Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 1269–1278
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Uridine diphosphate sugar-selective conjugation of an aldose reductase inhibitor (AS-3201) by UDP-glucuronosyltransferase 2B subfamily in human liver microsomes

Kenji Toide[a,b,*], Yoshiaki Terauchi[b], Toshihiko Fujii[b], Hiroshi Yamazaki[a], Tetsuya Kamataki[a]

[a]*Laboratory of Drug Metabolism, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan*
[b]*Pharmacokinetics & Physico-Chemical Property Research Laboratories, Dainippon Pharmaceutical CO., LTD., Osaka 564-0053, Japan*

Received 2 September 2003; accepted 12 November 2003

Abstract

N-Glucosidation is known as a major metabolic reaction for barbiturates in humans. However, the enzyme(s) involved in this *N*-glucosidation has not been clarified yet. Thus, to clarify the enzyme(s) involved in the *N*-glucosidation in human liver microsomes, we investigated the *N*-glucosyltransferase activity in recombinant UDP-glucuronosyltransferases (UGTs) using AS-3201, an aldose reductase inhibitor, as a substrate. AS-3201 was found to be biotransformed to both *N*-glucoside and *N*-glucuronide in human liver microsomes. The *N*-glucosyltransferase activities were detectable with multiple UGT isoforms (UGT1A1, UGT1A3, UGT1A4, UGT2B4, UGT2B7, and UGT2B15). In contrast, the *N*-glucuronyltransferase activities for the same substrate were seen with UGT1A (UGT1A1, UGT1A3, UGT1A4, and UGT1A9) but not UGT2B isoforms. We then determined the relative activity factor of each recombinant UGT and estimated the contribution of each UGT isoform to the *N*-glucosidation in human liver microsomes. The results showed that UGT2B isoforms mainly contribute to AS-3201 *N*-glucosidation in human liver microsomes. In addition, the activity of AS-3201 *N*-glucosyltransferase significantly correlated with that of amobarbital *N*-glucosyltransferase in microsomes from sixteen human livers ($r = 0.964$, $P < 0.01$), indicating that UGT2B isoforms were also involved in the barbiturate *N*-glucosidation in humans. The findings of this study clearly show that UGT2B specifically utilizes UDP-glucose but not UDP-glucuronic acid as a sugar donor for the conjugation of AS-3201 in human liver microsomes.

Keywords: Glucosidation; UDP-glucuronosyltransferase; UDP-glucose; Relative activity factor; Aldose reductase inhibitor

1. Introduction

UGTs play an important role in the metabolism of xenobiotics and endogenous compounds. UGTs have been classified into two families, UGT1 and UGT2; the latter being subdivided into the UGT2A and UGT2B subfamilies [1]. In humans, the *UGT1* genes are located on chromosome 2q37, and are encoded by at least 12 unique first exons, which are spliced to common exons from 2 to 5 [2]. UGT2Bs are encoded by separate genes, and are clustered on chromosome 4q13 [3,4].

The UGTs catalyze the conjugation of a variety of substrates with a sugar using UDP-sugar as a sugar donor. The UDP-sugar consists of UDP-GA, UDP-galactose, UDP-glucose, or UDP-xylose. Glucuronidation is one of the most important phase II drug-metabolizing reactions. A number of exogenous as well as endogenous compounds have been shown to undergo glucuronidation in humans. The UGT1 has been reported to catalyze the glucuronidation of bilirubin and various phenols and amines [5,6], whereas the UGT2B has been reported to catalyze the glucuronidation of opioids, bile acids, and steroids [6].

In contrast, glucosidation is known as a metabolic reaction for a relatively limited number of compounds, such as phenobarbital [7], amobarbital [8], sulphadimidine, sulphamerazine and sulphamethoxazole [9]. Accordingly,

* Corresponding author. Tel.: +81-6-6337-5888; fax: +81-6-6338-7656.
E-mail address: kenji-toide@dainippon-pharm.co.jp (K. Toide).

Abbreviations: UGT, UDP-glucuronosyltransferase; UDP-GA, UDP-glucuronic acid; LC/MS, liquid chromatography/mass spectrometry; ESI, electrospray ionization; $[M-H]^-$, deprotonated molecular ions; $[M+Cl]^-$, chlorinated molecular ions; RAF, relative activity factor; CYP, cytochrome P450; RAF_{UGT}, RAF of a recombinant UGT enzyme; ACT_{UGT}, the enzyme activity of a recombinant UGT.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.11.010

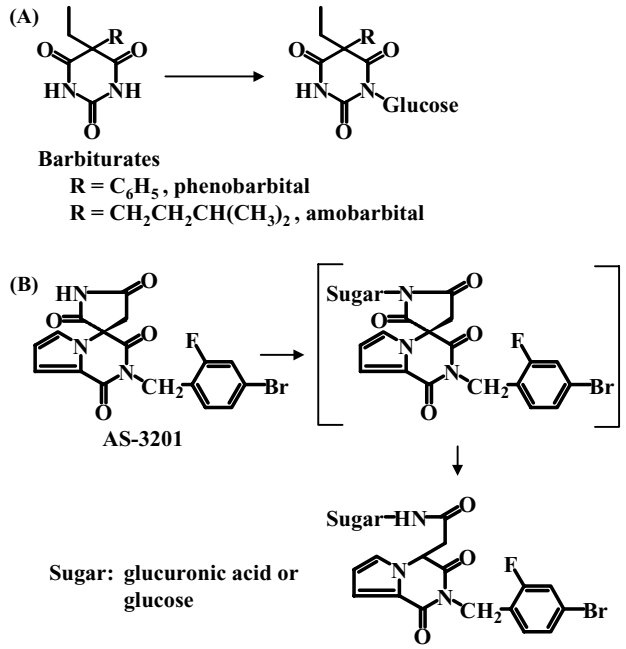

Fig. 1. *N*-Glucosidation of barbiturates (A) and AS-3201 (B) in humans.

the *N*-glucosides of barbiturates have been reported as the major urinary metabolites in humans (Fig. 1A), whereas the *N*-glucuronides have not been detected in human urine [7,8,10,11]. Thus, it was theorized that the enzyme(s) involved in barbiturate *N*-glucosidation might specifically utilize UDP-glucose as a sugar donor. However, to date, this enzyme(s) has not yet been clarified.

(*R*)-(−)-2-(4-bromo-2-fluorobenzyl)-1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrazine-4-spiro-3′-pyrrolidine-1,2′,3,5′-tetrone (AS-3201, Fig. 1B) is a structurally novel and potent aldose reductase inhibitor containing a succinimide ring [12,13]. The nitrogen atom in the succinimide ring is located between carbonyl groups, which is particularly similar to the target nitrogen atom for the glucosidation of barbiturates. *N*-Glucuronide and *N*-glucoside have been isolated and identified as the major metabolites of AS-3201 in humans, and it has been clarified that these conjugates formed through unstable intermediates [14].

In this paper, the enzyme(s) involved in the AS-3201 *N*-glucosidation was investigated, and compared with the enzyme(s) involved in the *N*-glucuronidation. We found that UGT2B enzymes catalyzed the *N*-glucosidation but not the *N*-glucuronidation of this chemical. Also, an application of RAF for recombinant human UGTs is reported for the first time.

2. Materials and methods

2.1. Materials

AS-3201 and [^{14}C]AS-3201 (1.41 MBq/mg) were synthesized at Dainippon Pharmaceutical. The radiochemical purity of [^{14}C]AS-3201 was more than 98%. Amobarbital, bilirubin, estradiol, estradiol 3-glucuronide, trifluoperazine, 4-methylumbelliferone, 4-nitrophenol, hyodeoxycholic acid, and azidothymidine were obtained from Sigma. UDP-GA and UDP-glucose were purchased from Wako Pure Chemical, recombinant UGTs (human UGT supersomes) and control microsomes (insect cell control supersomes) from Gentest, and pooled human liver microsomes (H0610) and human liver microsomes prepared from 16 individual donors from Xenotech. *cis*-4-Hydroxytamoxifen was kindly provided from Dr. Shinya Shibutani of State University of New York at Stony Brook, Stony Brook, NY. All other reagents and chemicals were of the highest grade commercially available.

2.2. Assays for AS-3201 N-glucuronidation and N-glucosidation

To determine enzyme activities for the AS-3201 *N*-glucosidation and *N*-glucuronidation, a reaction mixture (200 μL final volume) containing 0.1 M Tris–HCl (pH 7.1), 10 mM $MgCl_2$, 0.5 mg/mL of human liver microsomes or recombinant UGTs, UDP-glucose or UDP-GA, and AS-3201 or [^{14}C]AS-3201 was incubated at 37° for 2 hr. The incubation was performed using native human liver microsomes or UGT supersomes without activation by a detergent or alamethicin. Enzyme kinetic parameters were determined using AS-3201 and co-factors at concentrations of 25–500 and 50–2000 μM, respectively. The reaction was terminated by adding 50 μL of acetonitrile. After centrifugation at 15,000 *g* for 5 min, aliquots of the supernatant were subjected to HPLC or LC/MS. HPLC separations were performed using a Model 1100 liquid chromatograph system (Agilent) equipped with an Inertsil ODS-3V column (5 μm, 4.6 mm × 250 mm; GL-Science), maintained at 40°. The mobile phase consisted of solution A (10 mM ammonium acetate) and solution B (acetonitrile). At a constant flow rate (1 mL/min), a linear gradient from 15 to 60% of solution B was run over 35 min. The compounds were detected by radioactivity or absorbance at 296 nm. Radioactivity was counted by a flow-scintillation detector, FLO-ONE/Beta A-515 (Packard) using Ultima Flo-M (flow late, 2 mL/min; Packard) as a scintillation cocktail. The metabolites were quantified from the ratio of the peak radioactivity to the whole radioactivity eluted during the run time. LC/MS analysis was performed using a Model 1100 LC/MSD system (Agilent). Chromatographic separation was achieved with a Develosil ODS-UG5 column (2.0 mm × 100 mm; Nomura Chemical), maintained at 40°, using a two-solvent gradient system: solvent A (10 mM ammonium acetate) and solvent B (acetonitrile). At a constant flow rate (0.2 mL/min), a linear gradient from 15 to 55% of solvent B was run over 20 min. The mass spectrometer, with an ESI source, was operated in full scan mass spectrometry using the gas temperature of 320°, capillary voltage of 3.5 kV, and fragmentor of 75 in negative ion mode.

2.3. Amobarbital N-glucosidation assay

Amobarbital *N*-glucosyltransferase activity was assayed under the following conditions. The reaction mixture contained 0.1 M Tris–HCl (pH 7.1), 10 mM $MgCl_2$, 0.5 mg/mL of human liver microsomes, 2 mM UDP-glucose, and 50 μM amobarbital in a final volume of 200 μL. All reactions were conducted at 37° for 2 hr, and were terminated by adding 50 μL of acetonitrile. After centrifugation at 15,000 *g* for 5 min, aliquots of the supernatant were subjected to HPLC. HPLC was performed according to the method of Soine and Soine [15].

2.4. Other enzyme assays

Bilirubin glucuronyltransferase activity was determined by the method of Raijmakers *et al*. [16], 4-nitrophenol *O*-glucuronyltransferase and 4-methylumbelliferone *O*-glucuronyltransferase activities by the method of Hanioka *et al*. [17], azidothymidine *O*-glucuronyltransferase activity by the method of Sim *et al*. [18], and *cis*-4-hydroxytamoxifen *O*-glucuronyltransferase activity by the method of Nishiyama *et al*. [19]. The activities of estradiol 3-glucuronyltransferase and trifluoperazine *N*-glucuronyltransferase were determined according to the manufacturer's instructions for human UGT1A3 and UGT1A4 supersomes (Gentest). The concentrations of bilirubin, 4-nitrophenol, 4-methylumbelliferone, azidothymidine, *cis*-4-hydroxytamoxifen, estradiol, and trifluoperazine were 25, 100, 25, 250, 5, 10, and 125 μM, respectively. Hyodeoxycholic acid glucuronyltransferase activity was assayed in a reaction mixture containing 0.1 M Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.25 mg/mL of human liver microsomes, 2 mM UDP-GA, and 15 μM hyodeoxycholic acid in a final volume of 200 μL. The reactions were conducted at 37° for 15 min, and were terminated by addition of acetonitrile (50 μL). After centrifugation at 15,000 *g* for 5 min, aliquots of the supernatant were subjected to LC/MS. LC/MS analysis was performed using a Model 1100 LC/MSD system (Agilent). Chromatographic separation was achieved with a Develosil ODS-UG5 column (2.0 mm × 100 mm; Nomura Chemical), maintained at 40°, using a two-solvent gradient system: solvent A (10 mM ammonium acetate) and solvent B (acetonitrile). At a constant flow rate (0.2 mL/min), a linear gradient from 20 to 40% of solvent B was run over 15 min. The mass spectrometer with an ESI source was operated at the gas temperature of 320°, capillary voltage of 4 kV, and fragmentor of 175 in negative ion mode. Hyodeoxycholic acid glucuronide was detected at selected ion monitoring (*m*/*z* 567). Radominska *et al*. [20] have clarified that 6-*O*-glucuronide of hyodeoxycholic acid is formed in human liver microsomes. Therefore, the glucuronide formed in this condition is considered to be hyodeoxycholic acid 6-*O*-glucuronide.

To determine the apparent K_m values of glucuronidation towards these substrates, several concentrations of the substrates (10–250 μM for bilirubin, 4-methylumbelliferone, and hyodeoxycholic acid; 5–100 μM for estradiol and *cis*-4-hydroxytamoxifen; 25–500 μM for trifluoperazine and 4-nitrophenol; and 100–2500 μM for azidothymidine) were incubated with pooled human liver microsomes. The K_m value was determined using least-squares nonlinear regression analysis by plotting the metabolite formation rates against the substrate concentrations.

2.5. Determination of RAF values

The RAF is a parameter to estimate the activity of the enzyme in its native environment from the activity of the cDNA expressed enzyme, and was originally suggested for CYPs [21]. This value can be calculated by applying Eq. (1).

$$\mathrm{RAF} = \frac{\text{enzyme activity in human liver microsomes}}{\text{enzyme activity in cDNA expressed enzyme}} \quad (1)$$

The RAF of a recombinant CYP enzyme has been determined by the method of Crespi [21] using a specific enzyme activity for the CYP isoform. The UGTs, however, exhibit overlapping substrate specificities and the substrates are generally catalyzed by multiple UGT isoforms [6,21]. Thus, the RAF for recombinant UGT enzymes could not be determined using the method of Crespi [21]. In this study, the RAF for UGT was therefore determined on the following basis. When the RAF and the enzyme activity of a recombinant UGT isoform are represented as $\mathrm{RAF_{UGT}}$ and $\mathrm{ACT_{UGT}}$, respectively, the enzyme activity of the UGT isoform in human liver microsomes can be represented by Eq. (2).

$$\mathrm{RAF_{UGT}} \times \mathrm{ACT_{UGT}} = (\text{enzyme activity of a UGT isoform in human liver microsomes}) \quad (2)$$

The sum of the enzyme activity of each UGT isoform expressed in human liver microsomes is conceivable as the total UGT activity in human liver microsomes. Therefore, the UGT activity in human liver microsomes can be represented by Eq. (3).

$$\sum(\mathrm{RAF_{UGT}} \times \mathrm{ACT_{UGT}}) = (\text{UGT activity in human liver microsomes}) \quad (3)$$

We employed bilirubin, estradiol, trifluoperazine, 4-methylumbelliferone, 4-nitrophenol, hyodeoxycholic acid, azidothymidine, and *cis*-4-hydroxytamoxifen as substrates to determine the RAF of recombinant UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, and UGT2B15. The substitution of glucuronyltransferase activities towards these substrates in each recombinant UGT isoform to Eq. (3) lead to the use of eight equations containing the RAF for these recombinant UGT isoforms. The RAF values of recombinant UGT isoforms were obtained as the solutions of the equations, by substituting in the appropriate $\mathrm{ACT_{UGT}}$ values determined in this study.

2.6. Statistical analysis

Multiple linear regression, least-squares regression, and least-squares nonlinear regression were performed using the Origin 7J software (OriginLab).

3. Results

3.1. AS-3201 N-glucosidation in human liver microsomes

The formation of AS-3201 *N*-glucuronide and *N*-glucoside was detectable after incubation of AS-3201 with human liver microsomes in the presence of UDP-GA or UDP-glucose (Fig. 2A and B). The retention times of AS-3201 *N*-glucuronide and *N*-glucoside were 13.0 and 15.8 min, respectively. Peaks of *m/z* 568, 570, 604, and 606 were detected in the LC/MS spectrum of the *N*-glucuronide (Fig. 2C). The peaks of *m/z* 568 and 570, or 604 and 606 are isotopic clusters. The peaks of *m/z* 568 and 570 were considered to be derived from $[M-H]^-$, and

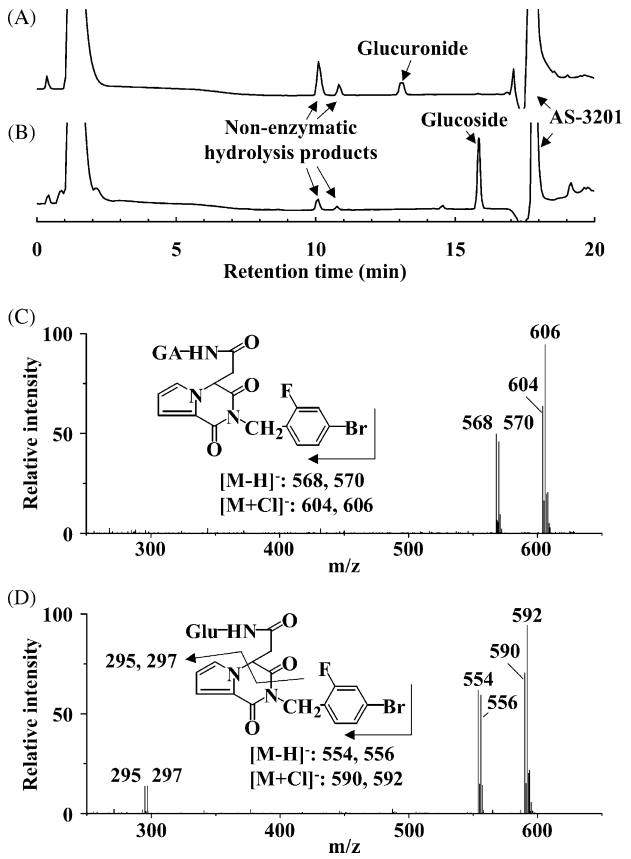

Fig. 2. HPLC chromatograms for the formation of AS-3201 *N*-glucuronide (A) and *N*-glucoside (B) monitored by absorbance at 296 nm. ESI-mass spectra of AS-3201 *N*-glucuronide (C) and *N*-glucoside (D) in the negative ionization mode. AS-3201 was incubated with pooled human liver microsomes in the presence of UDP-GA or UDP-glucose. The $[M-H]^-$ ions at *m/z* 568 and 570 or *m/z* 554 and 556 corresponded to AS-3201 *N*-glucuronide or *N*-glucoside, respectively. GA, glucuronic acid; Glu, glucose.

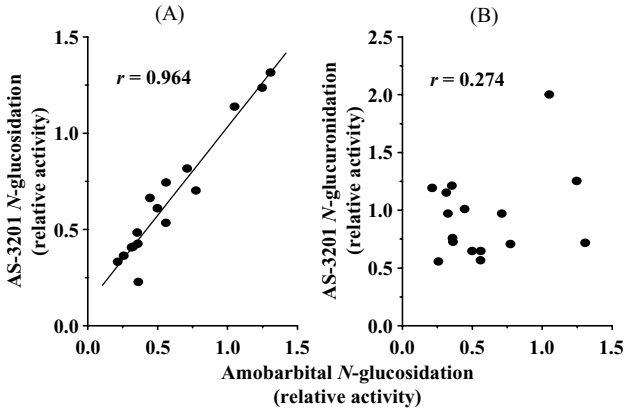

Fig. 3. Relationship between amobarbital *N*-glucosidation activities and activities for AS-3201 *N*-glucosidation (A) or *N*-glucuronidation (B) in human liver microsomes prepared from 16 individuals. Each enzyme activity was calculated from the HPLC peak area of each metabolite, and was represented relative to those with pooled human liver microsomes. The AS-3201 concentration was set at 37.5 μM for *N*-glucosidation and 125 μM for *N*-glucuronidation. Significant correlation between amobarbital *N*-glucosidation activity and AS-3201 *N*-glucosidation activity was observed ($r = 0.964$, $P < 0.01$).

m/z 604 and 606 from $[M+Cl]^-$ of the *N*-glucuronide. For *N*-glucoside, peaks of *m/z* 295, 297, 554, 556, 590, and 592 were detected in the LC/MS spectrum (Fig. 2D). The peaks of *m/z* 554 and 556 were considered to be derived from $[M-H]$, and *m/z* 590 and 592 from $[M+Cl]^-$ of the *N*-glucoside. The peaks of *m/z* 295 and 297 were considered to be the fragment ions derived from the *N*-glucoside, since they were also observed in the LC/MS spectrum of the aglycone (data not shown).

The enzyme activities for AS-3201 *N*-glucosidation and *N*-glucuronidation were determined with human liver microsomes prepared from sixteen individuals, and were compared with those for amobarbital *N*-glucosidation (Fig. 3). Relative enzyme activities for amobarbital *N*-glucosidation were highly correlated with those for AS-3201 *N*-glucosidation ($r = 0.964$, $P < 0.01$) but not with those for AS-3201 *N*-glucuronidation ($r = 0.274$). These results probably indicate that the *N*-glucosidation of AS-3201 and amobarbital are catalyzed by the same enzyme(s).

To determine kinetic parameters with respects to the concentration of the substrate and co-factors, various concentrations of [^{14}C]AS-3201 and either UDP-GA or UDP-glucose were incubated with pooled human liver microsomes. The enzyme activities were estimated by determining the radioactivity of each conjugate, as described in Section 2. The kinetic parameters for AS-3201 and co-factors were summarized in Table 1. The K_m values for AS-3201 and co-factor in the *N*-glucosidation were 75.3 and 392 μM, respectively. These values were almost three times lower than those in *N*-glucuronidation, suggesting that the affinities of the substrate and the co-factor to UGT protein(s) for the *N*-glucosidation were higher than those for *N*-glucuronidation.

Table 1
Kinetic parameters for glucosidation and glucuronidation of AS-3201 by human liver microsomes

	Substrate			Co-factor		
	K_m (μM)	V_{max} (nmol/hr/mg)	V_{max}/K_m (mL/hr/mg)	K_m (μM)	V_{max} (nmol/hr/mg)	V_{max}/K_m (mL/hr/mg)
Glucosidation	75.3	7.58	0.101	392	4.76	0.012
Glucuronidation	242	1.08	0.004	1320	0.84	0.001

The concentration of co-factors was fixed at 2 mM to determine the kinetic parameters towards AS-3201. The concentration of AS-3201 was fixed at 50 μM (*N*-glucosidation) or 200 μM (*N*-glucuronidation) to determine the kinetic parameters towards the UDP-GA or the UDP-glucose.

3.2. Determination of RAF for recombinant UGT isoforms

The K_m values of glucuronidation towards eight substrates in human liver microsomes were determined (Table 2). It was shown that the concentrations of the substrates employed in this study were lower than the K_m towards the substrates in human liver microsomes. Therefore, the enzyme activities observed under these conditions are in proportion to the intrinsic clearance of the reactions. It has been reported that the RAF calculated using the intrinsic clearance is the most appropriate value to estimate the activity of the enzyme in its native environment from the activity of the cDNA expressed enzyme [23].

Relative enzyme activities in each recombinant UGT isoform and pooled human liver microsomes are shown in Fig. 4. The glucuronyltransferase activities towards bilirubin and trifluoperazine were detectable with UGT1A1 and UGT1A4, respectively. The glucuronyltransferase activities towards other substrates were detectable with multiple UGT isoforms. When the activities of pooled human liver microsomes were defined as one, the substitution of these enzyme activities to Eq. (3) led to the following Eqs. (4)–(11).

$$\mathrm{RAF_{UGT1A1}} \times 3.19 = 1 \quad (4)$$

$$(\mathrm{RAF_{UGT1A1}} \times 3.09) + (\mathrm{RAF_{UGT1A3}} \times 0.33) = 1 \quad (5)$$

$$\mathrm{RAF_{UGT1A4}} \times 2.59 = 1 \quad (6)$$

$$(\mathrm{RAF_{UGT1A1}} \times 0.02) + (\mathrm{RAF_{UGT1A6}} \times 0.99) + (\mathrm{RAF_{UGT1A9}} \times 0.52) + (\mathrm{RAF_{UGT2B15}} \times 0.04) = 1 \quad (7)$$

$$(\mathrm{RAF_{UGT1A1}} \times 0.01) + (\mathrm{RAF_{UGT1A3}} \times 0.01) + (\mathrm{RAF_{UGT1A6}} \times 2.00) + (\mathrm{RAF_{UGT1A9}} \times 0.30) + (\mathrm{RAF_{UGT2B7}} \times 0.06) = 1 \quad (8)$$

$$(\mathrm{RAF_{UGT2B4}} \times 0.05) + (\mathrm{RAF_{UGT2B7}} \times 1.64) = 1 \quad (9)$$

$$(\mathrm{RAF_{UGT2B4}} \times 0.08) + (\mathrm{RAF_{UGT2B7}} \times 1.50) = 1 \quad (10)$$

$$(\mathrm{RAF_{UGT1A1}} \times 0.01) + (\mathrm{RAF_{UGT1A3}} \times 0.01) + (\mathrm{RAF_{UGT2B7}} \times 0.08) + (\mathrm{RAF_{UGT2B15}} \times 0.17) = 1 \quad (11)$$

The RAF values for recombinant UGT enzymes were obtained as the solutions of these equations (Table 2). The RAF values for UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, and UGT2B7 were lower than one, indicating that the activities of these recombinant UGT enzymes are higher than the activities in their native environment. On the other hand, the RAF values for UGT2B4 and UGT2B15 were 2.18 and 5.60, respectively.

Table 2
Relative glucuronosyltransferase activities towards eight substrates and the RAF value for each recombinant UGT isoform

	Substrate								RAF
	BIL	EST	TFP	MU	NP	HDC	AZT	OHT	
K_m (μM)	48.2	14.1	247	66.6	184	26.8	1550	8.74	
Substrate concentration (μM)	25	10	125	25	100	15	250	5	
UGT1A1	3.19	3.09	–	0.02	0.01	–	–	0.01	0.31
UGT1A3	–	0.33	–	–	0.01	–	–	0.01	0.09
UGT1A4	–	–	2.59	–	–	–	–	–	0.39
UGT1A6	–	–	–	0.99	2.00	–	–	–	0.36
UGT1A9	–	–	–	0.52	0.30	–	–	–	0.79
UGT2B4	–	–	–	–	–	0.05	0.08	–	2.18
UGT2B7	–	–	–	–	0.06	1.64	1.50	0.08	0.55
UGT2B15	–	–	–	0.04	–	–	–	0.17	5.60

Each enzyme activity represents relative activity to the activity obtained with pooled human liver microsomes. The definition of RAF was described in Section 2. The K_m values of glucuronidation towards these substrates with human liver microsomes are shown. BIL, bilirubin; EST, estradiol; TFP, trifluoperazine; MU, 4-methylumbelliferone; NP, 4-nitrophenol; HDC, hyodeoxycholic acid; AZT, azidothymidine; OHT, *cis*-4-hydroxytamoxifen; –, not detectable.

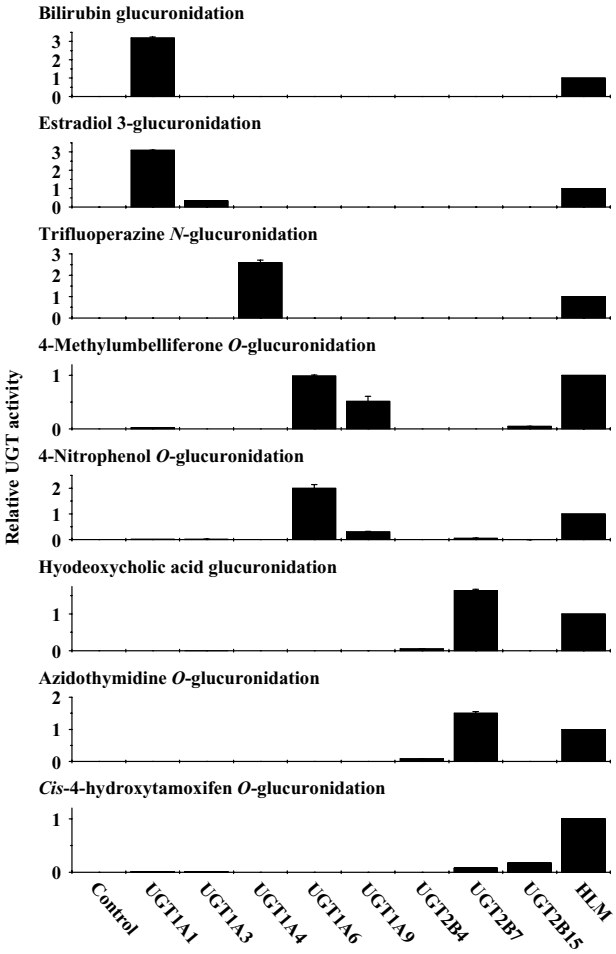

Fig. 4. Relative glucuronyltransferase activities towards eight substrates with each recombinant UGT isoform. Each activity was represented as the relative activity to the activity obtained with pooled human liver microsomes (mean ± SD, N = 3). Insect cell control supersomes were used as a control. HLM, pooled human liver microsomes.

3.3. UGT isoform(s) responsible for AS-3201 N-glucosidation

AS-3201 was incubated with recombinant UGT enzymes in the presence of UDP-glucose or UDP-GA. The substrate concentration was set at 37.5 μM for *N*-glucosidation and 125 μM for *N*-glucuronidation, both of which are almost a half of the K_m of *N*-glucosidation and *N*-glucuronidation in human liver microsomes. The enzyme activities observed under these conditions, therefore, are in proportion to the intrinsic clearance of the reactions. The *N*-glucosidation was detectable with UGT1A1, UGT1A3, UGT1A4, UGT2B4, UGT2B7, and UGT2B15 (Fig. 5A). The highest enzyme activity was seen with UGT1A4, followed by UGT2B15 and UGT1A3. These results indicate both UGT1A and UGT2B isoforms can catalyze AS-3201 *N*-glucosidation. On the other hand, the *N*-glucuronidations were detectable with UGT1A isoforms (UGT1A1, UGT1A3, UGT1A4, and UGT1A9) but not with UGT2B isoforms (Fig. 5B). Therefore, it appeared that UGT2B isoforms utilize UDP-glucose alone as a co-

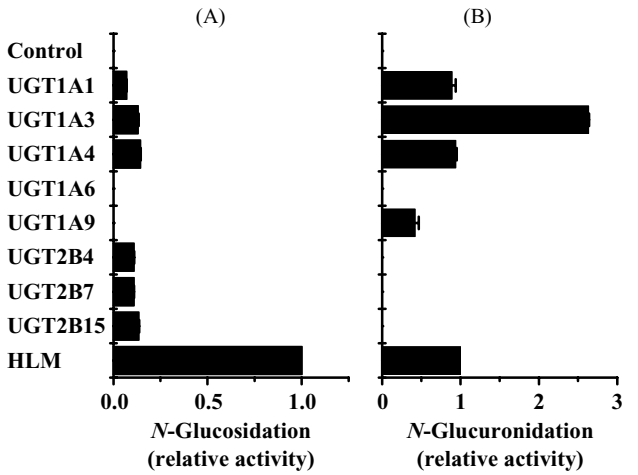

Fig. 5. Rate of AS-3201 *N*-glucosidation (A) and *N*-glucuronidation (B) catalyzed by recombinant UGT enzymes. Insect cell control supersomes were used as a control. The activity for each UGT isoform was represented as the relative activity to the activity obtained with pooled human liver microsomes (mean ± SD, N = 3). HLM, pooled human liver microsomes.

factor for AS-3201 conjugation. UGT1A8 and UGT1A10, which are expressed in extrahepatic tissues [24,25], catalyzed the *N*-glucosidation (data not shown).

To estimate the actual *N*-glucosyltransferase activity of each UGT isoform in human liver microsomes, the *N*-glucosyltransferase activity in each recombinant UGT enzyme was corrected by the RAF determined in this study (Fig. 6A). The highest enzyme activity was seen with UGT2B15, followed by UGT2B4 and UGT2B7. The enzyme activity in UGT1A isoforms was relatively low. These results show that UGT2B isoforms are mainly responsible for AS-3201 *N*-glucosidation in human liver microsomes. The *N*-glucuronyltransferase activity of each recombinant UGT enzyme was also corrected by the RAF

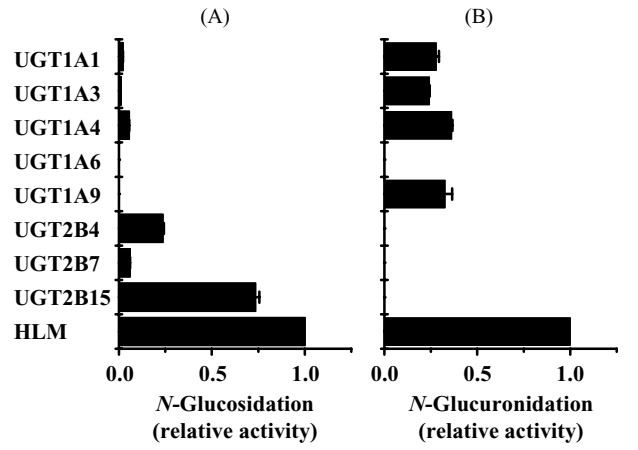

Fig. 6. Predicted rate of AS-3201 *N*-glucosidation (A) and *N*-glucuronidation (B) catalyzed by UGT isoforms in human liver microsomes. The predicted enzyme activities were determined using the RAF and the activities of recombinant UGT enzymes. The activity for each UGT isoform was represented as the relative activity to the activity obtained with pooled human liver microsomes (mean ± SD, N = 3). HLM, pooled human liver microsomes.

Table 3
The K_m (μM) of each UGT isoform for glucosidation and glucuronidation of AS-3201 by recombinant UGTs

	Glucosidation		Glucuronidation	
	AS-3201	UDP-glucose	AS-3201	UDP-GA
UGT1A1	158	648	293	1830
UGT1A3	109	627	320	1790
UGT1A4	315	1070	529	2420
UGT1A9	–	–	295	1550
UGT2B4	161	616	–	–
UGT2B7	148	513	–	–
UGT2B15	122	589	–	–

The concentration of co-factors (UDP-glucose and UDP-GA) was fixed at 2 mM to determine the K_m towards the AS-3201. The concentration of AS-3201 was fixed at 50 μM (*N*-glucosidation) or 200 μM (*N*-glucuronidation) to determine the K_m towards the UDP-GA or the UDP-glucose.

(Fig. 6B). The result shows that the actual *N*-glucuronyltransferase activity of UGT1A1, UGT1A3, UGT1A4, and UGT1A9 isoforms is nearly the same in human liver microsomes.

The K_m values of each recombinant UGT for the substrate (AS-3201) and the co-factors (UDP-glucose and UDP-GA) in the *N*-glucosidation of AS-3201 were determined (Table 3). The K_m values for both the substrate and the co-factors varied. However, the variation was within three times or less between the UGT1A and the UGT2B isoforms. In addition, the variation of the K_m values in the *N*-glucuronidation was within two times or less among the UGT1A isoforms. Therefore, the affinities of the substrate and the co-factors to the UGT protein were nearly the same among the UGT isoforms.

The enzyme activities for AS-3201 *N*-glucosidation in human liver microsomes prepared from sixteen individual subjects were compared with those for azidothymidine *O*-glucuronidation and *cis*-4-hydroxytamoxifen *O*-glucuronidation (Fig. 7). These enzyme activities were likely to be derived from UGT2B isoforms as shown in Table 2. A multivariate linear regression analysis showed that enzyme activities for AS-3201 *N*-glucosidation was significantly correlated with those for azidothymidine *O*-glucuronidation and *cis*-4-hydroxytamoxifen *O*-glucuronidation ($r = 0.750$, $P < 0.01$). Additionally, enzyme activities for amobarbital *N*-glucosidation were significantly correlated with those for azidothymidine *O*-glucuronidation and *cis*-4-hydroxytamoxifen *O*-glucuronidation ($r = 0.784$, $P < 0.01$, data not shown).

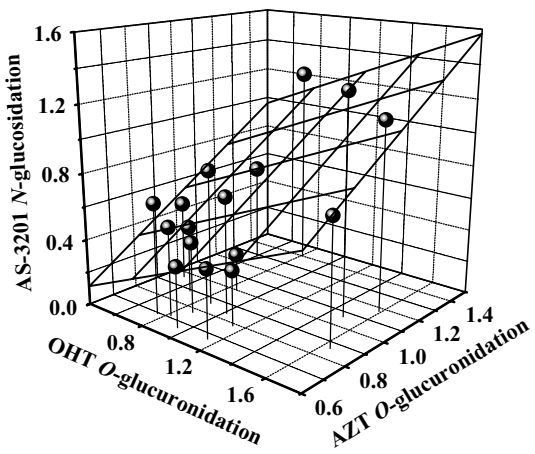

Fig. 7. Correlation among the activity of AS-3201 *N*-glucosidation and the activities of *cis*-4-hydroxytamoxifen (OHT) *O*-glucuronidation and azidothymidine (AZT) *O*-glucuronidation. A multivariate linear regression analysis shows the significant correlation between AS-3201 *N*-glucosidation activity and activities for OHT *O*-glucuronidation and AZT *O*-glucuronidation ($r = 0.750$, $P < 0.01$). Each activity represents relative to the activity obtained with pooled human liver microsomes.

4. Discussion

In the present study, we described several lines of evidence that the UGT2B isoforms are responsible for AS-3201 *N*-glucosidation but not *N*-glucuronidation in human liver microsomes. Our results indicated that UGT2B15 mostly contributes to AS-3201 *N*-glucosidation in human liver microsomes. UGT2B15 is known to catalyze the glucuronidation of a number of steroids, including 5α-dihydrotestosterone and androstane-3α,17β-diol [26,27]. A polymorphism of the *UGT2B15* gene in the coding region that results in an amino acid change at residue 85 from Asp to Tyr has been reported [28]. This polymorphism altered glucuronyltransferase activities towards 5α-dihydrotestosterone and androstane-3α,17β-diol [28]. It has been reported that the *N*-glucosidation is one of the major metabolic pathways for amobarbital in humans [8]. Therefore, the genotype of the *UGT2B15* gene might affect the pharmacokinetics of amobarbital, as well as AS-3201, in humans.

The RAF provides a means to relate the activity of the cDNA-expressed enzyme to the activity of the enzyme in its native environment. This was first proposed for recombinant CYP enzymes [21]. So far, several groups have reported the RAF values for recombinant CYP enzymes [23,29–31], whereas no one has reported the RAF of recombinant UGT enzymes. In this study, we provided a novel strategy to determine the RAF of recombinant UGT enzymes. We conceived that the sum of the enzyme activity of UGT isoforms expressed in human liver microsomes is equal to the UGT activity of human liver microsomes. Actually, the sum of the enzyme activity of UGT isoforms corrected by the RAF was almost the same activity of human liver microsomes in both the *N*-glucosidation and the *N*-glucuronidation. These results suggest that the RAF value determined in this study is appropriate to estimate the enzyme activity of the UGT isoform in human liver microsomes from the activity of the recombinant UGT enzyme.

It was reported in the literature that human UGT1A1 could catalyze bilirubin glucosidation, as well as bilirubin glucuronidation [32]. We found that human UGT1A1 was also able to catalyze both the *N*-glucosidation and the *N*-

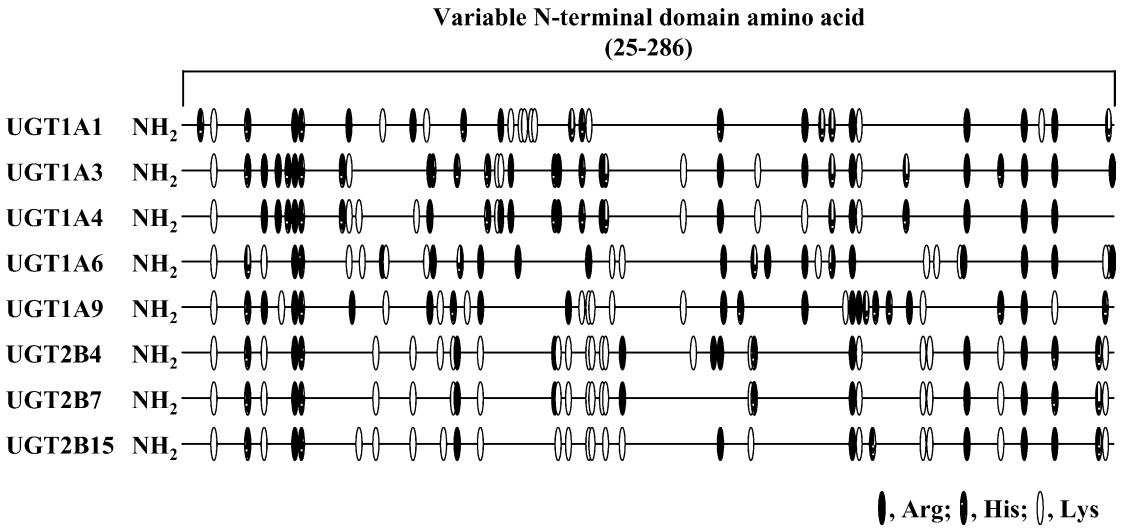

Fig. 8. Comparison of amino acid sequence in the N-terminal (25–286) of UGT proteins. The positions of Arg, His, and Lys residues for co-factor binding are shown.

glucuronidation of AS-3201. UGT1A3 and UGT1A4 are known as the UGT enzymes that catalyze a number of amine glucuronidations [33,34]. In this study, UGT1A3 and UGT1A4 catalyzed the AS-3201 *N*-glucosidation, as well as the AS-3201 *N*-glucuronidation, indicating these UGT1A isoforms as well as UGT1A1 utilize both UDP-glucose and UDP-glucuronic acid as a sugar donor for the conjugation of AS-3201. In contrast, UGT2B isoforms specifically utilize UDP-glucose, but not UDP-glucuronic acid, as a sugar donor for the conjugation of AS-3201. We then speculated that this property of UGT2B enzymes is determined by the amino acid sequence of UGT2B protein. The N-terminal and the C-terminal of the UGT protein contain an aglycone binding site and a transmembrane fragment, respectively [22]. Although UDP-sugar possibly interacts with both N- and C-terminal domains of the UGT protein, the carboxyl moiety of UDP-GA would interact with an Arg residue in the N-terminal [22,35]. In addition, the UDP moiety, which is common to UDP-sugar, would interact with the amino acid residue from 352 to 400 in the C-terminal. These findings suggest that the N-terminal sequence of UGT protein is important for the co-factor selective conjugation of AS-3201 by UGT2B. The amino acid sequence of the N-terminal is highly conserved among UGT2B proteins, but not between UGT1A and UGT2B proteins [1]. Arg residue for co-factor binding, as well as the other basic amino acid residues, locates itself on almost the same region in the UGT2B proteins (Fig. 8), suggesting that Arg residue interacting with the carboxyl moiety of UDP-GA is conserved in UGT2B proteins. Although the substrate possibly interacts with the C-terminal of UGT protein as well, the amino acid sequence of the C-terminal is highly conserved among UGT2B proteins. Therefore, the structure of the UGT2B protein and UGT2B protein–AS-3201 complex might be highly conserved among UGT2B isoforms, and the structure of UGT2B protein–AS-3201 complex might elucidate this enzyme property.

The difference between UDP-glucose and UDP-GA is only the C6 of the sugar moiety. However, the pK_a of glucose and glucuronic acid, calculated using the Pallas software (CompuDrug), were 15.6 and 2.65, respectively. This indicates that glucuronic acid, but not glucose, is ionized at physiological pH. Therefore, it is believed that the difference between UDP-glucose and UDP-GA judged with ionicity is one of the important determinants of co-factor selection for specific conjugation of AS-3201 by UGT2B enzymes.

Glucosidation is known as a metabolic reaction for a relatively limited number of compounds. Recently, it has been reported that an endothelin ETA receptor antagonist [36] and hyodeoxycholic acid [37] were conjugated with both glucuronic acid and glucose by UGT2B7 in human liver microsomes. These findings indicate that UGT2B7 can utilize both UDP-glucuronic acid and UDP-glucose as a sugar donor, and support the hypothesis that the structure of UGT2B enzyme–AS-3201 complex might elucidate the properties of UGT2B enzymes.

In conclusion, we showed evidence that UGT2B enzymes are involved in AS-3201 *N*-glucosidation, as well as amobarbital *N*-glucosidation, in human liver microsomes. These enzymes utilized UDP-glucose specifically, but not UDP-GA, as a sugar donor for the AS-3201 conjugation. Although the mechanism of this selectivity is not fully understood yet, it is believed that the tridimensional structure of UGT2B enzyme–AS-3201 complex might elucidate this enzyme property.

Acknowledgments

We thank Mr. Glen Argyle (Dainippon Pharmaceutical) for critical reading of the manuscript. This study was supported in part by a Grant-in-Aid (No. 99-2) from The Organization for Pharmaceutical Safety and Research

(OPSR), Ministry of Education, Science, Sports and Culture of Japan, and Ministry of Health, Labour and Welfare of Japan.

References

[1] Mackenzie PI, Owens IS, Burchell B, Bock KW, Bairoch A, Bélanger A, Fournel-Gigleux S, Green M, Hum DW, Iyanagi T, Lancet D, Louisot P, Magdalou J, Chowdhury JR, Ritter JK, Schachter H, Tephly TR, Tipton KF, Nebert DW. The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. Pharmacogenetics 1997;7:255–69.

[2] Owens IS, Ritter JK. Gene structure at the human *UGT1* locus creates diversity in isozyme structure, substrate specificity, and regulation. Prog Nucleic Acid Res Mol Biol 1995;51:305–38.

[3] Monaghan G, Clarke DJ, Povey S, See CG, Boxer M, Burchell B. Isolation of a human YAC contig encompassing a cluster of *UGT2* genes and its regional localization to chromosome 4q13. Genomics 1994;23:496–9.

[4] Turgeon D, Carrier JS, Lévesque E, Beatty BG, Bélanger A, Hum DW. Isolation and characterization of the human *UGT2B15* gene, localized within a cluster of *UGT2B* genes and pseudogenes on chromosome 4. J Mol Biol 2000;295:489–504.

[5] Bosma PJ, Seppen J, Goldhoorn B, Bakker C, Oude Elferink RP, Chowdhury JR, Chowdhury NR, Jansen PL. Bilirubin UDP-glucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man. J Biol Chem 1994;269:17960–4.

[6] Tukey RH, Strassburg CP. Human UDP-glucuronosyltransferases: metabolism, expression, and disease. Annu Rev Pharmacol Toxicol 2000;40:581–616.

[7] Tang BK, Kalow W, Grey AA. Metabolic fate of phenobarbital in man. *N*-Glucoside formation. Drug Metab Dispos 1979;7:315–8.

[8] Tang BK, Kalow W, Grey AA. Amobarbital metabolism in man: *N*-glucoside formation. Res Commun Chem Pathol Pharmacol 1978;21:45–53.

[9] Ahmad B, Powell JW. N1-glucosides as urinary metabolites of sulphadimidine, sulphamerazine and sulphamethoxazole. Eur J Drug Metab Pharmacokinet 1988;13:177–83.

[10] Tang BK. Drug glucosidation. Pharmacol Ther 1990;46:53–6.

[11] Paibir SG, Soine WH. High-performance liquid chromatographic analysis of phenobarbital and phenobarbital metabolites in human urine. J Chromatogr B Biomed Sci Appl 1997;691:111–7.

[12] Negoro T, Murata M, Ueda S, Fujitani B, Ono Y, Kuromiya A, Komiya M, Suzuki K, Matsumoto J. Novel, highly potent aldose reductase inhibitors: (*R*)-(−)-2-(4-bromo-2-fluorobenzyl)-1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrazine-4-spiro-3′-pyrrolidine-1,2′,3,5′-tetrone (AS-3201) and its congeners. J Med Chem 1998;41:4118–29.

[13] Kurono M, Fujiwara I, Yoshida K. Stereospecific interaction of a novel spirosuccinimide type aldose reductase inhibitor, AS-3201, with aldose reductase. Biochemistry 2001;40:8216–26.

[14] Toide K, Matsushita H, Hashizume T, Terauchi Y, Fujii T. *In vitro* metabolism of aldose reductase inhibitor AS-3201 in human liver microsomes. Ninth North American ISSX Meeting, 1999 Oct 24–28; Nashville, TN, Abstract No. 155.

[15] Soine PJ, Soine WH. High-performance liquid chromatographic determination of the diastereomers of 1-(α-D-glucopyranosyl)amobarbital in urine. J Chromatogr 1987;422:309–14.

[16] Raijmakers MT, Jansen PL, Steegers EA, Peters WH. Association of human liver bilirubin UDP-glucuronyltransferase activity with a polymorphism in the promoter region of the UGT1A1 gene. J Hepatol 2000;33:348–51.

[17] Hanioka N, Jinno H, Tanaka-Kagawa T, Nishimura T, Ando M. Determination of UDP-glucuronosyltransferase UGT1A6 activity in human and rat liver microsomes by HPLC with UV detection. J Pharm Biomed Anal 2001;25:65–75.

[18] Sim SM, Back DJ, Breckenridge AM. The effect of various drugs on the glucuronidation of zidovudine (azidothymidine AZT) by human liver microsomes. Br J Clin Pharmacol 1991;32:17–21.

[19] Nishiyama T, Ogura K, Nakano H, Ohnuma T, Kaku T, Hiratsuka A, Muro K, Watabe T. Reverse geometrical selectivity in glucuronidation and sulfation of *cis*- and *trans*-4-hydroxytamoxifens by human liver UDP-glucuronosyltransferases and sulfotransferases. Biochem Pharmacol 2002;63:1817–30.

[20] Radominska A, Little J, Pyrek JS, Drake RR, Igari Y, Fournel-Gigleux S, Magdalou J, Burchell B, Elbein AD, Siest G, Lester R. A novel UDP-Glc-specific glucosyltransferase catalyzing the biosynthesis of 6-*O*-glucosides of bile acids in human liver microsomes. J Biol Chem 1993;268:15127–35.

[21] Crespi CL. Xenobiotic-metabolizing human cells as tools for pharmacological and toxicological research. Adv Drug Res 1995;26:179–235.

[22] Radominska-Pandya A, Czernik PJ, Little JM, Battaglia E, Mackenzie PI. Structural and functional studies of UDP-glucuronosyltransferases. Drug Metab Rev 1999;31:817–99.

[23] Nakajima M, Nakamura S, Tokudome S, Shimada N, Yamazaki H, Yokoi T. Azelastine *N*-demethylation by cytochrome P-450 (CYP)3A4, CYP2D6, and CYP1A2 in human liver microsomes: evaluation of approach to predict the contribution of multiple CYPs. Drug Metab Dispos 1999;27:1381–91.

[24] Strassburg CP, Oldhafer K, Manns MP, Tukey RH. Differential expression of the UGT1A locus in human liver, biliary, and gastric tissue: identification of *UGT1A7* and *UGT1A10* transcripts in extrahepatic tissue. Mol Pharmacol 1997;52:212–20.

[25] Strassburg CP, Manns MP, Tukey RH. Expression of the UDP-glucuronosyltransferase 1A locus in human colon. Identification and characterization of the novel extrahepatic UGT1A8. J Biol Chem 1998;273:8719–26.

[26] Green MD, Oturu EM, Tephly TR. Stable expression of a human liver UDPglucuronosyltransferase (UGT2B15) with activity towards steroid and xenobiotic substrates. Drug Metab Dispos 1994;22:799–805.

[27] Beaulieu M, Lévesque E, Hum DW, Bélanger A. Isolation and characterization of a novel cDNA encoding a human UDP-glucuronosyltransferase active on C_{19} steroids. J Biol Chem 1996;271:22855–62.

[28] Lévesque E, Beaulieu M, Green MD, Tephly TR, Bélanger A, Hum DW. Isolation and characterization of UGT2B15(Y85): a UDP-glucuronosyltransferase encoded by a polymorphic gene. Pharmacogenetics 1997;7:317–25.

[29] Fitzsimmons ME, Collins JM. Selective biotransformation of the human immunodeficiency virus protease inhibitor saquinavir by human small-intestinal cytochrome P4503A4: potential contribution to high first-pass metabolism. Drug Metab Dispos 1997;25:256–66.

[30] Stormer E, von Moltke LL, Greenblatt DJ. Scaling drug biotransformation data from cDNA-expressed cytochrome P-450 to human liver: a comparison of relative activity factors and human liver abundance in studies of mirtazapine metabolism. J Pharmacol Exp Ther 2000;295:793–801.

[31] Venkatakrishnan K, von Moltke LL, Court MH, Harmatz JS, Crespi CL, Greenblatt DJ. Comparison between cytochrome P450 (CYP) content and relative activity approaches to scaling from cDNA-expressed CYPs to human liver microsomes: ratios of accessory proteins as sources of discrepancies between the approaches. Drug Metab Dispos 2000;28:1493–504.

[32] Senafi SB, Clarke DJ, Burchell B. Investigation of the substrate specificity of a cloned expressed human bilirubin UDP-glucuronosyltransferase: UDP-sugar specificity and involvement in steroid and xenobiotic glucuronidation. Biochem J 1994;303:233–40.

[33] Green MD, Tephly TR. Glucuronidation of amines and hydroxylated xenobiotics and endobiotics catalyzed by expressed human UGT1.4 protein. Drug Metab Dispos 1996;24:356–63.

[34] Green MD, King CD, Mojarrabi B, Mackenzie PI, Tephly TR. Glucuronidation of amines and other xenobiotics catalyzed by expressed human UDP-glucuronosyltransferase 1A3. Drug Metab Dispos 1998;26:507–12.
[35] Zakim D, Hochman Y, Kenney WC. Evidence for an active site arginine in UDP-glucuronyltransferase. J Biol Chem 1983;258: 6430–4.
[36] Tang C, Hochman JH, Ma B, Subramanian R, Vyas KP. Acyl glucuronidation and glucosidation of a new and selective endothelin ET(A) receptor antagonist in human liver microsomes. Drug Metab Dispos 2003;31:37–45.
[37] Mackenzie P, Little JM, Radominska-Pandya A. Glucosidation of hyodeoxycholic acid by UDP-glucuronosyltransferase 2B7. Biochem Pharmacol 2003;65:417–21.