Role of Human UGT2B10 in N-Glucuronidation of Tricyclic Antidepressants, Amitriptyline, Imipramine, Clomipramine and Trimipramine

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Running title

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List of non-standard abbreviations: TCA, tricyclic antidepressants; UGT, uridine diphosphate glucuronosyltransferase; UDPGA, uridine 5’-diphosphoglucuronic acid.
ABSTRACT:

The role of human UGT2B10 in the N-glucuronidation of a number of tricyclic antidepressants was investigated and compared to that of UGT1A4 in both Sf9 expressed system and human liver microsomes. The apparent $K_m (S_{50})$ values for the formation of quaternary N-glucuronides of amitriptyline, imipramine, clomipramine and trimipramine were 2.60, 16.8, 14.4, and 11.2 µM in UGT2B10 and 448, 262, 112, and 258 µM in UGT1A4, respectively. The kinetics of amitriptyline and imipramine glucuronidation in human liver microsomes exhibited biphasic character, where the high and low affinity components were in good agreement with our results in expressed UGT2B10 and UGT1A4, respectively. The kinetics of clomipramine and trimipramine glucuronidation in human liver microsomes were sigmoidal in nature and the $S_{50}$ values were similar to those found for expressed UGT1A4. The $in vitro$ clearances ($CL_{int}$ or $CL_{max}$) were comparable between UGT2B10 and UGT1A4 for glucuronidation of imipramine, clomipramine and trimipramine whereas $CL_{int}$ of amitriptyline glucuronidation by UGT2B10 was more than 10-fold higher than UGT1A4. Nicotine was found to selectively inhibit UGT2B10 but not UGT1A4 activity. At low tricyclic antidepressants concentration, nicotine inhibited their glucuronidations by 33~50% in human liver microsomes. Our results suggest that human UGT2B10 is a high affinity enzyme for tricyclic antidepressants glucuronidation and is likely to be a major UGT isoform responsible for the glucuronidation of these drugs at therapeutic concentrations $in vivo$. 
INTRODUCTION:

Glucuronidation, catalyzed by the uridine diphosphate glucuronosyltransferases (UGTs), is an important metabolic pathway for the detoxification and elimination of many endobiotics (bilirubin, bile acids) and xenobiotics (zidovudine, acetaminophen) (Miners et al., 2006). UGT enzymes transfer the glucuronic acid moiety from a cofactor uridine 5’-diphosphoglucuronic acid (UDPGA) to hydroxyl, carboxyl or amine groups of aglycone substrates (Tukey and Strassburg, 2004). The resulting β-glucuronide metabolites are usually water-soluble, less active or toxic and can be readily excreted from the body via bile and urine. At least 17 human UGT isoforms have been identified to date and can be categorized into two subfamilies, UGT1 and UGT2, based on similarities of their amino acid sequences and gene organization (Mackenzie et al., 2005). UGT enzymes are localized primarily in the endoplasmic reticulum and expressed mainly in the liver, though UGT1A7, UGT1A8, UGT1A10 and UGT2A1 are expressed only in extrahepatic tissues (Miners et al., 2006). UGT enzymes have generally been shown to display very broad and overlapping substrate selectivity (Kiang et al., 2005). Only a limited number of isoform-selective UGT substrates and inhibitors have been identified to date, while no isoform-selective UGT inhibitory antibody is available (Miners et al., 2006).

UGT-catalyzed glucuronidations are responsible for about one-third of all drugs metabolized by Phase II enzymes (Evans and Relling, 1999). Aliphatic tertiary amine or aromatic amine functional groups are quite common in drugs, such as in tricyclic antidepressants (TCAs) and anticonvulsants. UGT conjugations of these drugs can result in the formation of quaternary ammonium glucuronide metabolites. The quaternary ammonium glucuronides of a number of TCAs, including amitriptyline, imipramine, clomipramine and trimipramine (Fig. 1) have been identified as major phase II metabolites in human urine (Luo et al., 1995). The urinary...
excretion of amitriptyline N-glucuronide metabolite was 2.5 - 21% of the oral dose administered (Breyer-Pfaff et al., 1997). Human UGT1A3 and UGT1A4 were shown to contribute to the N-glucuronidation of imipramine and amitriptyline (Green et al., 1995, 1998). However, the contribution of UGT1A3 to drug conjugation may be insignificant to overall metabolism as it exhibited very high apparent K_m values compared with UGT1A4 (Green and Tephly, 1998; Kubota et al., 2007). Eadie-Hofstee plots of imipramine and amitriptyline N-glucuronide metabolites indicated that more than one enzyme or active site participated in the N-glucuronidation in human liver microsomes (HLM). UGT1A4 was suggested as the low affinity enzyme for conjugations of both drugs while the high affinity enzyme was not identified in these studies (Breyer-Pfaff et al., 1997, 2000; Nakajima et al., 2002).

It was well accepted that only UGT1A3 and UGT1A4 could catalyze quaternary N-glucuronidation reactions until recent reports demonstrated that UGT2B10 catalyzed direct quaternary N-conjugations at the aromatic nitrogen of nicotine, cotinine and nitrosamines with higher affinities than UGT1A4 (Kaivosaaari et al., 2007; Chen et al., 2008). UGT2B10 has been one of the least characterized UGT isoforms because early research indicated no activity towards a large variety of compounds (Jin et al., 1993). However, newly identified UGT2B10 substrates suggest that this enzyme could be more relevant than previously thought, especially in N-glucuronidation reactions. In the present study, we expressed human UGT2B10 enzyme in Sf9 insect cells and demonstrated that UGT2B10 catalyzes the quaternary N-glucuronidation of TCAs with higher affinity than UGT1A4. UGT2B10 is likely to be a major contributor to the glucuronidation of amitriptyline, imipramine, clomipramine and trimipramine at therapeutic concentrations in vivo.
Materials and Methods

Materials. Human liver QUICK-Clone™ cDNA was purchased from Clontech (Mountain View, CA). PfuUltra™ High-Fidelity DNA Polymerase was obtained from Agilent Technologies (La Jolla, CA). To enhance specificity, amplifications included AmpliWax® PCR Gems obtained from Applied Biosystems (ABI) (Foster City, CA); thermal cycling was performed on an ABI GeneAmp PCR System 9700. Zero Blunt® PCR Cloning Kit, Calf Intestine Alkaline Phosphatase, One-shot TOP10 chemically competent E. coli, Bac-to-Bac® Baculovirus Expression System, Cellfectin® II Reagent, Sf-900 II SFM (serum-free medium) insect cell media and Sf9 insect cells (Spodoptera frugiperda) were obtained from Invitrogen (Carlsbad, CA). DNA sequences were determined by using BigDye® Terminator v3.1 Cycle Sequencing Kits (ABI, Foster City, CA). Viability of Sf9 cells was determined by using a Vi-CELL™ Series Cell Viability Analyzer (Beckman-Coulter). BaculoELISA titer kit was purchased from Clontech (Mountain View, CA). Pooled HLM, recombinant UGT1A3 and UGT1A4 expressed in Sf9 insect cells were purchased from BD Gentest (Woburn, MA).

NuPAGE® 4-12% Bis-Tris pre-cast gels, MOPS SDS running buffer, Antioxidant, LDS Sample Buffer, Sample Reducing Agent, Transfer Buffer, PVDF membranes and SeeBlue® Plus2 pre-stained molecular weight standard were obtained from Invitrogen (Carlsbad, CA). UGT2B goat polyclonal antibody (sc-23479), donkey anti-goat IgG-HRP (sc-2033); Blotto, non-fat dry milk; TBST and TBS wash solutions, and Luminol Reagent were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Amersham Hyperfilm ECL was from GE Healthcare (Piscataway, NJ).
Amitriptyline, imipramine, clomipramine, trimipramine, nicotine, UDPGA were obtained from Sigma-Aldrich (St. Louis, MO). Hecogenin was purchased from Voigt Global Distribution Inc. (Lawrence, KS).

**Expression of UGT2B10 in Sf9 insect cells.** Full-length cDNA for human UGT2B10 was PCR-amplified from human liver QUICK-Clone™ cDNA with *PfuUltra™* High-Fidelity DNA Polymerase and AmpliWax PCR Gems following the manufacturers’ protocols. Forward and reverse primers for amplification of UGT2B10 cDNA were 5'-ATGGCTCTGAAATGGACTACAGTT and 5’-CCAGCTTCAAATCTCAGATATAAC, respectively. The PCR product was ligated into pCR-Blunt and sub-cloned into restriction enzyme digested and dephosphorylated pFastBac1. Clones and subclones were propagated in TOP10 *E. coli* cells. The cDNA sequence was confirmed by sequencing and GenBank accession number for UGT2B10 cDNA sequence is NM_001075.4.

The Invitrogen Bac-to-Bac Baculovirus Expression System was used to generate recombinant baculovirus according to the manufacture’s protocol. Briefly, pFastBac1 plasmid containing UGT2B10 was transfected into *E. coli* DH10Bac competent cells harboring Bacmid vector DNA and a helper plasmid designed to transpose the pFastBac1 insert into Bacmid, in situ. Recombinant Bacmid DNA was propagated, isolated and then used to transfect Sf9 insect cells via Cellfectin® II -mediated gene transfer to generate recombinant baculovirus. Recombinant baculovirus-containing culture supernatants were harvested (passage 1 viral stock), amplified and titered by using BaculoELISA kits.

High-titer passage 3 stock was used for UGT2B10 protein expression. Sf9 insect cell liquid cultures were grown in Sf-900 II SFM medium at 27°C with orbital shaking at 120 rpm. Sf9 cells were infected at ~ 1-1.5 x 10⁶ viable cells/mL. Cells were infected at a multiplicity of
infection (MOI) of 1.0 and harvested by centrifugation at 48 hrs post-infection. Microsomes were prepared by homogenization and 2-speed centrifugation (10,000 and 105,000g) and were reconstituted in phosphate buffered saline (pH 7.4). Microsomal protein concentrations were measured by using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Microsomes were stored at -70°C until use.

**Western Blotting.** UGT2B10 and uninfected control Sf9 cell microsomal fractions (20 µg each) were adjusted to contain 1x NuPAGE® LDS Sample Buffer and 1x NuPAGE® Sample Reducing Agent and then heated at 70 °C for 10 min. Samples were resolved on a NuPAGE Novex Bis-Tris 4-12% gel in NuPAGE MOPS SDS running buffer. SeeBlue Plus2 pre-stained standards were used as molecular weight marker. Cathode gel running buffer and electroblot transfer buffer contained NuPAGE Antioxidant. PVDF membranes were then probed with Santa Cruz Biotechnology’s goat polyclonal UGT2B antibody (1:200 dilution), followed by horseradish peroxidase-conjugated donkey anti-goat IgG (1:3500 dilution). UGT2B10 protein was visualized using the Luminol chemiluminescent reagent and Amersham Hyperfilm ECL.

**Activity Assays.** The enzyme reactions were conducted under linear conditions with respect to incubation time (up to 90 min) and protein concentration (up to 0.25 mg/mL). In all cases, 200 µL (or 400 µL for low substrate concentration incubations) of incubation mixtures containing 0.25 mg/mL microsomal protein, 50 µg/mg alamethicin, 50 mM pH 7.4 potassium phosphate buffer, and 5 mM MgCl₂ were kept on ice for 15 min before incubation to activate UGT enzymes. After 3 min pre-incubation, reactions were initiated with the addition of UDPGA (2 mM). Reactions were allowed to proceed for 90 min at 37°C, and terminated by addition of three volumes of acetonitrile/methanol (1:1). After centrifugation, the supernatant was transferred to 96-well plate. The samples were evaporated to dryness, reconstituted and the
glucuronide metabolites were quantified. Amitriptyline, imipramine, clomipramine and trimipramine were dissolved in methanol and the final incubation contained 1% methanol (v/v). Control incubations contained the same concentration of organic solvent.

To assess the contribution of each isoform to the formation of N-glucuronides, TCA compounds at 5 and 50 µM were first incubated with recombinant human UGT1A3, UGT1A4 or UGT2B10 in triplicates. The assays to study enzyme reaction kinetics were then performed in triplicates at 9 concentrations of TCA (0.15~100 µM) in recombinant UGT2B10 and 13 concentrations (0.15~500 µM) in recombinant UGT1A4 and pooled HLM.

**Selective Inhibition.** The selective inhibitory effects of hecogenin and nicotine on TCA glucuronidation were evaluated in recombinant UGT1A4 and UGT2B10. The incubation condition was the same as described above. Amitriptyline, imipramine, clomipramine and trimipramine were incubated at 5 µM in duplicate with UGT1A4 or UGT2B10 in the presence of 10 or 100 µM of hecogenin, or 10, 100 or 500 µM of nicotine. The formation of glucuronide metabolite in the presence of nicotine or hecogenin was compared to the formation in their absence (vehicle control).

The contribution of UGT1A4 and UGT2B10 on glucuronidation of TCA was further assessed in pooled HLM. Amitriptyline, imipramine, clomipramine and trimipramine were incubated at 5 and 200 µM with HLM in triplicate in the presence of 100 µM hecogenin, or 500 µM nicotine or both. The formation of glucuronide metabolite in the presence of nicotine or hecogenin was compared to the formation in their absence (vehicle control).

**Quantification of Quaternary N-glucuronide metabolites.** TCA glucuronides were determined with the method described for the quantification of trifluoperazine N-glucuronide (Uchaipichat et al., 2006) with a few modifications, as the UV absorption characteristics of
aliphatic N-glucuronide metabolite resemble the aglycone substrate (Hawes, 1998). The N-glucuronides were generated in HLM incubations at 100 μM of each TCA compound. After evaporation to dryness and reconstitution in 20% methanol, N-glucuronides were quantified using HPLC-UV spectroscopy. The UV spectra were recorded from 256 to 264 nm (encompassing the UV maxima), and calibration curves were prepared using corresponding TCA over a concentration range of 0.5 to 20 μM in incubation matrix. The quantified N-glucuronides were then diluted in matrix containing 200 nM hydroxytriazolam (internal standard) sequentially to serve as calibration standards in HPLC-MS analysis. The calibration curve of each metabolite acquired from accurate mass extracted ion chromatograms were used for quantification of N-glucuronides in sample incubations. The lower limits of quantification for the glucuronides of amitriptyline, imipramine, clomipramine, and trimipramine in prepared incubation mixtures were 0.016, 0.013, 0.027, and 0.024 μM, respectively.

Chromatographic separations of glucuronide metabolites from corresponding aglycones were performed on a Phenomenex (Torrance, CA) Luna 100 x 2.0 mm C18 (2) column (particle size 5 μm) connected to a Waters Acquity UPLC system equipped with photodiode array detector (Milford, MA). The components in the incubation were separated under a 10 min linear gradient condition at a flow rate of 0.3 mL/min using mobile phases of ammonium formate in water (20 mM, pH 5.0) (A) and acetonitrile (B). Starting conditions consisted of 20% B, and maintained for 0.5 min. The gradient was increased to 50% B over 5 min and a subsequent 1 min wash at 60% B. The mobile phase was returned to initial conditions to equilibrate for a further 4 min. Mass spectrometric analysis were carried out on a Thermo LTQ-Orbitrap mass spectrometer (Waltham, MA) using electrospray ionization (ESI) in positive mode, at sheath and auxiliary gas settings of 65 and 20, respectively, and a capillary temperature of 300°C. Full scan
accurate mass analyses were performed and the resolution of Orbitrap was set at 15000 for two scan events at the mass ranges of \( m/z \) 358-361 and 450-495. Accurate mass extracted ion chromatograms were obtained by measuring hydroxytriazolam (internal standard), and the N-glucuronides of amitriptyline, clomipramine, imipramine, and trimipramine at \( m/z \) 359.0466, 454.2223, 491.1924, 457.2333, and 471.249, respectively.

**Data Analysis.** Kinetic constants for TCA glucuronidation in recombinant UGT1A4, UGT2B10 and HLM were obtained by fitting the following kinetic equations to the experimental data using nonlinear regression (Prism 4, GraphPad Software Inc., San Diego, CA).

Michaelis-Menten equation for one-enzyme hyperbolic kinetics:

\[

\nu = \frac{V_{\text{max}} \times [S]}{K_m + [S]}

\]

Michaelis-Menten equation for two-enzyme hyperbolic kinetics:

\[

\nu = \frac{V_{\text{max}} \times [S]}{K_m + [S]} + \frac{V_{\text{max}2} \times [S]}{K_{m2} + [S]}

\]

where \( V_{\text{max}} \) is apparent maximal velocity and \( K_m \) is the concentration of substrate at which half-maximal velocity is achieved. \( V_{\text{max}2} \) and \( K_{m2} \) are constants for the second enzyme or binding site.

Hill equation, which describes sigmoidal kinetics:

\[

\nu = \frac{V_{\text{max}} \times [S]^n}{S_{50}^n + [S]^n}

\]

where \( S_{50} \) is the substrate concentration resulting in 50% of \( V_{\text{max}} \) (analogous to the \( K_m \) in previous equations) and \( n \) is Hill coefficient.

Substrate inhibition:

\[

\nu = \frac{V_{\text{max}}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_{si}}}

\]
where $K_{si}$ is the inhibition constant describing the reduction in rate.

Biphasic kinetics:

$$
\nu = \frac{V_{\text{max}} \times [S] + C_{\text{int2}} \times [S]^2}{K_m + [S]} \quad (5)
$$

The biphasic kinetic profile described by Equation 5 is different from that described in Equation 2. Biphasic kinetics has two distinct phases but does not follow saturation kinetics. At low substrate concentration, the kinetic profile is curved as with hyperbolic kinetics; however, at high substrate concentration, the velocity of the reaction continues to increase, developing a linear, upward slope. $C_{\text{int2}}$ represents the slope of the linear portion.

For reactions exhibiting Michaelis-Menten, substrate inhibition and biphasic kinetics, intrinsic clearance ($C_{\text{int}}$) was calculated as $V_{\text{max}}/K_m$. For reactions exhibiting sigmoidal kinetics, maximum clearance ($C_{\text{max}}$) was calculated using the following equation (Houston and Kenworthy, 2000).

$$
C_{\text{max}} = \frac{V_{\text{max}}}{S_{50}} \times \frac{(n-1)}{n(n-1)^{1/n}} \quad (6)
$$

Goodness of fit to kinetic models was assessed from standard error, 95% confidence intervals and $r^2$. Kinetic curves were also analyzed using Eadie-Hofstee plots when fitted with different kinetic models. Kinetic constants were reported as the mean ± standard error of the parameter estimated.
Results

Protein Expression. Full-length cDNA of human UGT2B10 was cloned and protein was expressed in Sf9 insect cells. Expression of UGT2B10 was confirmed by immunoblotting analysis using a commercially available anti-UGT2B antibody. According to the manufacturer, the anti-UGT2B antibody was raised against a peptide mapping near the C-terminus of UGT2B of human origin and therefore could be used for detection of a broad range of UGT2B family members of mouse, rat and human origin by Western Blotting. It also has been successfully used to detect human UGTs 2B4, 2B7, 2B10 and 2B11 by Western blot (Nishiyama et al., 2006). UGT2B10 protein exhibited an apparent molecular mass of ~ 51.8 kDa (Fig. 2) that was consistent with another report (Uchaipichat et al., 2004). Microsomes prepared from uninfected Sf9 cells exhibited no cross reactivity to anti-UGT2B antibody.

Quaternary N-Glucuronidation in Recombinant UGTs. Two TCA substrate concentrations, 5 and 50 µM, were used in the activity assays in recombinant UGT1A3, UGT1A4 and UGT2B10. At TCA concentration of 5 µM, UGT1A3 did not exhibit detectable N-glucuronidation activity towards imipramine or trimipramine. Low levels of amitriptyline and clomipramine N-glucuronidation, 3.2 and 0.27 pmol/min/mg, were detected in UGT1A3, respectively. However, glucuronide formation in UGT1A3 was at least 30-fold lower than that observed in UGT1A4 or UGT2B10. UGT2B10-catalyzed imipramine N-glucuronidation was approximately 2-fold more efficient than that catalyzed by UGT1A4. UGT2B10 and UGT1A4 exhibited comparable activity in metabolizing amitriptyline, clomipramine and trimipramine at 5 µM of substrate concentration. (Fig. 3A).

At 50 µM TCA concentration, UGT1A4 appeared to be the most active human UGT for TCA glucuronidation, while UGT1A3 exhibited the least activity (Fig. 3B). Results with
UGT1A3 are consistent with previous findings, indicating that UGT1A3 is likely insignificant to the overall metabolism of these drugs (Green and Tephly, 1998). UGT2B10-catalyzed imipramine N-glucuronidation was comparable with that catalyzed by UGT1A4, while UGT1A4 was approximately 5-fold more efficient than UGT2B10 in the glucuronidation of amitriptyline, clomipramine and trimipramine (Fig. 3B).

**Kinetics of Quaternary N-Glucuronidation.** The kinetics of TCA glucuronidation was characterized in HLM, recombinant UGT2B10 and UGT1A4 (Fig. 4). Kinetic parameters of glucuronidation for each substrate are shown in Table 1. Amitriptyline N-glucuronidation by HLM was best described with a two-enzyme Michaelis-Menten kinetics (eq. 2) (Fig. 4), with apparent $K_m$ values of 1.75 and 343 µM for the high and low affinity component, respectively. This is consistent with the previous reported $K_m$ values of 1.4 and 311 µM in HLM (Breyer-Pfaff et al., 1997). Substrate inhibition (eq. 4) and one-enzyme Michaelis-Menten kinetics (eq. 1) were observed for amitriptyline glucuronidation by UGT2B10 and UGT1A4, respectively (Fig. 4). The apparent $K_m$ values of amitriptyline N-glucuronidation were 2.60 and 448 µM in UGT2B10 and UGT1A4, in good agreement with $K_m$ values observed in HLM. The $K_{si}$ value for UGT2B10 (353 µM) was approximately 136-fold higher than the $K_m$ value. The intrinsic clearance for amitriptyline N-glucuronidation by UGT2B10 was more than 10-fold higher than that determined for UGT1A4.

Biphasic kinetics was observed for imipramine N-glucuronidation by HLM (eq. 5) (Fig. 4). The high affinity component exhibited apparent $K_m$ and $V_{max}$ values of 9.92 µM and 55.7 pmol/min/mg, while the low affinity component was not saturable at 500 µM substrate concentration and showed an apparent $CL_{int}$ of 0.98 µl/min/mg. Imipramine glucuronidation by UGT2B10 exhibited one-enzyme Michaelis-Mention kinetics (eq. 1) (Fig.4). The apparent $K_m$
and V\text{max} values of imipramine glucuronidation by UGT2B10 were 16.8 µM and 59.6 pmol/min/mg, in good agreement with the high affinity component in HLM. Sigmoidal kinetics was observed for imipramine glucuronidation by UGT1A4 (eq. 3), with apparent S\text{50} value of 262 µM (Table 1). The \textit{in vitro} clearances for imipramine glucuronidation by UGT2B10 and UGT1A4 were comparable, though it should be noted that CL\text{int} and CL\text{max} are not equivalent parameters.

Clomipramine N-glucuronidation by HLM and recombinant UGT1A4 exhibited sigmoidal kinetics (eq. 3) (Fig. 4). The kinetic constants were in good agreement between HLM and UGT1A4, exhibiting apparent S\text{50} values of 108 and 112 µM, V\text{max} values of 1750 and 1430 pmol/min/mg, Hill coefficient of 1.9 and 1.8, respectively. Clomipramine glucuronidation by UGT2B10 fit best to the one-enzyme Michaelis-Menten equation (eq. 1), with an apparent K\text{m} value of 14.4 µM, about 7.5-fold less than apparent S\text{50} that obtained from HLM or UGT1A4, though caution should be exercised in comparing K\text{m} and S\text{50} values.

In contrast to the sigmoidal kinetics observed with HLM, trimipramine glucuronidation by UGT2B10 and UGT1A4 exhibited one-enzyme Michaelis-Menten kinetics (eq. 1) (Fig. 4). The apparent K\text{m} values of trimipramine glucuronidation were 11.2 and 258 µM by UGT2B10 and UGT1A4. HLM exhibited apparent S\text{50} value of 277 µM, similar as apparent K\text{m} value by UGT1A4. The intrinsic clearance for trimipramine glucuronidation by UGT2B10 and UGT1A4 were similar (0.9 and 1.2 µl/min/mg for UGT2B10 and UGT1A4, respectively).

\textbf{Selective Inhibition of UGT Activities.} The inhibitory effects of hecogenin and nicotine on UGT1A4 and UGT2B10 activities were investigated using TCA as substrate at a 5 µM concentration. As shown in Fig. 5A and Fig. 5B, nicotine and hecogenin demonstrated highly selective inhibition of UGT2B10 and UGT1A4, respectively. Nicotine at 500 µM inhibited 76%,
89%, 88% and 90% of the glucuronidation of amitriptyline, imipramine, clomipramine and trimipramine by UGT2B10, while exhibited no inhibition of UGT1A4 activities. On the contrary, hecogenin at 100 µM inhibited 83%, 81%, 75% and 83% of the glucuronidation of amitriptyline, imipramine, clomipramine and trimipramine by UGT1A4, while exhibited less than 10% of inhibition of UGT2B10 activities.

The contribution of UGT1A4 and UGT2B10 to glucuronidation of TCAs was assessed in pooled HLM using hecogenin and nicotine as selective inhibitors. As shown in Fig. 6A, at 5 µM TCA concentration, 500 µM of nicotine significantly inhibited 33%, 50%, 36% and 40% of the glucuronidation of amitriptyline, imipramine, clomipramine and trimipramine by HLM. Hecogenin at 100 µM exhibited no inhibitory effect on amitriptyline glucuronidation, and inhibited 15%, 13% and 32% of glucuronidation of imipramine, clomipramine and trimipramine at 5 µM substrate concentration. At 200 µM TCA concentration (Fig. 6B), hecogenin at 100 µM significantly inhibited 45%, 75%, 72% and 49% of the glucuronidation of amitriptyline, imipramine, clomipramine and trimipramine by HLM, respectively. On the other hand, 500 µM nicotine did not inhibit TCA glucuronidation by HLM (Fig. 6B). Addition of hecogenin increased inhibitory effect of nicotine at 5 µM TCA concentration (Fig. 6A), while addition of nicotine did not increase the inhibitory effect of hecogenin at 200 µM TCA concentration (Fig. 6B).
Discussion

Human UGT2B10 has been largely considered as an “orphan” UGT isoform until recent findings demonstrated that UGT2B10 was primarily responsible for the quaternary N-glucuronidation of nicotine, cotinine and nitrosamines (Kaivosaari et al., 2007; Chen et al., 2008). The present study expanded the UGT2B10 substrate list to include a number of aliphatic tertiary amines, amitriptyline, imipramine, clomipramine and trimipramine. These observations may prompt other investigators to consider the importance of UGT2B10 in the metabolism and clearance of xenobiotics for drugs that undergo quaternary N-glucuronidation.

The quaternary N-glucuronides of TCAs were major Phase II metabolites identified in human urine (Luo et al., 1995). The steady-state plasma concentrations of amitriptyline, imipramine, clomipramine and trimipramine are approximately 0.2, 0.6, 0.2 and 0.2 µM respectively (Reis et al., 2009; Balley and Jatlow, 1976; Musa, 1989). In present study, the glucuronidation kinetics studied was assessed at a wide concentration range (0.15 ~ 500 µM) in HLM. In this way, in vitro observations would not be skewed without consideration of therapeutic concentrations of these drugs. Both amitriptyline and imipramine N-glucuronidation by HLM demonstrated biphasic kinetics, consistent with previous reports (Breyer-Pfaff et al., 1997; Nakajima et al., 2002). The investigators in previous studies suggested UGT1A4 as the low affinity enzyme in HLM, but were unable to identify the high affinity component. In the present study, the K_m values of amitriptyline and imipramine glucuronidation by recombinant UGT2B10 were in good agreement with those observed for high affinity component in HLM. This suggested that UGT2B10 is the high affinity enzyme in the glucuronidation of amitriptyline and imipramine. For clomipramine and trimipramine glucuronidation, the apparent K_m(S_{50}) values by recombinant UGT1A4 were similar as the apparent S_{50} values by HLM fitted with the
sigmoidal model. The apparent $K_m$ values of clomipramine and trimipramine glucuronidation by UGT2B10 were about 7.5 and 23-fold lower than $K_m(S_{50})$ by UGT1A4 or HLM. These results clearly demonstrated that UGT2B10 also exhibited higher affinity towards these two drugs than UGT1A4. However, the high affinity component associated with UGT2B10 was probably hidden by the sigmoidal kinetic character observed in HLM. Though the glucuronidation capacity ($V_{max}$) by UGT2B10 was lower than that by UGT1A4 for these TCAs, the in vitro clearance was comparable between UGT2B10 and UGT1A4 due to the very high affinity of UGT2B10 to TCAs. The intrinsic clearance of amitriptyline by UGT2B10 was more than 10-fold higher than that by UGT1A4. These results suggested that UGT2B10 was likely to be a high affinity but low capacity enzyme while UGT1A4 was a low affinity but high capacity enzyme in the glucuronidation of amitriptyline, imipramine, clomipramine and trimipramine. These TCA compounds are hydrophobic bases and have been reported to bind non-specifically to HLM and insect cell microsomes (Venkatakrishnan et al., 2000; McLure et al., 2000; Austin et al., 2002). It would be important to determine non-specific binding of these drugs in the in vitro incubation matrices to improve any extrapolation of the in vitro findings to predict in vivo observations (Obach, 1997).

In present study, we demonstrated that hecogenin selectively inhibited of UGT1A4 glucuronidation activity towards TCAs at concentration up to 100 µM. Nicotine demonstrated highly selective inhibition toward UGT2B10, and no inhibition towards UGT1A4 activities at concentration up to 500 µM in the in vitro conditions. Though the inhibitory effects of nicotine towards other UGT isoform activities requires further exploration, nicotine could be used as a selective inhibitor in vitro to differentiate UGT1A4 and UGT2B10 activities.
At low TCA concentration, nicotine significantly inhibited their glucuronidation activities in HLM, while hecogenin exhibited no inhibition of amitriptyline glucuronidation and much less inhibition of imipramine, clomipramine and trimipramine glucuronidation. The inhibition profiles at high TCA concentration were opposite to that observed at low TCA concentration, however, considering low therapeutic concentration of these drugs, observations at high TCA concentration might not be relevant in vivo. Similar inhibition profiles were also reported for amitriptyline (Dehal et al., 2001), where the authors showed that hecogenin significantly inhibited amitriptyline glucuronidation at high substrate concentration (200 µM), but not at low amitriptyline concentration (10 µM). The selective inhibition and enzyme kinetic results observed here for recombinant UGT1A4, UGT2B10, and HLM clearly demonstrated that UGT2B10 would be the major UGT isoform responsible for amitriptyline glucuronidation at therapeutic concentrations in vivo. UGT2B10 and UGT1A4 probably contribute equally in the glucuronidation of imipramine, clomipramine and trimipramine in vivo. Nicotine glucuronidation exhibited a high K_m value of 290 µM in recombinant UGT2B10 (Kaivosaari et al., 2007), which was much higher than the K_m values observed in this study for TCA glucuronidation by UGT2B10. This may be one of the reasons that 500 µM nicotine did not fully inhibit TCA glucuronidation in HLM.

Both UGT1A4 and UGT2B10 are primarily found in human liver, yet relative protein abundance of each UGT isoform in human tissues needs further evaluation. Several recent publications applied real time reverse transcriptase-polymerase chain reaction (RT-PCR) method to determine mRNA copy numbers of each UGT isoform in human liver and other tissues (Ohno and Nakajin, 2009; Izukawa et al., 2009). The RT-PCR method can provide useful information on the mRNA levels of each UGT isoform, but do not directly measure protein level. These
studies suggest UGT2B10 is one of the most abundant isoforms in liver and in the same range as UGT1A4, indicating that contributions of UGT2B10 to the N-glucuronidation of drugs could be of great importance in vivo. In addition, drugs that modulate UGT2B10 activity in patients might affect the clearance of TCAs in vivo.

In conclusion, this study demonstrated for the first time that UGT2B10 was able to catalyze aliphatic tertiary amines to form quaternary N-glucuronide. This work also revealed that UGT2B10 was the high affinity component for TCA glucuronidation in HLM. UGT2B10 would be a major UGT isoform responsible for the glucuronidation of amitriptyline, imipramine, clomipramine and trimipramine at therapeutic concentrations in vivo. Nicotine could potentially be used as selective UGT2B10 inhibitor in enzyme identification of xenobiotic N-glucuronidation pathways in humans.
References


Dehal SS, Gagne PV, Crespi CL and Patten CJ (2001) Characterization of a Probe Substrate and an Inhibitor of UDP Glucuronosyl Transferase (UGT) 1A4 Activity in Human Liver Microsomes (HLM) and cDNA-Expressed UGT Enzymes. *AAPS Pharm Sci.* 3(S1): 893.


Uchaipichat V, Mackenzie PI, Elliot DJ and Miners JO. (2006) Selectivity of substrate (trifluoperazine) and inhibitor (amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfinpyrazone) "probes" for human udp-glucuronosyltransferases. *Drug Metab Dispos.* **34**: 449-456


Footnote

1 current affiliation: GlaxoSmithKline, Respiratory Centre of Excellence in Drug Discovery.
Legends for figures.

Figure 1. Chemical structures of tricyclic antidepressants, amitriptyline, imipramine, clomipramine and trimipramine. Arrows indicate the site of quaternary N-glucuronidation.

Figure 2. Western blot analysis of protein expression level in microsomes prepared from Sf9 insect cells infected with human UGT2B10. Microsomes from uninfected insect cells were used as a negative control. Protein was probed with Santa Cruz Biotechnology’s goat polyclonal UGT2B antibody and horseradish peroxidase-conjugated donkey anti-goat IgG.

Figure 3. Quaternary N-glucuronides formation of amitriptyline, imipramine, clomipramine and trimipramine in human recombinant UGT1A3, UGT1A4 and UGT2B10 enzymes. The experiments were performed in triplicate at two substrate concentrations, 5 μM (A) and 50 μM (B). N.D. – not detectable.

Figure 4. Kinetic analysis of the glucuronidation of amitriptyline, imipramine, clomipramine and trimipramine by pooled human liver microsomes, recombinant UGT2B10, UGT1A4. The substrates were incubated at concentrations in the range of 0.15~500 μM for HLM and UGT1A4 and concentrations in the range of 0.15~100 μM for UGT2B10. The rates represent the mean (± S.D.) of triplicate incubations. Eadie-Hofstee plot is also presented as insert for each substrate.

Figure 5. The effect of hecogenin and nicotine on the glucuronidation activities of human recombinant UGT2B10 (A) and UGT1A4 (B) using amitriptyline, imipramine, clomipramine and trimipramine as substrates. Hecogenin was evaluated at 10 and 100 μM while nicotine was tested at 10, 100 and 500 μM. Each bar represents the mean percentage activity relative to vehicle control from duplicate measurements.

Figure 6. The effect of nicotine and hecogenin on the glucuronidation activities of amitriptyline, imipramine, clomipramine and trimipramine in pooled human liver microsomes at 5 μM (A) and
200 μM (B) substrate concentrations. Four conditions were evaluated, vehicle control, 100 μM hecogenin, 500 μM nicotine, or combination of 100 μM hecogenin and 500 μM nicotine. Each bar represents the mean percentage activity (± S.D.) relative to vehicle control from triplicate measurements. Two tailed equal variance Student’s t-test was performed between each inhibition condition and vehicle control (* p<0.05, ** p<0.01, *** p<0.001).
Table 1. Apparent kinetic parameters for glucuronidation of amitriptyline, imipramine, clomipramine and trimipramine by pooled human liver microsomes, human recombinant UGT2B10 and UGT1A4. Data are presented as mean ± standard error of parameter fit.

See Materials and Methods for individual kinetic equation.

<table>
<thead>
<tr>
<th></th>
<th>K_m (^{a}) or S_50 (^{b}) (µM)</th>
<th>V_{max} (pmol/min/mg)</th>
<th>K_{m2} (µM)</th>
<th>V_{max2}</th>
<th>K_{si} (µM)</th>
<th>n</th>
<th>Cl_{int2}</th>
<th>Cl_{int} (^{c}) or Cl_{max} (^{d})</th>
<th>Equation</th>
</tr>
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<tr>
<td><strong>UGT2B10</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>2.60 ± 0.48 (^{a})</td>
<td>120 ± 7</td>
<td>NA</td>
<td>NA</td>
<td>353 ± 137</td>
<td>NA</td>
<td>NA</td>
<td>46.2 (^{c})</td>
<td>Eq.4</td>
</tr>
<tr>
<td>Imipramine</td>
<td>16.8 ± 1.3 (^{a})</td>
<td>59.6 ± 1.3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>3.5 (^{c})</td>
<td>Eq.1</td>
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<tr>
<td>Clomipramine</td>
<td>14.4 ± 0.8 (^{a})</td>
<td>150 ± 2</td>
<td>NA</td>
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<td>NA</td>
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<td>10.4 (^{c})</td>
<td></td>
<td>Eq.1</td>
</tr>
<tr>
<td>Trimipramine</td>
<td>11.2 ± 0.9 (^{a})</td>
<td>9.90 ± 0.19</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.9 (^{c})</td>
<td></td>
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<tr>
<td><strong>UGT1A4</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Amitriptyline</td>
<td>448 ± 27 (^{a})</td>
<td>1890 ± 70</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>4.2 (^{c})</td>
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<tr>
<td>Imipramine</td>
<td>262 ± 46 (^{b})</td>
<td>700 ± 78</td>
<td>NA</td>
<td>NA</td>
<td>1.5 ± 0.1</td>
<td>NA</td>
<td>1.4 (^{d})</td>
<td></td>
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<tr>
<td>Clomipramine</td>
<td>112 ± 8 (^{b})</td>
<td>1430 ± 60</td>
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<td>6.4 (^{d})</td>
<td></td>
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<tr>
<td>Trimipramine</td>
<td>258 ± 26 (^{a})</td>
<td>313 ± 15</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.2 (^{c})</td>
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<td>Eq.1</td>
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<td><strong>Pooled HLM</strong></td>
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<tr>
<td>Amitriptyline</td>
<td>1.75 ± 1.45 (^{a})</td>
<td>85.6 ± 21.7</td>
<td>343 ± 65</td>
<td>1210 ± 80</td>
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<td>NA</td>
<td>52.4 (^{c})</td>
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<td>55.7 ± 15.8</td>
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<td>NA</td>
<td>0.98 ± 0.03</td>
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<td>6.6 (^{c})</td>
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<tr>
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<td>108 ± 6 (^{b})</td>
<td>1750 ± 50</td>
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<td>NA</td>
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<td>8.1 (^{d})</td>
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<tr>
<td>Trimipramine</td>
<td>277 ± 46 (^{b})</td>
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<td>1.2 ± 0.1</td>
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<td>0.7 (^{d})</td>
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</tbody>
</table>

NA – not applicable

\(^{a}\) – K_m fitting from Michaelis-Menten equation, substrate inhibition equation or biphasic equation

\(^{b}\) – S_50 fitting from Hill equation

\(^{c}\) – Cl_{int} (µL/min/mg) calculated as V_{max}/K_m

\(^{d}\) – Cl_{max} (µL/min/mg) calculated according to equation 6
Figure 1

Amitriptyline

Imipramine

Clomipramine

Trimipramine
Figure 3

A. 5 μM substrate

B. 50 μM substrate
Figure 4

Rate of Glucuronidation (pmol/min/mg)

HLM

UGT2B10

UGT1A4
Figure 5

A. Inhibition in UGT2B10

- Relative activity (%)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Vehicle control</th>
<th>10 μM Hecogenin</th>
<th>100 μM Hecogenin</th>
<th>10 μM Nicotine</th>
<th>100 μM Nicotine</th>
<th>500 μM Nicotine</th>
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<tr>
<td>Imipramine</td>
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<tr>
<td>Trimipramine</td>
<td></td>
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</table>

B. Inhibition in UGT1A4

- Relative activity (%)

<table>
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<tr>
<th>Substance</th>
<th>Vehicle control</th>
<th>10 μM Hecogenin</th>
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<th>10 μM Nicotine</th>
<th>100 μM Nicotine</th>
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</table>
Figure 6

A. 5 μM substrate concentration

Relative activity (%)

<table>
<thead>
<tr>
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<th>Hecogenin</th>
<th>Nicotine</th>
<th>Hec + Nic</th>
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Amitriptyline

Imipramine

Clomipramine

Trimipramine

B. 200 μM substrate concentration

Relative activity (%)

<table>
<thead>
<tr>
<th>Control</th>
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<th>Nicotine</th>
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Amitriptyline

Imipramine

Clomipramine

Trimipramine