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Influence of N-terminal domain histidine and proline residues on the substrate selectivities of human UDP-glucuronosyltransferase (UGT) 1A1, 1A6, 1A9, 2B7 and 2B10

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Running title: Influence of N-terminal domain Pro and His residues on UGT activity

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Abbreviations: LTG, lamotrigine; 4MU, 4-methylumbelliferone; 1NP, 1-naphthol; TFP, trifluoperazine; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

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Abstract

An N-terminal domain histidine (corresponding to position 39 of UGT1A1) is conserved in all UGT1A and UGT2B subfamily proteins except UGT1A4 (Pro-40) and UGT2B10 (Leu-34). Unlike most UGT1A and UGT2B xenobiotic metabolizing enzymes, UGT1A4 and UGT2B10 lack the ability to glucuronidate 4-methylumbelliferone (4MU) and 1-naphthol (1NP), both planar phenols, and naproxen (a carboxylic acid). However, only UGT1A4 glucuronidates the tertiary amines lamotrigine (LTG) and trifluoperazine (TFP). This study sought to elucidate the influence of specific N-terminal histidine and proline residues on UGT enzyme substrate selectivity. The conserved N-terminal domain histidine of UGT1A1, UGT1A6, UGT1A9 and UGT2B7 was mutated to proline and leucine-40 of UGT2B10 was substituted with histidine, and the capacity of the wild-type and mutant proteins to glucuronidate 4MU, 1NP, LTG, TFP and naproxen was characterized. Whereas UGT1A1(H39P), UGT1A6(H38P) and UGT1A9(H37P) lacked the ability to metabolize 4MU, 1NP and naproxen, all glucuronidated LTG. K_m values for UGT1A1(H39P) and UGT1A9(H37P) were 774 μM and 3812 μM , respectively, compared to 1579 μM for UGT1A4. UGT1A1(H39P) also glucuronidated TFP with a V_{max}/K_m value comparable to UGT1A4. In contrast to the wild-type enzyme, UGT2B10(L34H) glucuronidated 4MU and 1NP with respective K_m values of 260 μM and 118 μM . UGT2B7(H35P) lacked activity towards all substrates. The data confirm a pivotal role for an N-terminal domain proline in the glucuronidation of the tertiary amines LTG and TFP by UGT1A subfamily proteins while glucuronidation reactions involving proton abstraction generally, although not invariably, require a histidine at the equivalent position in both UGT1A and UGT2B enzymes.

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Introduction

UDP-Glucuronosyltransferase (UGT) enzymes catalyze the covalent linkage of glucuronic acid, donated by the co-factor UDP-glucuronic acid (UDPGA), to a typically hydrophobic substrate bearing a suitable functional group according to a second order nucleophilic substitution mechanism. Hydroxyl (aliphatic and aromatic), carboxylic acid and amine (primary, secondary and tertiary) functional groups most commonly serve as the nucleophilic 'acceptor' for glucuronic acid (Miners and Mackenzie, 1991; Radomska-Pandya et al., 1999). Since a myriad of compounds fulfill these minimal requirements for metabolism by UGT, glucuronidation provides an elimination and detoxification pathway for many drugs, non-drug xenobiotics and endogenous compounds. Nineteen functional human UGT proteins have been identified to date and these have been classified in two families (1 and 2) and three subfamilies, UGT1A, UGT2A and UGT2B based on sequence identity (Mackenzie et al., 2005). The individual UGT enzymes exhibit distinct, but overlapping, substrate and inhibitor selectivities (Kiang et al., 2005; Miners et al., 2004 and 2006; Radomska-Pandya et al., 1999; Sorich et al., 2006; Tukey and Strassburg, 2000).

The UDPGA binding site is highly conserved and located in the C-terminal domain of UGTs (Miley et al., 2007; Patana et al., 2007). Although an x-ray crystal structure for an entire UGT protein is lacking, multiple lines of evidence implicate the N-terminal domain in substrate (aglycone) binding and selectivity. All UGT1A enzymes contain an identical carboxyl terminus of 246 residues but unique N-terminal domains (residues 1 to 285-289) (Mackenzie et al., 2005), indicating that the latter region must determine substrate selectivity. Studies with chimeric UGT2B proteins have similarly associated the N-terminal domain with the substrate selectivities of several human, rabbit and rat UGT2B enzymes (Lewis et al., 2007; Li et al., 1997; Mackenzie 1990; Ritter et al., 1992 and 1993), while site-directed

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mutagenesis has identified specific N-terminal domain residues involved in the binding of substrates of human UGT 1A3, 1A4, 1A6, 1A10, 2B4, 2B15 and 2B17 (Barre et al., 2007; Dubois et al., 1999; Kubota et al., 2007; Li et al., 2007; Martineau et al., 2004; Senay et al., 2002; Xiong et al., 2006).

Particularly striking are differences in the ability of UGT1A and UGT2B enzymes to glucuronidate tertiary amines and planar phenols. Whereas UGT1A4 and UGT2B10 exhibit little or no activity towards the planar phenols 4-methylumbelliferone (4MU) and 1-naphthol (1NP), V_{\max} values with UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 2B7 and 2B15 exceed 25 pmol/min. mg (enzymes expressed in HEK293 cells) (Uchaipichat et al., 2004). In contrast, only UGT1A4 has been shown to convert the tertiary amines lamotrigine (LTG) and trifluoperazine (TFP) to their corresponding quaternary ammonium-linked glucuronides (Kubota et al., 2007). Although UGT2B10 lacks activity towards 4MU, 1NP, LTG and TFP, this enzyme catalyzes the N-glucuronidation of nicotine and cotinine (Chen et al., 2007; Kaivosaaari et al., 2007).

Recent studies with hybrid UGT1A3-UGT1A4 proteins demonstrated that the differing selectivities of these enzymes towards planar phenols and tertiary amines was determined by the first 44 residues, which correspond to amino acids 1 – 16 of the mature proteins (Kubota et al., 2007). A histidine in this region is conserved amongst all UGT1 and UGT2 family enzymes except UGT1A4 and UGT2B10, which have proline and leucine, respectively, at this position (Figure 1). (The His/Pro/Leu occurs at positions 39, 40, 38, 37, 35 and 34 of UGTs 1A1, 1A4, 1A9, 2B7 and 2B10, respectively.) Notably, the Pro40His mutation in UGT1A4 abolished the glucuronidation of LTG and TFP whereas the reciprocal (i.e. His40Pro) mutation in UGT1A3 conferred tertiary amine glucuronidation (Kubota et al.,

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2007). Unlike the glucuronidation of compounds containing a hydroxyl (and presumably carboxylate) group, quaternary ammonium glucuronide formation does not require proton abstraction. In this regard, the conserved His has been proposed as the catalytic base in glucuronidation reactions involving proton abstraction (Miley et al., 2007; Patana et al., 2008; Radomska-Pandya et al., 1999).

This study sought to characterize the importance of specific N-terminal domain proline and histidine residues in UGT substrate selectivity. The conserved histidine of UGT1A1, UGT1A6, UGT1A9 and UGT2B7 was substituted with proline and the glucuronidation of model tertiary amine (LTG and TFP), planar phenolic (4MU and 1NP) and carboxylic acid (naproxen) substrates by each mutant was characterized, as was the effect of the Leu34His mutation on UGT2B10 activity. The results demonstrate that introduction of proline in the N-terminal domain of UGT1A subfamily enzymes favors N-glucuronidation over the glucuronidation of the planar phenols and carboxylic acids. By contrast, activity studies with UGT2B10(L34H) and the UGT1A and UGT2B7 H→P mutants confirms a pivotal role for the near conserved N-terminal domain histidine in the glucuronidation of planar phenols.

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Materials and methods

Materials

Oligonucleotides were synthesized by Sigma Genosys (Castle Hill, Australia). Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA); Dulbecco's modified Eagle's medium (DMEM), MEM non-essential amino acids solution (10 mM; $\times 100$), and penicillin-streptomycin (penicillin 5,000 units/ml and streptomycin 5,000 $\mu\text{g/ml}$) from Invitrogen (Carlsbad, CA, USA); pBluescript II SK(+) from Stratagene (La Jolla, CA, USA); and the WB-UGT1A human UGT1A subfamily western blotting kit from BD Gentest (BD Biosciences, San Jose, CA, USA). LTG and lamotrigine N2-glucuronide were obtained from Wellcome Research Laboratories (Beckenham, UK). Cotinine, 4MU, 4-methylumbelliferone- β -D-glucuronide, 1NP, 1-naphthol- β -D-glucuronide, S-naproxen ('naproxen'), TFP (dihydrochloride salt), and UDPGA (trisodium salt) were purchased from Sigma-Aldrich (Sydney, Australia), and cotinine β -D-glucuronide from Toronto Research Chemicals (Toronto, Canada). Naproxen acyl glucuronide was provided by AstraZeneca (Sodertalje, Sweden). All other reagents and solvents were of analytical reagent grade.

Site-directed mutagenesis and expression of UGT proteins

The wild-type UGT 1A1, UGT1A6, UGT1A9, 2B7 and 2B10 cDNAs in pBluescript II SK(+) were used as templates for mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The primers used for mutagenesis are listed in Table 1. Mutations were confirmed by sequencing the entire cDNA (ABI Prism 3100 Genetic Analyzer with BigDye Terminator v3.1 Chemistry; Applied Biosystems, Foster City, CA, USA). UGT 1A1, UGT1A1(H39P), UGT1A4, UGT1A6, UGT1A6(H38P), UGT1A9, UGT1A9(H37P), UGT2B7, UGT2B7(H35P), UGT2B10 and UGT2B10(L34H) cDNAs were

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stably expressed in a human embryonic kidney cell line (HEK293), as described previously (Uchaipichat et al., 2004; Stone et al., 2003). Harvested cells were lysed by sonication using a Vibra Cell VCX130 Ultrasonic Processor (Sonics & Materials, Newtown, CT, USA) set to an amplitude of 40%. Cells were sonicated with four 2-sec ‘bursts’, each separated by 1 min cooling on ice. Lysed samples were centrifuged at 12,000g for 1 min at 4°C, and the supernatant fraction was separated and stored at -80°C until use.

Immunoblotting

Lysate protein from HEK293 cells expressing UGT1A (15µg), UGT2B7 (30 µg) and UGT2B10 (50 µg) enzymes and mutants was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gel and transferred onto a nitrocellulose membrane. Blots were probed with a commercial primary antibody specific for the human UGT1A subfamily or with an anti-UGT2B7 antibody (see below), followed by horseradish peroxidase-conjugated goat anti-rabbit antibody. The BM chemiluminescence blotting substrate (Roche Diagnostics GmbH, Mannheim, Germany) was used for immunodetection. Intensities of the immunoblots were measured with a Bio-Rad model GS-700 imaging densitometer. Antibody against UGT2B7 was prepared using amino acids 55-165 as the antigen. The peptide was synthesized with a 6-histidine tag at the C-terminus in *Escherichia coli* (BL21-DE3) and purified by affinity chromatography on a Ni-NTA column according to the protocol of the manufacturer (Qiagen). Antibody against the purified antigen was raised in rabbits and its specificity was assessed by immunoblotting against a panel of recombinant human UGTs. Apart from UGT2B7, the antibody weakly recognized UGT2B10 (see Results).

4MU and INP glucuronidation assay

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Incubations contained 0.1 M phosphate buffer (pH 7.4), 4 mM MgCl₂, 5 mM UDPGA, HEK293 cell lysate expressing UGT, and substrate (4MU or 1NP) in a total volume of 200 μ l. Activity screening studies with 4MU and 1NP were performed at the concentrations corresponding to the known K_m or S₅₀ value reported for each UGT enzyme and at 5 times the known K_m/S₅₀ (Uchaipichat et al., 2004) (Table 2), or at 100, 1000 and 5000 μ M with UGT2B10 and UGT2B10(L34H) as the enzyme source. Nine 4MU and 1NP concentrations in the range 50 - 1000 μ M and 25 - 500 μ M, respectively, were employed in kinetic studies with UGT2B10(L34H). Incubations with 1NP contained 0.5% DMSO. Of the enzymes investigated here, this concentration of DMSO has a significant effect only on the activity of UGT1A9 (27% decrease in control activity) (Uchaipichat et al., 2004). HEK293 cell lysate protein amount and incubation time varied for each enzyme, as reported previously (Uchaipichat et al., 2004), and reaction rates were linear with respect to both of these variables. Incubations conducted with UGT2B10 and UGT2B10(L34H) were performed for 120 min with 1 mg/ml of lysate protein. Reactions were initiated by the addition of UDPGA and, following incubation at 37°C for the specified time, terminated by the addition of 70% perchloric acid (2 μ l). Samples were centrifuged at 5000g for 10 min and a 30- μ l aliquot of the supernatant fraction was injected into the HPLC column. Chromatography conditions were as described by Udomuksorn et al. (2007). 4MU-glucuronide and 1NP-glucuronide were quantified by comparison of peak areas to those standard curves prepared from the authentic glucuronides over the concentration range 0.5 to 10 μ M.

TFP glucuronidation assay

The incubation mixture (200 μ l) contained 5 mM UDPGA, 4 mM MgCl₂, HEK293 cell lysate expressing UGT (0.25 mg/ml), and TFP in 50 mM Tris-HCl buffer (pH 7.4) (Uchaipichat et al., 2006). Screening for TFP glucuronidation was performed at a TFP

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concentration of 40 μM , which corresponds to the apparent K_m reported for UGT1A4 (Kubota et al., 2007), and at 100 μM . The kinetics of TFP glucuronidation were characterized using 9-10 substrate concentrations in the range of 5-200 μM for UGT1A4 and 10-300 μM for UGT1A1(H39P). Reactions were initiated by the addition of UDPGA, and incubations were performed at 37°C for 20 min. The reactions were terminated by the addition of 200 μl of 4% acetic acid/96% methanol and then centrifuged at 5000g for 10 min. A 40- μl aliquot of the supernatant fraction was injected into the HPLC column. Chromatography was performed according to Uchaipichat et al. (2006). TFP-glucuronide was quantified by comparison of peak areas to those of an external standard curve prepared over the concentration range 0.2 to 10 μM .

LTG glucuronidation assay

Incubations (200 μl) contained 5 mM UDPGA, 4 mM MgCl_2 , HEK293 cell lysate (1 mg/ml), and LTG in 0.1 M phosphate buffer (pH 7.4) (Rowland et al., 2006). Screening for LTG glucuronidation was performed at a LTG concentration of 1500 μM , which corresponds to the apparent K_m reported for UGT1A4 (Rowland et al., 2006), and 3000 μM . The kinetics of LTG glucuronidation were characterized using 12 substrate concentrations in the range of 25-3000 μM . Given the limited solubility of LTG, the maximal concentration possible in incubations is 3000 μM . Incubations contained 1% (v/v) acetonitrile, which has an appreciable effect only on the activity of UGT1A6 (22% reduction in control activity) (Uchaipichat et al., 2004). Following initiation of the reaction with UDPGA, incubations were performed at 37°C for 75 min. Reactions were terminated by the addition of 70% perchloric acid (2 μl), and then mixtures were centrifuged at 5000g for 10 min. A 40- μl aliquot of the supernatant fraction was injected into the HPLC column. Chromatography was performed according to Rowland et al. (2006). LTG-glucuronide was quantified by

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comparison of peak areas to those of an LTG-glucuronide standard curve prepared over the concentration range 2 to 20 μ M.

Naproxen acyl glucuronidation assay

Incubations contained 0.1 M phosphate buffer (pH 7.4), 4 mM $MgCl_2$, 5 mM UDPGA, HEK293 cell lysate expressing UGT (1 mg/ml), and naproxen in a total volume of 200 μ l (Bowalgaha et al., 2005). Activity screening studies were performed at naproxen concentrations corresponding to the known K_m or S_{50} value reported for each UGT enzyme and at 5 times the known K_m/S_{50} (Bowalgaha et al., 2005) (Table 2). Samples were incubated at 37°C for 40 min. Incubations with UGT2B7(H35P), UGT2B10 and UGT2B10(L34H) were additionally performed for 120 min with 10 mg/ml of lysate protein. Reactions terminated by the addition of an equal volume of ice-cold 4% (v/v) acetic acid in methanol. Samples were centrifuged at 5000g for 10 min and a 30- μ l aliquot of the supernatant fraction was injected into the HPLC column. Chromatography conditions and quantification of naproxen acyl glucuronide were as described by Bowalgaha et al. (2005).

Cotinine glucuronidation assay

Cotinine served as the positive control for UGT2B10 activity. The incubation mixture (200 μ l) contained 0.1 M phosphate buffer (pH 7.4), 5 mM UDPGA, 4 mM $MgCl_2$, HEK293 cell lysate expressing UGT2B10 or UGT2B10(L34H) (5 mg/ml), and cotinine (0.5, 1 or 2 mM). Incubations contained 1% (v/v) ethanol, which affects only the activity of UGT1A6 (29% reduction in control activity) (Uchaipichat et al., 2004). Reactions were initiated by the addition of UDPGA, and incubations were performed at 37°C for 3hr. The reactions were terminated by the addition of 3 μ l of 70% perchloric acid and then centrifuged at 5000g for 10 min. A 40 μ l aliquot of the supernatant fraction was injected into the HPLC column.

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Cotinine glucuronide was quantified by reversed phase HPLC using an Agilent 1100 HPLC system (Agilent Technologies, Sydney, Australia). The system consisted of an auto-sampler, gradient solvent delivery system, and UV detector (set at 254nm), fitted with a Zorbax Eclipse XBD-C8 (4.6 mm x 150 mm, 5 μ m particle size) analytical column (Agilent Technologies, Sydney, Australia) operating at 25°C. The mobile phase consisted of a 175 mM acetic acid and 4 mM 1-pentanesulfonic acid in 4% acetonitrile/water (A) and acetonitrile (B) gradient delivered at a flow rate of 1 ml/min. Initial conditions were 100 % A held for 6 min, followed by 60% A - 40% B over 0.1 min then held for 2 min, before returning to the starting conditions. Formation of cotinine glucuronide was confirmed by co-chromatography with the authentic compound. Unknown concentrations of cotinine glucuronide in incubations were determined by comparison of the peak areas with those of a cotinine β -D-glucuronide standard curve.

Data analysis

Data points represent the mean of duplicate measurements (<10% variance). Kinetic constants for 4MU, 1NP, TFP and LTG glucuronidation were obtained by fitting experimental data to the Michaelis-Menten or substrate inhibition equations using EnzFitter (Biosoft, Cambridge, UK).

Michaelis-Menten equation:

$$v = \frac{V_{\max} \times [S]}{K_m + [S]}$$

where v is the rate of reaction, V_{\max} is the maximum velocity, K_m is the Michaelis constant (substrate concentration at 0.5 V_{\max}), and $[S]$ is the substrate concentration.

Substrate inhibition:

$$v = \frac{V_{\max}}{1 + (K_m/[S]) + ([S]/K_{si})}$$

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where K_{si} is the constant describing the substrate inhibition interaction.

Goodness of fit to kinetic models was assessed by comparison of the F -statistic, coefficient of determination (r^2), parameter standard errors, and 95% confidence intervals. Kinetic constants are reported as the value \pm standard error of the parameter estimate.

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Results

His to Pro substitution in UGT1A proteins and UGT2B7

UGT enzymes and mutants were expressed in HEK293 cells. The expression of UGT1A1, UGT1A6, and UGT1A9 and their respective His→Pro mutants relative to UGT1A4 was confirmed by immunoblotting with an antibody specific for human UGT1A subfamily enzymes. The His to Pro substitutions did not appreciably affect expression (Figure 2A). The relative expression levels of UGT1A1, UGT1A1(H39P), UGT1A6, UGT1A6(H38P), UGT1A9, and UGT1A9(H37P) were 1:1.4:1.1:1.5:1.4:1.1. The expression of UGT1A4 relative to UGT1A1 was 1:1.34.

4MU, 1NP and naproxen activity screening studies were performed at concentrations corresponding to the K_m (or S_{50}) and five times the K_m/S_{50} for each of the parent enzymes (Bowalgaha et al., 2005; Uchaipichat et al., 2004). Screening studies with LTG were conducted at 1500 μM (the known K_m for UGT1A4) and 3000 μM , the maximum concentration achievable in incubations (Rowland et al., 2006). Similarly, screening experiments with TFP were performed at 40 μM (the known K_m) and 100 μM , the maximum concentration that avoids decreased activity due to the effects of substrate inhibition (Uchaipichat et al., 2006). UGT1A1, UGT1A6 and UGT1A9 glucuronidated 4MU, 1NP and naproxen, but not LTG and TFP (Table 2). Conversely, UGT1A4 glucuronidated LTG and TFP, but not 4MU, 1NP or naproxen. Whereas the His to Pro substitution in UGT1A1, UGT1A6 and UGT1A9 abolished activity towards the phenolic compounds 4MU and 1NP and the carboxylic acid containing substrate naproxen, the mutation conferred LTG glucuronidation activity to all three enzymes (Table 2). Of the three mutants, only UGT1A1(H39P) metabolized TFP. The low activity of UGT1A6(H38P) precluded full

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kinetic analysis of LTG glucuronidation. However, like UGT1A4, LTG glucuronidation by UGT1A1(H39P) and UGT1A9(H37P) followed Michaelis Menten kinetics (Figure 4). K_m values for UGT1A1(H39P) and UGT1A9(H37P) were about half and double those of UGT1A4, respectively (Table 3). V_{max}/K_m values, with V_{max} corrected for expression relative to UGT1A4, were 0.168, 0.039 and 0.211 $\mu\text{l}/\text{min. mg}$ for UGT1A4, UGT1A1(H39P) and UGT1A9(H37P), respectively. As reported previously for UGT1A4 (Uchaipichat et al., 2006), the kinetics of TFP glucuronidation by UGT1A1(H39P) were best fit to the substrate inhibition equation although deviation from this model was apparent at low substrate concentrations (Figure 3). The K_m was higher and the K_{si} and V_{max} values lower for UGT1A1(H39P) compared to the corresponding parameters for UGT1A4 (Table 3). V_{max}/K_m (corrected for expression relative to UGT1A4) values for TFP glucuronidation by UGT1A4 and UGT1A1(H39P) were 25.2 and 3.5 $\mu\text{l}/\text{min. mg}$, respectively.

Expression of UGT2B7(H35P), determined using an antibody raised against a peptide fragment of UGT2B7, was relatively low (1:0.42; Figure 2B). Although UGT2B7 glucuronidated 4MU, INP and naproxen at substrate concentrations corresponding to the known K_m/S_{50} and 5 times these values for each reaction (data not shown), UGT2B7(H35P) lacked activity with all substrates investigated here even when the HEK293 cell lysate protein content of incubations was increased 10-fold. It should be noted that the lower limits of quantification for the HPLC assays employed in this work translate to rates of glucuronide formation of 0.25 – 0.8 $\text{pmol}/\text{min. mg}$.

Leu to His substitution in UGT2B10

In the absence of a commercially available antibody for UGT2B10, expression of this enzyme and the L34H mutant was determined using the anti-UGT2B7 antibody which cross-

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reacts, albeit weakly, with UGT2B10. The expression level of UGT2B10 relative to UGT2B10(L34H) was 1:2.1 (Figure 2C). UGT2B10 activity was confirmed with cotinine as the substrate; rates of cotinine glucuronide formation were 1.2, 1.8 and 1.9 pmol/min. mg protein at substrate concentrations of 0.5, 1 and 2mM, respectively. UGT2B10 lacked activity towards 4MU, 1NP, naproxen, LTG and TFP. Lack of glucuronidation activity persisted when the content of UGT2B10 expressing HEK293 cell lysate present in incubations was increased to 20 mg/ml (to overcome the lower apparent expression compared to UGT2B10(L34H)). However, UGT2B10(L34H) glucuronidated both 4MU and 1NP (Figure 5). 4MU glucuronidation by UGT2B10(L34H) followed Michaelis Menten kinetics (K_m $260 \pm 6 \mu\text{M}$, V_{max} $17 \pm 0.2 \text{ pmol/min. mg}$), while 1NP glucuronidation by UGT2B10(L34H) exhibited substrate inhibition (K_m $118 \pm 25 \mu\text{M}$, K_{si} $135 \pm 19 \mu\text{M}$, V_{max} $14 \pm 1.4 \text{ pmol/min. mg}$). UGT2B10(L34H) did not metabolize naproxen, LTG, TFP or cotinine.

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Discussion

With the exception of UGT1A4 and UGT2B10, the N-terminal domain histidine corresponding to position 39 in UGT1A1 is conserved in UGT1A and 2B subfamily proteins (Figure 1). Proline and leucine occur at the corresponding position of UGT1A4 and UGT2B10. Recent work in this laboratory demonstrated that Pro-40 of UGT1A4 is essential for the glucuronidation of the tertiary amines LTG and TFP (Kubota et al., 2007). Furthermore, the His40Pro substitution in UGT1A3 conferred LTG and TFP glucuronidation activity, with concurrent near abolition of planar phenol glucuronidation. Results presented here further demonstrate the importance of the N-terminal domain proline and histidine in tertiary amine and planar phenol glucuronidation, respectively.

UGT1A1(H39P), UGT1A6(H38P) and UGT1A9(H37P) all glucuronidated LTG. Indeed, V_{\max}/K_m values for LTG glucuronidation by UGT1A4 and UGT1A9(H37P) were comparable. However, catalytic efficiencies were lower for UGT1A1(H39P) and UGT1A6(H38P). In contrast, only UGT1A1(H39P) metabolized TFP. Although an x-ray crystal structure for an entire UGT protein is currently not available, a homology model of UGT1A1 based on a plant glycosyltransferase places the conserved histidine in an α -helix (Locuson and Tracy, 2007). The structural rigidity of proline is known to disrupt α -helices. Hence, secondary and tertiary structure in the N-terminal domain is likely to differ between UGT1A proteins with the conserved histidine and UGT1A4 (and the His \rightarrow Pro mutants generated here). In turn, this may alter the architecture of the active site such that tertiary amine substrates may bind in a catalytically favorable orientation. However, differences between the His \rightarrow Pro mutants in the ability to glucuronidate TFP indicate that other regions of the UGT1A active site clearly influence the binding and/or turnover of tertiary amine substrates. Similarly, the ability of

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UGT2B10 to glucuronidate the tertiary amines cotinine and nicotine is not dependent on an equivalent N-terminal domain proline.

The glucuronidation reaction proceeds according to a second order nucleophilic substitution mechanism (Radomiska-Pandya et al., 1999). Proton abstraction is considered an obligatory step in the glucuronidation of all aliphatic alcohols, phenols, primary and secondary amines, acidic carbon atoms, and thiols, and the near conserved N-terminal domain histidine has been implicated as the most important catalytic base in this regard (see later discussion). However, the conversion of tertiary amines to quaternary ammonium-linked glucuronides does not involve proton abstraction. Based on the effects of site-directed mutation studies with UGT1A9 it has been suggested that Asp-143 in this protein may stabilize the transition state during the glucuronidation of the primary and secondary amine functional groups of 4-aminobiphenyl and retigabine (Patana et al., 2008). A similar mechanism may conceivably apply to the glucuronidation of tertiary amines.

The His35Pro substitution in UGT2B7 abolished 4MU, 1NP and naproxen glucuronidation. Moreover, UGT2B7(H35P) lacked activity towards the tertiary amines investigated here as substrates. Miley et al. (2007) reported recently that the His→Ala substitution at position 35 of UGT2B7 abolished activity towards androsterone, hyodeoxycholic acid and tetrachlorocatechol. This observation and data presented here are consistent with a pivotal role for His-35 in UGT2B7 activity. It should be noted, however, that the UGT2B7 enzyme appears to be 'sensitive' to structural change and most mutant and chimeric proteins generated from UGT2B7 exhibit low or absent activity (Lewis et al., 2007; Miley et al., 2007).

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Apart from UGT1A4, UGT2B10 is the only other 1A and 2B subfamily protein that lacks the conserved N-terminal domain histidine (Figure 1). Importantly, UGT2B10 lacks activity towards the xenobiotic alcohols (including 4MU and 1NP) and carboxylic acids and the hydroxy-steroids that typically show overlapping substrate selectivities between UGT enzymes (Jin et al., 1993), although very low activity has been reported with hydroxyeicosatetraenoic acids (Turgeon et al., 2003). More recently, UGT2B10 has been shown to glucuronidate certain tertiary amines, including cotinine, nicotine and tobacco-specific nitrosamines, but activities are relatively low (Chen et al., 2007 and 2008; Kaivosari et al., 2007). The UGT2B10(L34H) mutant was found here to glucuronidate 4MU and 1NP, but not cotinine. These observations suggest that Leu-34 is critical for the glucuronidation of cotinine (and possibly nicotine and tobacco-specific nitrosamines) by UGT2B10. While outside the scope of the present work, further studies are warranted to elucidate the role of Leu-34 and other N-terminal domain amino acids on UGT2B10 activity and substrate selectivity.

It has been proposed that the conserved N-terminal histidine acts as the catalytic base in the glucuronidation of planar phenols and other substrates where the reaction mechanism requires proton abstraction (Locuson and Tracy, 2007; Miley et al., 2007; Patana et al., 2008; Radomska-Pandya et al., 1999) and data presented here are generally consistent with this proposition. However, exceptions occur. Work in this laboratory found that the Pro40His mutation in UGT1A4 did not confer 4MU and 1NP glucuronidation (Kubota et al., 2007). Rather, substitution of Thr-36 of UGT1A4 with isoleucine (the corresponding amino acid in UGT1A3) resulted in significant 4MU and 1NP glucuronidation (V_{max} values approximately 100 pmol/min. mg). Although Li et al. (2007) reported that the His-40 mutant of UGT1A4 glucuronidated 4MU and 1NP, activities were low (< 1 pmol/min. mg) and almost certainly

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catalytically irrelevant when compared to the V_{\max} values for 4MU and 1NP glucuronidation by the individual wild-type human UGT enzymes and by UGT1A4(T36I). Interestingly, however, Li et al. (2007) further reported that the His38Arg mutation in UGT1A6 subtly altered substrate selectivity. Collectively, these observations suggest that the conserved N-terminal domain histidine may influence UGT substrate selection and a residue(s) elsewhere in the active site can function as the catalytic base.

The His→Pro substitution in UGT 1A1, 1A6, 1A9 and 2B7 abolished naproxen acyl glucuronidation. However, the UGT2B10(L34H) also lacked naproxen glucuronidation activity despite its capacity to metabolize planar phenols. Naproxen and other related carboxylic acid containing non-steroidal anti-inflammatory drugs that undergo extensive glucuronidation typically have pK_a values in the range 4 to 5. Thus, the carboxylate group is almost completely ionized at pH 7.4. The involvement of a catalytic base would clearly be dependent on whether glucuronidation involved conjugation of the carboxylate ion or the small fraction of the drug present in the neutral state.

It is known that Pro-40 is critical for the UGT1A4-catalyzed conversion of the tertiary amines LTG and TFP to their respective quaternary ammonium-linked glucuronides. This study shows for the first time that substitution of histidine for proline at positions 39, 38 and 37 of UGT1A1, UGT1A6 and UGT1A9, respectively, confers LTG glucuronidation activity to these enzymes. UGT1A1(H39P) additionally glucuronidated TFP. The data suggest that the His→Pro substitution induces a conformational change that favors the N-glucuronidation of these tertiary amines, at least in the UGT1A subfamily. It was also demonstrated for the first time that the inability of UGT2B10 to glucuronidate planar aromatic phenols such as 4MU and 1NP is due to the lack of the near conserved histidine in the N-terminal domain of this

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enzyme. The latter observation and the loss of 4MU and 1NP glucuronidation activity associated with the UGT1A and UGT2B7 His→Pro mutations are consistent with the hypothesis that the N-terminal domain histidine generally, but not invariably (Kubota et al., 2007; Li et al., 2007), acts as the catalytic base in the metabolism of compounds requiring proton abstraction for glucuronide formation. Taken together with previous reports (Barre et al., 2007; Dubois et al., 1999; Kubota et al., 2007; Li et al., 2007; Martineau et al., 2004; Senay et al., 2002; Xiong et al., 2006), the present study further demonstrates the pivotal roles of individual amino acids in determining the substrate selectivities of human UGT enzymes.

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Footnote

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Figure Legends

Figure 1. N-Terminal sequence identity of UGT1A and UGT2B proteins. The position of the conserved histidine residue in all proteins except UGT1A4 and UGT2B10 is indicated by the arrow.

Figure 2. Immunoblots of lysates of HEK293 cells stably expressing: (A) UGT1A enzymes and mutants; (B) UGT2B7 and UGT2B7(H35P); and (C) UGT2B10 and UGT2B10(L34H). HEK293 protein amounts were 15, 30 and 50 μ g of HEK293 for UGT1A proteins, UGT2B7 and UGT2B10, respectively. Untransfected HEK293 cells served as the negative control.

Figure 3. Eadie-Hofstee plots for trifluoperazine glucuronidation by: (A) UGT1A4 and (B) UGT1A1(H39P). Points are experimentally determined values (means of duplicate measurements at each concentration), while the solid lines are the computer-generated curves of best fit.

Figure 4: Eadie-Hofstee plots for lamotrigine glucuronidation by: (A) UGT1A4; (B) UGT1A1(H39P); and (C) UGT1A9(H37P). Points are experimentally determined values (means of duplicate measurements at each concentration), while the solid lines are the computer-generated curves of best fit.

Figure 5. Eadie-Hofstee plots for 4-methylumbelliferone (panel A) and 1-naphthol (panel B) glucuronidation by UGT2B10(L34H). Points are experimentally determined values (means of duplicate measurements at each concentration), while the solid lines are the computer-generated curves of best fit.

Table 1. Primers used for site-directed mutagenesis^a

Mutant	Template	Forward primer
1A1(H39P)	UGT1A1	5'-CCCAGTGGATGGCAGCC <u>C</u> CTGGCTGAGCATGCTTG-3'
1A6(H38P)	UGT1A6	5'-CCCTCAGGACGGAAGCC <u>C</u> CTGGCTTAGTATGAAGG-3'
1A9(H37P)	UGT1A9	5'-CCCATGGATGGGAGCC <u>C</u> CTGGTTCACCATGAGG-3'
2B7(H35P)	UHT2B7	5'-GGGCAGCAGAATACAGCC <u>C</u> TTGGATGAATATAAAGAC-3'
2B10(L34H)	UGT2B10	5'-GGGCCGCAGAATACAGCC <u>A</u> TTGGATGAATATGAAGAC-3'

^a Mutated bases are bolded and underlined. Reverse primers were complementary to the forward primers.

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Table 2. Rates of 4MU, 1NP, naproxen, TFP, and LTG glucuronidation activities by UGT1A1, UGT1A1(H39P), UGT1A6, UGT1A6(H38P), UGT1A9, UGT1A9(H37P), and UGT1A4.

UGT enzyme or mutant	Rate of glucuronide formation (pmol/min .mg)									
	4MU		1NP		Naproxen ^a		TFP		LTG	
	Low ^b	High ^b	Low ^b	High ^b	Low ^b	High ^b	40μM	100μM	1500μM	3000μM
1A1	89	178	89	167	3	8	NA	NA	NA	NA
1A1(H39P)	11	24	NA	13	NA	NA	89	121	21	30
1A6	31360	53710	4820	7030	75	95	NA	NA	NA	NA
1A6(H38P)	NA	NA	NA	NA	NA	NA	NA	NA	5	6
1A9	7970	16240	48	98	42	106	NA	NA	NA	NA
1A9(H37P)	NA	NA	NA	190	NA	NA	NA	NA	162	298
1A4	NA	NA	NA	NA	NA	NA	521	733	125	202

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Data are means of duplicate estimates (<10% variance)

NA, No activity detected.

^a Acyl glucuronidation pathway

^b 'Low' concentration corresponds to approximate K_m or S_{50} values: 4MU - 100 μ M for UGT1A1 and UGT1A6, and 10 μ M for UGT1A9; 1NP - 300 μ M for UGT1A1, 3 μ M for UGT1A6, and 1 μ M for UGT1A9; Naproxen - 1000 μ M for UGT1A1 and UGT1A9, and 800 μ M for UGT1A6. 'High' concentration is 5 times the low concentration.

Table 3. Kinetic parameters for TFP and LTG glucuronidation by UGT1A1(H39P), UGT1A6(H38P), UGT1A9(H37P), and UGT1A4.

UGT enzyme	TFP glucuronidation ^{a, b}			LTG glucuronidation ^{a, c}	
	K _m (μ M)	K _{si} (μ M)	V _{max} (pmol/min. mg)	K _m (μ M)	V _{max} (pmol/min. mg)
1A1(H39P)	305 \pm 1	87 \pm 1	827 \pm 2	774 \pm 25	32 \pm 1
1A6(H38P)	NA	NA	NA	ND	ND
1A9(H37P)	NA	NA	NA	3812 \pm 239	628 \pm 29
1A4	44 \pm 1	369 \pm 23	1164 \pm 24	1579 \pm 19	266 \pm 2

NA, No activity; ND, not determined.

^a Data presented as the parameter \pm standard error of parameter fit. Kinetic parameters are not corrected for relative expression or non-specific binding.

^b Fit to substrate inhibition equation.

^c Fit to Michaelis-Menten equation.

Figure 1

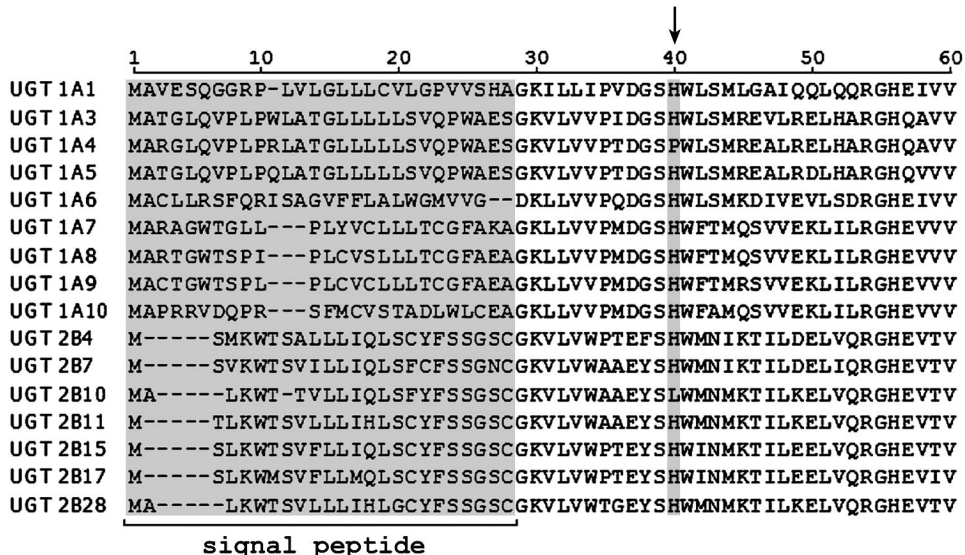


Figure 2

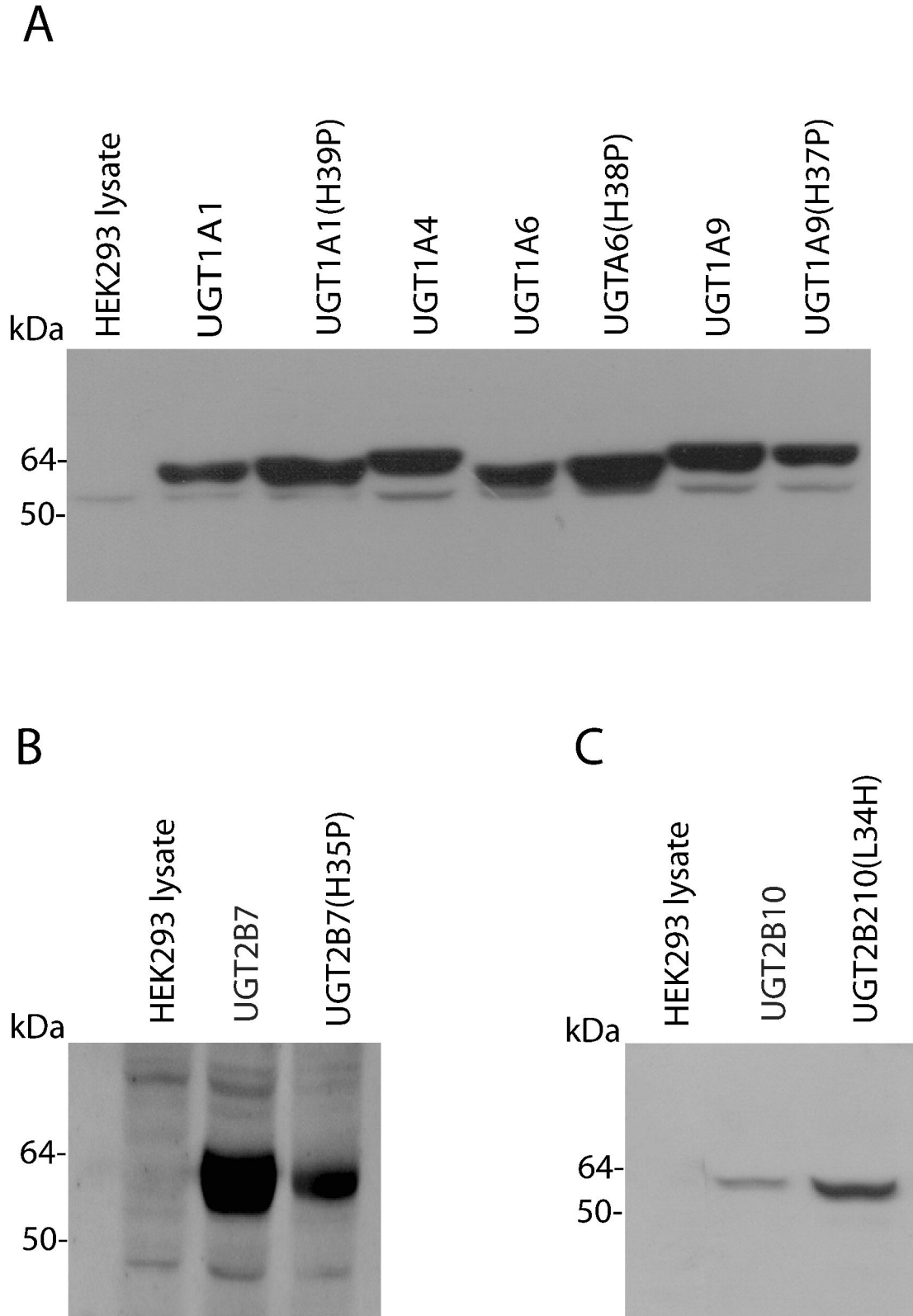


Figure 3

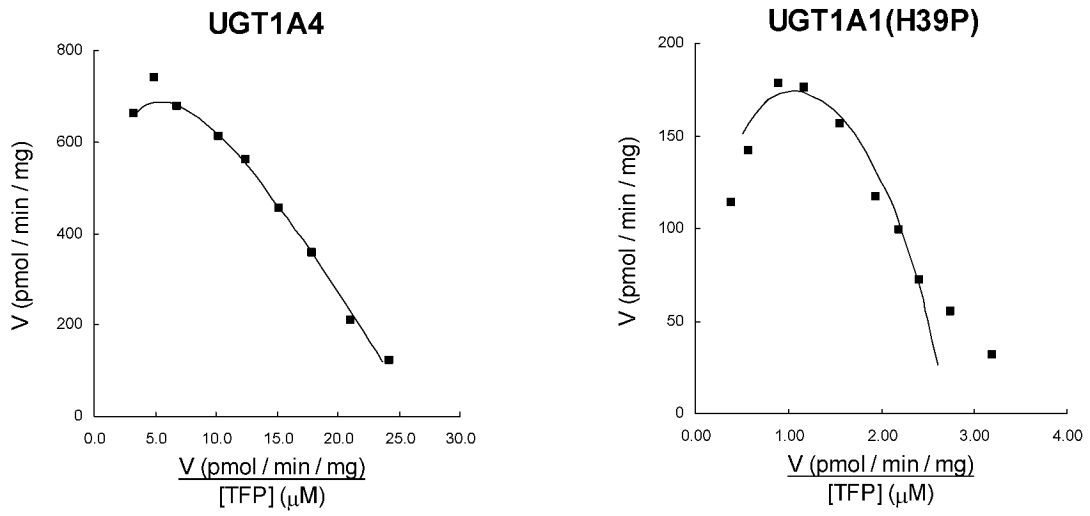


Figure 4

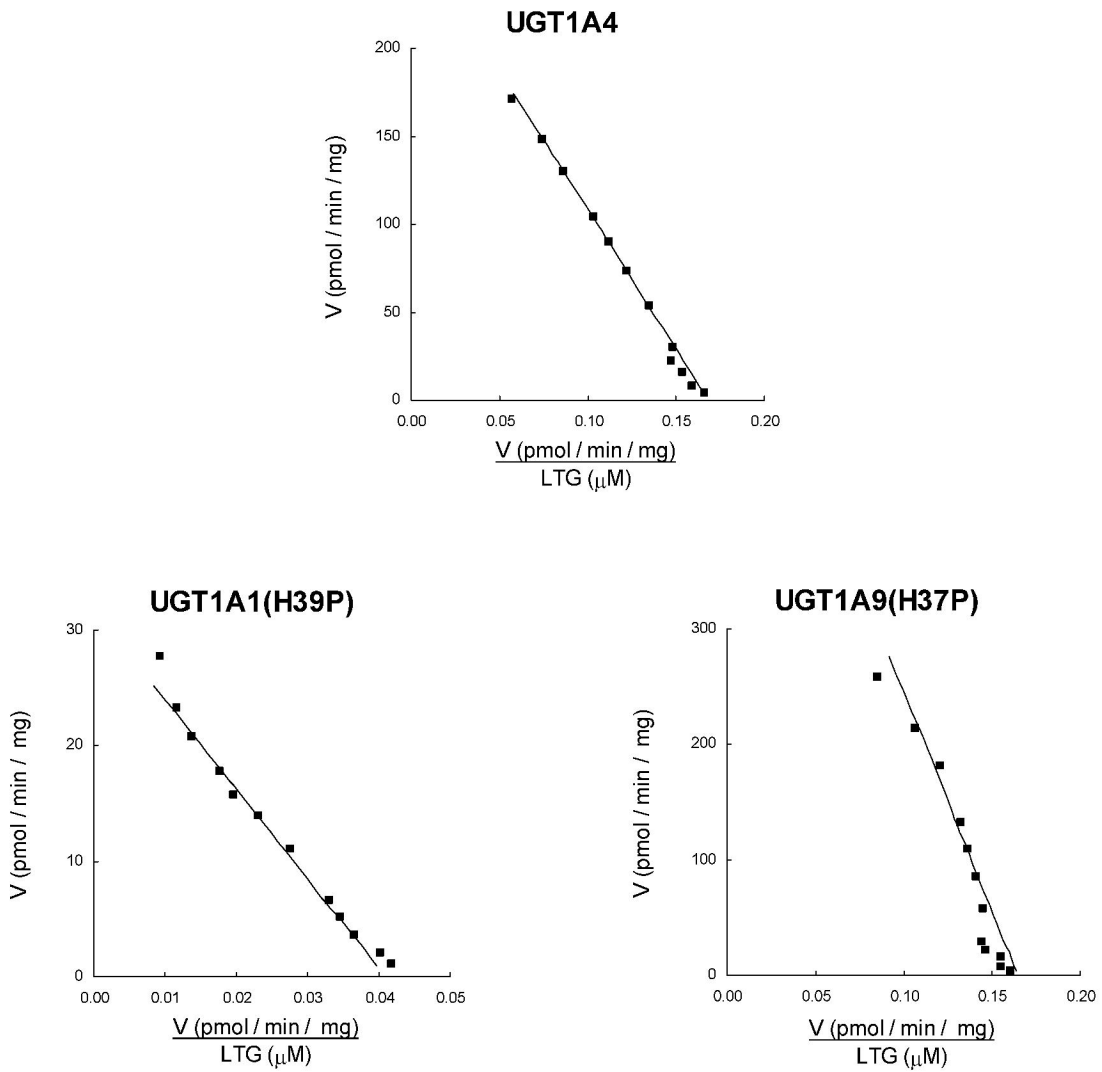


Figure 5

