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**Selectivity of substrate (trifluoperazine) and inhibitor (amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine and sulfinpyrazone) ‘probes’ for human UDP-glucuronosyltransferases**

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**Running title:** UGT substrate and inhibitor probes

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**Abbreviations used:** AUC, area under the plasma concentration-time curve; AZT, zidovudine, ie. 3'-azido-3'-deoxythymidine;  $f_{u_{inc}}$ , fraction unbound in incubation mixtures; GAZT, 3'-azido-3'-deoxythymidine 5'- $\beta$ -D-glucuronide; HEK293, human embryo kidney 293 cells; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; 4MU, 4-methylumbelliferone; 4MUG, 4-methylumbelliferone- $\beta$ -D-glucuronide; P450, cytochrome P450; TFP, trifluoperazine; TFPG, trifluoperazine glucuronide; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase

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## ABSTRACT

Relatively few selective substrate and inhibitor probes have been identified for human UDP-glucuronosyltransferases (UGT). This work investigated the selectivity of trifluoperazine (TFP), as a substrate, and amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine and sulfinpyrazone, as inhibitors, for human UGTs. Selectivity was assessed using UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15 expressed in HEK293 cells. TFP was confirmed as a highly selective substrate for UGT1A4. However, TFP bound extensively to both HEK293 lysate and to human liver microsomes in a concentration-dependent manner ( $f_{inc}$  0.20 – 0.59). When corrected for non-specific binding,  $K_m$  values for TFP glucuronidation were similar for both UGT1A4 (4.1  $\mu$ M) and human liver microsomes ( $6.1 \pm 1.2 \mu$ M) as the enzyme sources. Of the compounds screened as inhibitors, hecogenin alone was selective; significant inhibition was observed only for UGT1A4 ( $IC_{50}$  1.5  $\mu$ M). Using phenylbutazone and quinine as ‘models’, inhibition kinetics were variously described by competitive and noncompetitive mechanisms. Inhibition of UGT2B7 by quinidine was also investigated further since the effects of this compound on morphine pharmacokinetics (a known UGT2B7 substrate) have been ascribed to inhibition of P-glycoprotein. Quinidine inhibited human liver microsomal and recombinant UGT2B7 with respective  $K_i$  values of  $335 \pm 128 \mu$ M and 186  $\mu$ M. In conclusion, TFP and hecogenin represent selective substrate and inhibitor probes for UGT1A4, although the extensive non-selective binding of the former should be taken into account in kinetic studies. Amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine and sulfinpyrazone are non-selective UGT inhibitors.

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## INTRODUCTION

Glucuronidation is an important metabolic pathway for the inactivation and elimination of many compounds, including drugs from all therapeutic classes, dietary chemicals, environmental pollutants, and endogenous compounds (e.g., bilirubin, bile acids, hydroxysteroids). The glucuronidation reaction is catalyzed by the enzyme UDP-glucuronosyltransferase (UGT), which facilitates the covalent linkage (conjugation) of glucuronic acid, from its cosubstrate UDP-glucuronic acid (UDPGA), typically to substrates with carboxyl, hydroxyl (phenol or aliphatic alcohol) or amine functional groups (Miners and Mackenzie, 1991; Radomska-Pandya et al., 1999; Tukey and Strassburg, 2000; Miners et al., 2004).

UGT exists as an enzyme superfamily, and *UGT* genes have been classified into families and subfamilies based on the sequence identity of the encoded proteins (Mackenzie et al., 1997). To date, seventeen human UGT proteins have been identified: UGT 1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17 and 2B28. However, several of these (UGT 1A5, 2B4, 2B10, 2B11 and 2B28) appear to have low or absent activity with xenobiotic substrates. Available evidence suggests that the active UGT enzymes exhibit distinct but overlapping substrate selectivities (Radomska-Pandya et al., 1999; Tukey and Strassburg, 2000; Miners et al., 2004). In addition, the individual forms differ in terms of regulation of expression, and numerous factors (e.g., genetic polymorphism) are known to alter enzyme activity (Miners et al., 2004). Tissue-specific expression is also a feature of UGT; notably UGT 1A7, 1A8 and 1A10 are expressed only in the

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gastrointestinal tract while UGT2A1 appears to largely be involved in olfactory signal termination.

Given these features of UGT, *in vitro* methods for identifying the form(s) responsible for the metabolism of any given drug (i.e., reaction phenotyping) is essential for understanding and predicting factors responsible for altered metabolic clearance *in vivo*. Reaction phenotyping most commonly employs either human liver microsomes or hepatocytes as the enzyme source, as both contain the various forms relevant to hepatic drug clearance *in vivo* (Miners et al., 2004). Form-selective inhibitors are pivotal to reaction phenotyping, and the use of cytochrome P450 (P450) selective inhibitors has proved invaluable for the reaction phenotyping of P450-catalyzed reactions in human tissues (Miners et al., 1994; Clarke, 1998; Venkatakrishnan et al., 2001). In contrast to P450, the availability of UGT form selective-inhibitors is limited, although selective substrates have been identified for several UGTs (Miners et al., 2004). Consequently reaction phenotyping of UGT substrates has most commonly involved screening for activity by recombinant enzymes, which is a time consuming process and may yield ambiguous data (Miners et al., 2004).

In this study, we characterized the selectivity of amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine and sulfinpyrazone as inhibitors of human UGTs. There is evidence to suggest that these compounds may inhibit xenobiotic glucuronidation *in vitro* and/or *in vivo* (Ventafridda et al., 1987; Yue et al., 1990; Resetar et al., 1991; Rajaonarison et al., 1992; Kirkwood et al., 1998; Egfjord et al., 1999), or may be substrates for human UGTs *in vitro* and/or *in vivo* (Green and Tephly, 1996; Coffman et al., 1998; Gall et al., 1999; Mirghani et al., 2003).

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Quinidine is of further interest since it is utilized as an inhibitor of P-glycoprotein *in vivo* to assess the role of this transporter on drug bioavailability. Indeed, inhibition of P-glycoprotein mediated transport has been proposed as the mechanism for the increase in the area under the plasma concentration-time curve (AUC) of morphine (Kharasch et al., 2003), a drug metabolized predominantly by UGT2B7-catalyzed glucuronidation, in subjects co-administered quinidine.

Effects of the putative inhibitors on UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, and 2B15 were investigated using the non-selective substrate 4-methylumbelliferone (4MU) as the 'probe'. As UGT1A4 exhibits negligible activity towards 4MU (Uchaipichat et al., 2004), trifluoperazine (TFP) was characterized as a specific probe substrate for this enzyme and subsequently employed in the UGT1A4 inhibition studies. In addition, the inhibition kinetics of quinidine on human liver microsomal and recombinant UGT2B7 was further investigated using zidovudine (AZT) as the substrate.

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## MATERIALS AND METHODS

**MATERIALS** 4-Methylumbelliferone (4MU), 4-methylumbelliferone- $\beta$ -D-glucuronide (4MUG),  $\beta$ -glucuronidase (from *Escherichia coli*), alamethicin (from *Trichoderma viride*), amitriptyline (hydrochloride salt), androsterone, AZT (3'-azido-3'-deoxythymidine), canrenoic acid (potassium salt), GAZT (3'-azido-3'-deoxythymidine 5'- $\beta$ -D-glucuronide), hecogenin, phenylbutazone, TFP (dihydrochloride salt), trifluoroacetic acid, quinidine (hydrochloride monohydrate salt), quinine (hemisulfate salt hydrate), sulfinpyrazone, UDP-glucuronic acid (UDPGA; trisodium salt) and dialysis cellulose membrane (molecular weight cut off 12 000 Da) were purchased from Sigma-Aldrich (St Louis, MO, USA). Solvents and other reagents were of analytical reagent grade.

## METHODS

### Human liver microsomes

Microsomes were prepared from four human livers (HL10, 12, 29 and 40; obtained from the Department of Clinical Pharmacology human liver bank) according to Bowalgaha et al. (2005). Microsomes were activated by preincubation with alamethicin (50  $\mu$ g/mg of protein) on ice for 30 min prior to their use in incubations (Boase and Miners, 2002).

### Expression of UGT proteins.

UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15 cDNAs were stably expressed in a human embryonic kidney cell line (HEK293) as described previously

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(Sorich et al., 2002; Uchaipichat et al., 2004). Cells were separately transfected with the individual UGT cDNAs and were incubated in Dulbecco's Modified Eagle Medium (DMEM), which contained puromycin (1.5 mg/l), 10% fetal calf serum, and penicillin G sodium (100 units/ml)/streptomycin sulfate (100 µg/ml), in a humidified incubator with an atmosphere of 5% CO<sub>2</sub> at 37°C. After growth to at least 80% confluency, cells were harvested and washed in phosphate-buffered saline. The harvested cells were lysed by sonication using a Heat Systems-Ultrasonics sonicator set at microtip limit of 4. Cells expressing UGT1A proteins were sonicated with 4 x 2-sec 'bursts', each separated by 3 min cooling on ice. A similar method was applied to UGT2B subfamily enzymes, except sonication was limited to 1-sec bursts. The samples were then centrifuged at 12 000g for 1 min at 4°C, and the supernatant fraction was separated and stored at -80°C until use. The protein concentration of cell lysates was determined by the method of Lowry (Lowry et al., 1951) using bovine serum albumin as the standard.

It should be noted that immunoblotting studies with a commercial UGT1A antibody and a non-selective UGT antibody (raised against a purified mouse Ugt) have demonstrated that UGT is not expressed by untransfected HEK293 cells (Uchaipichat et al., 2004). Moreover, 4MU and TFP glucuronidation activities were below the limits of detection (see below) in control incubations performed with untransfected cells.

**TFP glucuronidation assay.**

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Trifluoperazine glucuronide (TFPG) formation was determined using the method of Uchaipichat et al (2005). The incubation mixture (200  $\mu$ l total volume) contained 50 mM Tris-HCl buffer (pH 7.4), 5 mM UDPGA, 5 mM  $MgCl_2$ , activated human liver microsomes (0.1 mg/ml) or UGT1A4 HEK293 cell lysate (0.25 mg/ml), and TFP (5-200  $\mu$ M). Reactions were initiated by the addition of UDPGA and incubations were performed at 37°C in a shaking water bath for 20 min. The reactions were terminated by the addition of 200  $\mu$ l 4% acetic acid/96% methanol and then centrifuged at 5000g for 10 min. A 40  $\mu$ l aliquot of the supernatant fraction was injected into the HPLC column. TFPG was quantified by comparison of peak areas to those of a TFP external standard curve prepared over the concentration range 0.2-10  $\mu$ M. The lower limit of detection, assessed as five times background, was 8 pmol TFPG/incubation.

#### **4MU glucuronidation assay.**

UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15 activities were determined with 4MU as the substrate. 4MU glucuronidation was measured according to a previously published procedure (Miners et al., 1988). Briefly, incubations contained UDPGA (5 mM),  $MgCl_2$  (5 mM), HEK293 cell lysate, phosphate buffer (0.1 M, pH 7.4) and 4MU in a total volume of 0.6 ml. Incubations for each individual isoform were performed using conditions reported previously by Sorich et al. (2002) for UGT1A1 and Uchaipichat et al. (2004) for UGT 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15. The lower limit of detection (as defined above) was 30 pmol 4MU glucuronide/incubation.

#### **AZT glucuronidation assay and quinidine inhibition of UGT2B7.**

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GAZT formation was measured using a modification of a previously described method (Boase and Miners, 2002). Incubation mixtures, in a total volume 200  $\mu$ l, contained phosphate buffer (0.1 M, pH 7.4),  $MgCl_2$  (4 mM), UDPGA (5 mM), AZT (250, 500, and 750  $\mu$ M for HLM or 200, 400, and 600  $\mu$ M for UGT2B7), quinidine (0, 100, 200, 300, and 400  $\mu$ M), and activated HLM (1 mg/ml) or HEK293 cell lysate expressing UGT2B7 (1.5 mg/ml). Reactions were initiated by the addition of UDPGA and incubations were carried out at 37°C in a shaking water bath for 60 min. Reactions were terminated by addition of 24%  $HClO_4$  (10  $\mu$ l) and mixtures were centrifuged at 5000g for 10 min. A 30  $\mu$ l of the supernatant fraction was injected into the HPLC column. HPLC was performed using an Agilent 1100 series (Agilent Technologies, Sydney, NSW, Australia) fitted with a Novapak  $C_{18}$  column (3.9 x 150 mm, 5 $\mu$ m; Waters Associates, Milford, MA, USA). The mobile phase, 0.12% v/v acetic acid in 10% acetonitrile/water, was delivered at a flow rate 1.2 ml/min. Column eluant was monitored at UV absorbance 267 nm. Under these conditions, retention times of GAZT and AZT were 3 and 6.2 min, respectively. Concentrations of GAZT in incubation samples were determined by comparison of peak areas to those of GAZT standard curve with concentrations in the range 1-20  $\mu$ M.

**Inhibition of 4MU or TFP glucuronidation by drugs and other chemicals.**

Amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine and sulfinpyrazone were screened as inhibitors of UGT activities using 4MU or TFP as the substrate. 4MU was used as the substrate for the inhibition studies with UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15, whereas TFP was used as the substrate for UGT1A4. Incubations with 4MU were performed at the

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concentration corresponding to the apparent  $K_m$  or  $S_{50}$  value of each enzyme (Sorich et al., 2002; Uchaipichat et al., 2004). Concentrations of amitriptyline, phenylbutazone, quinidine, quinine and sulfapyrazone used in screening experiments were 0, 10, 100, 500 and 1000  $\mu\text{M}$ . The concentrations of hecogenin used for inhibition screening were 0, 1, 10, 100 and 200  $\mu\text{M}$ , while concentrations of androsterone and canrenoic acid were 0, 1, 10, 100 and 500  $\mu\text{M}$ . Negative controls for the 4MU assay contained each putative inhibitor and substrate (i.e., 4MU) without UDPGA whereas negative controls for TFP assay contained inhibitor and UDPGA (without substrate). Amitriptyline and quinidine (available as salts) were dissolved in water but androsterone, canrenoic acid, phenylbutazone, quinine, sulfapyrazone and hecogenin required addition of an organic solvent, which was selected on the basis of known effect on enzyme activity (Uchaipichat et al., 2004). Up to 2% methanol (v/v) was selected for incubations with all UGTs except UGT 1A6 and 2B15. Incubations with these enzymes contained 1% methanol (v/v) for the dissolution of androsterone, canrenoic acid, phenylbutazone, quinine and hecogenin, while 1% DMSO (v/v) was used for incubations containing sulfapyrazone. Control incubations contained the same concentration of organic solvent.

Subsequent experiments that determined inhibitor constants ( $K_i$ ) for selected compounds included 4 inhibitor concentrations at each of three substrate concentrations. The  $K_i$  values of phenylbutazone and quinine were investigated for those enzymes exhibiting Michaelis-Menten 4MU glucuronidation kinetics (viz. UGT 1A1, 1A6, 1A7 and 1A10) and for UGT1A9 ('weak' substrate inhibition). In addition, AZT was used as the substrate for the determination of the  $K_i$  value for quinidine

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inhibition of human liver microsomal (HL 10, 12, 29 and 40) and recombinant UGT2B7.

**Non-specific binding of TFP to human liver microsomes and HEK293 cell lysate.**

Non-specific binding of TFP to human liver microsomes or HEK293 lysate was investigated using the equilibrium dialysis method of McLure et al. (2000). Briefly, one side of the dialysis apparatus contained TFP (10, 30, 60, 100 or 200  $\mu\text{M}$ ), pooled human liver microsomes (n=4) (0.1 mg/ml) or HEK293 cell lysate (0.25 mg/ml), and Tris-HCl buffer (50 mM, pH 7.4), while the other compartment contained Tris-HCl buffer alone. The dialysis cell assembly was immersed in a water bath maintained at 37°C and rotated at 12 rpm for 3 h. Control experiments were also performed with buffer or pooled human liver microsomes (0.1 mg/ml) or HEK293 cell lysate (0.25 mg/ml) on both sides of the dialysis cell at high (200  $\mu\text{M}$ ) and low (10  $\mu\text{M}$ ) TFP concentrations to ensure that equilibrium was attained. The contents of each compartment (200  $\mu\text{l}$ ) were collected, treated with 200  $\mu\text{l}$  of 4% (v/v) acetic acid in methanol, vortex mixed, and centrifuged (5000g for 10 min). A 5  $\mu\text{l}$  aliquot of the supernatant fraction was analyzed by HPLC. The HPLC system and conditions were essentially identical to those described previously for the measurement of TFPG (Uchaipichat et al., 2005), except that the mobile phase (50% mobile phase A / 50% mobile phase B) was delivered isocratically at a flow rate of 1 ml/min. Under these conditions, TFP eluted at 3.3 min. Standards in the concentration range 10-200  $\mu\text{M}$  were prepared in Tris-HCl buffer (50 mM, pH 7.4) and treated in the same manner as dialysis samples. The TFP concentrations of dialysis samples were determined by comparison of peak areas with those of the standard curve. Within-day assay

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imprecision was assessed by measuring TFP (10, 200  $\mu$ M) in 5 replicate samples containing buffer and pooled human liver microsomes (0.1 mg/ml) (from HL 10, 12, 29 and 40). Coefficients of variation in all cases were less than 4%.

### **Data analysis.**

Kinetic constants for TFP glucuronidation by liver microsomes or recombinant UGT1A4 were obtained by fitting experimental data to the Michaelis-Menten and 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 (Houston and Kenworthy, 2000):

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

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

Apparent  $K_i$  values were calculated by nonlinear regression analysis using Enzfitter software (Biosoft, Cambridge, UK) and determined by fitting experimental data to the expressions for competitive, noncompetitive and mixed inhibition models. Goodness of fit to kinetic and inhibition models was assessed from the F statistic,  $r^2$  values,

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parameter standard error estimates and 95% confidence intervals. The  $IC_{50}$  values were similarly determined by nonlinear regression analysis using Enzfitter. Kinetic constants and  $IC_{50}$  values are reported as the value  $\pm$  standard error of the parameter estimate.

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## RESULTS

### **Binding of TFP to human liver microsomes and HEK293 cell lysate**

To assess the impact of non-specific binding to incubation constituents on TFP glucuronidation kinetics, the fraction unbound of TFP in incubation mixtures ( $f_{u_{inc}}$ ) was determined as the drug concentration in the buffer compartment (free drug concentration) divided by the drug concentration in the HLM or HEK293 cell lysate compartment (total drug concentration). With both enzyme sources,  $f_{u_{inc}}$  was dependent on TFP concentration. As shown in Table 1, the  $f_{u_{inc}}$  values of TFP for mixtures containing HLM and HEK293 cell lysate increased with increasing TFP concentration. Over the concentration range 10-200  $\mu$ M, the extent of TFP binding to both enzyme sources decreased from approximately 80% to 41%. Due to the concentration-dependent binding, TFP concentrations in incubation mixtures were corrected for nonspecific binding and data were analyzed according to both the free and added TFP concentration.

### **TFP glucuronidation kinetics by human liver microsomes and UGT1A4 HEK293 cell lysate**

TFP (50 and 250  $\mu$ M) was screened for metabolism by all UGTs investigated here at a HEK293 lysate protein concentration of 0.25 mg/ml and incubation time 20 min. Activity was observed only with UGT1A4 (limit of detection 1.6 pmol/min.mg). Rates of TFP glucuronidation by UGT1A4 were 350 and 224 pmol/min.mg protein at substrate concentrations of 50 and 250  $\mu$ M, respectively. Subsequently, the kinetics

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of TFP glucuronidation by UGT1A4 were characterized over the substrate concentration range 5-200  $\mu\text{M}$ . Kinetic parameters for TFP glucuronidation by HLM and UGT1A4 are shown in Table 2. TFP glucuronidation kinetics by both enzyme sources were best described by a substrate inhibition model (Fig. 1). Based on added TFP concentrations,  $K_m$  values were  $61 \pm 22 \mu\text{M}$  and  $39 \mu\text{M}$  with HLM and UGT1A4 as the enzyme sources, respectively. The  $K_{si}$  values were about 2.3-fold (for HLM) and 5.2-fold (for UGT1A4) higher than the  $K_m$  values.  $V_{\max}$  values for TFP glucuronidation by HLM and UGT1A4 were  $965 \pm 180$  and  $789 \text{ pmol/min.mg protein}$ , respectively. When corrected for nonspecific binding, the  $K_m$  values for HLM ( $6.1 \pm 1.2 \mu\text{M}$ ) and UGT1A4 ( $4.1 \mu\text{M}$ ) were in good agreement (Table 2). Using the corrected substrate concentrations,  $K_{si}$  values were 35- to 70-fold higher than  $K_m$  (Table 2). The mean derived  $V_{\max}$  values were marginally lower for HLM and UGT1A4 (43% and 36%, respectively), compared to those determined on the basis of added substrate concentration.

### **Inhibition of UGT isoform activities**

The effect of amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine and sulfinpyrazone on UGT activities were investigated using 4MU or TFP as the substrate. 4MU was used as the substrate for the inhibition studies with UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15, whereas TFP was used as the substrate for UGT1A4. As shown in Fig. 2, hecogenin was a highly selective inhibitor of UGT1A4. The  $\text{IC}_{50}$  value for hecogenin inhibition of UGT1A4 was  $1.5 \mu\text{M}$  (Fig. 2D, Table 3). Hecogenin inhibition of UGT1A4 ranged

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from 40% at 1  $\mu\text{M}$  to 87% at 200  $\mu\text{M}$ , whereas inhibition of other UGT activities was less than 15%.

With the exception of hecogenin, all compounds generally exhibited nonselective inhibition of UGT activities (Fig. 2 and Table 3), although a degree of selectivity was observed for some compounds. Apart from UGT1A4, phenylbutazone and sulfinpyrazone exhibited greater inhibition of UGT1A family enzymes compared to UGT2B family enzymes (Fig. 2E and Fig. 2H). The  $\text{IC}_{50}$  values for inhibition of the UGT1A family enzymes ranged from 10-290  $\mu\text{M}$ , while the  $\text{IC}_{50}$  values for UGT2B enzymes were approximately 400-2900  $\mu\text{M}$  (Table 3). Interestingly, androsterone activated UGT1A8 activity (12-68%; Fig. 2B) while quinidine activated UGT1A3 activity (31-38%; Fig. 2F).

While screening quinine inhibition of 4MU glucuronidation by UGT isoforms, we observed that a soluble compound(s) present in the liquid scintillation glass vials (Chase Scientific Glass Inc., Rockwood, TN, USA), which were used for storing the quinine stock solution in methanol, was a highly selective inhibitor to UGT1A7 activity. Regardless of the quinine concentration added, UGT1A7 activity was almost completely abolished by the solution prepared in these vials. The effect of a methanolic extract prepared from the vials on 4MU glucuronidation by UGT1A7 was further investigated. UGT1A7 was inhibited by 80% by the methanolic extract (2% (v/v) final concentration of methanol in incubation mixture), whereas the methanolic extract from acid washed vials had a negligible effect on enzyme activity. Methanolic extracts from other brands of vials had little or no effect on UGT1A7 and other UGT activities.

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### **Inhibition kinetics of phenylbutazone and quinine**

Using phenylbutazone and quinine as ‘models’, the inhibition kinetics were characterized for those enzymes exhibiting Michaelis-Menten 4MU glucuronidation kinetics (viz. UGT 1A1, 1A6, 1A7, and 1A10) and for UGT1A9 (“weak” substrate inhibition) (Sorich et al., 2002; Uchaipichat et al., 2004). Data were consistent with noncompetitive inhibition, except for the inhibition of UGT1A1 by both drugs and quinine inhibition of UGT1A10 (all competitive inhibition) (Fig. 3, Table 4). Quinine inhibition of UGT 1A6 and 1A9 was not characterized kinetically due to the low degree of inhibition, while phenylbutazone inhibition of UGT1A6 was not adequately fitted to any of the inhibition models. The  $K_i$  values for inhibition by phenylbutazone and quinine ranged from 3.9  $\mu\text{M}$  (UGT1A7) to 272  $\mu\text{M}$  (UGT1A1) and 384  $\mu\text{M}$  (UGT1A1) to 784  $\mu\text{M}$  (UGT1A7), respectively.

### **Quinidine inhibition of AZT glucuronidation by human liver microsomes and UGT2B7**

The kinetics of quinidine inhibition of AZT glucuronidation by human liver microsomes and recombinant UGT2B7 was investigated. Quinidine was a competitive inhibitor of AZT glucuronidation by alamethicin-activated human liver microsomes and UGT2B7 (Fig. 4). The respective  $K_i$  values were  $335 \pm 128 \mu\text{M}$  (mean  $\pm$  SD) and 186  $\mu\text{M}$ .

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## DISCUSSION

Human UDP-glucuronosyltransferase (UGT) comprises an enzyme ‘superfamily’. Compared to other drug metabolizing enzymes, the substrate selectivity of the individual UGTs is poorly understood, due largely to the limited availability of isoform selective inhibitors (Miners et al., 2004). Selective inhibitors allow identification of the enzyme(s) responsible for the metabolism of any given compound by human liver microsomes (‘reaction phenotyping’) (Miners et al., 1994; Clarke, 1998; Venkatakrisnan et al., 2001). This study was performed to characterize the selectivity of inhibition of 10 human UGTs by eight compounds; amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfapyrazone. Inhibition was assessed using the non-selective UGT substrate 4-methylumbelliferone (4MU) for all enzymes except UGT1A4. TFP was characterized in this study as a ‘specific’ substrate of UGT1A4 and employed for inhibition studies of this enzyme. Hecogenin was demonstrated to be a selective inhibitor of UGT1A4. All other compounds were non-selective inhibitors, although phenylbutazone and sulfapyrazone exhibited ‘relative’ selectivity for UGT1A subfamily enzymes.

TFP is a tertiary amine which has been reported previously as a substrate of UGT1A4 (Green and Tephly, 1996), and recently it has been proposed that this compound is a selective substrate for UGT1A4 although the kinetics of inhibition were not characterized (Ghosal et al., 2004; Di Marco et al., 2005). The present study confirmed that, of the 10 human UGT enzymes investigated here, only UGT1A4 glucuronidated TFP. TFP glucuronidation by HLM and recombinant UGT1A4 both

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exhibited substrate inhibition, with similar  $K_m$  values (approximately 5  $\mu\text{M}$ ) based on free concentration. The TFP glucuronidation kinetics reported here contrast to results reported by BD Gentest ([http://www.bdbiosciences.com/discovery\\_labware/gentest/products/pdf/1A4\\_AAPS\\_S01T056R1.pdf](http://www.bdbiosciences.com/discovery_labware/gentest/products/pdf/1A4_AAPS_S01T056R1.pdf)), which were fitted to the Michaelis-Menten equation (despite kinetic plots resembling substrate inhibition). Importantly, it was shown that TFP bound extensively to HLM and HEK293 cell lysate in a concentration dependent manner. Even at the low protein concentrations used here (0.1 mg/ml of HLM and 0.25 mg/ml of cell lysate), binding to these enzyme sources ranged from 41% to 80%. This is consistent with previous studies indicating that non-specific binding to human liver microsomes may be substantial (and saturable) for lipophilic basic compounds (Obach, 1999; McLure et al., 2000; Austin et al., 2002). Interestingly, in this study we found TFP also binds to HEK293 cell lysate to the same extent as to human liver microsomes. The apparent  $K_m$  of TFP determined on the basis of the free concentration was 10-fold lower than that determined from added concentration.

Hecogenin exhibited highly selective inhibition of UGT1A4 and therefore represents a useful tool for the reaction phenotyping of human liver microsomal xenobiotic glucuronidation by this enzyme. These data are consistent with information provided by BD Gentest ([http://www.bdbiosciences.com/discovery\\_labware/gentest/products/pdf/1A4\\_AAPS\\_S01T056R1.pdf](http://www.bdbiosciences.com/discovery_labware/gentest/products/pdf/1A4_AAPS_S01T056R1.pdf)). Apart from TFP, we have also demonstrated that hecogenin (10  $\mu\text{M}$ ) essentially abolishes UGT1A4 catalyzed lamotrigine N2-glucuronidation (A Rowland, PI Mackenzie, JA Williams, JO Miners; manuscript in preparation) indicating that inhibition is unlikely to be substrate dependent. Since

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TFP glucuronidation by UGT1A4 does not follow Michaelis-Menten kinetics, further experiments to pursue the mechanism and inhibition constant were not conducted here. However, competitive inhibition might be expected since hecogenin is a known substrate of UGT1A4 (Green and Tephly, 1996).

It has been reported previously that phenylbutazone and sulfinpyrazone, which are both C-glucuronidated in humans (Dieterle et al., 1975; Aarbakke, 1978), inhibit the glucuronidation of AZT *in vitro* (Resetar et al., 1991; Rajaonarison et al., 1992). The present study confirmed that phenylbutazone and sulfinpyrazone inhibited UGT2B7, the enzyme responsible for AZT glucuronidation. However, these compounds additionally inhibited UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B15, although a degree of selectivity was also observed. Phenylbutazone and sulfinpyrazone exhibited preferential inhibition of UGT1A family enzymes (except UGT1A4). Apparent  $K_i$  values for phenylbutazone inhibition of UGT 1A1, 1A7, 1A9, and 1A10 ranged from 3.9 to 272  $\mu\text{M}$ . The  $K_i$  value for phenylbutazone inhibition of AZT glucuronidation (a known substrate of UGT2B7) by HLM was reported previously as 1.4 mM (Resetar et al., 1991).

Amitriptyline, canrenoic acid, quinidine and quinine were similarly non-selective inhibitors of UGTs. These results are consistent with previously published data which demonstrated inhibition of xenobiotic glucuronidation *in vitro* and/or *in vivo* (Ventafriidda et al., 1987; Yue et al., 1990; Resetar et al., 1991; Rajaonarison et al., 1992; Kirkwood et al., 1998; Egfjord et al., 1999). Inhibition by androsterone was also screened in this study, since it is apparently a selective substrate for UGT2B7

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(Jin et al., 1997; Coffman et al., 1998; Gall et al., 1999). While androsterone was a reasonably potent inhibitor of UGT2B7 (about 80% inhibition at 100  $\mu\text{M}$ ), it also inhibited UGT 1A1, 1A3, 1A4, 1A9, 1A10, and 2B15. In contrast, androsterone activated UGT1A8 activity. Overall, these data demonstrate that amitriptyline, androsterone, canrenic acid, quinidine, and quinine are a non-selective UGT inhibitors, and thus have little potential for UGT reaction phenotyping. However, the current findings indicate that the drugs listed above may potentially contribute to inhibitory interactions *in vivo*. It should be noted in this regard that glucuronidation kinetic parameters measured using human liver microsomes as the enzyme source generally under-predict clearance and the extent of inhibitory interactions *in vivo* by an order of magnitude (Boase and Miners, 2002; Uchaipichat et al., 2005). For example, although the  $\text{IC}_{50}$  for amitriptyline inhibition of UGT2B7 measured here was relatively high (129  $\mu\text{M}$ ), the AUC and elimination half-life of the UGT2B7 substrate morphine were reported to increase by 86% and 69%, respectively, in patients co-administered amitriptyline (50 mg/day) (Ventafridda et al., 1987).

The inhibition kinetics of AZT glucuronidation, catalyzed by human liver microsomal and recombinant UGT2B7, by quinidine was further investigated. Quinidine is utilized as a P-glycoprotein inhibitor, and it has been reported recently (Kharasch et al., 2003) that quinidine increased the apparent oral clearance of morphine, a known selective substrate of UGT2B7 (Court et al., 2003; Stone et al., 2003). In the present study, quinidine competitively inhibited AZT glucuronidation by HLM and UGT2B7, with respective  $K_i$  values of 335 and 186  $\mu\text{M}$ . While these concentrations are higher than the quinidine therapeutic plasma concentration range (2-5  $\mu\text{g/ml}$ , equivalent to 6-

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15  $\mu$ M) (Woosley and Shand, 1978), *in vitro* data tend to underestimate inhibition of drug glucuronidation *in vivo* (as noted above). Thus, inhibition of morphine first pass extraction may contribute, at least in part, to the quinidine-morphine interaction *in vivo*.

The present study found that a methanol-soluble compound(s) in a particular brand of liquid scintillation glass vials was a highly selective inhibitor of UGT1A7. The samples from these and acid washed (as a control) glass vials were further analyzed using LC/MS. Spectra from both samples were similar, except for the presence of polyethylene glycol (possibly used as a detergent in the production process) and three (as yet unidentified) compounds with mass to charge ratios of 294, 240, and 214. These observations provide a warning that some compounds present in glassware used for performing drug metabolism studies *in vitro* may inhibit enzyme activity.

There is evidence for multiple substrate binding sites within the active site of a number of UGTs (Uchaipichat et al., 2004; Stone et al., 2003). To gain further insight into substrate-inhibitor interactions, the mechanism of phenylbutazone and quinine inhibition of UGT 1A1, 1A6, 1A7, 1A9 and 1A10 was investigated. Notably, phenylbutazone inhibition of UGT 1A7, 1A9 and 1A10 and quinine inhibition of UGT1A7 was best described by the non-competitive model, which may suggest distinct binding sites for substrate and inhibitor in these enzymes.

In summary, TFP was confirmed as a selective 'probe' substrate of UGT1A4, while hecogenin was confirmed as a highly selective inhibitor on this enzyme. Thus, TFP

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and hecogenin represent useful tools for the reaction phenotyping of UGT1A4 substrates. There is a possibility that amitriptyline, canrenoic acid, phenylbutazone, quinidine, quinine, and sulfinpyrazone may inhibit the metabolism of coadministered glucuronidated drugs. In particular, quinidine also has the capacity to inhibit a number of human UGTs when used as a P-glycoprotein inhibitor *in vivo*.

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## **FOOTNOTE**

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## LEGENDS FOR FIGURES

**FIGURE 1:** *Representative kinetic plots (rate (v) vs [S] and Eadie-Hofstee) for trifluoperazine glucuronidation by human liver microsomes and UGT1A4*

Panels A and B: TFP glucuronidation by alamethicin-treated microsomes (HL40).

Panels C and D: TFP glucuronidation by UGT1A4.

**FIGURE 2:** *The effects of amitriptyline (panel A), androsterone (panel B), canrenoic acid (panel C), hecogenin (panel D), phenylbutazone (panel E), quinidine (panel F), quinine (panel G), sulfinpyrazone (panel H) on the activities of human UGTs.*

TFP (40  $\mu$ M) was used as substrate for the inhibition studies with UGT1A4, whereas 4MU was used as the substrate for all other isoforms. Concentrations of 4MU corresponded to the known  $K_m$  or  $S_{50}$  for each UGT. Panels to the right show inhibitor concentrations. Each bar represents the mean percentage activity relative to control from duplicate measurements.

**FIGURE 3:** *Representative Dixon plots for phenylbutazone and quinine inhibition.*

Phenylbutazone inhibition of UGT1A1 (panel A) and UGT1A9 (panel B), and quinine inhibition of UGT1A1 (panel C) and UGT1A7 (panel D).

**FIGURE 4:** *Representative Dixon plots for quinidine inhibition of zidovudine (AZT) glucuronidation by alamethicin-activated human liver microsomes (HL40; panel A) and UGT2B7 (panel B).*

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**TABLE 1**

*Nonspecific binding of trifluoperazine to human liver microsomes (HLM) and  
HEK293 cell lysate*

[TFP] ( $\mu$ M)	Fraction unbound ( $f_{u_{inc}}$ ) <sup>a</sup>	
	HLM (0.1 mg/ml)	HEK293 cell lysate (0.25 mg/ml)
10	0.21	0.20
30	0.26	N.D.
60	0.42	0.44
100	0.49	0.50
200	0.59	0.57

N.D., not determined

<sup>a</sup> Each value represents the mean from duplicate samples

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**TABLE 2**

*Kinetic constants for trifluoperazine glucuronidation by alamethicin-treated human liver microsomes and by UGT1A4<sup>a,b,c</sup>.*

	$K_m$ ( $\mu\text{M}$ )	$K_{si}$ ( $\mu\text{M}$ )	$V_{max}$ (pmol/min.mg protein)
HL10	84 $\pm$ 1.1 (7.6 $\pm$ 0.1)	65 $\pm$ 1.2 (105 $\pm$ 2)	820 $\pm$ 5.9 (394 $\pm$ 2)
HL12	51 $\pm$ 1 (6.0 $\pm$ 0.2)	122 $\pm$ 3.6 (158 $\pm$ 6.4)	1180 $\pm$ 11 (721 $\pm$ 7.1)
HL29	77 $\pm$ 1.9 (6.2 $\pm$ 0.1)	84 $\pm$ 2.4 (164 $\pm$ 4.5)	1048 $\pm$ 16 (487 $\pm$ 3.2)
HL40	35 $\pm$ 0.5 (4.7 $\pm$ 0.3)	287 $\pm$ 6.5 (417 $\pm$ 54)	815 $\pm$ 5.7 (578 $\pm$ 13)
<b>Mean <math>\pm</math> SD</b>	<b>61 <math>\pm</math> 22</b> <b>(6.1 <math>\pm</math> 1.2)</b>	<b>140 <math>\pm</math> 101</b> <b>(211 <math>\pm</math> 140)</b>	<b>965 <math>\pm</math> 180</b> <b>(545 <math>\pm</math> 139)</b>
UGT1A4	39 $\pm$ 3.5 (4.1 $\pm$ 0.04)	206 $\pm$ 25 (285 $\pm$ 3.5)	789 $\pm$ 43 (508 $\pm$ 1.1)

<sup>a</sup> Best fit to substrate inhibition model for all data.

<sup>b</sup> Data presented as mean  $\pm$  standard error of parameter fit.

<sup>c</sup> Data in parenthesis are kinetic parameters corrected for nonspecific binding.

**TABLE 3**

*IC<sub>50</sub> values for inhibition of human UDP-glucuronosyltransferases by amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine and sulfinpyrazone*

UGT <sup>a</sup>	IC <sub>50</sub> (μM) <sup>b</sup>							
	Amitriptyline	Androsterone	Canrenoic acid	Hecogenin	Phenylbutazone	Quinidine	Quinine	Sulfinpyrazone
<b>1A1</b>	1345 ± 37	125 ± 6	372 ± 1	N.D. <sup>c</sup>	294 ± 4	776 ± 14	861 ± 7	46 ± 1.6
<b>1A3</b>	1120 ± 22	65 ± 31	1387 ± 27	N.D. <sup>c</sup>	266 ± 8	N.D. <sup>d</sup>	388 ± 41	267 ± 4
<b>1A4</b>	856 ± 24	106 ± 5	503 ± 24	1.5 ± 0.1	1227 ± 95	1619 ± 17	1355 ± 56	651 ± 13
<b>1A6</b>	152 ± 4	N.D. <sup>c</sup>	545 ± 20	N.D. <sup>c</sup>	10 ± 6	2515 ± 480	3290 ± 240	187 ± 11
<b>1A7</b>	355 ± 22	N.D. <sup>c</sup>	693 ± 30	N.D. <sup>c</sup>	11 ± 1.5	507 ± 10	970 ± 69	16 ± 2
<b>1A8</b>	111 ± 10	N.D. <sup>d</sup>	362 ± 4	N.D. <sup>c</sup>	40 ± 1.6	551 ± 13	977 ± 17	158 ± 1
<b>1A9</b>	443 ± 6	7 ± 4.2	1205 ± 53	N.D. <sup>c</sup>	13 ± 0.2	278 ± 20	> 5000	11 ± 0.7
<b>1A10</b>	153 ± 10	102 ± 4	318 ± 9	N.D. <sup>c</sup>	48 ± 1.8	536 ± 2	717 ± 18	58 ± 0.6
<b>2B7</b>	129 ± 4	16 ± 4	707 ± 25	N.D. <sup>c</sup>	2962 ± 727	159 ± 21	322 ± 11	2741 ± 45
<b>2B15</b>	72 ± 0.4	48 ± 3	249 ± 4	N.D. <sup>c</sup>	394 ± 6	133 ± 14	212 ± 4	402 ± 9

<sup>a</sup> Basal control activities of UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15 at the substrate concentrations employed (see Methods) were 65, 400, 300, 70000, 15000, 2800, 3000, 5300, 70 and 15 pmol/min.mg, respectively.

<sup>b</sup> Data presented as mean ± standard error of parameter fit

<sup>c</sup> N.D., not determined due to low degree of inhibition (IC<sub>50</sub> > 200 μM)

<sup>d</sup> N.D., not determined due to enzyme activation

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**TABLE 4**  
*Inhibitor constants for phenylbutazone and quinine inhibition of 4-methylumbelliferone glucuronidation by human UGTs*

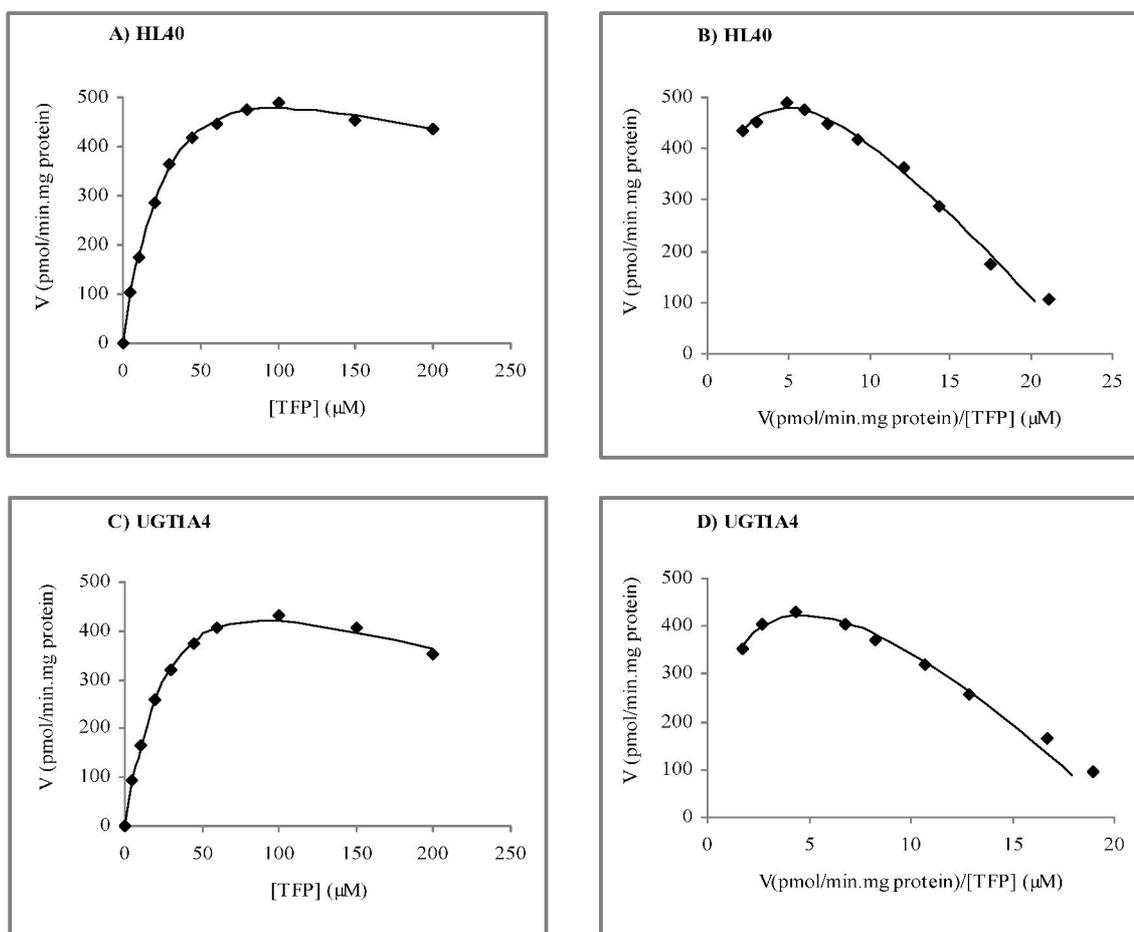
UGT	$K_i$ ( $\mu\text{M}$ ) <sup>a, b</sup>	
	Phenylbutazone	Quinine
1A1	272 $\pm$ 8.6	384 $\pm$ 6.8
1A6	N.F.	N.D.
1A7	3.9 $\pm$ 0.3	784 $\pm$ 3.5
1A9	5.7 $\pm$ 0.2	N.D.
1A10	12 $\pm$ 0.4	492 $\pm$ 31

N.D., not determined due to low degree of inhibition ( $\text{IC}_{50} > 1 \text{ mM}$ )

N.F., data were not consistent with competitive, noncompetitive, or mixed inhibition models.

<sup>a</sup> Noncompetitive inhibition, except for phenylbutazone inhibition of UGT1A1 (competitive inhibition) and quinine inhibition of UGT 1A1 and 1A10 (competitive inhibition).

<sup>b</sup>  $K_i \pm$  standard error of parameter fit



**FIGURE 1**

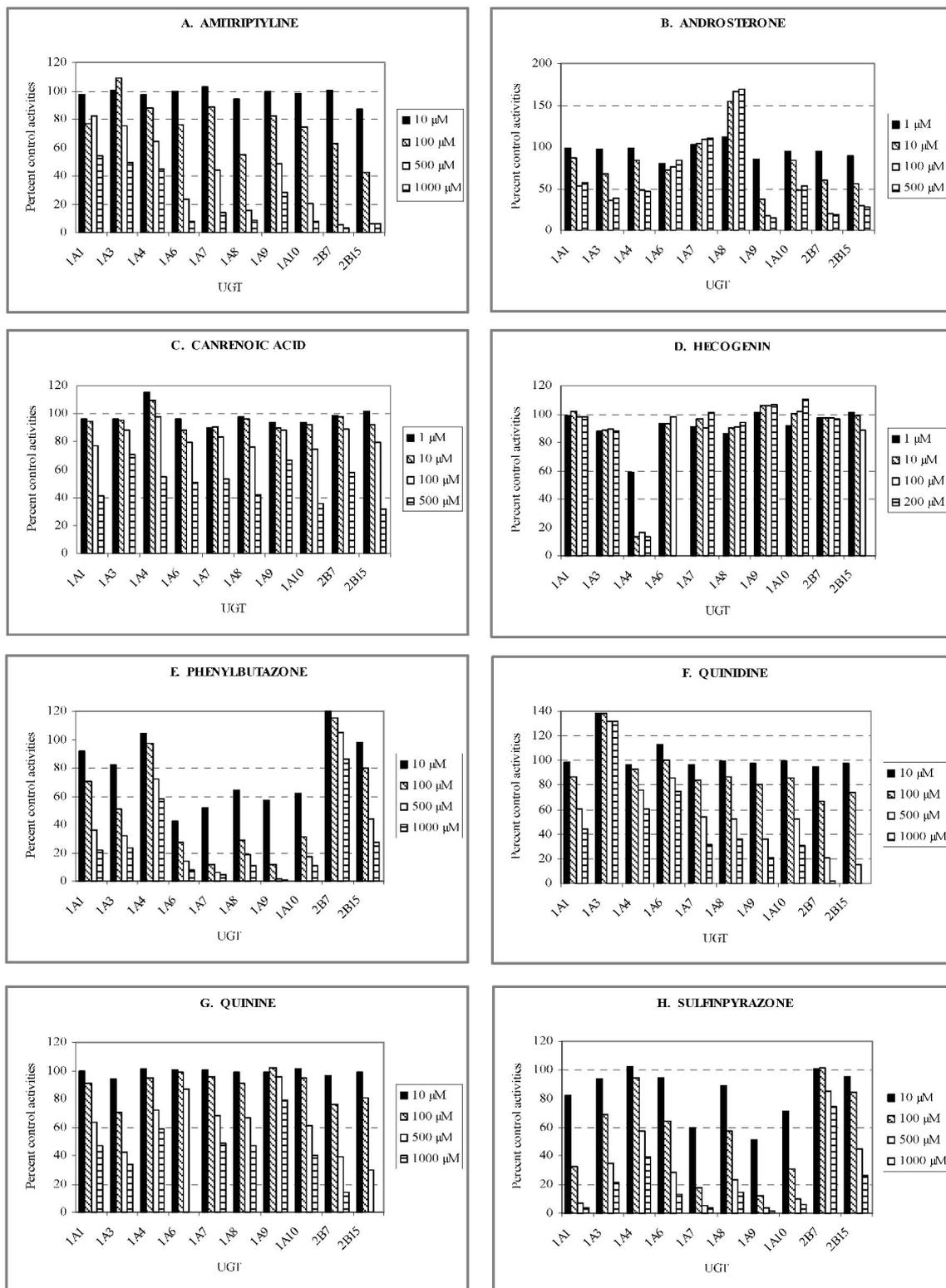
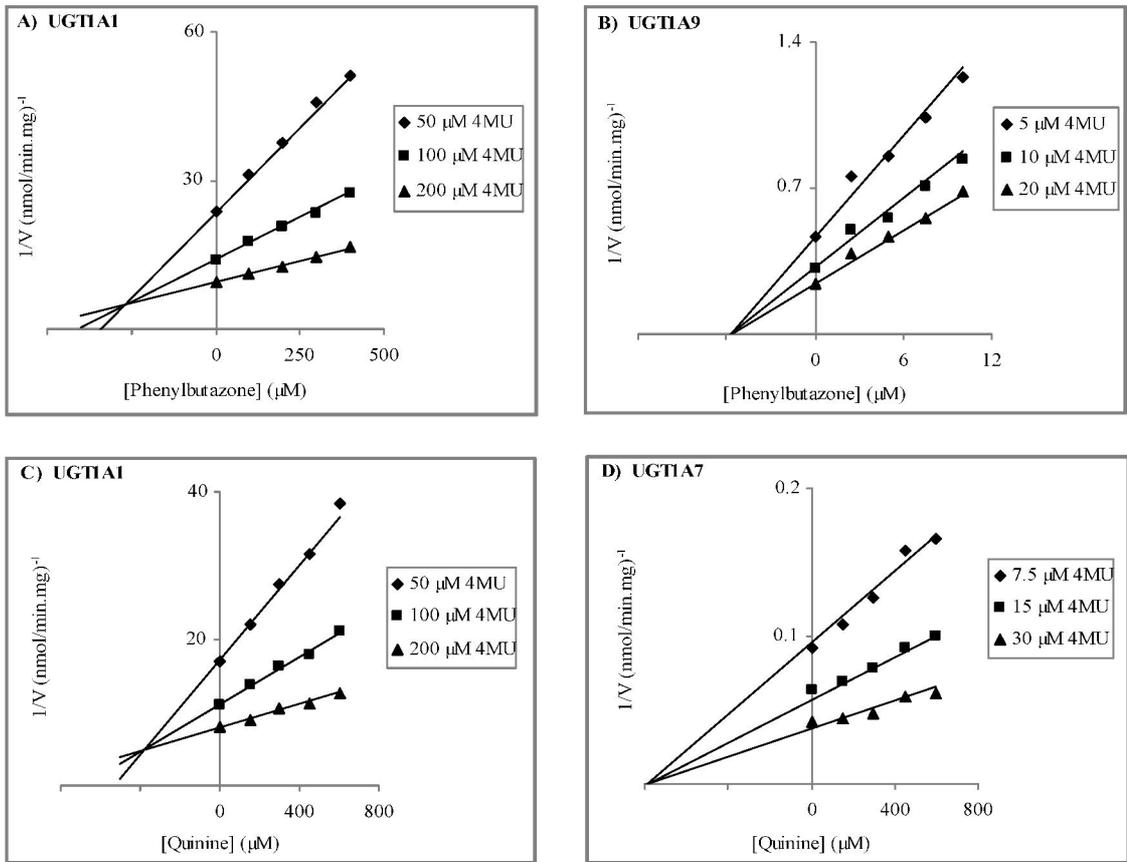
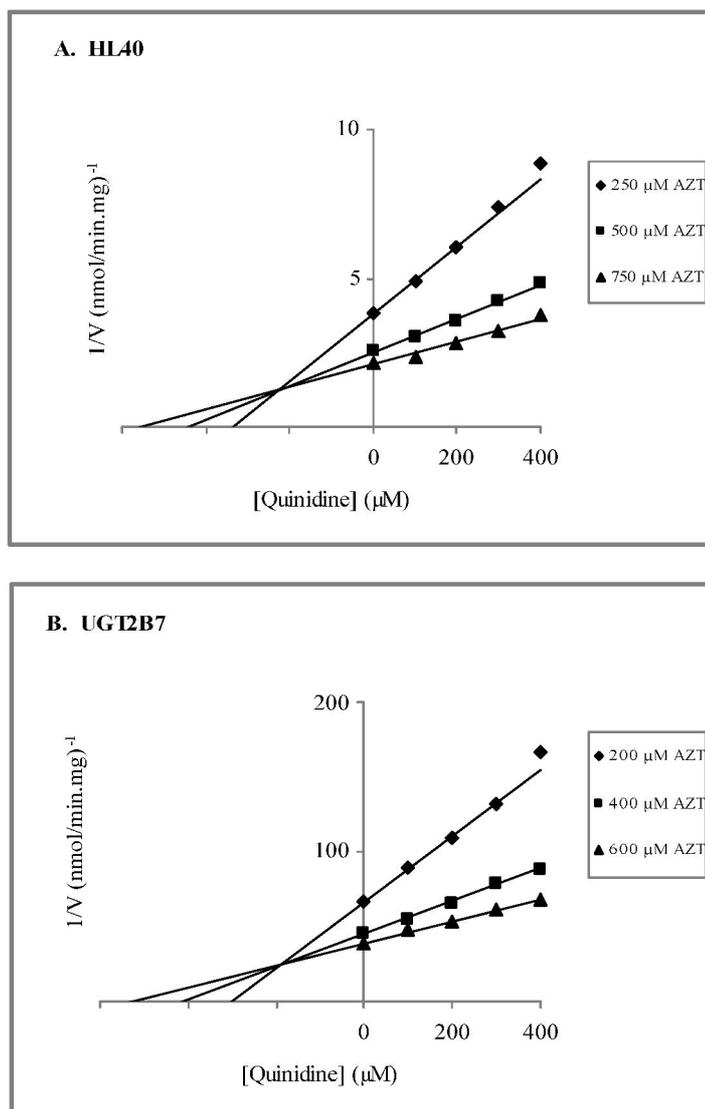


FIGURE 2



**FIGURE 3**



**FIGURE 4**