SELECTIVITY OF SUBSTRATE (TRIFLUOPERAZINE) AND INHIBITOR (AMITRIPTYLINE, ANDROSTERONE, CANRENOIC ACID, HECOGENIN, PHENYL BUTAZONE, QUININE, AND SULFINPYRAZONE) “PROBES” FOR HUMAN UDP-GLUCURONOSYLTRANSFERASES

Verawan Uchaipichat, Peter I. Mackenzie, David J. Elliot, and John O. Miners

Department of Clinical Pharmacology, Flinders University and Flinders Medical Centre, Adelaide, Australia

Received September 13, 2005; accepted December 19, 2005

ABSTRACT:
Relatively few selective substrate and inhibitor probes have been identified for human UDP-glucuronosyltransferases (UGTs). This work investigated the selectivity of trifluoperazine (TFP), as a substrate, and amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfipyrazone, as inhibitors, for human UGTs. Selectivity was assessed using UGTs 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 human liver microsomes in a concentration-dependent manner (Kd 0.20–0.59). When corrected for nonspecific binding, Kd values for TFP glucuronidation were similar for both UGT1A4 (4.1 µM) and human liver microsomes (6.1 ± 1.2 µM) as the enzyme sources. Of the compounds screened as inhibitors, hecogenin, alone, was selective; significant inhibition was observed only for UGT1A4 (IC50 1.5 µM). Using phenylbutazone and quinine as “models,” inhibition kinetics were variously described by competitive and noncompetitive mechanisms. Inhibition of UGT2B7 by quinine was also investigated further, because the effects of this compound on morphine pharmacokinetics (a known UGT2B7 substrate) have been ascribed to inhibition of P-glycoprotein. Quinine inhibited human liver microsomal and recombinant UGT2B7, with respective Kd values of 335 ± 128 µM and 186 µM. In conclusion, TFP and hecogenin represent selective substrate and inhibitor probes for UGT1A4, although the extensive nonspecific binding of the former should be taken into account in kinetic studies. Amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfipyrazone are nonselective UGT inhibitors.

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, 2000). 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, 17 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) seem to have low or absent activity with xenobiotic substrates. Available evidence suggests that the active UGT enzymes exhibit distinct but overlapping substrate selectivities (Radominska-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 gastrointestinal tract, whereas UGT2A1 seems to be largely involved in olfactory signal termination.

Given these features of UGTs, 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, because 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 general.

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; AUC, area under the plasma concentration-time curve; AZT, zidovudine, i.e., 3′-azido-3′-deoxythymidine; fu, fraction unbound in incubation mixtures; GAZT, 3′-azido-3′-deoxythymidine 5′-β-D-glucuronide; HEK293, human embryo kidney 293 cells; HLM, human liver microsome; HPLC, high-performance liquid chromatography; 4MU, 4-methylumbelliferone; P450, cytochrome P450; TFP, trifluoperazine; TFPG, trifluoperazine glucuronide; UDPGA, UDP-glucuronic acid.
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, canrenonic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfipyrazone as inhibitors of human UGTs. There is evidence to suggest that these compounds may inhibit xenobiotic glucuronidation in vitro and/or in vivo (Ventaiyak et al., 1987; Yue et al., 1990; Resetar et al., 1991; Rajanarison et al., 1992; Kirkwood et al., 1998; Eggerdl et al., 1999) or may be substrates for human UGTs in vitro and/or in vivo (Green and Teply, 1996; Coffman et al., 1998; Gall et al., 1999; Mirgiani et al., 2003). Quinidine is of further interest because it is used as an inhibitor of P-glycoprotein in vivo to assess the role of this transporter in 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 coadministered quinidine.

Effects of the putative inhibitors on UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, and 2B15 were investigated using the nonselective substrate 4-methylumbelliferone (4MU) as the “probe.” Since UGT1A4 exhibits negligible activity toward 4MU (Uchaipichat et al., 2004), trifluoperazine (TFP) was characterized as a specific probe substrate for this enzyme and subsequently used in the UGT1A4 inhibition studies. In addition, the inhibition kinetics of quinidine on human liver microsomal and recombinant UGT2B7 was further investigated using zidovudine (3′-azido-2′,3′-dideoxythymidine, AZT) as the substrate.

Materials and Methods

Materials. 4MU, 4-methylumbelliferone-β-D-glucuronide, β-glucuronidase (from Escherichia coli), alamethicin (from Trichoderma viride, amitriptyline (hydrochloride salt), androsterone, AZT, canrenonic acid (potassium salt), GAZT (3′-azido-3′-deoxyxymidine 5′-β-D-glucuronide), hecogenin, phenylbutazone, TFP (dihydrochloride salt), trifluoroacetic acid, quinidine (hydrochloride salt), quinine (hemin sulfate salt, hydrate), sulfipyrazone, UDPGA (trisodium salt), and dialysis cellulose membrane (molecular mass cutoff 12,000 Da) were purchased from Sigma-Aldrich (St. Louis, MO). GAZT formation was measured using a modification of a previously described technology, Sydney, NSW, Australia) fitted with a Novapak C18 column (3.9 × 30 mm; Waters, Milford, MA). The mobile phase was 0.12% v/v acetic acid in 10% acetonitrile/water, was delivered at a flow rate of 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 a GAZT standard curve prepared over the concentration range 0.2 to 10 μM. The lower limit of detection, assessed as 5 times background, was 8 pmol TFP glucuronidation.

AZT Glucuronidation Assay. The inhibition study with TFP was performed in a similar manner with TFP (5–200 μM). The lower limit of detection (as defined above) was 30 pmol 4MU glucuronide/incubation.

AZT Glucuronidation Assay and Quinidine Inhibition of UGT2B7. GAZT formation was measured using a modification of a previously described method (Boase and Miners, 2002). Incubation mixtures, in a total volume of 200 μl, contained 0.1 M phosphate buffer (pH 7.4), 4 mM MgCl₂, 5 mM UDPGA, AZT (250, 500, and 750 μM for HLMs or 200, 400, and 600 μM for UGT2B7), quinidine (0, 100, 200, 300, and 400 μM), and activated 1 mg/ml HLMs or HEK293 cell lysate expressing UGT2B7 (1.5 mg/ml). Reactions were terminated by 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. HPLC was performed using an Agilent 1100 series (Agilent Technologies, Sydney, NSW, Australia) fitted with a Novapak C18 column (3.9 × 150 mm). The mobile phase, 0.12% v/v acetic acid in 10% acetonitrile/water, was delivered at a flow rate of 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 to 20 μM.

Inhibition of 4MU or TFP Glucuronidation by Drugs and Other Chemicals. Amitriptyline, androsterone, canrenonic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfipyrazone 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 concentration corresponding to the apparent Kₘ or S₀p value of each enzyme (Sorich et al., 2002; Uchaipichat et al., 2004). Concentrations of amitriptyline, phenylbutazone, quinidine, quinine, and sulfipyrazone used in screening experiments were 0, 1, 10, 50, and 100 μM. The concentrations of hecogenin used for inhibition screening were 0, 1, 10, 100, and 200 μM, whereas concentrations of androsterone and canrenonic acid were 0, 1, 10, 100, and 500 μM. 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, canrenone acid, phenylbutazone, quinidine, sulfipyrazone, and hecogenin required addition of an organic solvent, which was selected on the basis of known effect on enzyme activity (Uchailpichat 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, canrenone acid, phenylbutazone, quinidine, and hecogenin, whereas 1% dimethyl sulfoxide (v/v) was used for incubations containing sulfipyrazone. Control incubations contained the same concentration of organic solvent.

Subsequent experiments that determined inhibitor constants ($K_i$) for selected compounds included four inhibitor concentrations at each of three substrate concentrations. The $K_i$ values of phenylbutazone and quinidine 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 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). In brief, one side of the dialysis apparatus contained TFP (10, 30, 60, 100, or 200 μM), 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), whereas 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 μM) or low (10 μM) TFP concentrations to ensure that equilibrium was attained. The contents of each compartment (200 μl) were collected, treated with 200 μl of 4% (v/v) acetic acid in methanol, vortex mixed, and centrifuged (5000g for 10 min). A 5-μl 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 (Uchailpichat et al., 2005), except that the mobile phase (50% mobile phase A/50% mobile phase B) was delivered isocratically 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 to 200 μM 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 imprecision was assessed by measuring TFP (10 or 200 μM) in five 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%. The effects of amitriptyline, androsterone, canrenone acid, phenylbutazone, quinidine, and sulfipyrazone on UGT activities (Fig. 1) were assessed from the peak areas with those of the standard curve. Within-day assay imprecision was assessed 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, $f_{unb}$ was dependent on TFP concentration. As shown in Table 1, the $f_{unb}$ values of TFP for mixtures containing HLMs and HEK293 cell lysate increased with increasing TFP concentration. Over the concentration range 10 to 200 μM, the extent of TFP binding to both enzyme sources decreased from approximately 80% to 41%. Because of the concentration-dependent binding, TFP concentrations in incubation mixtures were corrected for non-specific binding and data were analyzed according to both the free and added TFP concentration.

**TFP Glucuronidation Kinetics by Human Liver Microsomes and UGT1A4.** TFP glucuronidation kinetics by both enzyme sources were best described by a substrate inhibition model (Fig. 1). Based on added TFP concentrations, $K_{si}$ values were 61 ± 22 μM and 39 μM with HLMs and UGT1A4 as the enzyme sources, respectively. The $K_{si}$ values were approximately 2.3-fold (for HLMs) and 5.2-fold (for UGT1A4) higher for 1% dimethyl sulfoxide (v/v) and UGT1A4 HEK293 Cell Lysate. TFP (50 and 250 μM) was screened for metabolism by all UGTs investigated here at a HEK293 lysate protein concentration of 0.25 mg/ml and an incubation time of 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 μM, respectively. Subsequently, the kinetics of TFP glucuronidation by UGT1A4 were characterized over the substrate concentration range 5 to 200 μM. Kinetic parameters for TFP glucuronidation by HLMs 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, $K_{si}$ values were 61 ± 22 μM and 39 μM with HLMs and UGT1A4 as the enzyme sources, respectively. The $K_{si}$ values were 35- to 70-fold higher than $K_m$ (Table 2). The mean derived $V_{max}$ values were marginally lower for HLMs and UGT1A4 (43% and 36%, respectively), compared with those determined on the basis of added substrate concentration.

**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 ($f_{unb}$) 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, $f_{unb}$ was dependent on TFP concentration. As shown in Table 1, the $f_{unb}$ values of TFP for mixtures containing HLMs and HEK293 cell lysate increased with increasing TFP concentration. Over the concentration range 10 to 200 μM, the extent of TFP binding to both enzyme sources decreased from approximately 80% to 41%. Because of 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.

**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 ($f_{unb}$) 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, $f_{unb}$ was dependent on TFP concentration. As shown in Table 1, the $f_{unb}$ values of TFP for mixtures containing HLMs and HEK293 cell lysate increased with increasing TFP concentration. Over the concentration range 10 to 200 μM, the extent of TFP binding to both enzyme sources decreased from approximately 80% to 41%. Because of the concentration-dependent binding, TFP concentrations in incubation mixtures were corrected for non-specific binding and data were analyzed according to both the free and added TFP concentration.

**TFP Glucuronidation Kinetics by Human Liver Microsomes and UGT1A4.** TFP glucuronidation kinetics by both enzyme sources were best described by a substrate inhibition model (Fig. 1). Based on added TFP concentrations, $K_{si}$ values were 61 ± 22 μM and 39 μM with HLMs and UGT1A4 as the enzyme sources, respectively. The $K_{si}$ values were 35- to 70-fold higher than $K_m$ (Table 2). The mean derived $V_{max}$ values were marginally lower for HLMs and UGT1A4 (43% and 36%, respectively), compared with those determined on the basis of added substrate concentration.

**Inhibition of UGT Isoform Activities.** The effects of amitriptyline, androsterone, canrenone acid, hecogenin, phenylbutazone, quinidine, and sulfipyrazone 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 $IC_{50}$ value for hecogenin inhibition of UGT1A4 was 1.5 μM (Fig. 2D; Table 3). Hecogenin inhibition of UGT1A4 ranged from 40% at 1 μM to 87% at 200 μM, 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; Table 3), although a degree of selectivity was observed for some compounds.

---

**Table 1**

Non-specific binding of trifluoroperazine to HLMs and HEK293 cell lysate

<table>
<thead>
<tr>
<th>[TFP]</th>
<th>HLMs (0.1 mg/ml)</th>
<th>HEK293 Lysate (0.25 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM</td>
<td>0.21</td>
<td>0.20</td>
</tr>
<tr>
<td>30 μM</td>
<td>0.26</td>
<td>N.D.</td>
</tr>
<tr>
<td>60 μM</td>
<td>0.42</td>
<td>0.44</td>
</tr>
<tr>
<td>100 μM</td>
<td>0.49</td>
<td>0.50</td>
</tr>
<tr>
<td>200 μM</td>
<td>0.59</td>
<td>0.57</td>
</tr>
</tbody>
</table>

N.D., not determined

* Each value represents the mean from duplicate samples.
Apart from UGT1A4, phenylbutazone and sulfinpyrazone exhibited greater inhibition of UGT1A family enzymes compared with UGT2B family enzymes (Fig. 2, E and H). The IC₅₀ values for inhibition of the UGT1A family enzymes ranged from 10 to 290 μM, whereas the IC₅₀ values for UGT2B enzymes were approximately 400 to 2900 μM (Table 3). Interestingly, androsterone activated UGT1A8 activity (12–68%; Fig. 2B) whereas 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), which were used for storing the quinine stock solution in methanol, was a highly selective inhibitor of 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.

**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. UGT1A1, 1A6, 1A7, and 1A10) and for UGT1A9 (“weak” substrate inhibition) (Sorich et al., 2002; Uchai-pichat 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). Quinidine inhibition of UGT1A6 and 1A9 was not characterized kinetically because of the low degree of inhibition, whereas phenylbutazone inhibition of UGT1A6 was not adequately fitted to any of the inhibition models. The Kᵢ values for inhibition by phenylbutazone and quinine ranged from 3.9 μM (UGT1A7) to 272 μM (UGT1A1) and 384 μM (UGT1A1) to 784 μM (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
FIG. 2. The effects of amitriptyline (A), androsterone (B), canrenoic acid (C), hecogenin (D), phenylbutazone (E), quinidine (F), quinine (G), and sulfipyrazone (H) on the activities of human UGTs. TFP (40 μM) 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.
AZT glucuronidation by alamethicin-activated human liver microsomes and UGT2B7 (Fig. 4). The respective $K_i$ values were $335 \pm 128 \mu M$ (mean ± S.D.) and $186 \mu M$.

**Discussion**

Human UGT comprises an enzyme “superfamily.” Compared with 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; Venkatakrishnan et al., 2001). This study was performed to characterize the selectivity of inhibition of 10 human UGTs by eight compounds: amitriptyline, androsterone, canrenonic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfinpyrazone. Inhibition was assessed using the nonselective UGT substrate 4MU for all enzymes except UGT1A1 (C) and UGT1A7 (D) is shown.

**Table 3**

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

$^a$ Basal control activities of UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, and 2B15 at the substrate concentrations used (see Materials and Methods) were 65, 400, 300, 70,000, 15,000, 2800, 3000, 5300, 70, and 15 pmol/min·mg, respectively.

$^b$ Data are presented as mean ± standard error of parameter fit.

$^c$ N.D., not determined due to low degree of inhibition (IC$_{50}$ > 200 μM).

$^d$ N.D., not determined due to enzyme activation.

![Fig. 3](https://example.com/fig3.png)
consistent with competitive, noncompetitive, or mixed inhibition models.

Inhibition constant for phenylbutazone and quinine inhibition of 4-methylumbelliferone glucuronidation by human UGTs

<table>
<thead>
<tr>
<th>UGT</th>
<th>Phenylbutazone</th>
<th>Quinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>272 ± 8.6 μM</td>
<td>384 ± 6.8 μM</td>
</tr>
<tr>
<td>1A6</td>
<td>N.F.</td>
<td>N.D.</td>
</tr>
<tr>
<td>1A7</td>
<td>3.9 ± 0.3 μM</td>
<td>784 ± 3.5 μM</td>
</tr>
<tr>
<td>1A9</td>
<td>5.7 ± 0.2 μM</td>
<td>N.D.</td>
</tr>
<tr>
<td>1A10</td>
<td>12 ± 0.4 μM</td>
<td>492 ± 31 μM</td>
</tr>
</tbody>
</table>

N.D., not determined due to low degree of inhibition (IC50 > 1 mM); N.F., data were not consistent with competitive, noncompetitive, or mixed inhibition models.

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

b Values are $K_i$ = standard error of parameter fit.

A. HL40

![Representative Dixon plots for quinidine inhibition of zidovudine (AZT) glucuronidation by alamethicin-activated human liver microsomes (HL40; A) and UGT2B7 (B).](http://www.bdbiosciences.com/discovery_labware/gentest/products/pdf/1A4_AAPS_S01T056R1.pdf)

confirmed that, of the 10 human UGT enzymes investigated here, only UGT1A4 glucuronidated TFP. TFP glucuronidation by HLMs and recombinant UGT1A4 both exhibited substrate inhibition, with similar $K_{i0}$ values (approximately 5 μM) based on free concentration. The TFP glucuronidation kinetics reported here contrast with results reported by BD Gentest (http://wwwbdbiosciences.com/discovery_labware/gentest/products/pdf/1A4_AAPS_S01T056R1.pdf), which were fitted to the Michaelis-Menten equation (despite kinetic plots resembling substrate inhibition). It is noteworthy that it was shown that TFP bound extensively to HLMs and HEK293 cell lysate in a concentration-dependent manner. Even at the low protein concentrations used here (0.1 mg/ml HLMs and 0.25 mg/ml cell lysate), binding to these enzyme sources ranged from 41% to 80%. This is consistent with previous studies indicating that nonspecific 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 that TFP also binds to HEK293 cell lysate to the same extent as to human liver microsomes. The apparent $K_i$ 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). Apart from TFP, we have also demonstrated that hecogenin (10 μM) essentially abolishes UGT1A4-catalyzed lamotrigine N2-glucuronidation (A. Rowland, P. I. Mackenzie, J. A. Williams, and J. O. Miners, manuscript in preparation), indicating that inhibition is unlikely to be substrate-dependent. Because 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 μM. The $K_i$ value for phenylbutazone inhibition of AZT glucuronidation (a known substrate of UGT2B7) by HLMs was reported previously as 1.4 mM (Resetar et al., 1991).

Ammitriptyline, canrenonic acid, quinidine, and quinine were similarly nonselective inhibitors of UGTs. These results are consistent with previously published data that demonstrated inhibition of 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). Inhibition by androsterone was also screened in this study, since it is apparently a selective substrate for UGT2B7 (Jin et al., 1997; Coffman et al., 1998; Gall et al., 1999). Although androsterone was a reasonably potent inhibitor of UGT2B7 (about 80% inhibition at 100 μM), it also inhibited UGT 1A1, 1A3, 1A4, 1A9, 1A10, and 2B15. In contrast, androsterone activated UGT1A8 activity. Overall, these data demonstrate that amitriptyline, androsterone, canrenonic acid, quinidine, and quinine are nonselective 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 micro-
some as the enzyme source generally underpredict 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 IC50 for amitriptyline inhibition of UGT2B7 measured here was relatively high (129 μM), the AUC and elimination half-life of the UGT2B7 substrate morphine were reported to be increased by 86% and 69%, respectively, in patients coadministered amitriptyline (50 mg/day) (Ventafripta et al., 1987).

The inhibition kinetics of AZT glucuronidation, catalyzed by human liver microsomal and recombinant UGT2B7, by quinidine was further investigated. Quinidine is used 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 HLMs and UGT2B7, with respective Ks values of 335 and 186 μM. Although these concentrations are higher than the quinidine therapeutic plasma concentration range (2–5 μM) (Wooley 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 to the quinidine-morphine interaction in vivo. There is evidence for multiple substrate binding sites within the active site of a number of UGTs (Stone et al., 2003; Uchaipichat et al., 2004). To gain further insight into substrate-inhibitor interactions, the mechanism of phenylbutazone and quinine inhibition of UGT1A1. The samples from these and acid washed (as a control) glass vials were further analyzed using liquid chromatography/mass spectrometry. 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 (Stone et al., 2003; Uchaipichat et al., 2004). To gain further insight into substrate-inhibitor interactions, the mechanism of phenylbutazone and quinine inhibition of UGT1A1. The samples from these and acid washed (as a control) glass vials were further analyzed using liquid chromatography/mass spectrometry. 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 (Stone et al., 2003; Uchaipichat et al., 2004). To gain further insight into substrate-inhibitor interactions, the mechanism of phenylbutazone and quinine inhibition of UGT1A1. The samples from these and acid washed (as a control) glass vials were further analyzed using liquid chromatography/mass spectrometry. 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.

References


Address correspondence to: Professor John O. Miners, Department of Clinical Pharmacology, Flinders Medical Centre, Bedford Park, SA 5042, Australia. E-mail: john.miners@flinders.edu.au