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Dopamine is a low affinity and high specificity substrate for the human UDP-glucuronosyltransferase 1A10*

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SULT, sulfotransferase; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase

Abstract

The purpose of this work was to identify human UDP-glucuronosyltransferases (UGTs) capable of glucuronidating dopamine. Using a sensitive LC-MS/MS method we screened all 19 known human UGTs and found that only one enzyme, UGT1A10, catalyzed dopamine glucuronidation at substantial rates, yielding both dopamine-4-*O*-glucuronide (37.1 pmol/min/mg) and dopamine-3-*O*-glucuronide (32.7 pmol/min/mg). Much lower (<2 pmol/min/mg) or no dopamine glucuronidation activity was found for all other UGTs tested at 1 mM dopamine concentration. Evaluation of UGT1A10 expression pattern in human tissues by quantitative RT-PCR confirmed that it is mainly expressed in small intestine, colon and adipose tissue, while only low levels were found in trachea, stomach, liver, testis and prostate, but not in the brain. Dopamine glucuronidation assays using microsomes from human liver and intestine corroborated these findings since activity in intestinal microsomes was markedly higher than in liver microsomes. Moreover, the glucuronidation regio-selectivity in intestinal microsomes was similar to recombinant UGT1A10 and both enzyme sources exhibited sigmoidal kinetics with substrate affinity (K_A) values in the range of 2-3 mM. Examination of four UGT1A10 mutants, F90A, F90L, F93A, and F93L, revealed lower dopamine glucuronidation in all of them, particularly in F90A and F93A. Nonetheless, the substrate affinities of the 4 mutants were similar to UGT1A10. Interestingly, mutant F93L exhibited regio-selectivity, conjugating dopamine at the 4-OH position about 3 times more efficiently than at the 3-OH. These results shed new light on the structure and function of UGT1A10 and indicate that dopamine may be a useful probe substrate for this enzyme.

UDP-glucuronosyltransferases (EC 2.4.1.17; UGTs) catalyze the glucuronidation reaction, involving the transfer of glucuronic acid moiety from UDP-glucuronic acid (UDPGA) to an aglycone substrate. There are 19 distinct human UGT isoforms and they are divided into three subfamilies: UGT1A, UGT2A and UGT2B (Mackenzie et al., 2005). The aglycone substrates of the UGTs are both endogenous compounds like bilirubin and steroid hormones, as well as xenobiotics, including many drugs. Most of these aglycones are very lipophilic and the conjugation with glucuronic acid stimulates their excretion from the cell and from the body through bile or urine. In most cases glucuronidation also renders the aglycone substrate biologically inactive (Tukey and Strassburg, 2000; King et al., 2000; Wells et al., 2004).

The aglycone substrate specificity of individual UGTs is very complex and one of the major objectives in UGT research is gaining a detailed understanding of the substrate specificity of all the human UGTs. Most of them can glucuronidate many different substrates with variable chemical structures. This, together with high sequence homology among the UGTs, also means there is frequently partial overlap of the substrate specificity of individual isoforms. However, there are compounds that are mainly glucuronidated by a single or very few UGTs (Court, 2005), demonstrating that alongside similarities, there are also significant differences in their substrate specificity. Such specific substrates, or probe substrates, are important tools for the study of glucuronidation in microsomes from different tissues and in cell lines since nearly all of them contain several different UGTs, and currently we have no good way to determine the amount of each UGT in a given sample of this kind.

Dopamine (Fig.1A) is an important neurotransmitter and its homeostasis in central and peripheral nervous system is important for many physiological processes as well as diseases (Eisenhofer et al. 2004). Dopamine is also found in the circulation, where the sulfated dopamine is more abundant than the free form (Johnson et al., 1980). Its sulfonation is catalyzed by sulfotransferase (SULT) 1A3, an enzyme with a strong preference for

catecholamines (Eisenhofer et al., 1999). SULT1A3 is mainly found in the gastrointestinal tract, where almost half of the endogenous dopamine is formed (Eisenhofer et al., 1997; Eisenhofer et al., 1999), but it is also found in the brain (Whittemore et al., 1985). Dopamine glucuronides were previously found in the human cerebrospinal fluid (Tyce et al., 1986), and one of the aims of the current study was to find out which UDP-glucuronosyltransferases (UGTs) are responsible for dopamine glucuronidation in human.

The majority of UGTs are expressed in the liver, but they are also found in various other tissues. UGT1A10 was considered an extra-hepatic UGT that is mainly expressed in the intestine (Strassburg et al., 1997; Strassburg et al., 1998; Mojarrabi and Mackenzie, 1998; Cheng et al., 1999; Strassburg et al., 1999; Zheng et al., 2002). Recent findings, however, detected low expression levels of UGT1A10 in liver and hepatocytes (Li et al., 2007; Nakamura et al., 2008). Based on the latter, it might be speculated that low expression levels of UGT1A10 that were not detected previously, could also occur in the brain. Such a finding, for a highly active enzyme like UGT1A10 (Xiong et al., 2006; Starlard-Davenport et al., 2007; Itäaho et al., 2008), would certainly have functional implications. Therefore, in the present study we have re-examined the expression of UGT1A10 in various human tissues, including the brain.

In previous works focusing on structure-function relationships of the UGTs, we have investigated the activities of UGT1A10 towards phenol compounds (Xiong et al., 2006) and different estrogens (Starlard-Davenport et al., 2007; Itäaho et al., 2008). Specifically it was suggested that F90 and F93 of UGT1A10 participate in the binding of phenolic substrates through ring stacking (Xiong et al., 2006). Consequently, it would be interesting to study how Ala and Leu mutations at positions 90 and 93 of UGT1A10 affect its activity towards dopamine.

It is often assumed that among the two major systems of phase II drug metabolism, sulfonation is a high affinity but low capacity process, whereas glucuronidation offers high capacity but low affinity. We have previously analyzed dopamine sulfonation by SULT1A3 (Itäaho et al., 2007) and in the present study we have analyzed the glucuronidation of this neurotransmitter. The results yield a new possible probe for UGT1A10, and shed new light on the activity of this important, primarily intestinal, enzyme.

Materials and methods

Reagents and Enzymes

Dopamine hydrochloride, Uridine 5′-diphosphoglucuronic acid trisodium salt and D-Saccharic acid 1,4-lactone were purchased from Sigma-Aldrich. Sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate were from Fluka (Buchs, Switzerland and Steinheim, Germany). Radiolabeled [^{14}C]UDPGA was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Dopamine-4-*O*-glucuronide was synthesized in our laboratory as described elsewhere (Uutela et al., in press). Pooled human liver microsomes and intestinal microsomes, as well as recombinant UGT2B15, were purchased from BD Gentest (Woburn, MA). The other recombinant UGTs were produced in our laboratory as his-tagged proteins in baculovirus-infected insect cells (Kurkela et al., 2003; Kurkela et al., 2007). The activity of UGT2B10 was studied using freshly harvested cells due to possible partial inactivation upon membranes/microsomes preparation (Kaivosaari et al. 2007). The other recombinant UGTs, as well as four UGT1A10 mutants that were described recently (Xiong et al., 2006; Starlard-Davenport et al., 2007), were studied as membranes that had been stored in -70˚C until used. HPLC grade acetonitrile was from Rathburn Chemicals (Walkerburn, U.K.) and formic acid (98-100%) was from Riedel-deHaën (Seelze, Germany).

HPLC and MS Methods and Equipment

The samples were analysed by high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS) as described elsewhere (Uutela et al., in press) with minor modifications in HPLC conditions. Agilent 1100 series HPLC equipment (Agilent Technologies, Waldbronn, Germany) was used with Supelco Discovery® HS F5 (4×150 mm, 3 μm) column (Supelco, Bellafonte, PA). A mixture of acetonitrile and 0.1 % aqueous formic acid was used as the mobile phase. The gradient was started after 1 min of isocratic flow of 5

% acetonitrile. The acetonitrile proportion was raised up to 20 % in 10 min and then decreased again to 5 % for the rest of the run. The flow rate was 0.9 ml/min and 5 minutes equilibration was used between samples. Retention times for dopamine-3-glucuronide (Fig. 1B) and dopamine-4-glucuronide (Fig. 1C) were (Mean ± S.D.) 5.3 ± 0.18 min and 4.7 ± 0.13 min, respectively, and the total run time was 13 min.

An API3000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) with a turbo ion spray source was used for detection, as described elsewhere (Uutela et al., in press). Purified air (Atlas Copco CD 2, Wilrijk, Belgium) was used as a nebulizing gas and nitrogen generated with Whatman 75-72 generator (Haverhill, MA) was used as a turbo, curtain and collision gas. The turbo gas flow rate was 6 l/min and its temperature was 280°C. Analyses were carried out in the positive ion mode and the ion spray (5500 V) and orifice (declustering) voltages (24 V) were optimized. The collision energies were 28 V and 18 V and the collision cell exit potentials were 11.1 V and 12.5 V for the selected reaction monitoring (SRM) pairs 330.1/137.1 and 330.1/154.1, respectively. The data were collected and processed by Analyst 1.4.2 software.

Quantification of Glucuronides

Dopamine-4-*O*-glucuronide was used as an external standard to quantify both glucuronide isomers, and standards were analysed with every sample set. The correlation coefficient was >0.99 in all cases and the linear range was from 10 nM to 1.0 μM. The limit of detection was 1 nM and the lowest enzymatic activity detected was 0.02 pmol/min/mg. In order to verify that the signals of the two dopamine glucuronides were similar, the ratio of the peak areas of both glucuronides, dopamine-4-glucuronide and dopamine-3-glucuronide, was determined using HPLC (Agilent Technologies, Waldbronn, Germany) coupled with radiochemical detector (Model 9701, Reeve Analytical, Glasgow, U.K.). In order to produce both dopamine

glucuronide isomers as radioactive compounds, the samples containing dopamine (2 mM) and recombinant UGT1A10 (1.2 mg/ml) were incubated in the presence of 6 μM radiolabeled [^{14}C]UDPGA and 50 μM unlabeled UDPGA at 37°C for 3 hours. The other conditions were as described below. Same amounts of the same incubated samples were injected onto two separate HPLC systems, one coupled with a radiochemical detector and the other coupled with MS. The peak areas for each of the two glucuronides were found to be of substantially the same size in both systems (data not shown).

Screening of Microsomes, Recombinant UGTs and Mutants of UGT1A10

The activities of human liver and intestinal microsomes, 19 human recombinant UGTs, as well as 4 mutants of UGT1A10, towards dopamine were screened. The reaction mixtures contained 0.4-1.6 mg/ml protein, 1 mM UDPGA, 5 mM saccharolactone, 2 % dimethyl sulfoxide and 5 mM $MgCl_2$ in 50 mM phosphate buffer (pH 7.4). The total incubation volume was 250 μl and the dopamine concentration was 1 mM. The activities of the 4 mutants of UGT1A10 were screened in the presence of both 1 mM and 5 mM dopamine. The duplicated samples were incubated at 37°C for 60 min. The reactions were terminated by adding 25 μl of chilled 4 M perchloric acid and cooling the tubes in a cold block, followed by centrifugation for 5 minutes at 16,100 × *g* to remove the precipitated proteins. 100 μl of the supernatant were injected to LC-MS/MS.

Enzyme Kinetic Studies

Kinetic constants were determined for dopamine glucuronidation by UGT1A10 and the four mutants, as well as human liver and intestinal microsomes. The samples were prepared in triplicate similarly as the screening samples, except that the protein concentrations were 100-400 μg/ml and the incubation times were 30-45 min, which were within the linear range of the

glucuronide formation. Two concentrations of the co-substrate UDPGA were tested with the most active sample, recombinant UGT1A10. The dopamine glucuronidation rates were essentially identical under both conditions, indicating that 1 mM was a saturating UDPGA concentration in this experimental system (data not shown). Hence, 1 mM UDPGA was used for all the other UGT assays. Eight dopamine concentrations, ranging from 0.1 to 10 mM, were used in the kinetic analyses.

Normalization of Enzymatic Activities

The expression levels of all recombinant UGT enzymes were determined by immunodetection as described previously (Kurkela et al., 2007). The commercial UGT2B15 was the only enzyme the expression level of which could not be normalized due to the absence of a His-tag. The other enzymatic activities presented in this study were normalized with respect to the expression level of UGT1A10. The relative expression levels per mg protein in the tested membrane batch of the recombinant UGTs that catalyzed dopamine glucuronidation were as follows: 1A1 (4.0), 1A3 (1.1), 1A6 (1.7), 1A7 (12), 1A8 (10), 1A9 (2.0), 2A1 (4.7), 1A10 (1.0), 2A3 (1.3), 2B7 (5.7), 2B11 (3.2), 2B17 (0.53), 10F90A (2.4), 10F90L (0.48), 10F93A (3.7), and 10F93L (2.1).

Enzyme Kinetic Analyses

The normalized initial velocity data from the enzyme kinetic studies were fitted either to the Michaelis-Menten equation [$v = V_{max} \times S/(K_m + S)$, where v is the initial velocity of the reaction, S is the substrate concentration, V_{max} is the maximal velocity and K_m is the substrate concentration at 0.5 V_{max}], to the substrate inhibition equation [$v = V_{max} \times S/(K_s + S + S^2/K_i)$, where K_i is the constant describing the substrate inhibition interaction], or to the Hill equation [$v = V_{max} \times S^n/(K_A{}^n + S^n)$, where K_A is the substrate concentration at 0.5 V_{max} and n is the Hill

coefficient], by nonlinear regression using GraphPad Prism version 4.03 for Windows (GraphPad Software Inc., San Diego, CA). The best kinetic model was selected considering the randomness of the residuals, the standard errors of the estimates and the correlation coefficients. None of the reactions followed the substrate inhibition equation.

UGT1A9 and UGT1A10 mRNA Quantitation in Human Tissues

Total RNA was extracted using Trizol reagent (Sigma-Aldrich, St Louis, MO, USA) from 47 human livers and pooled. Liver donors were all of European-American ancestry and included both males (n=36) and females (n=11). Use of the tissue was approved by the institutional review board of Tufts University School of Medicine. Total RNA from human adrenal gland (n=62), whole brain, cerebellum (n=24), fetal brain (n=59), colon (n=3), kidney, lung, placenta, prostate (n=47), small intestine (n=5), testis (n=19), thyroid (n=65), trachea, and uterus (n=10) were purchased from BD-Clontech (USA), while total RNA from human adipose tissue, stomach, pancreas, and ovary were from Ambion (USA). To generate cDNA, 1 μg of total RNA was treated with DNase (Promega, USA) and reverse transcribed (Superscript II, Invitrogen, USA) with random hexamer primer (0.1 μg). Quantitative PCR reactions (25 μL) included Sybr Green 2X master mix (Applied Biosystems, USA), 10 μL of 1:10 diluted cDNA (except 1:30 dilution for liver cDNA; and 1:500 dilution for cDNA assayed with 18S rRNA primers), and 200 nM of each primer. Primer pair sequences were as follows: CCC CTC GAT GCT CTT AGC TGA GTG T (18S-rRNA-forward), CGC CGG TCC AAG AAT TTC ACC TCT (18S-rRNA-reverse), GGA GCC ACT GGT TCA CCA TGA G (UGT1A9-forward), AGA TCC TCC AGG GTA TAT GAA GTT GAA (UGT1A9-reverse), TTG ATA CCT GTG GCT TAA TTG TTG CT (UGT1A10-forward), GGC ATC TGA GAA CCC TAA GAG ATC AT (UGT1A10-reverse). Real-time PCR analysis (Model 7300, Applied Biosystems, USA) was performed with the following PCR method: 95˚C for 10

min, 40-45 cycles of 95˚C for 30 sec and 60˚C for 60 sec. Amplification specificity was ensured initially by sequencing of representative PCR products, and in each run by PCR product duplex melting temperature analysis. Negative controls included exclusion of cDNA template and reverse transcription enzyme. mRNA concentrations were calculated using standard curves of PCR threshold cycle number versus concentration of template derived from serial dilutions of purified PCR product. Curves were linear ($R^2 > 0.99$) over the concentration range 10^{-9} to 10^{-14} M for 18S rRNA and 10^{-14} to 10^{-18} M for other gene products, which defined the upper and lower quantitation limits of the respective assays. For each tissue, cDNA reactions were performed in triplicate and quantitative PCR reactions were performed at least in duplicate. Results were expressed as the mean (± SE) number of mRNA copies per 10^9 copies of 18S rRNA. Assay precision as reflected by the coefficient of variation of replicates averaged 26 % and 31 % for UGT1A9 and UGT1A10, respectively.

Results

Screening of Recombinant UGTs

The 19 human UGTs of subfamilies 1A, 2A and 2B were screened for dopamine glucuronidation activity. The substrate concentration in the screening studies was somewhat high (1 mM), and we employed a sensitive detection method (LC-MS/MS). Under these conditions, a few UGTs exhibited detectable activity but only one, UGT1A10, glucuronidated dopamine at significant rates (Fig. 2). The regio-selectivity of dopamine glucuronidation was also studied and in the case of UGT1A10 there was only a minor difference between the two hydroxyls of the dopamine molecule; dopamine-4-glucuronide was produced slightly more than dopamine-3-glucuronide. No dopamine-N-glucuronide was detected in these assays.

Very low dopamine glucuronidation activity was detected with UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 2A1, 2A3, 2B7, 2B11, 2B15 and 2B17 (Fig. 2). The regio-selectivity of these UGTs mostly differed from UGT1A10 and some exhibited a strong preference for the 3-OH (Fig. 2). UGTs 2A3 and 2B11 even appeared to be totally selective for the 3-OH, but since their activity was so low, barely above the detection limit, it is possible that the dopamine-4-glucuronide they might have produced remained undetected. In addition, we evaluated possible formation of dopamine N-glucuronide by recombinant UGTs, particularly UGTs 1A4 and 2B10, but none was detected (data not shown).

We also measured dopamine glucuronidation activity using microsomes from human liver and human intestine (Fig. 2). The activity per mg protein in the assay in intestinal microsomes was 5 to10 times higher than in liver microsomes, dependent on which dopamine glucuronide was compared. Interestingly human liver microsomes exhibited a clear preference for the 3-OH glucuronidation of dopamine, which is a very similar regio-selectivity to that of the very low activity of the liver UGTs 1A6, 1A9, 2B7 and 2B17 (Fig. 2). On the other hand,

human intestinal microsomes exhibited very little preference for the 4-OH glucuronidation of dopamine, and had a near identical regio-selectivity to that of recombinant UGT1A10 (Fig. 2).

After discovering the dopamine glucuronidation activity of UGT1A10, we used this substrate to study four active-site mutants of this enzyme, F90A, F90L, F93A, and F93L, that we have recently evaluated using different substrates (Xiong et al., 2006; Starlard-Davenport et al., 2007). The mutants were assayed in the presence of both 1 and 5 mM dopamine and revealed relatively low dopamine glucuronidation activity in comparison to wild-type UGT1A10 (Fig. 3). Nevertheless, substitution of either F90 or F93 with alanine, as in F90A and F93A, lowered the activity towards the 4-OH of dopamine much more than replacement of either of these two phenylalanine residues with leucine. The two F to L mutants, F90L and F93L, differed sharply from each other with respect to dopamine glucuronidation at the 3-OH, however (Fig. 3). While mutant 10F90L, like wild-type UGT1A10, exhibited similar glucuronidation activity towards both hydroxyls of dopamine, mutant 10F93L exhibited approximately 3 times higher glucuronidation activity towards the 4-OH than towards the 3-OH of dopamine.

Enzyme Kinetic Studies

The enzyme kinetics of dopamine glucuronidation by UGT1A10, mutants 10F90A, 10F90L, 10F93A, 10F93L, as well as human liver and intestinal microsomes were analysed with varied dopamine concentrations. Dopamine glucuronidation by UGT1A10 were best described by the Hill equation (Fig. 4, Table 1) and the same type of sigmoidal kinetics was exhibited by the two mutants at phenylalanine 90, whereas mutants 10F93A and 10F93L exhibited Michaelis-Menten kinetics (Fig. 5, Table 1). In the case of the native samples, the activity of human intestinal microsomes followed the Hill equation (Fig. 6, Table 1). Unfortunately, enzyme

kinetic parameters could not be determined for human liver microsomes since enzyme saturation could not be achieved at the highest substrate concentration tested (10 mM).

The kinetic analyses revealed that the dopamine affinity for all these UGTs was low. Nonetheless, it is noteworthy that the K_A values of recombinant UGT1A10 and human intestinal microsomes were in the same range, 2-3 mM (Table 1). Moreover, the mutagenesis of F90 and F93 to either Ala or Leu did not affect the affinity of the enzyme for dopamine, even if all of them lowered the V_{max} values (Table 1).

UGT1A9 and UGT1A10 mRNA Quantitation in Human Tissues

Since UGT1A10 appeared to be the most active human enzyme in dopamine glucuronidation we evaluated the tissue specificity of expression of this isoform. Although studies with some tissues were done previously, there were inconsistencies in results, particularly whether or not this enzyme is expressed in liver (Strassburg et al., 1997; Strassburg et al., 1998; Mojarrabi and Mackenzie, 1998; Cheng et al., 1999; Strassburg et al., 1999; Zheng et al., 2002; Li et al., 2007; Nakamura et al., 2008). Also, since dopamine is an important neurotransmitter in the brain, we wanted to find out if UGT1A10 is expressed in the brain. In the same tissues, we also analysed the expression pattern of UGT1A9, an enzyme that is highly homologous to UGT1A10 in its nucleotides and amino acid sequence, but differs from it markedly in its tissue distribution. Results presented in Table 2 confirm the previous findings of Li et al. (2007) in that although UGT1A10 is largely an extrahepatic enzyme, this UGT is also expressed in the liver at very low level. Nonetheless, UGT1A10 is mainly expressed in the intestine and in adipose tissue. In addition to liver, very low expression of UGT1A10 was detected in testis and prostate, and slightly higher expression in trachea (Table 2). These findings of low expression levels of UGT1A10 outside the intestine highlight the absence of any detectable mRNA for UGT1A10 (or UGT1A9) in the brain. UGT1A9, in contrast to UGT1A10, is

mainly expressed in the liver and in the kidneys. Noteworthy, UGT1A9 is also expressed at considerable levels in the adipose tissue, small intestine and colon (Table 2).

Discussion

Following the discovery of dopamine glucuronide in rat brain (Uutela et al., in press) we have examined the dopamine glucuronidation activity of the 19 human UGTs of subfamilies 1A, 2A and 2B. The results demonstrated that UGT1A10 catalyzes this reaction at much higher rate than any other human UGT (Fig. 2). The relatively high activity of UGT1A10 was not reported in a previous study by Taskinen et al. (2003), simply because UGT1A10 was not included in that study. In addition, we have detected very low dopamine glucuronidation activity in many more UGTs than previously, probably due to the improved detection method that was employed in the present study. Importantly, our results with the recombinant human UGTs are in good agreement with the results from human liver and intestinal microsomes (Table 1 and Fig. 6) since the expression of UGT1A10 in the intestine is much higher than its expression in the liver (Table 2; see also Mojarrabi and Mackenzie, 1998; Cheng et al., 1999).

The dopamine glucuronidation activity we have found in human intestinal microsomes is in apparent contrast to the results of an earlier study on catechol glucuronidation (Antonio et al., 2003). No detectable dopamine glucuronidation activity by human intestinal microsomes was reported in that work and we suggest that the reason(s) for that may lie in the experimental conditions, particularly lower substrate concentration during the assays (500 μM versus 1000 μM in the present work), and lower detection sensitivity. In addition, it is possible that variability in the quality of intestinal microsomes also contributed to the differences between the studies.

Although our original aim was to expose possible contribution of glucuronidation to dopamine metabolism in different tissues, the results steered the focus of this research to a new direction. Dopamine appears to be a useful probe substrate for UGT1A10. This compound largely follows the two criteria for a good probe compound, namely that it is selective for one isoform and exhibits a similar affinity for the individual enzyme as for

human tissues or microsomes (Court, 2005). The fact that we have discovered that many other isoforms glucuronidated dopamine at detectable rate tells more about the high sensitivity of the assay system (LC-MS/MS) than about the activity of these UGTs. For example, the normalized activity of the second best UGT after UGT1A10, UGT1A6 glucuronidating dopamine at the 3-OH, was less than 1.3 % of the respective activity of UGT1A10 (Fig. 2). Moreover, the recombinant UGT1A10 is very similar to human intestinal microsomes with respect to kinetics and regio-selectivity of dopamine glucuronidation (Fig. 4 and Fig. 6) strongly suggesting that UGT1A10 is by far the main contributor to this activity in the native sample as well.

The kinetic studies revealed that the dopamine affinity for both recombinant UGT1A10 and human intestinal microsomes was low. This finding might suggest that glucuronidation does not play a significant role in dopamine metabolism in human. Nonetheless, the rather low substrate affinity should not prevent the use of dopamine as a probe substrate for UGT1A10, as low affinity to serotonin did not prevent the latter compound being a useful probe substrate for UGT1A6 (Krishnaswamy et al., 2003). The availability of such a probe substrate for UGT1A6 has provided means to study this enzyme and its interactions with other UGTs (Kurkela et al., 2007; Fujiwara et al., 2007), and is likely to be instrumental in future studies as well.

Dopamine glucuronidation by UGT1A10 was not appreciably regio-selective, but there was a slight preference for 4-OH. While one should be cautious when discussing the regio-selectivity of enzymes that exhibit very low dopamine glucuronidation rates, it is tempting to draw attention to some of our observations concerning these UGTs. UGTs 1A7, 1A8 and 1A9 are the most homologous to UGT1A10 and the three of them strongly favoured the 3-OH over the 4-OH (Fig. 2). These observations are in line with a previous study on the regio-selectivity of 11 human UGTs in dobutamine glucuronidation (Alonen et al., 2005). Overall, the regio-

selectivity of individual UGTs in case of dopamine was similar to the corresponding findings for the two catecholic hydroxyls of dobutamine. Interestingly, there are only 16 amino acid residues in UGT1A10 that differ from the residues at the corresponding positions in any of the UGTs 1A7, 1A8 or 1A9. Hence, dopamine glucuronidation could be used to identify the amino acids that are responsible for the differences in the activity and regio-selectivity among these closely homologous UGTs.

Of the hepatic UGTs that catalyze dopamine glucuronidation at low but detectable rates, 1A1, 1A3 and 2B15 exhibited no clear regio-selectivity, or very low preference for the 4-OH, whereas 1A6, 1A9, 2B7 and 2B17 exhibited strong preference for the 3-OH (Fig. 2). Based on these results, dopamine glucuronidation by human liver microsomes was expected to yield more dopamine-3-glucuronide than dopamine-4-glucuronide. This expectation was in full agreement with the experimental results, concerning the regio-selectivity as well as the low dopamine glucuronidation activity in human liver microsomes (Fig. 6).

UGT1A10 is mainly expressed along the gastro-intestinal tract (Table 2, see also Strassburg et al., 1998; Mojarrabi and Mackenzie, 1998). The most active enzyme in dopamine sulfonation, SULT1A3, is also mainly expressed in the intestine (Eisenhofer et al., 1999; Sundaram et al., 1989). In addition, almost half of the endogenous dopamine is produced in the mesenteric organs, where most of the dopamine sulfates are produced (Eisenhofer et al., 1997). UGT1A10 is probably much less important for dopamine metabolism than SULT1A3, whose affinity to dopamine is about 1000 times higher (K_m at 2-3 μM range) (Itäaho et al., 2007). In rat, the glucuronidation is a more important metabolic pathway for dopamine than in human (Wang et al., 1983), most likely because sulfotransferase corresponding to human SULT1A3 does not exist in rodents (Eisenhofer et al., 1999; Honma et al., 2001).

Recent studies have found that two phenylalanine residues in UGT1A10, F90 and F93, may be directly involved in substrate binding (Xiong et al., 2006). The effect of mutating these Phe residues into Ala (Xiong et al., 2006), or Ala and Leu (Starlard- Davenport et al., 2007) on several enzymatic activities have been examined. The results were quite complex, revealing effects on either the V_{max} or the K_m, depending on the substrate. In the present study all the four mutations lowered the normalized V_{max} of the reaction, without significantly affecting its K_m values (Table 1, Fig. 5). Surprisingly, the extent of V_{max} reduction, at least for the formation of dopamine-4-glucuronide, appeared to be dependent more on the type of Phe substitution than on its position. Replacing either F90 or F93 with Ala decreased V_{max} much more than a Leu substitution at either position (Table 1, Fig. 5). A possible explanation for these findings is that leucine is more similar than alanine to phenylalanine, when it comes to hydrophobicity and size. In any case, the situation is even more complex when looking at the regio-selectivity of the mutants. Whereas at position 90 the regio-selectivity of the F to L mutant was similar to the wild-type, mutant 10F93L exhibited very different regio-selectivity, glucuronidating dopamine at the 4-OH about 3 times faster than at the 3-OH (Table 1, Fig. 5). It thus appears that more studies should be conducted in order to fully elucidate the significance of the residues at position 90 and 93 on binding different substrates to UGT1A10.

In summary, the results of this study highlight the activity of the human UGT1A10 and, thereby, the contribution of intestinal UGTs to first-pass metabolism of drugs and other xenobiotics. We have identified a new possible probe substrate for UGT1A10 and used it to study mutants of this enzyme. The results revealed distinct effects that are likely to be dependent on differences in the size of the side chain, as much as differences in their positions within the protein. These findings should facilitate better understanding of the substrate specificity of the human UGTs and the factors that determine it.

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***Footnote**

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Figure legends

Fig. 1. The structures of dopamine (A), dopamine-3-glucuronide (B) and dopamine-4-glucuronide (C).

Fig. 2. Dopamine glucuronidation by different UGT enzymes. Glucuronidation activities were determined with 1 mM dopamine and 1 mM UDPGA and the samples were incubated at 37°C for 60 min. Normalized glucuronidation rates (with respect to the expression level of UGT1A10) of the enzymes for which the relative expression level could be determined, are shown on the left side of the figure. The reaction rates in the samples for which normalization was not possible or relevant (UGT2B15 "supersomes", HLM, HIM) are on right side of the figure, and they could be directly compared with UGT1A10. The insert shows an enlargement of the normalized rates for UGTs that exhibited very low activity. All values are means of two samples.

Fig. 3. Dopamine glucuronidation by four mutants of human UGT1A10. Glucuronidation rates were determined with 1 mM and 5 mM dopamine in the presence of 1 mM UDPGA. The samples were incubated in duplicate at 37°C for 60 min and mean values are shown. Reaction rates were normalized for the expression levels with respect UGT1A10.

Fig. 4. Glucuronidation of dopamine by recombinant human UGT1A10. Samples were incubated in the presence of 1 mM UDPGA and 200 μg/ml protein at 37˚C for 30 min. All samples were prepared in triplicate. The initial rate data were fitted to the Hill equation and the kinetic constants are shown in table 1.

Fig. 5. Enzyme kinetics of dopamine glucuronidation by four mutants of human UGT1A10. Samples were incubated in the presence of 1 mM UDPGA and 100-400 μg/ml protein at 37˚C for 45 min. All samples were prepared in triplicate. The normalized initial rate data were fitted to the Michaelis-Menten (10F93A and 10F93L) or Hill equation (10F90A and 10F90L). The enzyme kinetic constants are listed in table 1.

Fig.6. Glucuronidation of dopamine by human intestinal microsomes (HIM) and liver microsomes (HLM). Samples were incubated in the presence of 1 mM UDPGA and 200 μg/ml protein at 37°C for 45 min. All samples were prepared in triplicate. The initial rate data of HIM were fitted to the Hill equation (see table 1 for kinetic constants). The kinetic constants for HLM could not be determined, but the Cl_{int} values were determined as the slopes of the regression lines fitted to the initial rates at low dopamine concentrations. Cl_{int} values for the formation of dopamine-3-glucuronide and dopamine-4-glucuronide were 1.7 nl/min/mg and 0.9 nl/min/mg, respectively.

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Tables

Table 1. The estimated kinetic parameters for glucuronidation of dopamine. The K_m and K_A values and the V_{max} values of UGT1A10 and human intestinal microsomes are presented as best-fit values ± standard error. The V_{max} values of the UGT1A10 mutants are normalized for their expression levels compared to wild-type recombinant UGT1A10. Enzyme kinetic parameters for human liver microsomes could not be determined since enzyme saturation could not be achieved at the highest substrate concentration tested.

	Dopamine-3-*O*-glucuronide			Dopamine-4-*O*-glucuronide		
	K_m or K_A (μM)	V_{max} (pmol/min/mg)	n	K_m or K_A (μM)	V_{max} (pmol/min/mg)	n
1A10[H]	1950 ± 171	116 ± 4.89	1.52	2190 ± 205	140 ± 6.50	1.49
10F90A[H]	1890 ± 211	6.62	1.50	1990 ± 240	3.38	1.65
10F90L[H]	2590 ± 150	36.7	1.60	2310 ± 103	36.2	1.74
10F93A[MM]	2630 ± 353	14.9		2910 ± 352	13.0	
10F93L[MM]	3110 ± 479	15.8		2670 ± 344	54.1	
HIM[H]	2870 ± 267	25.3 ± 1.13	1.26	3410 ± 398	28.1 ± 1.56	1.17
HLM	Could not be determined.			Could not be determined.		

[H]The data was fitted to the Hill equation, [MM]The data was fitted to the Michaelis-Menten equation.

Table 2. UDP-glucuronosyltransferase 1A9 and 1A10 mRNA expression in human tissues (copies per 10^9 copies of 18S rRNA). The values are mean ± SE.

Tissue	1A9	1A10
Adipose	86 ± 15	313 ± 57
Adrenal gland (n=62)	3 ± 0	-
Brain	-	-
Brain, cerebellum (n=24)	-	-
Brain, fetal (n=59)	-	-
Colon (n=3)	188 ± 55	1210 ± 299
Kidney	2157 ± 269	-
Liver, adult (n=47)	3239 ± 42	6 ± 0
Lung	-	-
Ovary	-	-
Pancreas	-	-
Placenta	3 ± 0	-
Prostate (n=47)	8 ± 1	6 ± 1
Small intestine (n=5)	319 ± 109	660 ± 254
Stomach	4 ± 1	21 ± 3
Testis (n=19)	13 ± 3	6 ± 1
Thyroid (n=65)	1 ± 0	-
Trachea	32 ± 7	27 ± 8
Uterus (n=10)	-	-

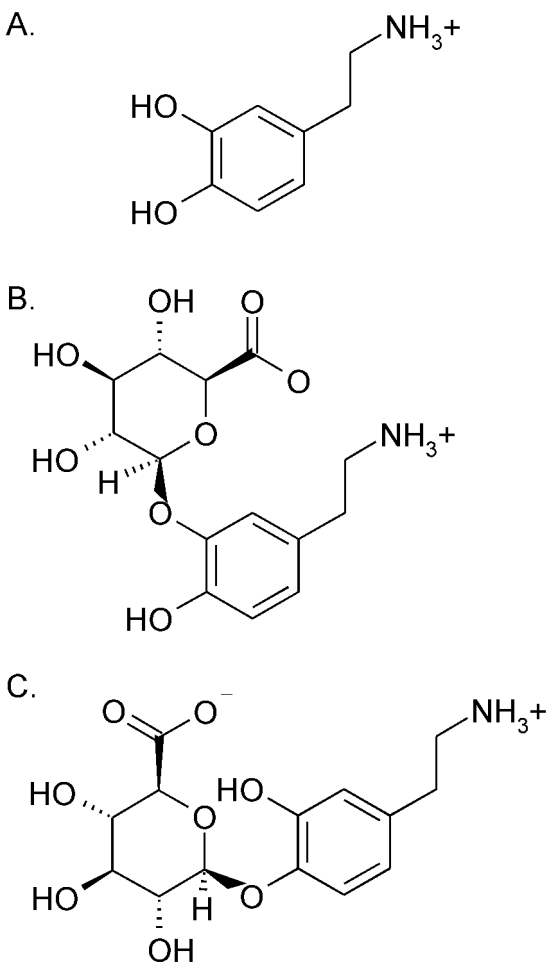

Figure 1.

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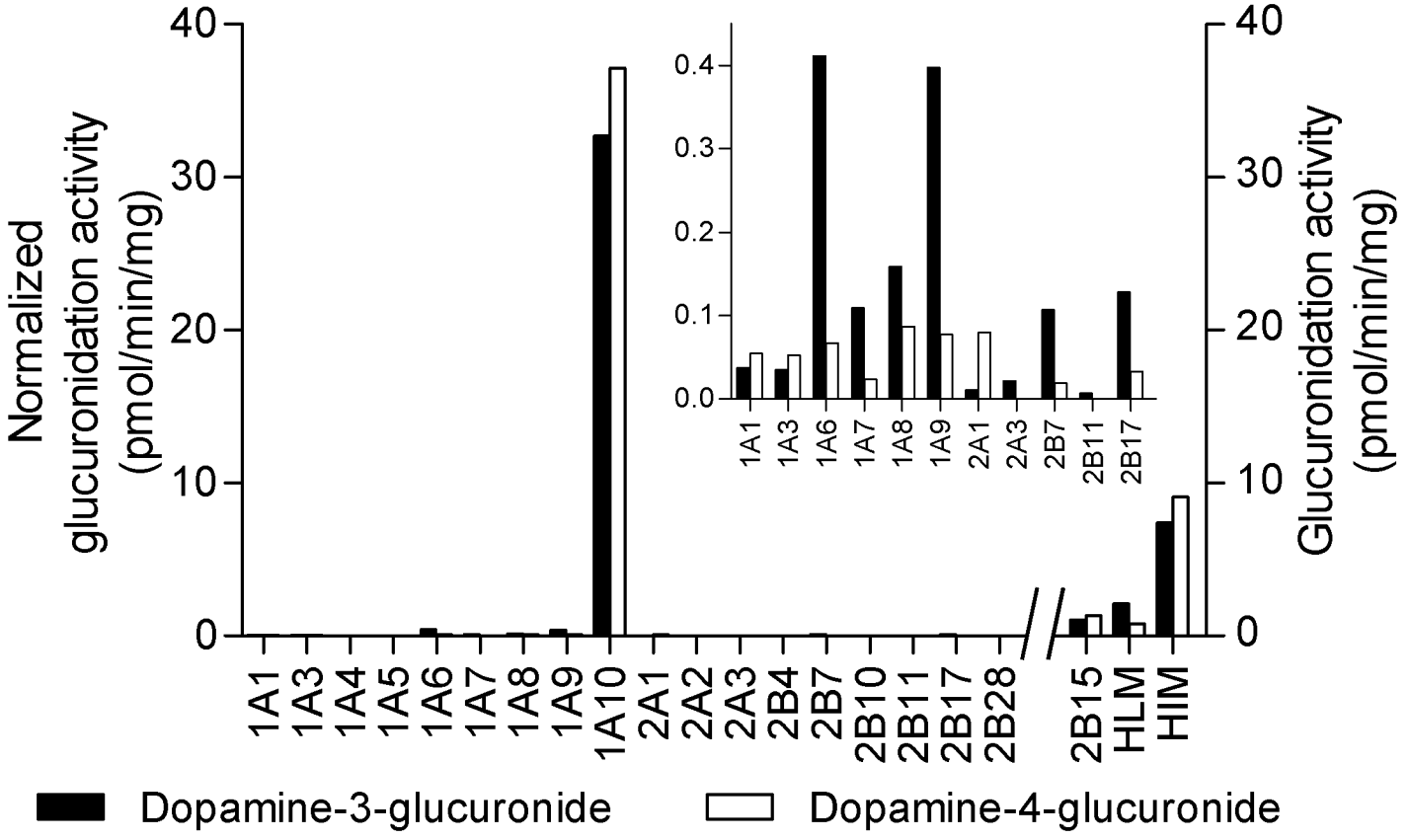

Figure 2.

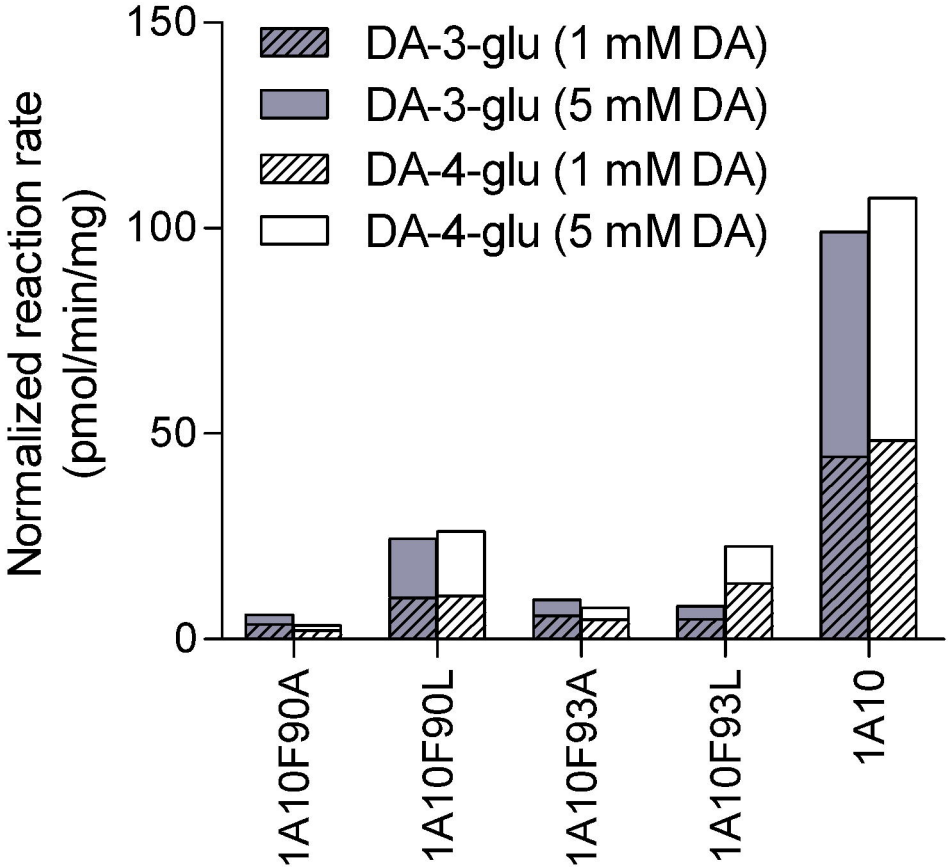
150
100
50
0
Normalized reaction rate
(pmol/min/mg)
DA-3-glu (1 mM DA)
DA-3-glu (5 mM DA)
DA-4-glu (1 mM DA)
DA-4-glu (5 mM DA)
1A10F90A
1A10F90L
1A10F93A
1A10F93L
1A10

Figure 3.

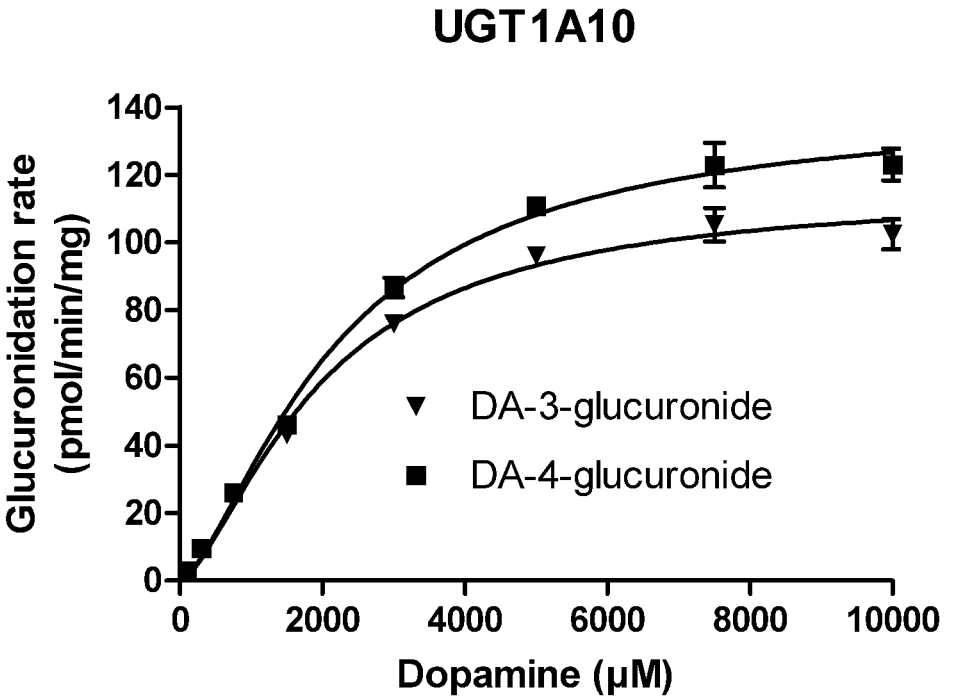
UGT1A10
Glucuronidation rate (pmol/min/mg)
140
120
100
80
60
40
20
0
0
2000
4000
6000
8000
10000
Dopamine (μM)
DA-3-glucuronide
DA-4-glucuronide

Figure 4.

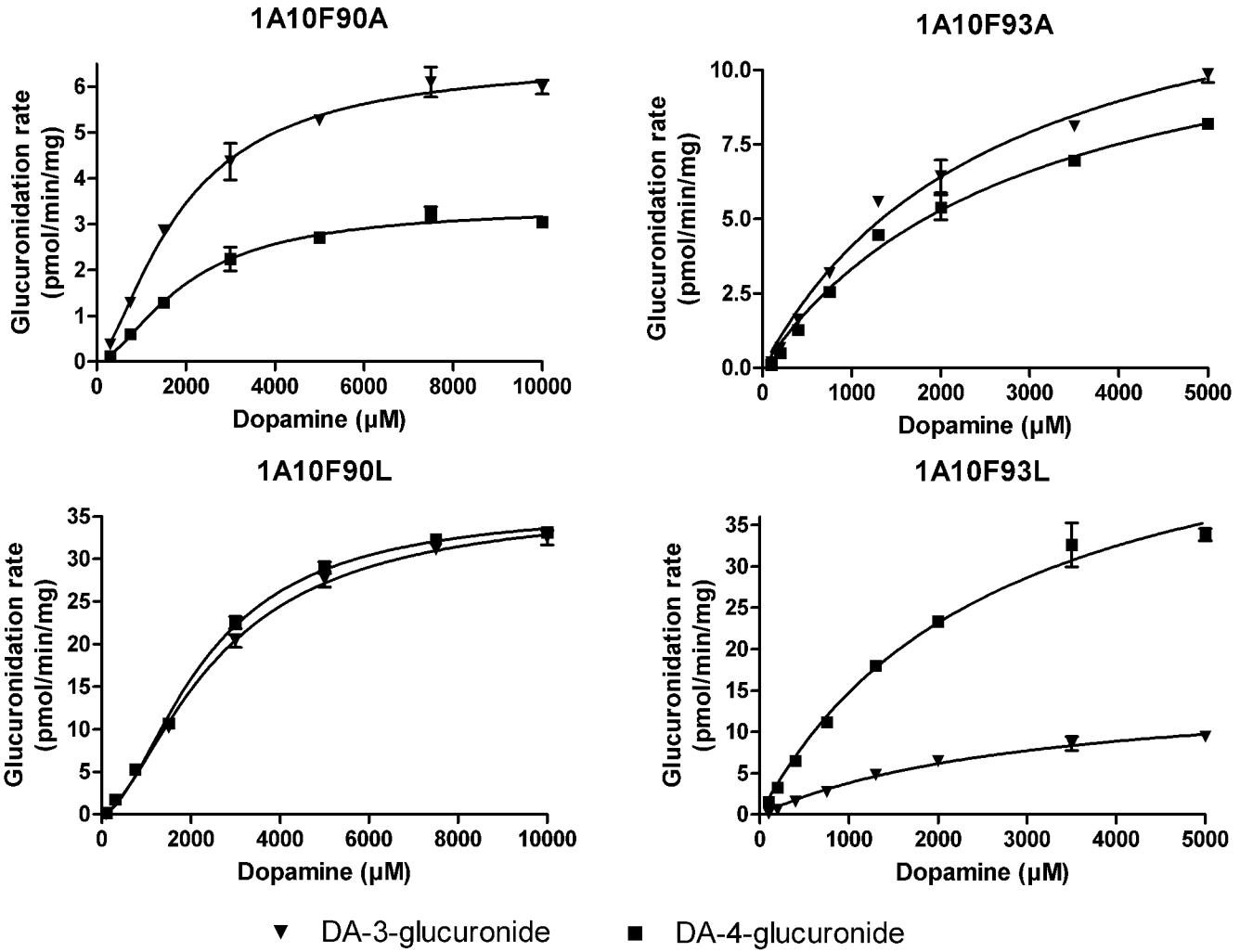
1A10F90A
Glucuronidation rate (pmol/min/mg)
Dopamine (μM)
1A10F93A
Glucuronidation rate (pmol/min/mg)
Dopamine (μM)
1A10F90L
Glucuronidation rate (pmol/min/mg)
Dopamine (μM)
1A10F93L
Glucuronidation rate (pmol/min/mg)
Dopamine (μM)
DA-3-glucuronide
DA-4-glucuronide

Figure 5.

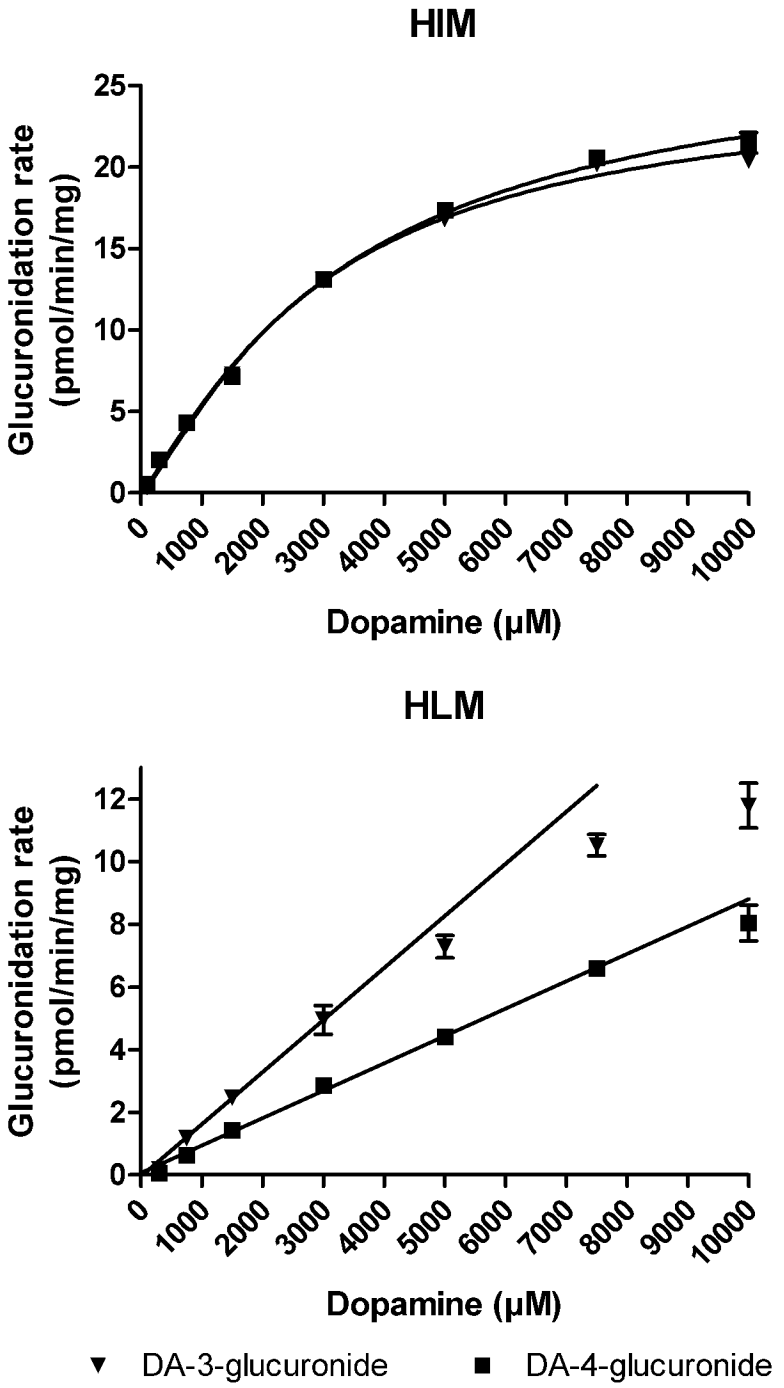
HIM
Glucuronidation rate (pmol/min/mg)
25
20
15
10
5
0
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
Dopamine (μM)
HLM
Glucuronidation rate (pmol/min/mg)
12
10
8
6
4
2
0
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
Dopamine (μM)
DA-3-glucuronide
DA-4-glucuronide

Figure 6.