ± 0.1
UGT1A10	76.2 ± 14.6	0.02 ± 0.002	0.28 ± 0.05
HEK293 cells			
Wild-type UGT1A9	4.4 ± 0.5	3.9 ± 0.3	891 ± 45
Mut 1	10.1 ± 0.3 **	7.3 ± 1.1 **	723 ± 109
Mut 2	3.6 ± 0.5	3.2 ± 0.3	913 ± 176
Mut 3	5.6 ± 0.6	10.4 ± 2.1 **	1883 ± 427 **
Mut 4	9.1 ± 2.2 **	12.8 ± 1.0 **	1441 ± 242
Mut 5	2.1 ± 0.4	5.5 ± 0.2	2678 ± 456 **
Mut 6	5.9 ± 1.3	4.0 ± 0.4	681 ± 78
Mut 7	4.4 ± 1.1	3.4 ± 0.6	789 ± 73

The substrate concentrations were 0.5-100 μM for HEK293 expression systems, UGT1A7 and UGT1A9 Supersomes and 25-1000 μM for UGT1A8 and UGT1A10 Supersomes. Data are mean ± SD of three independent determinations. The V_{max} values were normalized with the relative UGT expression level. ** $P < 0.01$ compared with wild-type UGT1A9.

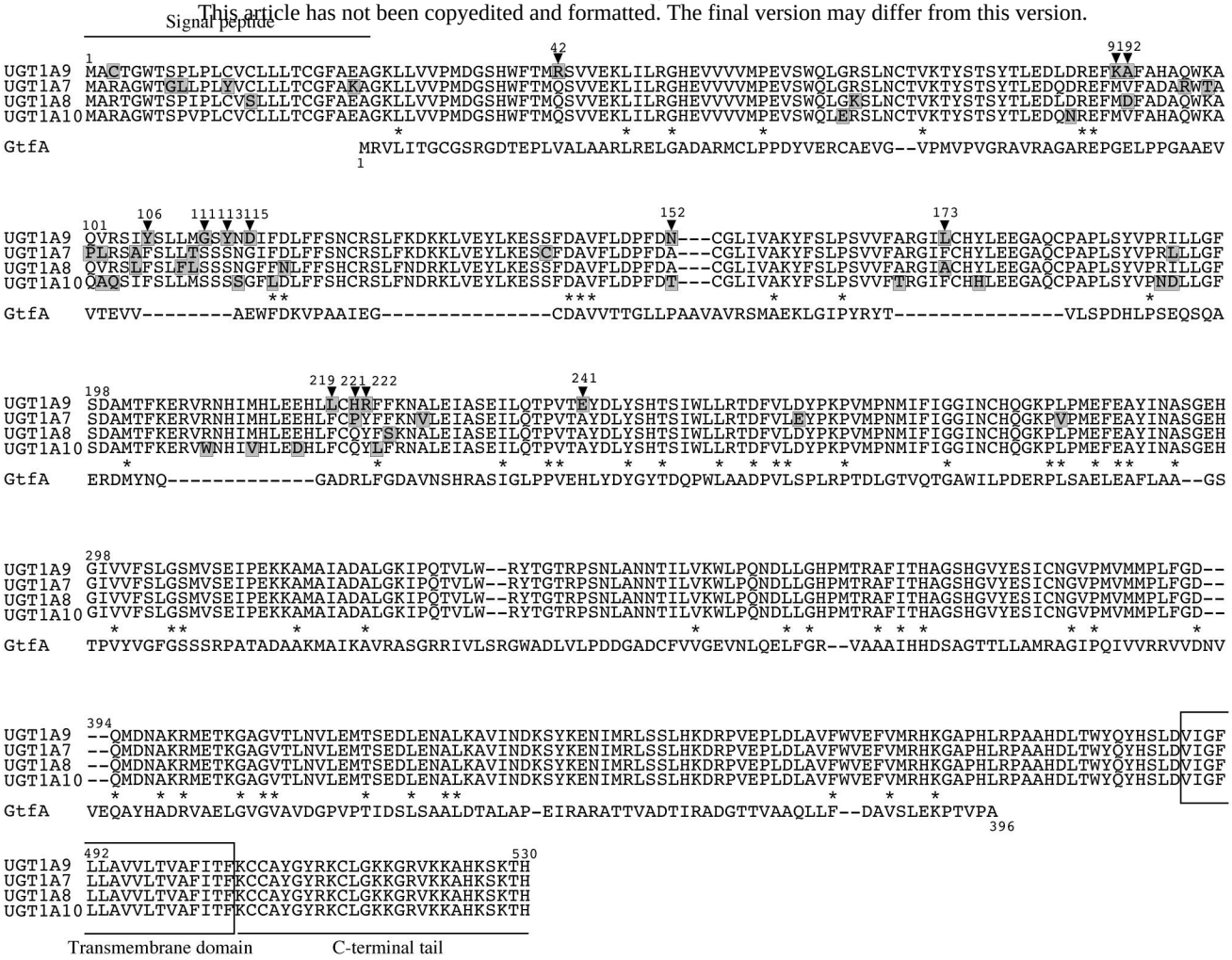
DMD Fast Forward. Published on October 2, 2008 as DOI: 10.1124/dmd.108.022913
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3. Kinetic parameters for *p*-NP *O*-glucuronidation by UGT1A9, UGT1A7, UGT1A8, and UGT1A10 expressed in insect cells (Supersomes) and wild-type and mutants UGT1A9 expressed in HEK293 cells.

	K_m (μM)	V_{max} (nmol/min/unit)	V_{max} / K_m (μL/min/unit)
Supersomes			
UGT1A9	13.9 ± 0.8	0.5 ± 0.05	33 ± 2
UGT1A7	268.7 ± 27.8	0.2 ± 0.08	0.59 ± 0.18
UGT1A8	> 10000	-	
UGT1A10	1966.7 ± 723.4	0.03 ± 0.001	0.015 ± 0.004
HEK293 cells			
Wild-type UGT1A9	16.9 ± 1.2	1.0 ± 0.1	58 ± 4
Mut 1	30.0 ± 4.3 *	1.1 ± 0.1	38 ± 5
Mut 2	17.8 ± 6.4	0.7 ± 0.06	45 ± 16
Mut 3	10.2 ± 5.7	1.6 ± 0.1 **	192 ± 100 **
Mut 4	49.2 ± 2.7 **	2.0 ± 0.1 **	41 ± 4
Mut 5	15.7 ± 0.8	1.5 ± 0.3 **	96 ± 23
Mut 6	15.2 ± 5.3	0.6 ± 0.08 *	41 ± 9
Mut 7	18.8 ± 5.4	0.6 ± 0.04 *	32 ± 12

The substrate concentrations were 5-200 μM for HEK293 expression systems and UGT1A9 Supersomes, 50-2000 μM for UGT1A7 Supersomes, and 0.25-10 mM for UGT1A10 Supersomes. Data are mean ± SD of three independent determinations. The V_{max} values were normalized with the relative UGT expression level. * $P < 0.05$, ** $P < 0.01$ compared with wild-type UGT1A9.

DMD Fast Forward. Published on October 2, 2008 as DOI: 10.1124/dmd.108.022913
This article has not been copyedited and formatted. The final version may differ from this version.



DMD Fast Forward. Published on October 2, 2008 as DOI: 10.1124/dmd.108.022913
This article has not been copyedited and formatted. The final version may differ from this version.

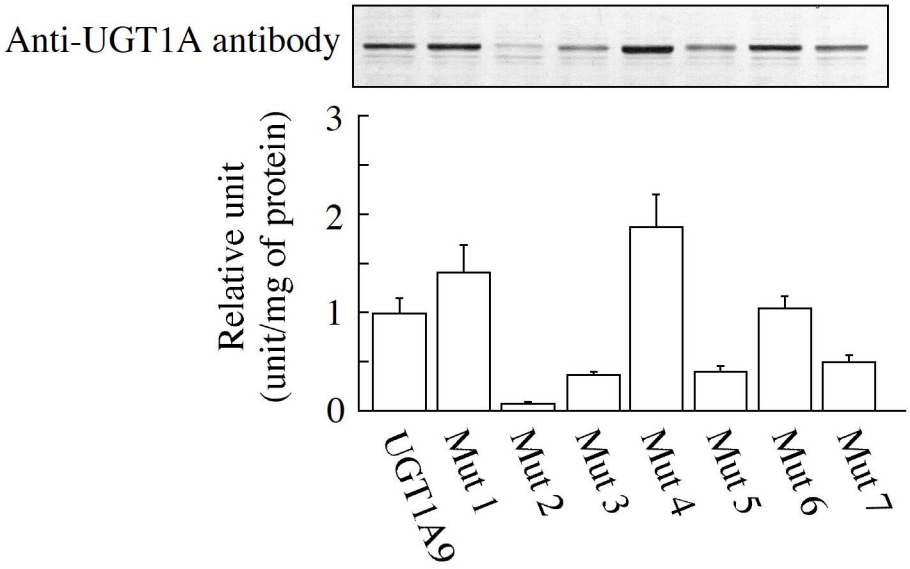


Fig. 3

DMD Fast Forward. Published on October 2, 2008 as DOI: 10.1124/dmd.108.022913
This article has not been copyedited and formatted. The final version may differ from this version.

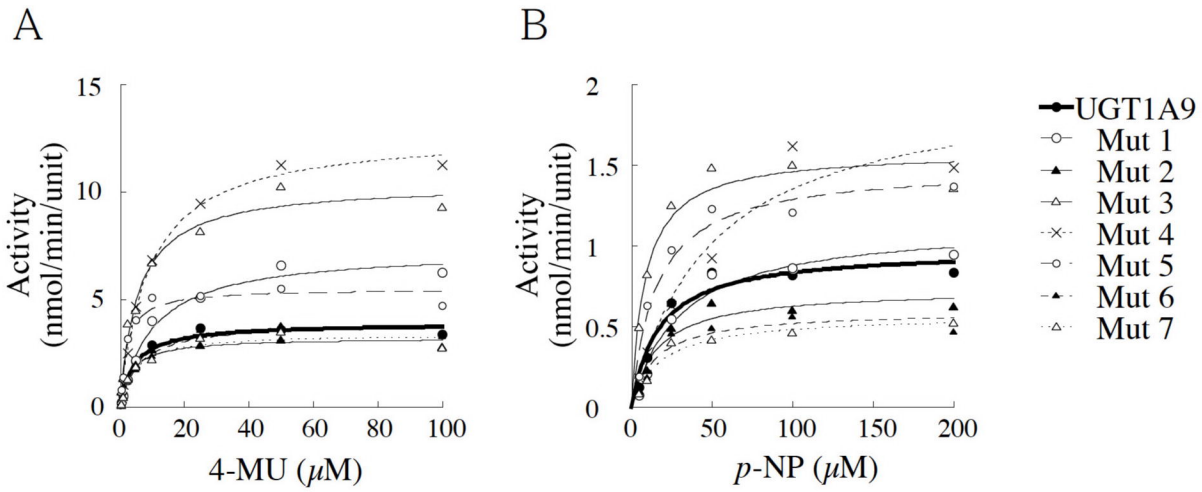


Fig. 4

DMD Fast Forward. Published on October 2, 2008 as DOI: 10.1124/dmd.108.022913
This article has not been copyedited and formatted. The final version may differ from this version.

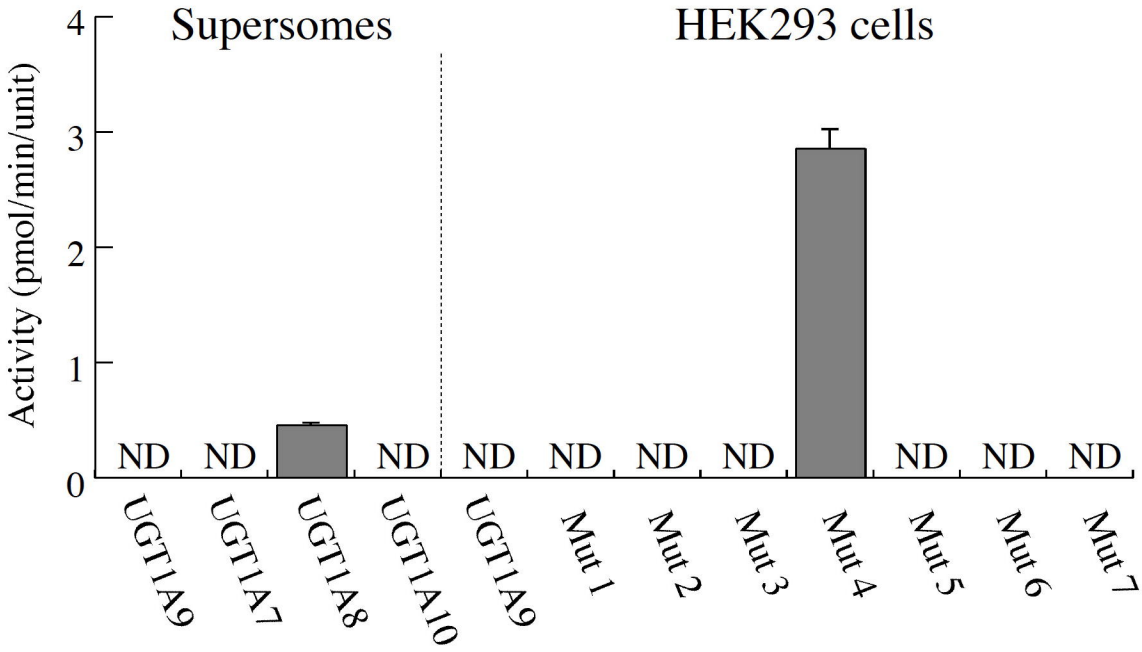


Fig. 5

DMD Fast Forward. Published on October 2, 2008 as DOI: 10.1124/dmd.108.022913
This article has not been copyedited and formatted. The final version may differ from this version.